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Document Structure

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Section 1: An Overview of Forensic Biology Workflow

Casework in the Forensic Biology discipline is routinely processed in batches. Typical batch size is 5-10 cases but may be larger or smaller as needed. Each batch of casework has a corresponding LIMS-DNA packet, where documentation of batch QA controls and lab processing is recorded. The Forensic Biology Procedures Manual describes the protocols used for lab processes, interpretation, report writing, and technical and administrative reviews.

Batched casework moves through this general laboratory workflow:

1. Biological screening: presumptive tests for blood and/or semen
2. Prioritizing analysis, sampling, and extraction: analyst uses the available documentation, such as Request for Laboratory Services and sexual assault kit paperwork, along with biological screening results (if applicable) to make decisions about which evidence is best suited for DNA analysis. Evidence is prepared for the DNA extraction process, and a protocol is selected based on type of evidence.
3. Quantification and quant-based decisions: the quantification process determines the amount of DNA present in a sample, as well as the amount of male DNA and the quality of the DNA. Based on the quantification results, analysis of samples may stop or proceed to amplification
4. Amplification and genetic analysis: Samples are amplified by short tandem repeat (STR) polymerase chain reaction (PCR) analysis, or by Y-STR analysis, which is PCR specific to the markers on the Y-chromosome. Capillary electrophoresis is used to analyze the amplified DNA.
5. Interpretation and report writing analyst assesses the profiles, makes comparisons, and calculates statistics, as appropriate.
6. Review: All reports undergo technical and administrative reviews prior to release

If an analyst completes only part of the workflow and analysis and/or interpretation of the evidence is completed by another analyst, the first analyst must document and self-review the full extent of their work. This documentation may include bench notes, LIMS-DNA documentation, and / or the part of the FB Analysis report which addresses their results and conclusions, as appropriate. The same principle applies to technical reviews, where specific elements of a given case may be technically reviewed by different analysts. The review documentation must clearly indicate if technical review has been performed by more than one reviewer.

This manual is designed to address typical situations that arise during forensic casework. Analysts must seek prior approval of protocol changes that impact the use of evidence. The DNA Technical Manager may authorize adaptations of these protocols to specific case situations and will document this approval with a Biology Deviation Request Form.

Forensic Biology Abbreviations and Definitions

The following abbreviations are commonly used in bench notes and on other documents generated during analysis.

Abbreviations and Definitions	
Phenolphthalein/Kastle-Meyer	PH, Pheno, or KM
Fluorescence/Alternate Light Source	F or ALS
Acid Phosphatase activity	AP, AP Spot (test)
Nucleated Epithelial Cells	NECs
Prostate-Specific Antigen	PSA or p30
Human Hemoglobin	hHb, Species
Positive test result	Pos or (+)
Negative test result	Neg or (=)
Reddish-brown color observed	R-B
Case, item, date & initials	CIDI
Scientific Crime Detection Laboratory	SCDL
Forensic Biology Procedure Manual	FBPM
Forensic Biology General Laboratory Maintenance Manual	FBGLM
CODIS Manual	COD
Victim Forensic History	VFH
Suspect Forensic History	SFH
LIMS	Laboratory Information Management System

Abbreviations and Definitions (continued)	
ICS	Internal Control Specimen
SAK	Sexual assault kit
F1, E or Epi	Epithelial fraction (Fraction 1)
F2, S or Sp	Sperm fraction (Fraction 2)
F2, SS	Sperm/substrate fraction (Fraction 2, which applies to both sperm and sperm/substrate)
F2sub, Sub	Substrate fraction (Fraction 3)
RB	Reagent Blank
RBS or RBF2	Reagent blank sperm / Reagent blank sperm & substrate
RBE or RBF1	Reagent blank epithelial
RBsub or RBF3	Reagent blank substrate
RBQ	Reagent blank questioned (direct)
RBD	Reagent Blank Direct with DTT
RBK	Reagent Blank Known
GTD	GenTegra-DNA
Quant/QT	Quantification / Quantifiler Trio
No Male	No male DNA detected
Low Male	Female: male ratio is 4:1 or higher. Y-STRs recommended.
Low DNA	Quantitation indicates that consumption of sample is recommended.
NFA	No further analysis
INC	Inconclusive
PTC	Permission to consume
PCR	Polymerase Chain Reaction
STR / Y-STR	Short Tandem Repeat / STR analysis specific to Y chromosome markers. When noted on a Quantifiler Trio report, indicates that the extract is suitable to proceed to STR or Y-STR analysis
GF(E)	GlobalFiler(Express)
PPY23	PowerPlex Y23

Abbreviations and Definitions (continued)	
e-gram	Electropherogram
BL	Noisy baseline
PHR	Peak Height Ratio
PH or PkHt	Peak Height
RFU	Relative Fluorescent Units
AT	Analytical threshold
ST	Stochastic threshold
IT	Interpretational threshold
PU	Pull-Up
DO	Complete Drop-Out at a Locus
PDO (or IHO for RapidHIT)	Partial Drop Out at a Locus (or Inconclusive homozygote)
OL (or OB for RapidHIT)	Off Ladder (or Out Of Bin)
OMR	Outside Marker Range
OS	Off Scale/Saturation
NP	No genetic profile obtained
NR	Data not reported
NS	Data not used for statistics
NA	Locus not amplified (when comparing between kits)
A/TA	Artifact (i.e. stutter) or true allele
IF	Instrument failure
ILS	ILS failure
ART	Artifact
TRI	Tri-allele
OBL / 2 OBL	Obligate allele / 2 obligate alleles
UND	Undetermined
NS_{mix}	Data not used for mixture statistic

Abbreviations and Definitions (continued)	
ND	Not deduced
NC	No Calculation
CR	Contributor ratio
SP	Shared peak (used in Y-STR deduction)
E	Elimination allele (used in Y-STR deduction)
P	Deduced probative allele (used in Y-STR deduction)
( )	Minor component allele
[ ]	Major/Minor components not separated
D5Mut	D5 Mutation
HT	Allele below homozygote threshold, w/o a detected sister allele

Note: Notations such as strikes or comments that are made electronically within the GeneMapper ID-X software prior to the date in the footer of each page are attributable to the logged-in analyst, as noted on the bottom of the printed electropherogram; those notations do not require any additional documentation. Notations added later require date and initials of the analyst, either individually or collectively (e.g. Minor allele notation added 6-26-20 cmd)

Section 2 Biological Screening

This section includes the following topics:

2.1 Hair and Fiber Evidence

Hair and fiber evidence may be recovered from evidentiary items by the following methods:

Tape Lifting
Clear plastic latent fingerprint tape (or other suitable adhesive tape) can be applied adhesive side down to the surface of the evidence item. Press the tape down, and then pull away. Hairs and fibers will adhere to the adhesive on the tape. Place the tape adhesive side down on the shiny side of freezer paper (or on another appropriate surface).

Scraping
The item to be examined is suspended above the examination surface and gently scraped with a clean metal spatula. Scraping in a downward direction allows surface debris to fall onto the examination surface for collection. The debris is transferred to an appropriate storage container.

Hand-picking
Clean forceps are used to collect trace evidence from evidence items. The isolated material is then placed in an appropriate container. Isolated trace evidence should be sealed in a suitable container and clearly marked with identifying information.

Isolated trace evidence may be placed within the originating item or it may be repackaged as an entirely new item of evidence.

The collection and any subsequent examination of trace evidence is documented in the laboratory bench notes.

The laboratory does not currently provide microscopic comparisons for hair and fiber evidence submitted or recovered in casework. If required, trace evidence may be sent to another laboratory for such analysis.

Screening Hairs
Screening of hairs is no longer performed. Possible hairs recovered in casework will be examined visually. Observations such as length and color should be included in bench note documentation.

Range of analyst discretion for Hair and Fiber Evidence

Document actions as appropriate, but no explanation required if directions followed as described in above section(s):

Analyst discretion is used to determine whether trace evidence is collected/evaluated. Depending on individual case circumstances, it may be appropriate to collect/evaluate trace evidence. Collection and examination of trace evidence need not occur in cases where the trace is not likely to have probative value.

Permissible with documentation of actions **and** reason for change to collection method:

Collection can be performed, but not in agreement with the specified directions.

2.2 Transfer Sources of DNA

Items not examined for the presence of biological evidence may be sampled by swabbing the item for contact/touch sources of DNA. Standard screening protocols apply.

- Document the item by digital imaging.
- Moisten a sterile cotton-tipped swab using sterile water.
- Swab the area(s) of interest (those likely to have the most contact with bare skin).
- If the sample is not proceeding immediately to DNA analysis, the isolated sample(s) are packaged and retained as a separate item of evidence.
- If the sample is proceeding immediately to DNA analysis, any remaining extract is packaged and retained as a separate item of evidence after DNA analysis is complete.

Range of analyst discretion for Transfer Sources of DNA

Document actions as appropriate, but no explanation required if directions followed as described in above section(s):

Analyst discretion is used to determine whether evidence is swabbed for contact/touch DNA.

Permissible with documentation of actions **and** reason for change to collection method:

Collection can be performed, but not in agreement with the specified directions.

2.3 Blood Stain Analysis

Blood may be present in the form of pools, spatters, or stains. The detection and characterization of blood is achieved by visual examination and subsequent presumptive testing for peroxidase-like activity of hemoglobin. Stains testing positive to a presumptive test for blood may be further characterized by determining if the stain is animal or human in origin, or by DNA profiling to provide information as to the blood's source.

Suspected bloodstains can be located by several methods, including visual and stereoscopic examination, and chemical presumptive testing.

For items with negative results: a second analyst must witness on the first ten negative cases for newly qualified analysts and the first negative case for each analyst each calendar year.

Visual Examination

Most dried bloodstains on cloth can be detected visually because their color is different from that of the material on which the blood has been deposited. Bloodstains range in color from a reddish-brown to black.

Blood deposited on dark colored items may be difficult to locate visually. The use of a stereomicroscope (6.3 – 40 x) may assist in the location of possible bloodstains. Bloodstains on clothing and other porous substrates tend to be wicked into the substrate unlike dirt and debris, which will be found on and around the fibers. This can easily be seen stereoscopically.

Bloodstains deposited on non-porous substrates will dry on the surface as a film or a crust.

Chemical Examination

Suspected blood stains can be tested for peroxidase-like activity with Phenolphthalein reagent and hydrogen peroxide. Stains can be sampled with a dry or moistened swab or piece of filter paper, or by teasing a few stained fibers from the substrate.

Stains testing positive to this presumptive test for blood may be further characterized as to their origin (human or higher primate) using Human Hemoglobin analysis by immunoassay.

Detection of Heme with Phenolphthalein (Kastle-Meyer Test)

This is an oxidative test for the presumptive identification of blood based on the catalytic activity of the heme group of hemoglobin. If the sample is of a limited nature and the presumptive test is likely to consume enough sample to prevent successful DNA typing, then the presumptive test should not be performed. The limited nature of the sample will be documented in the analyst's bench notes.

Color catalytic tests are very sensitive, but not specific. The positive color test alone should not be interpreted as positive proof of blood. The major sources of "false positive"

reactions are chemical oxidants and vegetable peroxidases. Color development before the addition of H_2O_2 may be due to the presence of chemical oxidant. Fruit and vegetable peroxidases react like blood but slower and weaker. Additionally, the presumptive test for blood is not species specific. Blood and other tissues from animals besides humans will also give a positive reaction with this test.

Procedure

- Positive & Negative Controls: A human blood standard control is swabbed and tested each day that the presumptive test reagents are used. A swab moistened with the same lot of sterile water used for the questioned samples is also tested to function as a reagent control.
- Questioned samples: Moisten a sterile cotton-tipped swab with sterile water. Rub the swab over the suspected bloodstain. Stains may also be sampled with a dry swab or a folded piece of filter paper, or by teasing a few stained fibers from the substrate.
- Add 1-2 drops of phenolphthalein working solution to the swab. Wait a few seconds and observe any color changes.
- Add 1-2 drops of a 3% hydrogen peroxide solution.
- Record all lot numbers, expiration dates and control results in the FB Reagents form on the case request for each day the test is performed, which will in turn populate the bench notes.
- **For items with negative results:** a second analyst must witness on the first ten negative items for newly qualified analysts and the first negative item for each analyst each calendar year. Record this in the Verifications/Performance Monitoring/Hair tab in the FB Analysis form. (There may still be a requirement for a case activity in these instances – refer to current version of QAM.)

Interpretation of Results

The positive control must yield a positive result (i.e. rapid pink color change) and the negative control give no color change, for the test results on evidentiary samples to be valid.

Positive: The appearance of a rapidly developing pink color after the addition of 3% hydrogen peroxide (H_2O_2) is a presumptive positive test for the presence of blood. A pink color forming after ten seconds should not be considered as a positive result, as auto-oxidation can occur in air and light.

Negative: No color change immediately (within a few seconds) after addition of 3% hydrogen peroxide (H_2O_2). A negative result is indicative of the absence of detectable quantities of heme or its derivatives. `

Inconclusive: In some instances, a particular substrate makes it very difficult or impossible to see a color change from clear to pink. Typically, this occurs when the red or pink color of the substrate (e.g. red fabric dyes or some food products) is transferred to the testing swab. However, any time that the color change or lack thereof is not readily apparent may be appropriately deemed inconclusive.

Detection of Human Hemoglobin (hHb) using OneStep ABAcard HemaTrace
This test is a simple, rapid and a sensitive confirmatory test for human (and other higher primates) blood.

Procedure

- Place a portion of the suspected bloodstain or swab into an entire labeled tube of the extraction buffer provided.
- Allow the sample(s) to extract for 1 – 5 minutes.
- If refrigerated, allow the sample(s) to warm to room temperature.
- Remove the device/card and dropper from the sealed pouch.
- Add 4-5 drops of the extract, using the dropper provided, into the sample region (S) on the card.
- Allow the sample to stand for 10 minutes. Positive results can be seen as early as 2 minutes depending on the hHb concentration.
- Document the lot number of the ABAcard and expiration date and the test results in the FB Reagents form on the case request for each day the test is performed, which will in turn populate the bench notes.
- **For items with negative results (when Hematrace testing performed up front):** a second analyst must witness on the first ten negative items for newly qualified analysts and the first negative item for each analyst each calendar year. Record this in the Verifications/Performance Monitoring/Hair tab in the FB Analysis form.

Interpretation of Results

The control line in the control area (C) is an internal procedural control. A distinct pinkish line will always appear if the test has been performed correctly and the reagents are working properly. If the control line does not appear, the test is invalid, and a new test must be performed.

Positive: The formation of two pink lines, one in the test area (T) and in the control area (C) is a positive result, indicating that the human hemoglobin concentration is at least 0.05µg/ml. Another analyst should confirm visually weak results. This verification will be documented in the bench notes.

Negative: The formation of only one pink line in the control area C indicates a negative test result. This may indicate that:

- No human hemoglobin is present above 0.05µg/mL, or
- Presence of “high dose hook effect”. Presence of “high dose hook effect” may give a false negative result due to the presence of high concentrations of human hemoglobin in the sample. If suspected, based on visual inspection of the stain, the sample should be retested using a 1:100 or 1:1,000-fold dilution of the sample.

  Inconclusive: There is no formation of a pink line in either the test area (T) or the control area (C) of the card. Repeat the test and re-examine the test procedure carefully.

Range of analyst discretion for Blood Stain Analysis

Document actions as appropriate, but no explanation required if directions followed as described in above section(s):

Analyst discretion is used to determine which stains to presumptive test and/or isolate when multiple stains are present on a (non-swab) item.

Permissible with documentation of actions **and** reason given for change to collection method:

Collection can be performed, but not in agreement with the specified directions.

Permissible with reason given:

Collection could be performed but no stains were isolated due to case-specific circumstances.

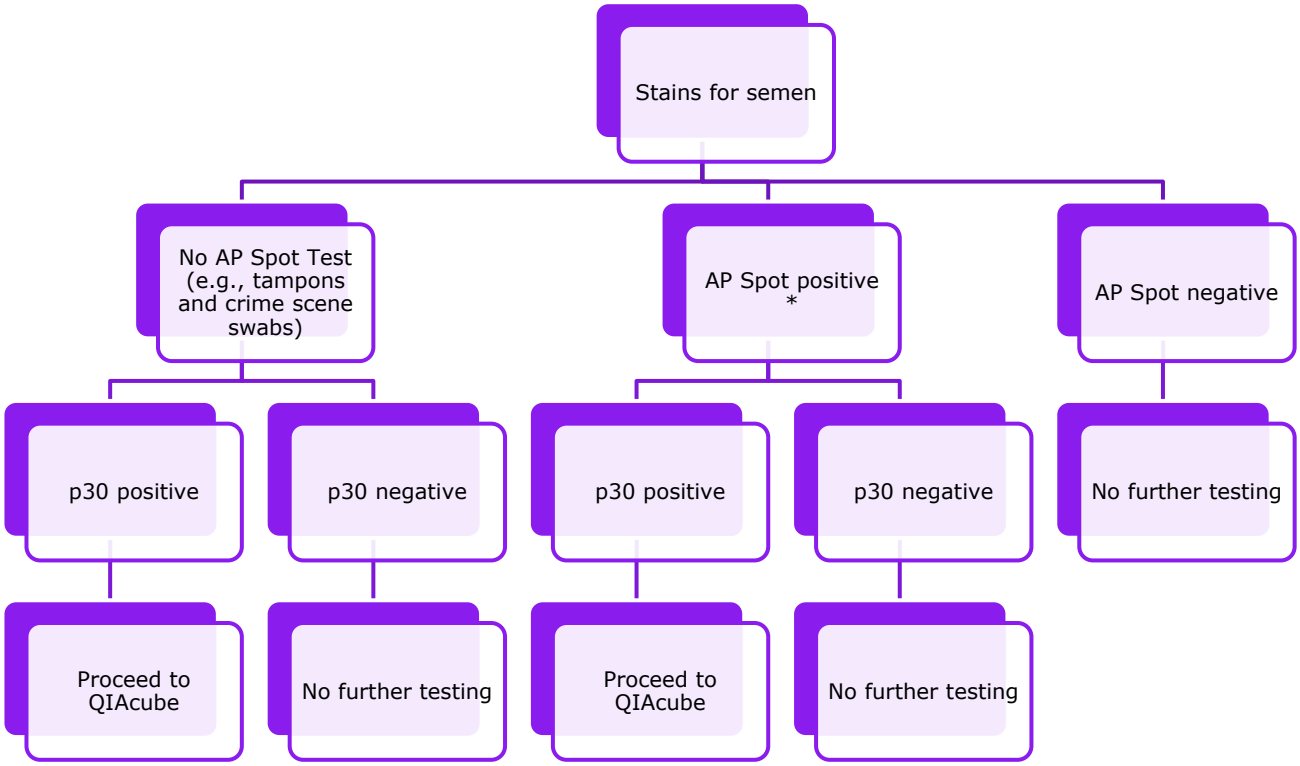
Biology Deviation Request Form required:

Test or interpretation of test results cannot be performed according to directions.

2.4 Semen Stain Analysis

The detection and identification of semen is achieved by visual examination, presumptive chemical testing for the presence of acid phosphatase and by testing for human seminal protein p30. See the flowchart below outlining the processing of semen stains and crime scene swabs (not swabs from evidence collection kits).

Note: This decision tree does not apply to proficiency tests, where all screening tests should be performed.



* Underwear not worn until 24 hours post-event can go directly to the QIAcube, without p30 testing

Suspected semen stains can be located by several methods, including careful visual examination, the use of tactile senses, fluorescence under alternate light sources, and chemical presumptive testing.

For items with negative results: a second analyst must witness on the first ten negative items for newly qualified analysts and the first negative item for each analyst each calendar year. Record this in the Verifications/Performance Monitoring/Hair tab in the FB Analysis form.

Visual and Tactile Examination

Most dried semen stains on cloth are detectable visually because their color is different from that of the material on which the semen has been deposited. Semen stains that have been exposed to warm, moist conditions may assume a yellow coloration due to the growth of bacteria. Seminal fluid frequently has a stiffening effect on fabric, forming a crusty stain.

Alternate Light Source Examination using the Crime-lite®82S Forensic Light Source

Reference: Crime-lite®82S Forensic Light Sources User Manual

The Crime-lite®82S Forensic Light Source normally emits blue light. The blue narrowband has a bandwidth of 420-470nm, with a peak at 445nm. Semen stains frequently fluoresce when examined with alternative wavelengths of light. They can appear as a bright fluorescence on a dark background or as a dark area when the background itself fluoresces. Mark the suspect stains with a pen and proceed with chemical presumptive tests.

General Safety Guidelines

· It is essential that proper eye protection be provided for and worn by anyone operating an intense light source such as the Crime-lite®82S Forensic Light Source. Permanent eye damage can occur from specular (direct illumination to the eye) or reflected or refractive light hitting the eye. Choose goggles based on specific wavelength. Do not allow use of inappropriate or incorrect goggles. Some goggles offer only a narrow band of protection.

· Remove all unnecessary reflective surfaces from the area or exam room. Avoid looking at reflections in shiny and/or spherical objects such as door knobs, watch crystals, tools, jewelry, window panes, mirrors or any other surface that may reflect light.

· Exposing the skin to the beam of light (directly from the unit) can cause burns and other skin damage. The direct emission of the light is very warm. The light source also warms up as the instrument is being used for a prolonged period of time. In the event of prolonged use that begins to overheat the light source, a switch will be activated to

disconnect the power. The light source can be used again once the instrument is allowed to cool.

Proper Operation of the System

The light source is powered by an attachable and rechargeable battery pack at the bottom of the light source with the use of a handheld adaptor. The handheld adaptor should be attached to the bottom of the handle of the light source. When ready for use, attach the battery pack to the handheld adaptor to the bottom of the light source. Turn on the light source using the switch. The beam of light should appear within a few seconds. Alternatively, the handheld adaptor can be removed from the light source and attached to the battery pack, and a corded adaptor can be used to lessen the weight of the light source. The corded adaptor is plugged into the bottom of the handle of the light source and on top of the combined handheld adaptor and battery pack.

When the light source is not in use, turn off the light source using the switch. The battery pack should be removed from the adaptor and docked in the charging station to maintain battery life.

Generally, the optimum setting for the detection of physiological stains such as semen, saliva, urine, and blood is at 450nm. Forensic odontologists and pathologists prefer this setting for bite mark and bruising detection and photographic documentation.

Prior to the examination of casework evidence, a known semen stain control is examined with the blue narrowband affixed. This ensures that the system is functioning properly. The stain must exhibit the expected fluorescence for the unit to be used in casework. This check is performed each day that the unit is in use and is documented in the FB Reagents form on the case request for each day the test is performed.

Special case – inherently fluorescent substrates

Certain fabrics may show background/inherent fluorescence. Therefore, even if the ALS is inconclusive due to overwhelming background fluorescence, the item should still be swabbed and AP spot tested or, alternatively cut and p30 tested. In such cases, it is useful to focus on areas that would be consistent with drainage (such are the crotch area of underwear or pants) or to section the entire item if the item would not have drainage (such as a shirt or towel)

- Section the area (into three or four sections), swab, and test each section for AP Spot test activity. Areas that are positive can then be cut and combined for p30 as one stain (noted in bench note documentation)
- Alternatively, areas can be cut directly for p30.

Chemical Examination

Semen stains can be located by detecting the presence of acid phosphatase activity. Visible stains can be lightly swabbed with a water-moistened cotton swab and tested by AP Spot Test.

Detection of Acid Phosphatase using AP Spot Test
This is a presumptive test for the detection of seminal fluid. Samples with positive results are further analyzed by performing a p30 test. Negative results typically require no further analysis.

Procedure
• Prepare working solution: Add 5 mL of dH_2O to 0.13 grams of AP Spot Test. Mix the solution thoroughly. Store the working solution at room temperature protected from light (e.g., wrap the container in aluminum foil). Cap the working solution when not in use to minimize oxidation. Note: Different volumes of working solution may be prepared if the ratio of AP Spot Test to dH_2O remains constant.
• Positive & Negative Controls: A human semen standard control and sterile water blank must be tested each day that the reagents are used in casework. The positive control should portray a purple-ish color and the negative control should not exhibit a color change. The controls must function as expected for the test results on questioned stains to be valid.
• Moisten a sterile swab with a minimal amount of sterile deionized water. Rub the questioned stain with moistened swab.
• Add 1-2 drops of AP Spot Test working solution
• Examine for color change in up to three minutes

For non-swab items with negative results: a second analyst must witness on the first ten negative items for newly qualified analysts and the first negative item for each analyst each calendar year. Record this in the Verifications/Performance Monitoring/Hair tab in the FB Analysis form.

The lot numbers, expiration dates of the reagents and the results of the test are documented in the FB Reagents form on the case request for each day the test is performed. Note: this test may also be used to ‘map’ the location of semen stains by pressing a large section of moistened filter paper on the test item. After the paper is removed from the item, add the reagents to the paper.

Interpretation of Results
Positive: The development of a purple-ish color of any intensity within three minutes is a preliminary indication of the presence of acid phosphatase, a component of semen.

Negative: Any color change after three minutes, or no purple-ish color reaction.

Inconclusive: The color of the stain or its substrate interferes with the reading of the test color change. Inconclusive results should be treated as if potentially positive and continue to p30 testing.

Additional Notes

- This procedure is not specific for semen. Possible sources of acid phosphatase other than semen, i.e., vaginal fluids, or feces, generally produce slow, weak reactions (pale pink).
- Old stains and stains containing very little semen may also produce a slow, weak reaction. For stains that are 10 years old or older, the analyst may let the AP spot test reaction continue beyond the three-minute cut-off but for no longer than ten minutes. The negative control swab must be analyzed for the same length of time.
- If greater than three minutes, the time used must be noted in the bench notes (and FB Reagents form notes for relevant controls).
- An explanation for the extended time must also be included in the case bench notes.

Detection of p30 by the Abacus ABAcard® (with option to make microscopic slide)
This is a rapid and sensitive detection method for p30, a component of seminal fluid.

This test is normally performed on stains which are AP Spot Test positive and on body swabs collected within 48 hours where semen may be present.

Procedure

A. Place the sample in a 1.5 mL QIAcube microcentrifuge tube.

B. Add enough sterile water to the sample to just cover the material (300-500 µL works well for one full swab tip), 150 µL at a minimum.

C. Incubate at 37° C for at least 30 minutes but no more than two hours.

D. Centrifuge briefly to remove liquid from inside cap. Move substrate to a spin basket and spin for 5 minutes at ~14500 rpm.

E. Remove the device and dropper from the sealed pouch. The dropper is not used and can be discarded.

F. Using a pipette, add 100 µL of extract into the sample region (S) on the card.

G. Allow the sample to stand for 10 minutes. Positive results can be seen as early as 1 minute depending on the p30 concentration.

H The lot number and expiration date of the p30 cards and the results of the controls and case sample are documented in the FB Reagents form on the case request for each day the test is performed, which in turn populates the bench notes.

Interpretation of Results

Positive: The formation of two pink lines, one in the test area (T) and in the control area (C) is a positive result, indicating the concentration of p30 is at least 4ng/ml. Another analyst should confirm visually weak results. This verification will be documented in the Verifications/Performance Monitoring/Hair tab.

Negative: The formation of only one pink line in the control area (C) indicates a negative test result. This may indicate that (a) No p30 is present above 4ng/ml or (b) presence of "high dose hook effect". Presence of "high dose hook effect" may give a false negative result due to the presence of high concentrations of p30 in the sample. If this is suspected, based on preliminary acid phosphatase test results, the sample should be retested using a 1:10 to a 1:1,000-fold dilution of the sample in question using the remaining sample.

Inconclusive: There is no formation of a pink line in either the test area (T) or the control area **(c)** of the card. Repeat the test and reexamine the test procedure carefully.

I. After the p30 test has been completed:

- If the p30 test is positive, return the substrate to the tube to proceed to differential extraction. Store at approximately 4 °C until ready to start extraction.
- If the p30 test for a consumed sample is negative and not proceeding to further analysis, the substrate must be air-dried and repackaged.
- If the p30 test for a non-consumed sample is negative and not proceeding to further analysis, the substrate may be discarded.

J. Slides are not routinely made, but can be made at this stage upon case-specific request:

Re-suspend cell pellet by vortexing, then spin briefly to remove liquid from cap. Remove 3 µL to a microscope slide and proceed to stain.

Staining of Slides

This is a differential staining process to aide in the conclusive identification of spermatozoa.

- Heat fix cells to a microscope slide by placing the slide in a 37°C oven for 15 minutes.
- Cover the slide/debris with Nuclear Fast Red stain and let stand for at least 10 minutes.
- Gently wash away the Nuclear Fast Red with deionized water.
- Immediately cover the slide/debris with Picroindigocarmine stain (PICS) and let stand for 15-30 seconds.
- Gently wash the slide with reagent grade ethanol. Allow to air dry.
- Add Permount and a cover slip.

- Examine the slide microscopically (200-400x) and record the results in the FB Analysis form based on the interpretations given below.

Interpretation of Results

Nuclear material is stained red by the Nuclear Fast Red dye. Sperm heads are usually well differentiated with the acrosome staining significantly less densely than the distal region of the head. Picroindigocarmine stains the epithelial membranes green. Nuclei inside the epithelial cells may appear purple. Yeast cells also stain red; however, the stain is uniform throughout the cells and extends into polyp-like structures, which are occasionally observed with yeast cells.

If no spermatozoa are observed on a slide, a second analyst should examine the slide for a microscopic verification. If the same conclusion is reached on the same slide, the verification by the second analyst is documented in the Verifications/Performance Monitoring/Hairs tab in the FB Analysis form.

Range of analyst discretion for Semen Stain Analysis

Document actions as appropriate, but no explanation required if directions followed as described in above section(s):

- Analyst discretion is used to determine which stains to presumptive test and/or isolate when multiple stains are present on a (non-swab) item.

Permissible with documentation of actions **and** reason given for change:

- Stain collection can be performed, but not in agreement with the specified directions.
- AP Spot Test reading is taken past three minutes.
- Microscopic sperm search is performed when there was no case-specific request.

Permissible with reason given:

- Stain collection could be performed but no stains were isolated due to case-specific circumstances.

Biology Deviation Request Form required:

- Test or interpretation of test results cannot be performed according to directions.

Section 3 Prioritizing analysis, sampling, and extraction

This section includes the following topics:

Protocols and special triage guidelines specific to cases with sexual assault kits (SAKs) are described in the next section.

Analysts rely upon available information to make assessments about which items are most appropriate for analysis on a case-by-case basis. The extraction process for a single sample generally accommodates up to two full swabs worth of material (such as two full swabs, four half-swabs, and so on) per tube.

Typically, swabs within a single packet are either sampled equally (such as taking half of each of two swabs for a questioned sample), or clearly unequally (such as using all of one swab from a sleeve with two swabs in a reference buccal sample) . Documentation on the packaging must be clear if it is not obvious that swabs were not sampled equally.

- If all swabs within a sealed envelope are equally sampled, it is sufficient to write identifying information on the sealed envelope without tagging the swabs as well.
- If a package contains 2 separate swab packets, each with one swab, and only one packet is opened for sampling, it is sufficiently obvious where sampling occurred and labeling of swab packets is not required.
- For any instance where swabs are sampled unequally and un-obviously (e.g., a portion of only one of two swabs in the same packet where both swabs were previously sampled), the sampled swab should be tagged with a label containing the case number and item number.

Elution volumes of 40 µL are routinely used for all questioned extracts, as sample size permits.

Bench notes must document the amount of sample used to create the extract.

Sample consumption policy for forensic casework (Forensic FBI QAS 7.4 / Database FBI QAS Standard 7.4)

When a sample is used in its entirety to create an extract, the bench notes must clearly indicate that the sample has been used in its entirety. Half the extract must be retained unless written permission from Department of Law for consumption of the sample has been obtained and documented in LIMS.

During analysis, report writing, and technical review, DNA extracts can be stored for several weeks at 2ºC to 8ºC. Questioned sample extracts, substrates (in their spin baskets), and reference sample extracts should be retained at 2ºC to 8ºC until completion of technical review.

Following successful completion of technical review, substrates are discarded. If original untested reference sample remains, reference sample extracts are routinely discarded after successful completion of technical review. Regardless of whether untested original material remains, all remaining questioned sample extracts should be dried down with GenTegra-DNA for long-term storage.

Policy regarding sample consumption of database items can be found with the sampling directions later in this section.

3.1 Overview of QA samples in a casework batch

An extraction set is a set of samples taken through ***the same extraction protocol*** (e.g., sperm/substrate, epithelial and/or direct) ***at the same time by the same analyst***. An extraction set may include more than one QIAcube or EZ Biorobot run, if they are run concurrently on the same instrument make and model. A casework batch usually involves multiple extraction sets. Each extraction set requires its own controls, described in the table below

Protocol	***Type***	***Consists of:***	***Assessed by:***	***Retain/Discard***
Questioned sample extraction	Internal Control Specimen (ICS)	Prepared sample previously typed (staff buccal or reference material)	Quant for all ICSs, amplify and verify type for each ICS (one amplification per differential extraction is sufficient)	Discard after technical review
	Reagent blank	Three blanks, each created under the most stringent conditions of any sample in the set	Blank with highest relevant quant: dry down and amplify by STR. Second blank: if batch includes any casework that could ever potentially need Y-STR amp, dry down and amp by Y-STR (If NA, dry down and retain the second blank) Third blank: dry down and retain.	Retain any questioned blanks not consumed
Known sample extraction	ICS	Casework reference previously typed or staff buccal	Quant for all ICSs, amplify and verify type for each ICS.	Discard after technical review
	Reagent blank	One blank, created under the most stringent conditions of any sample in the set	Amplify 15 µL STR and 17.5 µL by Y-STR if any knowns were run by Y-STR	Discard after technical review (may retain if future Y-STR analysis is likely or if any K's retained)

Reagent blanks (QAS 9.5.1.1)

- Triplicate reagent blanks are created for each extraction set of questioned samples taken through the same extraction protocol (e.g. sperm/substrate, epithelial and/or direct) on the same day and the same instrument model(s) by the same analyst at the same time. Runs from different instrument models (i.e., QIAcube classic and QIAcube connect, or EZ1 and EZ2 instruments) cannot be combined into a single extraction set.
- Exception: proficiency tests which are worked alone and not part of a batch of casework, only require one questioned reagent blank. For proficiency tests run as standalone batches, it is not necessary to dry down and consume the reagent blanks; and the same reagent blank may therefore be used for one full-volume amplification using STR analysis and one full-volume amplification using Y-STR analysis.
- Reagent blank names include LIMS-DNA set name and a control number.
- All reagent blanks are quantified and dried down. Exception: proficiency tests which are worked alone and not part of a batch of casework, only require GenTegra if a corresponding proficiency test extract is also dried down.
- Typically, it is only necessary to create a single reagent blank for reference samples. Because reference samples are rarely consumed and reference extracts are routinely discarded, it is not necessary to create multiple reagent blanks for reference samples.
- Reagent blanks must be made to the most stringent conditions of any sample in the extraction set. If you are in doubt of how to set up a reagent blank, please confer with the Technical Manager before proceeding.
- Documentation for the reagent blanks is in the LIMS-DNA packet.

Internal Control Specimen

- Each extraction set must contain an ICS. An ICS may have more than one fraction, such as a sperm/epithelial fraction for a differential extraction.
- Note: Hair extractions should be considered as their own extraction set, with their own hair ICS and reagent blanks.
- All ICSs are extracted and quantified.
- At least one ICS per extraction set is amplified and verified. Additional ICSs may need to be amplified and verified for quality assurance purposes or troubleshooting. See ICS interpretation section for more details.
- This control is used to check for the efficacy of the extraction process and to reveal any large-scale issues with mislabeling / sample switching. As such, it should not be placed in the exact middle of an extraction set.
- Documentation of each ICS, including its source, is included in the LIMS-DNA packet.

3.2 Types of evidence – triage, sampling, and extraction

Reference: EZ1 DNA Investigator Handbook

Case-Specific Triage Guidelines

Sexual Assault Kit Routine Guidelines for Initial Processing - Female victim kits

- Always review RLS and Forensic History first for case-specific details affecting triage decisions; may need to contact law enforcement for clarification
- Guidelines below are routine, but exceptions will be encountered. Reasons for exceptions must be documented in bench notes.

Sample type	Routinely sampled?	Timeframe	Sampling and processing
Internal body swabs with suspected semen – under 48 hours	Y for vaginal/ cervical N for oral and rectal	Up to 48 hours for vaginal and/or cervical; up to 24 hours for oral; up to 48 hours for rectal	• half of each swab • differential if p30 + / direct w/DTT if p30 negative • substrate processed separately
Internal body swabs with suspected semen – over 48 hours	Y for vaginal/ cervical N for oral and rectal	>48 hours to 168 hours for vaginal and/or cervical; up to 72 hours for rectal; never for oral >24 hours	• half of each swab • direct extraction w/DTT
External body swabs with suspected semen	Y for most N for perineum/anal	Up to 48 hours	• half of each swab • differential extraction if p30 +; direct with DTT if p30 negative • sperm and substrate combined
External body swabs with suspected semen	Y	>48 hours to 72 (or 96) hours	• half of each swab • direct extraction w/DTT
Body swabs with suspected saliva	Y (breast) N (all other)	Up to 48 hours	• half of each swab • R/L separate • direct extraction
Body swabs for contact DNA (bruise, strangulation, etc.)	Y (breast) N (all other)	Up to 48 hours	• half of each swab • R/L (or equivalent) separate • direct extraction
Hand/finger swabs	N	Up to 48 hours	• Half of each swab • R/L separate • fingernail scrapings separate • finger/hand swabs may be combined by hand or limited (e.g. index only) • direct extraction
Fingernail scrapings	N	Up to 48 hours	• All of swabs/scrapings • R/L separate • Direct extraction
Known sample(s)	Y	NA	• Portion of swab, or all of one if >1 collected

Male suspect kits

Sample type	Routinely sampled?	Timeframe	Sampling and processing
Penile swabs	Y	Up to 48 hours	• half of each swab

			• Differential extraction with sperm and substrate combined
Bite marks or suspected saliva	N	Up to 48 hours	• Half of each swab • direct extraction
Miscellaneous contact swabs	N	Up to 48 hours	• Half of each swab • Direct extraction
Hand/finger swabs	N	Up to 48 hours	• Half of each swab • R/L separate • fingernail scrapings separate • finger/hand swabs may be combined by hand or limited (e.g., index only) • direct extraction
Fingernail scrapings	N	Up to 48 hours	• All of swabs/scrapings • R/L separate • Direct extraction
Known sample(s)	Y	NA	• Portion of swab, or all of one if >1 collected. • extraction and quant required at screening if victim is female (if applicable)

Triage Practices for Sexual Assault cases with Sexual Assault Kit (SAK) evidence

A condensed summary of these guidelines can be found above.

For the purposes of these triage guidelines, any collection of questioned swabs from the body of a victim or suspect, collected in association with an alleged sexual assault, is treated as a SAK, regardless of whether the packaging includes the specific phrase “Sexual Assault Kit”.

The goal in these cases is to deliver prompt results on a limited number of samples to assist each investigation at an early stage. In cases where the SAK(s) are likely to provide the most probative evidence, the relevant swabs in the SAK(s) will be the only evidence examined in the initial testing process. Additional testing of more questioned samples from the SAKs or additional items (such as clothing or bedding) will only be routinely performed after consultation with Department of Law and/or the submitting officer. In high-priority cases, that consultation may happen before any testing begins, and therefore may be considered with more samples worked in the first round of testing.

Because the length of time between alleged events and kit collection can have a significant impact on triage decision, cases with conflicting information about timelines may benefit from outreach to law enforcement for more information.

For SAKs collected within 48 hours of events, p30 testing will be used on swabs where semen is suspected. Swabs with p30 positive results will proceed to differential extraction; and swabs with negative p30 results will proceed to a direct extraction with DTT. Swabs where semen is not suspected will proceed to a direct extraction.

For SAKs collected more than 48 hours after events, a Direct to DNA approach will be used: selected swabs will proceed straight to DNA extraction without biological screening. Samples likely to contain spermatozoa proceed to a direct extraction with DTT, while samples collected for possible saliva or contact (such as breast swabs and finger swabs) proceed to direct EZ Biorobot extraction protocol.

Each sample typically includes up to two swabs worth of material. All questioned extract remaining after analysis is retained, regardless of whether the swabs were consumed.

Samples typically extracted from SAKs include:

a. Vaginal and cervical swabs (process separately) with possible semen evidence
    i. Differential extraction if within 48 hours and p30 positive; direct with DTT if p30 negative or over 48 hours
    ii. Sample half of each swab
    iii. In differential extractions, sperm and substrate fractions are worked separately.
    iv. These are processed when collected up to seven days after the alleged event. Factors affecting length of time for viable testing include victim menstruation.
b. Oral swabs with possible semen evidence
    i. Oral swabs are only routinely processed when allegations of penile / oral penetration are indicated, when swabs are collected within 24 hours, and when there is a possibility of spermatozoa being present. It is not essential that allegations specify ejaculation, but the scenario should in some way indicate the possibility of the presence of spermatozoa / semen.
    ii. Sample half of each swab.
    iii. Differential extraction if within 24 hours and p30 positive; direct with DTT if within 24 hours and p30 negative.
    iv. In differential extractions, sperm and substrate fractions are worked separately.

c. Rectal swabs with possible semen evidence
    i. Rectal swabs are only routinely processed when allegations of penile / rectal penetration are indicated, when swabs are collected within 72 hours, and when there is a possibility of semen / spermatozoa being present. It is not essential that allegations specify ejaculation, but the scenario should in some way indicate the possibility of the presence of semen / spermatozoa.
    ii. Sample half of each swab.
    iii. Differential extraction if within 48 hours and p30 positive; direct with DTT if p30 negative and within 72 hours.
    iv. In differential extractions, sperm and substrate fractions are worked separately.
d. "External" external female genitalia swabs (includes outer aspect of labia majora, mons, etc.) where semen OR semen and saliva suspected, miscellaneous semen swabs from non-genital areas
    i. These are routinely processed when collected up to 72 hours after the alleged event but may be worked when collected up to 96 hours after, depending on case specific circumstances.
    ii. Sample half of each swab.
    iii. Differential extraction if within 48 hours and p30 positive, otherwise direct with DTT
    iv. Sperm and substrate are worked as a combined fraction if differential
e. "Internal" external female genitalia swabs (includes remainder of vulva swabs, inner labia majora/labia minora/introitus/etc.) where semen suspected.
    i. These are routinely processed when collected up to 72 hours after the alleged event but may be worked when collected up to 96 hours after, depending on case specific circumstances.
    ii. Sample half of each swab.
    iii. Differential extraction if within 48 hours and p30 positive, otherwise direct with DTT
    iv. Sperm and substrate are worked as a combined fraction if differential
f. Swabs with suspected saliva (includes breast swabs, bite marks, "external" external and "internal" external genitalia if specified in scenario)
    i. Only processed if reason is indicated by forensic history
    ii. Sample half of each swab.
    iii. Right and left breast swabs are NOT routinely combined. Multiple swabs from one breast (e.g., right nipple and right breast) may be combined at analyst discretion.
    iv. These are only routinely processed if collected within 48 hours of the alleged event but may be worked when collected up to 96 hours after. Timeframes may be affected by circumstances described in forensic history, such as hygiene / showering, victim was deceased, etc.

v. Processed by direct extraction

g. Miscellaneous contact swabs, vaginal swabs where digital penetration only is specified
   i. Only processed if reason is indicated by forensic history
   ii. Sample half of each swab.
   iii. These are only routinely processed if collected within 48 hours of the alleged event but may be worked when collected up to 96 hours after. Timeframes may be affected by circumstances described in forensic history, such as hygiene / showering, victim was deceased, etc.
   iv. Proceed with direct extraction

h. Finger/hand swabs
   i. Only processed if reason is indicated by forensic history
   ii. Swabs from left and right hand are processed separately. Fingernail scrapings are not combined with finger/hand swabs.
   iii. Half of each swab is sampled.
   iv. These are only processed if collected within 48 hours of the alleged event but may be worked when collected up to 96 hours after. Timeframes may be affected by circumstances described in forensic history, such as hygiene / showering, age of victim, etc.
   v. Proceed with direct extraction

i. Fingernail clippings/scrapings
   i. Only processed if reason is indicated by forensic history
   ii. Swabs from left and right hand are processed separately. Fingernail scrapings are not combined with finger/hand swabs.
   iii. All of swabs are sampled, along with a swab of the bindle if appropriate.
   iv. These are only processed if collected within 48 hours of the alleged event but may be worked when collected up to 96 hours after. Timeframes may be affected by circumstances described in forensic history, such as hygiene / showering, age of victim, etc.
   v. Proceed with direct extraction

j. External male genitalia swabs
   i. Penile swabs are routinely processed when collected up to 48 hours after the alleged event but may be worked when collected up to 96 hours after, depending on case specific circumstances. Scrotum swabs are processed only if indicated by case scenario (such as condom use).
   ii. Sample half of each swab
   iii. Differential extraction or direct extraction performed, dependent on case scenario.
   iv. Sperm and substrate are worked as a combined fraction if differential

k. Perineum swabs and/or anal swabs
   i. Perineum or anal swabs collected separately from other external genitalia areas are not routinely worked unless a rectal assault is alleged.

ii. These are processed when collected up to 72 hours after the alleged event but may be worked when collected up to 96 hours after, depending on case specific circumstances.
iii. Sample half of each swab.
iv. Differential extraction if within 48 hours and p30 positive, otherwise direct with DTT

l. Special Case: On occasion, permission is given by Department of Law to consume a sample for Y-STR analysis, where only half the sample has been previously extracted. Click here for a protocol specific to this situation .
m. Reference samples
i. Sampling and processing of reference samples is described later in this section.

Range of analyst discretion for Triage Practices for Sexual Assault cases with Sexual Assault Kit (SAK) evidence

Document actions and decisions (such as items sampled, amount sampled, and extraction methods used) as appropriate, but no explanation required if directions followed as described in above section(s).

Permissible with documentation of actions **and** reason given for change:

- Case-specific scenarios and forensic history information may impact the decision of which samples to process, as well as whether to use differential or direct extraction. Examples: specific allegations of digital penetration only, forensic exam showing rectal trauma, victim is deceased, pre-pubescent victim genitalia swabs (using direct extraction with DTT instead of differential extraction), etc. The decision to not work a specific damaged item within a kit (mold, water damage, etc.) also applies here.
- Additional testing of more questioned samples from the SAKs or additional items (such as clothing or bedding) may be performed at analyst discretion on a case-specific basis even without consultation.
- Evidence guidelines for collection of SAKs are designed to collect as much potentially viable biological evidence as possible at the earliest possible opportunity. Sometimes the processing timelines for routine casework are narrower than the collection timeframes. Samples may be processed outside the recommended processing timeframes at analyst discretion.
- When length of time between alleged events and kit collection is uncertain due to missing or conflicting information about timelines, and/or when length of time is very close to or exactly at a decision point, the analyst may use discretion in choosing which processing method is more appropriate.

Biology Deviation Request Form required:

- Decisions that could potentially impact options for current or future testing, such as combining samples that are not routinely combined (e.g., left- and right-hand swabs). This does not apply to actions performed at specific instruction by Department of Law (e.g., permission to consume evidence is granted).

Triage Practices for cases with allegation of strangulation

Prioritization of neck swabs and fingernail scrapings collected within 48 hours is typical for cases where strangulation is alleged.

Because the length of time between alleged events and evidence collection can have a significant impact on triage decision, cases with conflicting information about timelines may benefit from outreach to law enforcement for more information.

Range of analyst discretion for Triage Practices for cases with allegation of strangling

Document actions and decisions (such as items sampled, amount sampled, and extraction methods used) as appropriate, but no explanation required if directions followed as described in above section(s).

Permissible with documentation of actions **and** reason given for change:

- Case-specific scenarios and forensic history information, such as recent hygiene, may impact the decision of which samples to process.
- Case-specific scenarios and forensic history information, such as recent hygiene, may impact the decision to work samples past the 48-hour cut-off.

Biology Deviation Request Form required:

- Decisions that could potentially impact options for current or future testing, such as combining samples that are not routinely combined (e.g., left- and right-hand fingernail scraping). This does not apply to actions performed at specific instruction by Department of Law (e.g., permission to consume evidence is granted).

Processing guidelines by sample type

NOTE: When swabs are cut in half, the goal is to take no more than half of any DNA present on the swab. Cutting is typically done vertically and should attempt to leave at least half of any visible material/stain on the swab.

Questioned blood stains

- Typical bloodstains on fabric are sampled by cutting approximately 0.5sq cm of the stain using a sterile, disposable scalpel. The size of the cutting may vary depending on the size and condition of the stain, but whenever possible, half or less of a bloodstain should be sampled. The entire stain may be sampled for very small or potentially degraded stains.
- Known bloodstains on thick fabric or filter paper may be cut or sampled using a 3mm punch.
- Bloodstains may also be sampled by swabbing with a damp, sterile swab if the substrate is difficult to cut, or potentially contains PCR inhibitors (e.g., cigars or denim).
- Bloodstained swabs are usually sampled by cutting a portion of the stained area of the swab.
- Typical extraction is by questioned direct protocol

Likely saliva swabs/isolated samples

- Swabs with little or no visible staining collected from areas likely to contain saliva (e.g., cups, bottles, beverage cans, cigars, etc.) will be sampled by cutting half of each swab.
    - Exception: cigarette butts are rarely swabbed. Instead, a small cutting (~1 cm^2) of paper is removed from the filter end. Cigarette butts are digitally imaged prior to sampling.
- When the sampled swab material is too large for a single tube, it should be split into multiple tubes and the extracts ultimately recombined. Ensure that all reagent blanks from that extraction set are treated in the same way.
- Typical extraction is by questioned direct protocol

Contact swabs/isolated samples (does NOT include body swabs)

- Typically, contact swabs will be sampled in their entirety, since collection is less likely to be uniform across the swab(s) as well as being low in DNA quantity.
    - If many swabs were collected from a single area, they may each be sampled in part due to size constraints on the amount of sample that fits in the extraction tube(s).
- When the sampled swab material is too large for a single tube, it should be split into multiple tubes and the extracts ultimately recombined. Ensure that all reagent blanks from that extraction set are treated in the same way.
- Typical extraction is by questioned direct protocol

Fingernail Swabs/Scrapings

- Samples are received either as swabs or debris that is scraped into a paper bindle.
- Swabs from under the nails are generally sampled by cutting the swab tips into the sample tube. However, as in the case of contact swabs, the entire swab may be used with at least half of the extract being retained for future use.
- When sampling debris in a paper bindle, a moistened swab is used to swab the scraper, visible debris, and the inside of the paper bindle. Typically, one swab is used per hand. The entire swab is then cut into the sample tube. When the bindle contains a large amount of debris, a representative amount may be sampled.
- Typical extraction is by questioned direct protocol

Fingernails or Fingernail Clippings

- These samples are routinely consumed in their entirety (rather than attempting to sample half), and half the extract retained.
- Fingernails or fingernail clippings should be imaged prior to sampling.
- If either washing or swabbing method described below is used, that must be documented in the bench notes. Further details (such as number of minutes the sample was agitated) are not required in the documentation.

GOAL: Obtain a profile foreign to the owner AND owner profile
Extraction process will create **two** extracts.

1. **Create Extract A – Nail surface**

- Option 1: Swabs from the nail(s) may be collected for profiling of tissue and cellular debris external to the nail itself. The entire swab tips are cut into the sample tube, and extraction is by questioned direct protocol
- Option 2: If surface material, i.e. blood or dirt, is observed on the nail, the nail may be washed by immersing in 480µL of G2 buffer in a micro-centrifuge tube with gentle agitation (~5 to 15 minutes at 900 RPM or less) and heat (~56ºC). The item may benefit from longer agitation, depending on age and condition of the item. The washing is then transferred to a new micro-centrifuge tube and digested by adding 20µL Proteinase K and continuing through the questioned direct protocol

2. **Create Extract B - Nail**

- Extract the cleaned nail using the direct with DTT protocol.

GOAL: Obtain a profile foreign to the owner
Extraction process will create **one** extract.

- Option 1: Swabs from the nail(s) may be collected for profiling of tissue and cellular debris external to the nail itself. The entire swab tips are cut into the sample tube, and extraction is by questioned direct protocol. After swabbing, clippings are dried and re-packaged with the evidence.
- Option 2: If surface material, i.e. blood or dirt, is observed on the nail, the nail may be washed by immersing in 480µL of G2 buffer in a micro-centrifuge tube with gentle agitation (~5 to 15 minutes at 900 RPM or less) and heat (~56ºC). The item may benefit from longer agitation, depending on age and condition of the item. The washing is then transferred to a new micro-centrifuge tube and digested by adding 20µL Proteinase K and continuing through the questioned direct protocol. After washing, clippings are dried and re-packaged with the evidence.
- Option 3: Clippings are extracted by questioned direct protocol. After extraction, clippings are dried and re-packaged with the evidence.

GOAL: Obtain a profile from the owner of the clippings
Extraction process will create **one** extract.

- Option: If surface material, i.e. blood or dirt, is observed on the nail, the nail may be washed by immersing in 480µL of G2 buffer in a micro-centrifuge tube with gentle

agitation (~5 to 15 minutes at 900 RPM or less) and heat (~56ºC). The item may benefit from longer agitation, depending on age and condition of the item. The washing is then discarded.
- Extract the (cleaned) nail using the direct with DTT protocol.

Hairs
Possible hairs are not routinely extracted in the first round of testing.

For mounted hairs, score the edge around the cover slip with a sterile scalpel. Remove the cover slip by prying it off or by soaking the slide in xylene substitute. Use a pipette to wash the mounting medium away with xylene substitute. Pick up the hair with clean forceps and wash in a beaker, on a watch glass, or on a microscope slide with absolute ethanol, then wash the hair with sterile, de-ionized water in a clean container.

Note: Hair extractions should be considered as their own extraction set, with their own hair ICS and reagent blanks. A reference (known) hair sample is processed in the batch alongside the questioned hair sample(s) as an internal control. Processing of the control hair should be performed similarly to the processing of the casework hair(s).

Note: Possible hairs are typically extracted individually. Exceptions may be made based on the nature of the evidence – for example, a clump of hairs attached with skin tissue may be worked in combination. However, these exceptions must be clearly documented in the bench notes.

Note: Hairs are consumed in their entirety (rather than attempting to sample half), and half the extract retained.

- Examine the hair, microscopically if necessary, for the presence of surface materials (i.e. blood or dirt) and document the findings. The hair may be placed on a clean piece of white paper or on a microscope slide for examination.
- If surface material is observed on the hair, the hair should be washed by immersing in 480µL of G2 buffer in a micro-centrifuge tube with gentle agitation and heat. This may be appropriate when attempting to obtain a DNA profile of the surface material separately from the hair itself.
- The washing is then transferred to a new micro-centrifuge tube and may be extracted by adding 20µL Proteinase K and continuing with the questioned direct extraction protocol.
- Cut approximately 1cm of each end of the hair and combine for digestion. Using clean forceps, place the hair sample into a sterile 1.5mL tube.
- Alternatively, to minimize the number of transfers, hairs may be cut directly into and digested in a screw-cap tube.
- Hair extraction is performed using the direct with DTT protocol.
- The remaining portion of the hair is re-packaged with the evidence.

Swabs other than body swabs for differential extraction (such as surface stains)

- Sample size is half of each swab. If homogeneity of collection can be assumed for a set of two swabs, then the analyst can sample all of one swab.
- Substrates must be extracted, either by combining the substrate with the sperm pellet or extracting it separately.
- A flowchart titled "Biological screening of stains for semen incorporating the QIAcube decision tree" summarizes the analysis decisions for semen stains.
- For samples which are likely to contain moderate or small amounts of epithelial DNA, such as less-intimate clothing stains, the substrate may be added back to the sperm pellet when it comes off the QIAcube.
- Extraction is performed using the differential extraction protocol.

Isolated stains with possible semen

- For isolated stains, sample size will depend on the size of the cutting but should include no more than half the stain.
- For stains on items of clothing, bedding, etc., analysts follow the flowchart in the to determine which samples are appropriate for extraction using the differential extraction protocol.
- Adult underwear will be processed only if a kit was not collected within 48 hours and underwear were known to be those worn immediately post-event. For cases involving minors, it may be appropriate to analyze underwear even if less than 48 hours and/or unknown when worn.
- If an item has been consumed for analysis, the substrate must be extracted, either combined with the sperm pellet or worked separately:
    - Items likely to have very high amounts of epithelial cells, such as underwear cuttings from crotch area, should be processed without combining the sperm pellet with the substrate.
    - Items likely to have relatively lower amounts of epithelial DNA, such as cuttings from clothing other than underwear, may have the substrate added to the sperm pellet for a single extract.

Condoms

- Condoms may contain evidence that can be isolated and compared to the DNA profiles of suspects or victims in sexual assault cases. Condoms should be sampled as follows:
    - Document the condition of the condom. Imaging may be used if necessary, to document unusual characteristics, such as extensive dirt, debris, tearing, etc.
    - Label one pair of sterile cotton swabs "outside". Moisten the swabs using sterile deionized water. Swab the "outside" surface of the condom, as received.
    - Repeat for the "inside" surface of the condom.
    - If the samples are not proceeding immediately to DNA analysis, they are packaged and retained as a separate item of evidence.

Note: Acid phosphatase and p30 testing should not be conducted when screening condoms. The laboratory has previously encountered condoms which gave incorrect AP test results.

Half of condom swabs are routinely sampled, with substrates combined with sperm pellets. If homogeneity of collection can be assumed for a set of two swabs, then the analyst can sample all of one swab. Extraction is performed using the differential extraction protocol.

Tampons and sanitary pads

- For screening a tampon:
  - image the item, then either
    - Option 1: Swab and AP Spot test – if positive, test small cutting(s) for p30. If AP Spot test is negative, no further analysis is required.
    - Option 2: Go directly to p30 test. If positive, sample proceeds to differential extraction. If negative, no further analysis.
- For screening a sanitary pad:
  - Image the pad
  - ALS the pad. If there are distinct areas of fluorescence, swab and AP Spot test
    - If AP Spot Test positive, cut and test for p30. Positive area(s) should be circled and noted on evidence as well as described in bench notes
    - If AP Spot test negative, no further testing
  - Note: some brands of sanitary pad show background fluorescence. Therefore, even if the ALS is negative, the item should still be swabbed and AP Spot tested or cut and p30 tested. In such cases, it is useful to focus on areas that are bloody since blood can mask fluorescence. Some options for testing an ALS negative pad are:
    - Section the pad (into three or four sections), swab, and test each section for AP Spot test activity. Areas that are positive can then be cut and combined for p30 as one stain (note in bench note documentation)
    - Swab areas with blood and AP Spot test – if AP Spot test is positive, cut for p30. If AP Spot test is negative, no further analysis is required.
    - Cut directly for p30.

Fetal tissue

NOTE: a second qualified analyst must witness item sampling for fetal tissue.

Fetal tissue may be received by the laboratory for DNA analysis in criminal paternity cases. The following are general guidelines for processing fetal tissue samples. The sample collection process should be documented and may vary from what is described on a case-by-case basis.

- Samples are generally received frozen in a specimen cup and should be completely thawed at room temperature prior to any processing.
- The thawed sample can be transferred in its entirety to a weigh boat (or similar consumable) for ease of viewing. If fetal parts are clearly visible, it is not necessary to transfer other non-fetal tissue that may be present.
    - If fetal tissue is clearly visible, use a sterile scalpel to cut a small portion (ex. a digit from a hand or foot) and place it in a sterile microcentrifuge tube.
    - If fetal tissue is not clearly visible, the analyst should assess all the tissue material and select 3-5 samples of possible fetal tissue. Each is placed in a separate microcentrifuge tube
- 2-3 washes of the tissue sample(s) should be performed to remove any possible maternal blood
    - Add ~500uL of sterile water to each sample
    - Vortex and centrifuge briefly
    - Remove wash using a pipette and discard as biohazardous material
    - Repeat 1-2 more times, as necessary until the wash is clear
- Proceed with questioned direct protocol

Other tissue samples
Refer to the EZ1 DNA Investigator handbook for instructions on the appropriate sample size based on tissue type. Consult with the Technical Manager before proceeding if you suspect that evidence may not be best analyzed using the above guidelines.

NOTE: a second qualified analyst must witness item sampling for human remains.

Casework reference samples

- Typically, known buccal swabs require only 1/3 of one swab or a small portion of two swabs. However, if two or more swabs are available, all of one swab may be used.
- Typically, dried blood cards require ~0.5 cm^2 of stain for extraction.
- Typically, references are extracted using the known direct extraction protocol.
- If two or more swabs are available and one entire swab head is used, extraction may be by the Global Filer Express protocol, along with a corresponding reagent blank and internal control standard. Note: This method should only be attempted for buccal swabs collected within 10 years since the direct amplification procedure is less effective on older (or previously analyzed) swabs. (Note: If this protocol is used, the technical reviewer must also be qualified in performing Global Filer Express analysis. Alternatively, the swab may be processed by RapidHIT if it is no more than five years old (see RapidHIT protocols for further guidance).
- If the reference sample is particularly old or potentially degraded (some database samples and references collected by the Medical Examiner), it may be more appropriate to use the questioned direct extraction protocol, which is optimized for maximal DNA recovery. If more than one elution volume is used for an extraction set of reference samples, the reagent blank must be run using the smallest elution volume.

Database reference samples

- Typically, database samples include two buccal swabs. Ideally, one entire swab is used to generate a profile for CODIS entry via GlobalFiler Express direct amplification, and the second swab is retained for possible future retesting (either for quality assurance or profile confirmation purposes).
- When only one swab is present, the entire swab may be consumed in an attempt to obtain a profile. The sample must be noted as Consumed in Analysis in the LIMS.
- When two swabs are present, but the first swab does not yield a full profile at expanded CODIS core loci, the entire second swab may be extracted by the casework questioned direct protocol (i.e., good faith efforts). The sample must be noted as Consumed in Analysis in the LIMS.

Range of analyst discretion for Processing guidelines by sample type

Document actions and decisions (such as items sampled, amount sampled, and extraction methods used) as appropriate, but no explanation required if directions followed as described in above section(s).

Permissible with documentation of actions **and** reason given for change:

- Case-specific scenarios and forensic history information, such as recent hygiene, may impact the decision to change prioritization of evidence (such as hairs).
- Case-specific scenarios and forensic history information, such as recent hygiene, may impact the decision to work samples past the designated time cut-off, if applicable.

Biology Deviation Request Form required:

- Sampling or processing decisions that could potentially impact options for current or future testing, such as combining samples that are not routinely combined (e.g., left- and right-hand fingernail scraping). This does not apply to actions performed at specific instruction by Department of Law (e.g., permission to consume evidence is granted).

3.3 Direct (non-differential) and Direct with DTT Extraction for Questioned samples

Note: Swabs or cuttings may be placed in either a 2.0 mL microfuge tube or a 1.5 mL QIAcube microfuge tube, since either of these will accommodate a spin basket. An additional transfer step will need to take place before the sample can be put on the EZ Biorobot instrument. See Option 2 below for use of Qiagen Lyse & Spin baskets.

Note: Carrier RNA may be added ***either*** in the pre-digest solution (step 1) ***or*** immediately prior to putting the sample on the EZ1 or EZ2 biorobot. It is not necessary to document when the cRNA is added.

Note: To prevent overflow during the EZ Biorobot processing, all digests should be transferred to a capless 2 mL tube.

Note: The direct with DTT extraction option should be used with samples that may contain semen but are not processed by differential extraction, due to length of time between event and sample collection or other case-specific circumstances. This option should also be used with hair samples and with fingernail clippings where the source or owner of the nail is to be determined. All samples using direct with DTT constitute their own extraction set, with an ICS and appropriate reagent blanks. This protocol is the same as the protocol for Direct Extraction for Questioned Samples, with modifications noted in italics:

1. Prepare the pre-digest solution:
   (Number of samples + 3) x 480 µl G2 buffer
   (Number of samples + 3) x 20 µl Proteinase K
   (Number of samples + 3) x 1 µl carrier RNA – if adding cRNA at this step

   (Number of samples + 3) x 40 µl DTT for direct with DTT extraction

2. Add enough pre-digest solution to each sample cutting to allow for at least 200µl of free liquid in the tube after absorption by the substrate.
3. Incubate at 56° C for 1.5 – 2 hours at 900 rpm on a thermomixer.
   Incubate at 70° C for direct with DTT extraction.
4. Centrifuge briefly to remove liquid from inside the lid.
5. Transfer the substrate to a spin basket and replace in the tube.
6. Centrifuge at ~14500 rpm for 5 minutes.
7. Remove substrate in spin basket to a separate labeled tube.
8. Vortex and spin down briefly.
9. Transfer up to 500 µl of the digest into a sterile 2.0 mL screw-cap tube.
   Transfer up to 540 µL of digest for direct with DTT extraction
10. Add 1 µl carrier RNA solution (if not added pre-digest) and 400µl of pre-warmed (at least 10 minutes at 56° C) Buffer MTL to the transferred digest solution.
11. Proceed to EZ1 or EZ2 protocol

Option 1 – Extracting large samples in multiple tubes

When to use: if it is necessary to extract a sample that consists of more than two swabs worth of substrate material, then use of more than one tube may be required to digest and extract the entire sample efficiently.

- Divide the swab or other substrate material among enough tubes such that the material in each tube can fit in a spin basket and can move somewhat freely when 500 µL of pre-digest solution are added to the tube.
- Each reagent blank in the set must be prepared using the same number of EZ Biorobot channels. For example, if a set of swabs are extracted among three tubes and then combined into a single extract, then each reagent blank should also be made using three EZ Biorobot tubes which are then combined into a single extract.

Option 2 – Qiagen Lyse & Spin baskets

WHEN TO USE: Lyse & Spin baskets can be used for questioned direct or direct with DTT extractions following the above protocols, with these modifications:

Substrates and digest buffers are added directly into the spin basket inside the tube. The digest takes place in the spin basket.
Digests are performed on a 2.0 mL Thermo-mixer, using the appropriate designated Lyse & Spin temperature set point.
Following digest, centrifuge at ~14500 rpm for 1 - 5 minutes.
The closed tube can be cut at the hinge, and the capless tube placed on the EZ Biorobot.

3.4 *Differential Extraction with QIAcube Classic Automated Wash Protocol*

Note: Swabs or cuttings may be placed in either a 2.0 mL microfuge tube or a 1.5 mL QIAcube microfuge tube, since either of these will accommodate a spin basket. If a 2.0 mL tube is used, an additional transfer step will need to take place before the sample can be put on the QIAcube instrument.

Before starting:

1. Refer to QIAcube Loading Chart for correct loading of sample tubes in the Rotor Adaptor and Shaker

2. Heat a thermomixer to 56° C for the epithelial digest, and a thermomixer to 70° C for the sperm digest. (1.5 mL or 2 mL thermomixer should be chosen to correspond to the tube sizes to be placed in it.)

Sample preparation for differential extraction:

3. Add 480 µL G2 and 20 µL Proteinase K to each sample. Optional: a G2 buffer / Proteinase K master mix may be prepared with (number of samples +3) * 480 µL G2 and (number of samples + 3) * 20 µL Proteinase K; distribute 500 µL of master mix to each sample. Optional: 1 µL cRNA per sample can be added at this point instead of immediately prior to EZ Biorobot, either individually or via master mix.

4. Incubate at 56° C for 1.5 – 2 hours at 900 rpm on a thermomixer.

5. Centrifuge briefly to remove liquid from inside the lid.

6. Transfer the substrate to a spin basket and replace in the tube.

7. Centrifuge at ~14500 rpm for 5 minutes.

8. Remove substrate in spin basket to a separate labeled tube.

9. If the sample is in a 2.0 mL dolphin tube, vortex to thoroughly re-suspend, and centrifuge briefly to remove liquid from inside the lid, then transfer all of tube contents to a 1.5 mL QIAcube tube.

Loading the QIAcube:

10. Fill tip racks with adequate supply of disposable filter tips, 1000 µL, wide-bore.

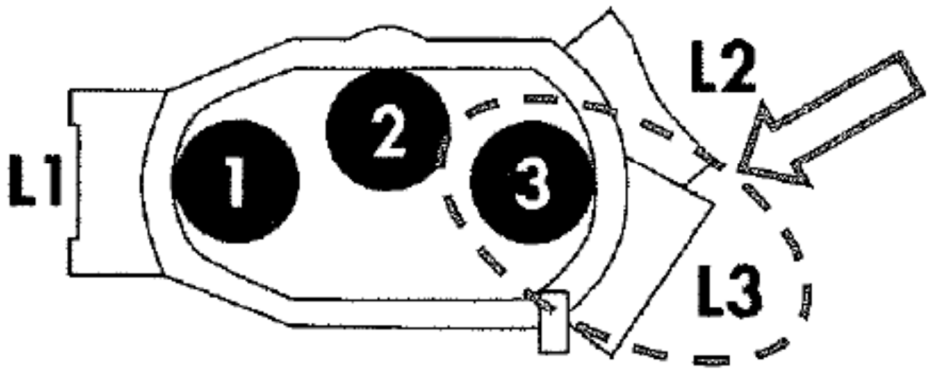
11. Aliquot Buffer G2 in 30 mL reagent bottle in Reagent Bottle Rack position 1. Do not fill above the fill line. Minimum volumes of G2 buffer are listed in the table below:

# samples	Buffer G2 (µL)
7 or fewer	16920
8	18980
9	21040
10	23100
12	27220

12. Add sterile water to the fill line of the reagent bottle in position 2. This bottle may either be kept capped during part A, or not loaded until part B, of the QIAcube run.

13. Place clean, labeled, straight-sided 2 mL tubes (such as screwcap EZ1 sample tubes, uncapped) in Shaker. These tubes will ultimately contain the epithelial fractions. Refer to the QIAcube Loading Chart for correct tube position. Each tube must also have a plug placed in its corresponding lid slot, since this is how the optical sensor detects that a tube is present.

14. Place lysed samples from step 9 in position 3 of the QIAcube rotor adaptor, with lid in position L3 (see image below of rotor adaptor).



15. Place rotor adaptors containing samples into QIAcube centrifuge. Refer to QIAcube Loading Chart for correct position.

Running the automated wash protocol:

16. Remove G2 bottle cap.

17. Ideally, QIAcube instruments should always be left on. If it isn't already on, turn on the QIAcube by pressing the power button.

18. Run QIAcube Separation and Lysis protocol, “Separation and Lysis 12 A”. The run protocol can be located as follows: under DNA, select Pipetting; select Epithelial and Sperm Cell, select Lyse 12 A mod. Start and follow prompts.

19. At the conclusion of “Separation and Lysis 12 A” protocol, refill the tip racks as needed and ensure that both the Buffer G2 reagent bottle and sterile water reagent bottle are in place and uncapped. Epithelial fractions are now located in the shaker and can be removed from the QIAcube at this point.

20. Run “Separation and Lysis 12 B”.

21. After protocol “Separation and Lysis 12 B” is complete, each sperm fraction will be found in the 1.5 mL tube in rotor adaptor position 3.

Sperm fraction processing:

22. Re-suspend the sperm pellet by vortexing; centrifuge briefly to remove liquid from inside the lid.

23. Add the following reagents to each sperm fraction:

140 µL Buffer G2

20 µL Proteinase K

40 µL 1M DTT

Optional: these reagents may be prepared and distributed as a master mix.

(number of samples + 3) * 140 µL Buffer G2

(number of samples + 3) * 20 µL Proteinase K

(number of samples + 3) * 40 µL 1M DTT

Distribute 200 µL of master mix to each sample.

Optional: If a substrate is being added to its sperm pellet, it is added after the sperm pellet digest reagents have been added to the tube.

Optional: If a substrate is being processed separately, the above reagents are added to a tube and vortexed briefly, followed by addition of the substrate.

24. Incubate at 70° C at 900 rpm in a thermomixer for at least 10 minutes.

Preparing for EZ1 or EZ2 protocol:

25. Pre-warm Buffer MTL at 56° C for at least 10 minutes.

26. Epithelial fraction: add 1 µL cRNA and 400 µL warm Buffer MTL to the 2 mL sample tube.

27. Sperm (and/or substrate) fraction: if applicable, remove substrate by spin basket (as described in steps 5-8). Add 1 µL cRNA and 400 µL warm Buffer MTL to the sample tube.

28. Proceed to EZ1 or EZ2 protocol

QIAcube clean-up:

After running a protocol, perform the regular maintenance procedure:

- Wipe down platform with a Kimwipe moistened with ethanol and then distilled water.
  - Do not directly spray the inside of the QIAcube with water or ethanol.
  - Do not use bleach, which can react with the reagents.
- Empty the waste drawer.
  - If necessary, wipe down with a Kimwipe moistened with ethanol and then distilled water.
- Remove used disposable labware and unwanted samples and reagents from the worktable. Discard in biohazardous waste.
  - Plastic rotor adaptors are single use only.
- Replace the lids of reagent bottles and close tightly.
- Re-rack the tips if there are any partially used tip racks.

Running the QIAcube Connect:

The differential extraction protocol used on this instrument is run in two parts: 3A and 3B. The break between the two sections allows the user to remove the epithelial fractions from the instrument for processing while the sperm pellet washes are performed in part 3B.

Protocol 3A:

Under Setup, choose DNA under Applications.

Under Select kit, choose Pipetting.

Under Select material, choose Epithelial and Sperm Cell

Under Select Protocol, choose 3. Separation Lysis A

No options are available under Define parameters, choose Next.

Under Select number of samples, choose the number of samples you will be loading.

Follow the series of prompts to load consumables and digests onto the instrument:

* Buffer bottle is not required for Protocol 3A

* Empty waste drawer and fill tip racks with adequate supply of disposable filter tips, 1000 µL, wide bore.

* Place clean, labeled, straight-sided 2 mL tubes (such as screwcap EZ1 sample tubes, uncapped) in Shaker. These tubes will ultimately contain the epithelial fractions. Refer to the Loading Chart on the screen for correct tube position. Each tube must also have a plug placed in its corresponding lid slot, since this is how the optical sensor detects that a tube is present.

* Load the centrifuge rotor adaptor as shown in the on-screen diagram

Start the run. Protocol 3A typically takes 18 minutes or less.

At the conclusion of protocol 3A, remove the epithelial fraction tubes if desired.

Select Protocol 3B and follow all screen prompts for loading reagents and consumables.

Aliquot Buffer G2 in 30 mL reagent bottle in Reagent Bottle Rack position 1. Do not fill above the fill line. Minimum volumes of G2 buffer are listed in the table below. Prepare the sperm digest buffer master mix in a skirted 2 mL tube, based on the number of samples:

		Sperm Digest Master Mix			
# Samples	**Buffer G2**	**G2 (µl)**	**PK (µl)**	**DTT (µl)**	**cRNA (µl)**
2	6100	259.5	17.3	69.2	2.31
3	7900	374.25	24.95	99.8	3.33
4	9700	489	32.6	130.4	4.35
5	11500	603.75	40.25	161	5.37
6	13300	718.5	47.9	191.6	6.39
7	15100	833.25	55.55	222.2	7.41
8	16900	948	63.2	252.8	8.43
9	18700	1062.75	70.85	283.4	9.45
10	20500	1177.5	78.5	314	10.47
12	24100	1407	93.8	375.2	12.51

Run Protocol 3B.

At the conclusion of the protocol, remove the sperm fractions from the rotor adaptors.

Optional: If a substrate is being added to its sperm pellet, it is added after the sperm pellet digest reagents have been added to the tube.

Optional: If a substrate is being processed separately, sperm digest reagents are added to a tube and vortexed briefly, followed by addition of the substrate. Sperm digest reagents include 140 µL Buffer G2, 20 µL Proteinase K, 40 µL 1M DTT and 1 µL carrier RNA per sample. The same volume of sperm digest reagents (200 µL if prepared as a master mix) must be added to the corresponding sperm fraction reagent blank after it is removed from the Qiacube Connect instrument.

Incubate at 70° C at 900 rpm in a thermomixer for at least 10 minutes.

Preparing for EZ1 or EZ2 protocol:

Pre-warm Buffer MTL at 56° C for at least 10 minutes.

Epithelial fraction: add 1 µL cRNA (if not added previously) and 400 mL warm Buffer MTL to the 2 mL sample tube.

Sperm (and/or substrate) fraction: if applicable, remove substrate by spin basket (as described in steps 5-8). Add 1 µL cRNA (if not added previously) and 400 mL warm Buffer MTL to the sample tube.

Proceed to EZ1 protocol or EZ2 protocol.

QIAcube Connect clean-up:

After running a protocol, perform the regular maintenance procedure:

- Wipe down platform with a Kimwipe moistened with ethanol and then distilled water.
- Do not directly spray the inside of the QIAcube with water or ethanol.
- Do not use bleach, which can react with the reagents.
- Empty the waste drawer.
- If necessary, wipe down with a Kimwipe moistened with ethanol and then distilled water.
- Remove used disposable labware and unwanted samples and reagents from the worktable. Discard in biohazardous waste.
- Plastic rotor adaptors are single use only.
- Replace the lids of reagent bottles and close tightly.
- Re-rack the tips if there are any partially used tip racks.
- Following the last run of the day, perform a UV run. Under Tools, select the UV Run tab. Select two cycles (1 cycle = 12 minutes) and Start.

3.5: Direct Extraction for known samples

1. Prepare the pre-digest solution:
   (Number of samples + 3) x 230 µl G2 buffer
   (Number of samples + 3) x 230 µl sterile de-ionized water
   (Number of samples + 3) x 10 µl Proteinase K

2. Add enough pre-digest solution to each sample cutting to allow for at least 200µl of free liquid in the tube after absorption by the substrate.

3. Incubate at 56ºC for at least one hour. The incubation may be performed in either an incubator or in a thermomixer set at 900 rpm and appropriate temperature.

4. Following incubation, transfer 200µl of the digest buffer into a sterile 2 mL screw-cap tube.

5. Proceed to EZ1 or EZ2 protocol

Note: In an effort to yield as much DNA as possible form a low-level or degraded reference sample, the questioned direct protocol may also be used.

3.6: EZ1 protocol and EZ2 protocol

EZ1 Protocol

Insert the DNA Investigator card into the slot located on the front of the BioRobot EZ1 Advanced-XL.

Turn on the power switch on the back of the instrument.

Directions are displayed on the screen on the front of the instrument. Press the START button

For questioned samples select Large Volume protocol, TE elution buffer, and 40 µL elution volume. Exceptions documented in DNA-LIMS packet.

For known samples select Trace protocol, TE elution buffer, and 200 µL elution volume. Exceptions documented in DNA-LIMS packet.

Follow the instructions displayed on the screen to set up the reagent cartridges, digest tubes, tip holders, and elution tubes.

Note: Ensure that the reagent cartridges snap into place in the cartridge rack and that the caps are removed from the sample tubes and elution tubes prior to loading on the deck. The caps from the sample tubes may be discarded. The caps from the elution tubes are retained and replaced upon completion of the protocol.

If fewer than 14 samples are being extracted, reagent cartridges and consumables need to be used only for the occupied channels.

Upon completion of the protocol, remove the elution tubes containing the purified DNA and cap the tubes.

Clean the instrument by wiping down with ethanol, followed by distilled water, after each use. Do **NOT** use bleach, as it may react with the extraction reagents.

Clean the piercing unit after each use by selecting option 2 (“Man”) on the main menu, then option 3 (“Clean”). Then wipe each piercing unit down with ethanol, followed by distilled water.

EZ2 Protocol (EZ2 Recovery protocol can be found in the appendices)

Instruments are typically left on, but if the instrument is off, turn on the power switch on the front of the instrument (lower right). The instrument will walk through a series of screen prompts to format the run. Directions are displayed on the screen on the front of the instrument.

Choose the Setup icon at the top of the screen.

Under Applications, select DNA.

Under Select kit, choose DNA Investigator Kit.

Under Select protocol, choose from the following protocols:

- DNA Investigator Trace: used for reference samples
- DNA Investigator Large Volume: used for questioned samples where pre-warmed buffer MTL is added to sample digests *before* they are put on the instrument

Under Define parameters, select the following:

- Elution buffer – TE
- Rack type – Tip rack
- Elution volume – 40 µl for questioned samples, 200 µl for known samples. Exceptions documented in LIMS-DNA packet.

Under Select sample positions, click on each of the channels that will be used. There is a toggle switch to select all, if applicable. Note: If fewer than 24 samples are being extracted, reagent cartridges and consumables need to be used only for the occupied channels.

Under Enter Sample IDs, choose Generate missing sample IDs. (These could be entered manually, if preferred).

Follow the instructions displayed on the screen to set up the reagent cartridges, digest tubes, tip holders, and elution tubes.

Note: Ensure that the reagent cartridges snap into place in the cartridge rack and that the caps are removed from the sample tubes and elution tubes prior to loading on the deck. The caps from the sample tubes may be discarded. The caps from the elution tubes are retained and replaced upon completion of the protocol.

Under Run setup selection overview, check all conditions, then select Skip load check – this will start the run without performing a load check. Load checks are not required.

Upon completion of the protocol (~18 – 24 minutes), remove the elution tubes containing the purified DNA and cap the tubes.

Maintenance following daily use:

Clean the instrument by wiping down with ethanol, followed by distilled water, after each use. Do NOT use bleach, as it may react with the extraction reagents.

Clean the piercing unit after each use: Under the Maintenance menu, select Daily. Use the Move down button to move the piercing unit into place for cleaning. Wipe each piercing unit down with ethanol, followed by distilled water. Use the Move up button to return the piercing unit to its home position.

At the end of the run(s) for the day, perform a UV run. Under the Maintenance menu, select one cycle and Start. The UV run takes 34 minutes 3 seconds.

3.7: Guidelines for addressing non-consumed evidence from previous analysis

Current routine practice in analysis of sexual assault kits from female victims includes sampling half of most body swabs and retaining the remaining half for possible future re-testing. However, previously issued reports sometimes offered the option of fully consuming evidence and combining the new extract with the old extract from the same item to increase the chance of obtaining a Y-STR profile suitable for comparison. This is no longer an option under current protocols.

Similarly, previously issued reports where an item of evidence was fully consumed to create an extract may have indicated that analysis was only possible of the extract was fully consumed.

Upon request, an analyst may review quantification data for retained extracts from previous analysis to see if an extract is eligible for amplification while retaining at least half of the evidence (or extract, if evidence was fully consumed), under current amplification guidelines. If quantification data and case specifics support amplification, the analyst may proceed to amplification, regardless of the previous report statement indicating that permission to consume was required.

Section 4 DNA Quantification and Quant-Based Decisions

Questioned samples and known samples not amplified by Global Filer Express are routinely quantified in single reactions to preserve extract. A single quantitation reaction is typically used for each questioned reagent blank but is not required if the extract is amplified. Quantitation is not mandatory for database samples or forensic known buccal samples amplified by Global Filer Express (QAS 9.4.1). As per QAS 9.4, quantitation is required for all questioned samples.

As per QAS 9.5.2, a standard curve must be included with each quantification run.

Range of analyst discretion for timing of adding Gentegra-DNA

Gentegra-DNA may be added to extracts *at any point before or after quantification*. Since all questioned extracts are routinely either consumed or retained, it may be helpful to add the GenTegra-DNA to all questioned extracts as soon as the samples have been quantified. However, this will affect sample concentrations; and re-calculations of the extract concentrations would be needed prior to amplification set-up. The procedure for drying down samples using Gentegra-DNA is in the appendices.

References:

- *ABI Prism 7000 Sequence Detection and Applied Biosystems 7500 Real Time PCR System User Bulletin*
- *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*
- *Applied Biosystems Quantifiler HP and Trio DNA Quantification Kits User Guide*

This section covers the following topics:

- Procedure for setting up and running a quantification plate using Quantifiler Trio on the 7500
- Criteria to evaluate quantification standards
- Assess the quality and potential probative value of each casework extract based on its QT results
- Identify casework extracts where analysis stops based on quantification results
- Choose appropriate samples for STR and Y-STR amplifications

4.1 Procedure for Quantifiler Trio on the 7500

Preparing the 7500 Real-Time PCR Instrument for a Run

- Open the HID Real-Time PCR Analysis software:
  - Log in under analyst name or initials
- Select the Quantifiler Trio icon on the left
- On the left is a menu with three parts: Experimental Properties, Plate Set Up and Run Method
  - Run method is correct as default – do not alter these settings.
  - Under Experimental Properties, enter a name for the project. For LIMS-DNA casework, the project should be named the same as the quant set. For database, the project name should include the batch name or quantification date. Default settings below are correct and should not be changed (Instrument: 7500 (96 wells); Experiment Type: Quantitation – HID Standard Curve; Reagents: TaqMan Reagents; Ramp Speed: Standard (~1 hour to complete a run).
  - Comments section may be used to add instrument number or other relevant information.
  - Under Plate Set Up, the default settings include Standards and NTC.
    - For database: Under the Define Samples tab, add samples. These can be left as Sample 1, Sample 2, and so on, with sample codes manually written on printouts later; or they can be entered with codes. Under the Assign Targets and Samples tab, highlight a box (or boxes) on the map, then check the box from the list on the left (Heading: Assign samples to the selected wells) for the corresponding reaction well(s). Data collected from wells that are assigned to the same sample will be averaged by the software.
    - Note: when LIMS-DNA export is used, samples do not need to be defined during Plate set-up, but best practice is to double check the plate map to ensure that all samples exported properly.
    - Standard Curve wells are already correctly assigned. As required in the plate set-up, the template default also includes two NTC reactions.
    - The Quantifiler Trio report prints out results ordered by rows (i.e. A1, A2, A3, A4…).
    - Selected samples are automatically assigned four targets: T.Large Autosomal (T-L) , T.Small Autosomal (T-S), T.IPC, and T. Male (T.Y).
- Save the experiment in the experiments folder (Applied Biosystems > 7500 > experiments > folder with analyst initials)

Preparing the DNA Quantitation Standards

Note: The volumes listed below are minimum volumes to optimize accuracy in pipetting. Larger quantities of standard curve may be prepared by scaling all volumes as needed. Prepared standard curves can be stored for up to two weeks at 2 to 8 °C with documentation of the date made, the expiration date, and the reagent lot numbers. Note: the directions below will make 30 µL of standard 1. The directions may be modified to create a different volume of standard 1 if desired (example: 40 µL Quantifiler THP DNA Dilution Buffer and 40 µL Quantifiler THP DNA Standard)

- Bring the reagents to room temperature before pipetting.
- Label five sterile microcentrifuge tubes for standards 1 through 5.
- Dispense 20 µL of Quantifiler THP DNA Dilution Buffer into Std 1 and 90 µL into each of the remaining tubes
- Prepare Standard 1:
    - Vortex the Quantifiler THP DNA Standard for 3 – 5 seconds.
    - Using a new pipette tip, add 20 µL of Quantifiler Trio DNA Standard to the tube for standard 1.
    - Mix the dilution thoroughly.
- Prepare Standards 2 through 5:
    - Using a new pipette tip, add 10 µL of the prepared standard 1 to the tube for the next standard.
    - Mix the standard thoroughly.
    - Repeat until you complete the dilution series.

The approximate concentrations of DNA in prepared Standards 1 through 5 are as follows:
Standard 1 ≈ 50ng/µL
Standard 2 ≈ 5 ng/µL
Standard 3 ≈ 0.5 ng/µL
Standard 4 ≈ 0.05 ng/µL
Standard 5 ≈ 0.005ng/µL

Preparing the Reactions

- Bring the reagents to room temperature before pipetting.
- It is recommended that preparation and aliquotting of the master mix take place in a designated hood in order to minimize the introduction of contamination as well as dust or particulate matter. Addition of DNA to the plate routinely occurs at the analyst's bench.
- While preparing the reactions, keep the 96-well reaction plate in a base (not directly on the bench top) to protect the plate from scratches and particulate matter.
- Calculate the volume of each component needed to prepare a master mix, including a few additional reactions. This calculation is made automatically on the designated 96-well plate QPCR worksheet that is used for well mapping (database) or by LIMS-DNA (casework).
    - Quantifiler™ Trio Primer Mix at 8 μL per reaction.
    - Quantifiler™ THP PCR Reaction Mix at 10 μL per reaction.
- Vortex the Quantifiler Trio Primer Mix and PCR Reaction mix for 3-5 seconds and centrifuge briefly before opening the tube.
- Pipette the required volumes of the components into a sterile tube. Vortex and centrifuge briefly.
- Dispense 18 µL of reaction mix into each reaction well.
- Add 2 µL of sample, standard or control to the applicable wells. 2µL of dilution buffer is added to each NTC well.
- Seal the plate with the Optical Adhesive Cover. Use an applicator to create a firm seal between each of the wells. NOTE: Do not write on the optical film; if possible do not write on the plate at all prior to a run.
- Centrifuge the plate at 3000 rpm for at least 20 seconds to remove any bubbles.

Sample Loading

- Push the tray door on the 7500 instrument to open it.
- Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder, with the notched A12 position in the top right of the tray.
- Close the tray door. Apply pressure to the right side of the tray door at an angle.

Starting the Run

- Click the green box labeled START RUN in the upper left-hand corner.

Analyzing the Data

- When the run is complete and the quality checks described in section 4.2 have been completed, export the raw data file from the 7500 and input into the LIMS-DNA workbook.

4.2 Criteria to evaluate quantification standards (QAS 9.6.1)

NOTE: Verification of Quantifiler Trio assesses conformance of the new lot with validation ranges for quality metrics. A kit lot which falls outside accepted ranges may be deemed suitable for use and may include optional or required adjustments specific to that lot. Adjustments made in accordance with kit verification do not require Biology Deviation Request Forms but do require documentation when the adjustments fall outside the ranges outlined in this manual.

Checking the Standard Curve

The large autosomal, small autosomal, and male DNA Standard curves need to be assessed for their R^2 values, slopes, and Y-intercept values. No more than two data points may be removed from a standard curve to achieve passing quality metrics, and it is not acceptable to remove both end points from either end of the standard curve.

R^2 is a measure of the closeness of fit between the standard curve regression line and the individual data points of the quantification standard reactions. A value of 1.0 indicates a perfect fit between the regression line and the data points. Passing value for R^2 is >=0.98.

Slope is an indicator of amplification efficiency. A slope value of -3.3 corresponds to true doubling of the amplicon at each cycle. Acceptable slope values fall in the range of -3.0 to -3.6.

Y-intercept is the theoretical expected quantity for 1 ng/µL of DNA. Y-intercept is used to compare performance among different QT kit lot numbers. If an average Y-intercept value drops by one C_T value, the standard curve will shift to the left and samples that previously quantified at 2 ng/µL will now quantify at 1 ng/µL. This will cause an underestimation of DNA quantity, which may lead to higher peak heights and possible increased baseline artifacts in analysis of amplified samples. Conversely, if the average Y-intercept value increases by one C_T value, the standard curve will shift to the right and samples that previously quantified at 1 ng/µL will now quantify at 2 ng/µL. This will cause an overestimation of DNA quantity, which will lead to lower peak heights and possible stochastic effects in the analysis of amplified samples.

Validation ranges for Y-intercept values are 25.8 – 26.3 for the male standard curve, 24.9 – 25.6 for the large autosomal human standard curve, and 26.8 – 27.3 for the small autosomal human standard curve. During QT kit verification, the Y-intercepts for the three curves are noted in the 7500 logbooks. At the time of kit verification, the Technical Manager or designee will check that Y-intercept values are falling within the validation ranges. If they do not, the kit may still be deemed acceptable, since amplification targets may be modified on a plate- and sample-specific basis. This decision will be included in the kit verification documentation. Analysts will track subsequent Y-intercept values for all curves run in the 7500 logbooks.

Checking the Internal PCR Control (IPC)
IPC values are documented in the Experiment Results Report for each reaction. As per the manufacturer, the IPC C_T value for each reaction is expected to fall in the range of 26 to 30. Values lower than 26 may be observed, but these are not considered failing and do not require any notation since there is no expected impact on downstream processing. The LIMS-DNA Supplemental Workbook highlights any values 30 and above. Values outside the expected range do not require any notation.

Checking the Passive Reference (Mustang Purple)
The passive reference signal flag (on the QC summary page of the Experimental Results Report, under the flag BADROX; or flag can be checked directly in the software) indicates if any samples exhibited a bad passive reference signal. The results of this check are recorded in the LIMS-DNA Supplemental Workbook and in the LIMS-DNA packet.

Checking the No Template Controls (NTC)
In the Results Table of the Experiment Results Report or in the software, confirm that the quantity of DNA for both Trio Human (large and small) as well as Trio Male is negative (C_T = undetermined). A detected quantity of DNA in both NTC wells could indicate contamination of the master mix. Low-level results (<0.001) in only one NTC well is not consistent with systemic contamination and does not require consultation.

Checking the Initial Template Quantity
Quantities of DNA, including large autosomal (T-L), small autosomal (T-S) and male DNA (T-Y) are listed for each reaction in the Results Table. The Quantity (Mean) column provides the average of any wells assigned to the same sample identifier in 4.1. When STR analysis will not be performed based on the quantification results (as described below), this shall be reflected in the bench notes for that sample.

Range of analyst discretion for Evaluating quantification standards

Permissible with documentation of actions:

- In case of slope, Y-intercept, or R^2 failure: Analyst may remove up to two data points from the standard curve, as long as those two points do not effectively remove an end point from the standard curve, to bring quality metrics within passing range. The quantification documentation should indicate which wells were removed.
- In case of IPC failure: When a C_T for an IPC is greater than 30 for a casework questioned sample, the sample should be assessed for possible amplification inhibition. Such samples are automatically flagged in the LIMS-DNA workbook. Note that excessive quantities of DNA can exhibit excessive concentration inhibition.
- In case of lot-specific kit issues: Corrections and/or adjustments can be made based on concerns identified during reagent verification of a specific lot of Quantifiler Trio, but a

note must be added in the quantification documentation (e.g., “Low Y-int noted – OK per lot verification” added as a comment)

Biology Deviation Request Form required:

- In case of slope failure: Slope values outside the specified range require approval by the DNA Technical Manager, issued via a Biology Deviation Request Form. If approval is not given, the plate must be re-run and sample data from the plate with the failing slope will not be used.
- In case of Y-intercept failure: If a Y-intercept falls farther than + / - 1 from the verified value, the Technical Manager must be notified to determine a course of action. If the Y-intercept is not approved by the Technical Manager via Biology Deviation Request Form, then the plate must be re-run and sample data from the plate with the failing Y-intercept will not be used.
- In case of passive reference failure: The Technical Manager is to be consulted when any samples fail this check. Approval or approval with modification by the Technical Manager will be issued with a Biology Deviation Request Form. If approval is not issued, then the plate must be re-run and sample data from the plate with the failing passive reference will not be used.
- In case of NTC failure: If the quantity of either Trio Human or Trio Male is greater than zero (not listed as Undetermined) in both wells, consult the DNA Technical Manager for a course of action. Approval or approval with modification by the Technical Manager will be issued with a Biology Deviation Request Form. If approval is not issued, then the plate must be re-run and sample data from the plate with the failing NTCs will not be used.

4.3 Quantifier Trio calculations and considerations for downstream assessment

These quant-based decision trees can be found in this section:

- ***Quant decision tree for suspect cases where male DNA is probative***
- ***Y-STR Quant decision tree (Questioned samples)***
- ***Consumed sample quant decision tree for non-suspect cases where male DNA is probative***
- ***Quant decision tree for extracts where female DNA is probative***
- ***Consumed sample quant decision tree for extracts where any profile (male or female) would be probative***

Ideal target ranges for amplification

Based on validation studies, the ideal amplification target template amount of high-quality DNA is 1.0 ng for GlobalFiler amplification and 0.5 ng for PowerPlex Y23 amplification. These values (or corresponding values adjusted for lower quality DNA) are the preferred target values. However, values as low as 0.5 ng for GlobalFiler and 0.25 ng for PowerPlex Y23 can be reasonably expected to yield profiles suitable for comparison. Thus, the ideal target range for GlobalFiler is 1.0 ng to 0.5 ng, and 0.5 ng to 0.25 ng for PowerPlex Y23. While the upper end of the ideal target range is generally preferred, analysts may target anywhere within the ideal target range (or adjusted for DNA quality) without additional documentation.

Degradation Index

How to calculate Degradation Index (DI)

- Degraded or inhibited DNA samples may benefit from amplification at higher target input DNA amounts. Quantifiler Trio results can be used to calculate a degradation index.

  Small autosomal target / large autosomal target = degradation index (DI)
  T-S / T-L = DI

- DI may be calculated by hand (such as for legacy samples) but is typically calculated automatically in the LIMS-DNA Supplemental Workbook.

How Degradation Index impacts amplification decisions

- Throughout this section, quantitation cutoff values are used for stopping analysis and receiving permission to consume. For samples with a DI less than or equal to 1.20, there is no impact on quantification cutoff values.
- For samples with a DI from 1.20 to 2.00, the cutoff values for stopping analysis or receiving permission to consume will double.
- For samples with a DI greater than 2.00, the new cutoff value for stopping analysis or receiving permission to consume is the original cutoff multiplied by the DI.
- This applies to both STR and Y-STR analysis.

Assessing whether an extract is likely to be single source, either entirely or effectively, for the purposes of STR amplification:

How to assess likely single source based on quantification results.

- **NOTE**: either of the below situations relies upon the assumption that a sample is likely to have DNA from only one contributor of the gender in question. Some case scenarios (e.g. multiple possible assailants or the possible presence of a consent partner as well as an assailant) preclude this assumption.
- Single-source (and effectively single-source) female: Intimate samples from a female's evidence collection kit (that is, body swabs or underwear from an evidence kit) with no male DNA detected are likely to be single source. Alternatively, samples with detectable male DNA present in a ratio of greater than 4.00:1.00 female: male are likely to be effectively single source / suitable for comparison for the major (non-male) component. As such, they are typically better candidates for Y-STR analysis.
- Single-source male: Samples where the male DNA quantitation value is higher than the total human quantitation value are likely to only yield results suitable for comparison for the male contributor(s).

Range of Analyst Discretion in single-source assessment:

- No additional documentation is needed - sample is assessed to be "typical" single source as described in the scenarios above.
- Permissible with documentation for the reason – case scenario specific information influenced this decision (e.g., multiple assailants, consent partner, etc.)

How a single source sample may impact amplification decisions.

- Based on case allegations, it is sometimes possible to assess whether a fraction from a differential extraction is likely to have probative value. For instance, in a case where a female alleges a sexual assault by a male, any fraction which does not include detectable male DNA, as determined by quantification results, will not be probative to the allegation. In other instances, such as an assault with a male victim and an alleged male suspect, gender alone cannot be used to assess whether a fraction may have probative value.

- Intimate sample fractions are not routinely processed when quantification results indicate a gender different from the putative perpetrator's gender. For example, in a case where a female alleges sexual assault by a male, an epithelial fraction where no male DNA was detected would not be amplified. Although these often have robust quantities of DNA present, they are occasionally low in quantity. These samples would only be run as a QA check, and it is not necessary to offer the option to consume them. If such an extract has a T-S concentration less than 0.0040 ng/µL, the extract need not be amplified. If such an extract has a T-S value between 0.0040 and 0.0530 ng/µL, a full-volume amplification will be performed without drying down the extract if amplification is needed for QA purposes.

Range of Analyst Discretion in amplification decisions:

- No additional documentation is needed - sample is amplified or not amplified based on obvious criteria as described above (e.g., not amplified when no male detected in a case with an alleged male assailant, etc.)
- Permissible with documentation for the reason – amplification for QA purposes should be noted as such.

Ratio of female to male DNA detected in an extract. (minor male)

How to calculate a ratio of female to male (minor male)

- When female DNA is in excess, an estimate of the ratio of female to male DNA can be made by subtracting the male quantification value from the small autosomal concentration to obtain the female contribution, then dividing by the male quantification value. This ratio is automatically calculated in the LIMS-DNA workbook.
    - Female: Male ratio (minor male): (T-S – T-Y) / T-Y
- When male DNA quantity is <0.0200 ng/µL, stochastic effects can limit the accuracy of assessment of the true female to male ratio.

Determining suitability for future testing for probative male

- These are general guidelines and may not apply to the specific details of a given case. ***Unless noted otherwise, the guidelines below apply to typical samples from female victim evidence kits in which ½ of each of the relevant swabs was extracted. When half the original evidence remains untested, as in these examples, extracts may be fully consumed.***
- Extracts with no male DNA detected will be retained with no further testing. A common exception would be a sample where a female profile may have probative value, such as swabs from a condom.
- Extracts with male DNA present at ratios greater than 4.00:1.00 female: male are not suitable for routine STR analysis but may be suitable for Y-STR analysis.
- Extracts with likely mixtures: Extracts likely to contain mixtures are suitable for STR DNA analysis when ALL three conditions listed below are met.
    - male DNA is present in a ratio of 4.00:1.00 female: male or less.
    - small autosomal concentration DNA equal to or greater than 0.0130 ng/µL
    - male DNA concentration is equal to or greater than 0.0050 ng/µL.
- Extracts for Y-STR analysis are routinely amplified as low as 0.1 ng (T-Y = 0.0125 ng/µL; this may be adjusted to account for degradation). However, targeting as low as 0.02 ng is permissible on a case-specific basis. Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
    - NOTE: One factor to consider in this assessment is the T-Y intercept of the quantification standard curve. **A T-Y Y-intercept which is lower than usual (for example, less than ~26.2) indicates a higher chance of obtaining a usable low-template Y-STR profile.**

- Consumed evidence minimum concentrations: If permission is granted to use all of a consumed-sample extract, the following cut offs apply for no further analysis:
    - If T-Y < 0.0007, no further analysis
    - For DI of 1.20 to 2.00: T-S < 0.0040 for likely single source and 0.0140 for likely mixtures
    - For DI above 2: T-L < 0.0020 for likely single source and 0.0070 for likely mixtures

  Note: There is no protocol in place for combining an existing extract with a newly created extract for the same item, since that would require corresponding reagent blanks to undergo the same process.

Range of Analyst Discretion in suitability for further analysis for probative male extracts:

- No additional documentation is needed - sample is assessed to be "typical" sample or common exception as described in the scenarios above.
- Permissible with documentation for the reason – Based on non-suspect case or some event-specific scenarios, extracts proceed to STR testing when ratios are 5.00:1.00 female to male or less.
- Permissible with documentation for the reason – Based on some event-specific scenarios, extracts proceed to Y-STR testing with amplification target below 0.1 ng (or equivalent adjusted for degradation).
- Require a Biology Deviation Request Form – Exceptions for STR amplification outside the 5.00:1.00 female to male or less range, as well as choosing to amplify below cut-offs for no further analysis. These are often related to severity of crime or limitations of evidence.

Ratio of female to male DNA (minor female)

Extracts which have a T-Y value that is greater than the T-S value can be reasonably assumed to have so much male DNA present that any female DNA will not be sufficient for comparisons to probative female reference samples. However, when the T-Y is less than T-S, the situation is not as obvious. Because Quantifiler Trio (QT) uses multi-copy targets, which may vary in number between individuals, the ratio of T-S to T-Y for a single-source DNA extract from a male individual may not be exactly 1:1. Based on validation studies, the T-Y reading can differ by +/- 20% from the T-S reading; but the ratio of T-S to T-Y remains consistent for each male individual, regardless of tissue type (blood, semen, etc.). When a reference sample is analyzed by QT, a correction factor can be calculated for T-Y, which then allows for a better prediction of detectable quantities of female DNA in a questioned sample collected from the male's body.

How to calculate an approximate ratio of female to male (minor female)

1. Calculate Y_{Corr} from male reference sample quant results:

   Y_{corr} = T-S / T-Y

2. For casework extract:.
   $T\text{-}Y_{original} * Y_{corr} = T\text{-}Y_{new}$

3. Use the new corrected value for T-Y to calculate a male: female contributor ratio

   $[T\text{-}Y_{new} / (T\text{-}S – T\text{-}Y_{new})]$

The following is a more detailed example of this calculation:

Ycorr calculations

1. Use QT data for the male reference sample to calculate a T-Y correction factor of T-S / T-Y

   Example: A male reference sample has QT readings of T-S = 0.80 and T-Y = 0.70

   $Y_{corr} = T\text{-}S / T\text{-}Y = 0.80 / 0.70 = 1.14$

2. For a questioned sample extract of body swabs from that male, multiply the T-Y by the Y correction factor.

   Example: A left hand swab from the above male has QT readings of T-S = 1.60 and T-Y = 1.20. After multiplying by Y_{corr}, the new T-Y value is 1.20 * 1.14 = 1.37 [$T\text{-}Y_{original} * Y_{corr} = T\text{-}Y_{new}$]

3. Use the new corrected value for T-Y to calculate a male: female contributor ratio

   Example: For the above sample, the ratio of male to female is 1.37 / (1.60 – 1.37) = 6:1 [$T\text{-}Y_{new} / (T\text{-}S – T\text{-}Y_{new})$]

Determining suitability of future testing for probative female

Samples with a male: female ratio of 4.00:1.00 or smaller are likely to have detectable amounts of female DNA and may proceed to amplification.

Range of Analyst Discretion in suitability for further analysis for probative female extracts:

- No additional documentation is needed - sample is suitable as described above.
- Permissible with documentation for the reason – Samples with a ratio between 4.00:1.00 and 5.00:1.00 have only a fair chance of having detectable amounts of female DNA and should be amplified only when no other samples are readily available.

- Require a Biology Deviation Request Form – Samples with a ratio greater than 5.00:1.00, which are unlikely to yield an interpretable minor component profile.

4.4 Identify casework extracts where analysis stops based on quantification results

Extracts from consumed samples that contain insufficient DNA for analysis

Based on validation studies, an extract containing a single source of DNA has a limited chance of being suitable for comparison when the input template DNA is less than 0.0630 ng. Based on the low chance of success, samples likely to be single-source (such as blood stains, hairs, and some sperm and epithelial fractions) with a T-S value of less than 0.0020 ng/µL can either be amplified by Y-STR, or no further analysis will be performed on the sample. 0.0020 ng/µL is the concentration where, if the extract were dried down with GenTegra-DNA and reconstituted with 15 µL, input template DNA would be ~0.0760 ng.

For two source mixtures, sample below 0.25 ng of input template DNA have a limited chance of being suitable for comparison. Based on the low chance of success, samples likely to contain mixtures (such as body swabs and contact DNA samples) with a T-S of less than 0.0070 ng/µL can either be amplified by Y-STR's (if appropriate) or no further analysis will be performed on the sample. 0.0070 ng/µL is the concentration where, if the extract were dried down with GenTegra-DNA and reconstituted with 15 µL, input template DNA would be ~0.2660 ng.

NOTE: Extracts where T-S, T-L and T-Y are all 0.0003 or lower are considered inconclusive for the presence of human DNA.

Range of analyst discretion:
Requires a Biology Deviation Request Form - In case-specific circumstances, it may be appropriate to amplify extracts below specified cutoffs.

Extracts where all the evidence was used to create the extract

Analysts may use up to 18 µL of extract for amplification, if minimum quantities of DNA are present (T-S > 0.0040 ng/µL for STR, or T-Y > 0.0015 ng/µL for Y-STR, adjusted higher if DI > 1.20 or for STR likely mixtures)

In instances where quantitation results for a consumed sample indicate that very limited amounts of DNA are present (T-S of 0.0040 – 0.0020 ng/µL for STR, or T-Y 0.0015 – 0.0007 ng/µL for Y-STR, or higher if DI > 1.20 or STR likely mixture), results suitable for comparison are only likely to be obtained if the extract is fully consumed.

In situations where amplification of the consumed sample extract with very limited DNA may be beneficial, the analyst's report can indicate that the analyst should be contacted to discuss options. Full consumption of the extract requires written authorization from Department of Law prior to amplification.

Extracts where half or less of the evidence was used to create the extract

If the existing extract does not contain sufficient DNA to perform amplification (either STR or Y-STR), the extract will be retained, and no further analysis will be performed.

Range of analyst discretion with extracts made from half or less of the evidence

- Permissible with documentation for the reason if future testing options are not impacted:
    - If abundant original evidence is present, the analyst may choose to perform another extraction, as long as at least half the original material (that is, half the original quantity of the sample, not half of the remaining material after the first sampling) remains unused.
    - Extracts are routinely amplified for Y-STRs down to 0.1 ng, but an extract may be amplified for Y-STRs down to 0.02 ng (or equivalent based on degradation) based on case-specific circumstances.
- Requires a Biology Deviation Request Form - In case-specific circumstances, it may be appropriate to amplify extracts below specified cutoffs.

Extracts from the fractions of a differential extraction of an intimate sample

Differential extractions from body swabs and/or intimate items of clothing (such as underwear in an evidence collection kit) are intended to maximize separation of owner and non-owner DNA. Based on the quantification results, only the more/most probative fraction from differentially extracted items is routinely amplified.

Case scenarios must be considered to determine whether biological sex can be used to assess the probative value of a given fraction. In cases where the suspect and victim are the same biological sex, the male/female results from quantification cannot be used as justification for not amplifying an extract. Other considerations, such as amount of DNA and degradation index, may be used to assess which extract(s) should be amplified.

If the sperm and substrate fractions are each extracted and quantified separately for a given sample, it is only necessary to amplify one of them. The decision of which extract to amplify will be based on the Quantifiler Trio results. Priorities (as applicable) are as follows:

1. Amplification at appropriate target quantity is possible while still retaining half the extract.
2. More likely to yield a single source foreign profile.
3. Has the higher percentage of foreign DNA.
4. Smaller degradation index

Range of analyst discretion in selection of differential fraction(s) to amplify

- No additional documentation is needed – extract is chosen based on the criteria listed above.
- Permissible with documentation for the reason if future testing options are not impacted
    - Examination of the amplified fraction(s) indicates possible sample switch or contamination issues, and other fractions are then amplified to further assess the concern.
    - More than one potentially probative fraction is amplified with the same technology
    - Amplification choice does not adhere to the priority list above for a reason specific to casework scenario.
- Require a Biology Deviation Request Form - Decisions that could potentially impact options for current or future testing. This does not apply to actions performed at specific instruction by Department of Law (e.g., permission to consume evidence is granted).

Quant-based decision flow charts color coding on the following pages:

Title highlights:

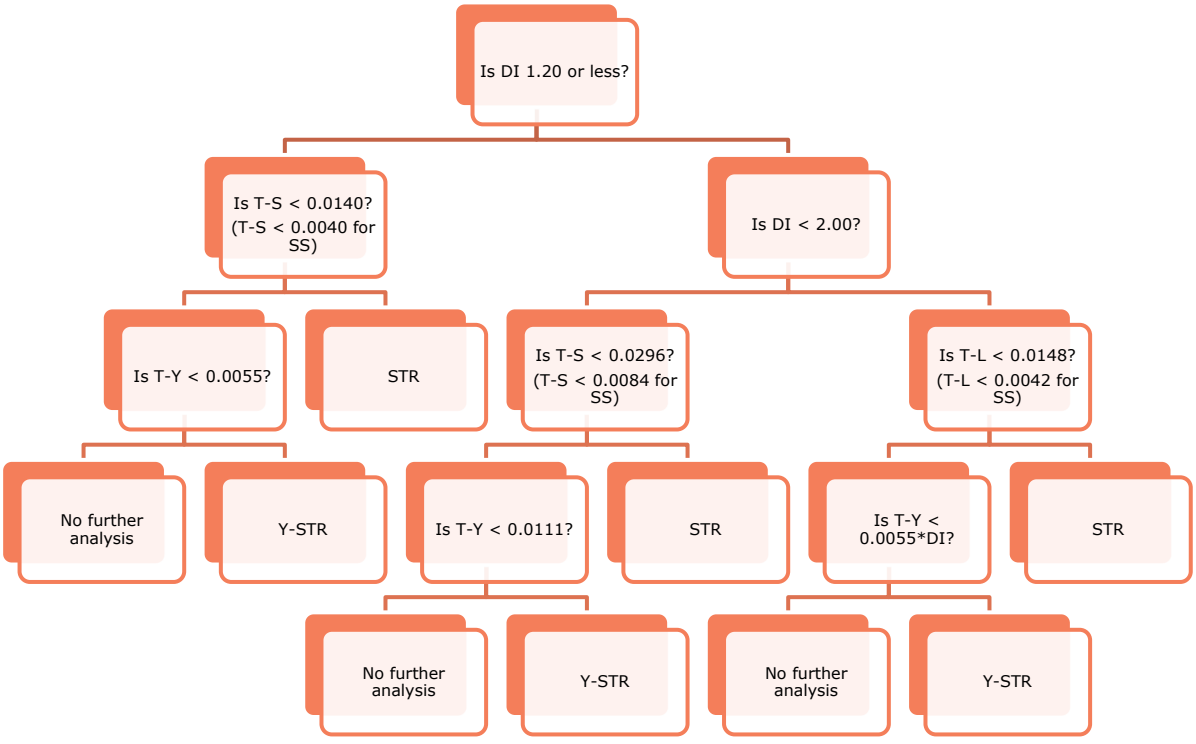
- Blue – Y-STRs are an option with ROUTINE cut-off values
- Yellow – Y-STRs are not an option
- Green – Y-STRs are an option with LOWEST POSSIBLE cut-off values

Flowcharts:

- Orange – consumed samples
- Purple – not-consumed samples

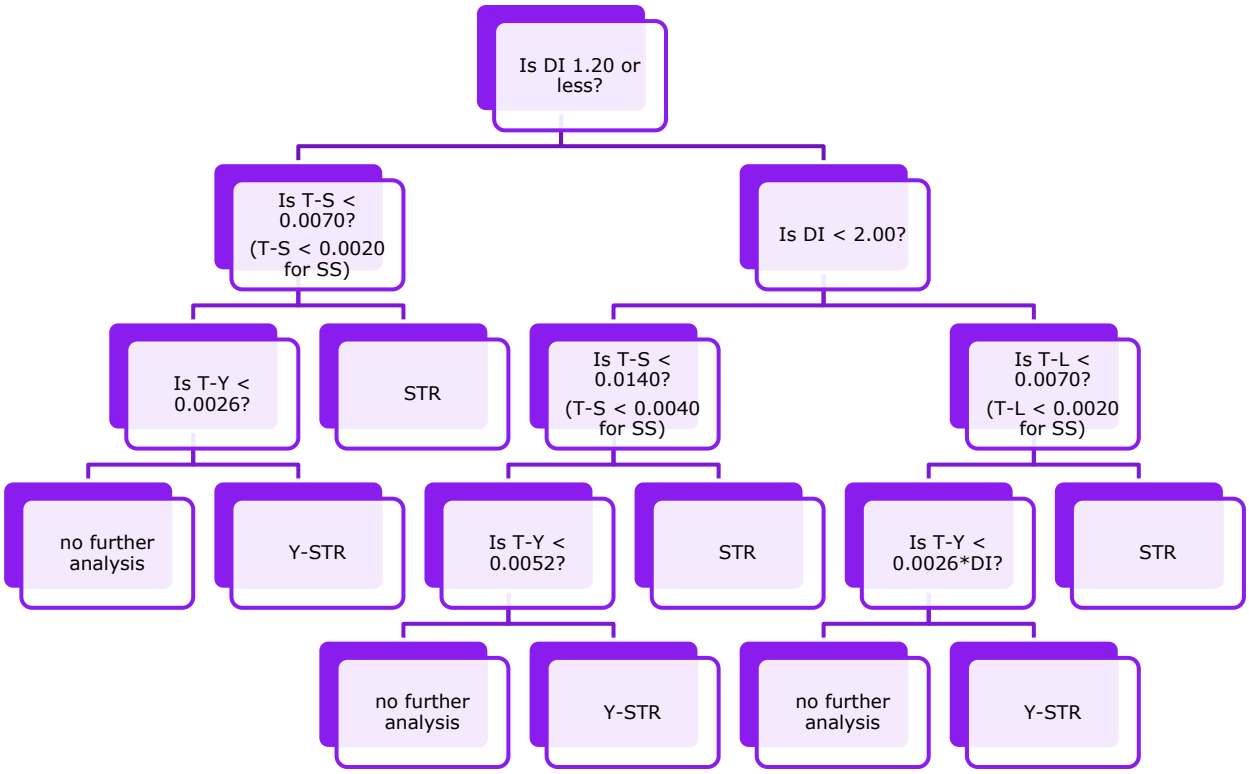
CONSUMED samples: Quant decision tree for suspect case extracts where male DNA is probative and ROUTINE Y-STRs are an option

- Y-STR analysis only performed in sexual assault and homicide cases
- Samples with a female: male ratio of 5.00:1.00 or greater are not eligible for STR analysis for a probative male – see Y-STR decision tree.
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Cut-offs assume that lowest possible Y-STR amps are not being considered – see other flowcharts if they are.
- Yes = Left; No = Right



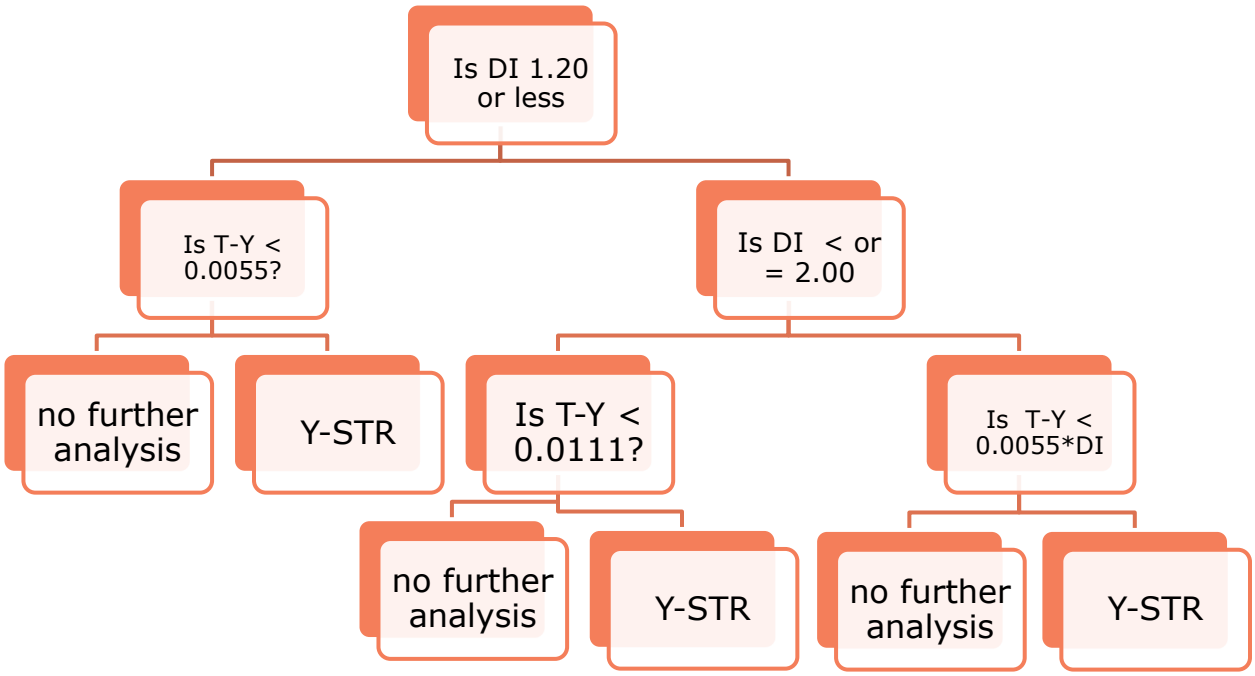
NON-CONSUMED samples: Quant decision tree for <u>suspect</u> case extracts where male DNA is probative and ROUTINE Y-STRs are an option

- Y-STR analysis only performed in sexual assault and homicide cases
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- Cut-offs assume that lowest possible Y-STR amps are not being considered – see other flowcharts if they are.
- Samples with a female: male ratio of 5.00:1.00 or greater are not eligible for STR analysis for a probative male – see Y-STR decision tree.
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL
- Yes = Left; No = Right



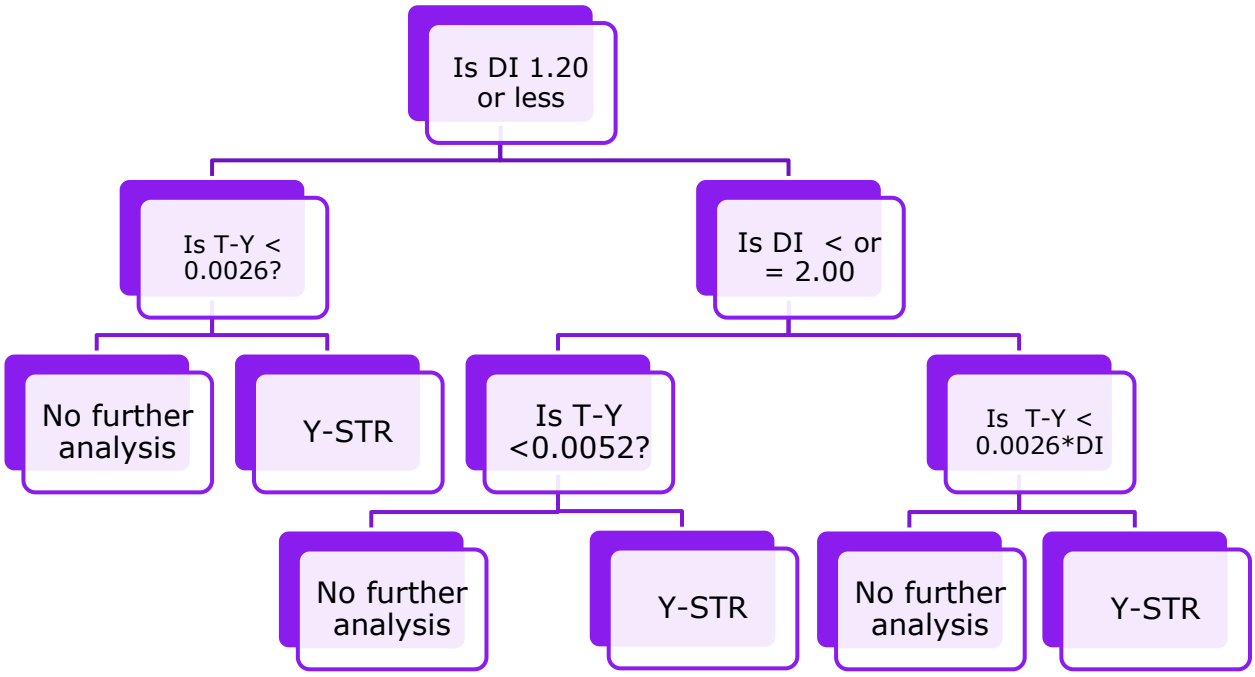
CONSUMED questioned samples: ROUTINE Y-STR Quant decision tree

- If T-Y < 0.0007 for a consumed sample extract, no further analysis.
- Y-STR analysis only performed in sexual assault and homicide cases
- If STR analysis is a possibility, it should be attempted first
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Cut-offs assume that lowest possible Y-STR amps are not being considered – see other flowcharts if they are.
- Yes = Left; No = Right



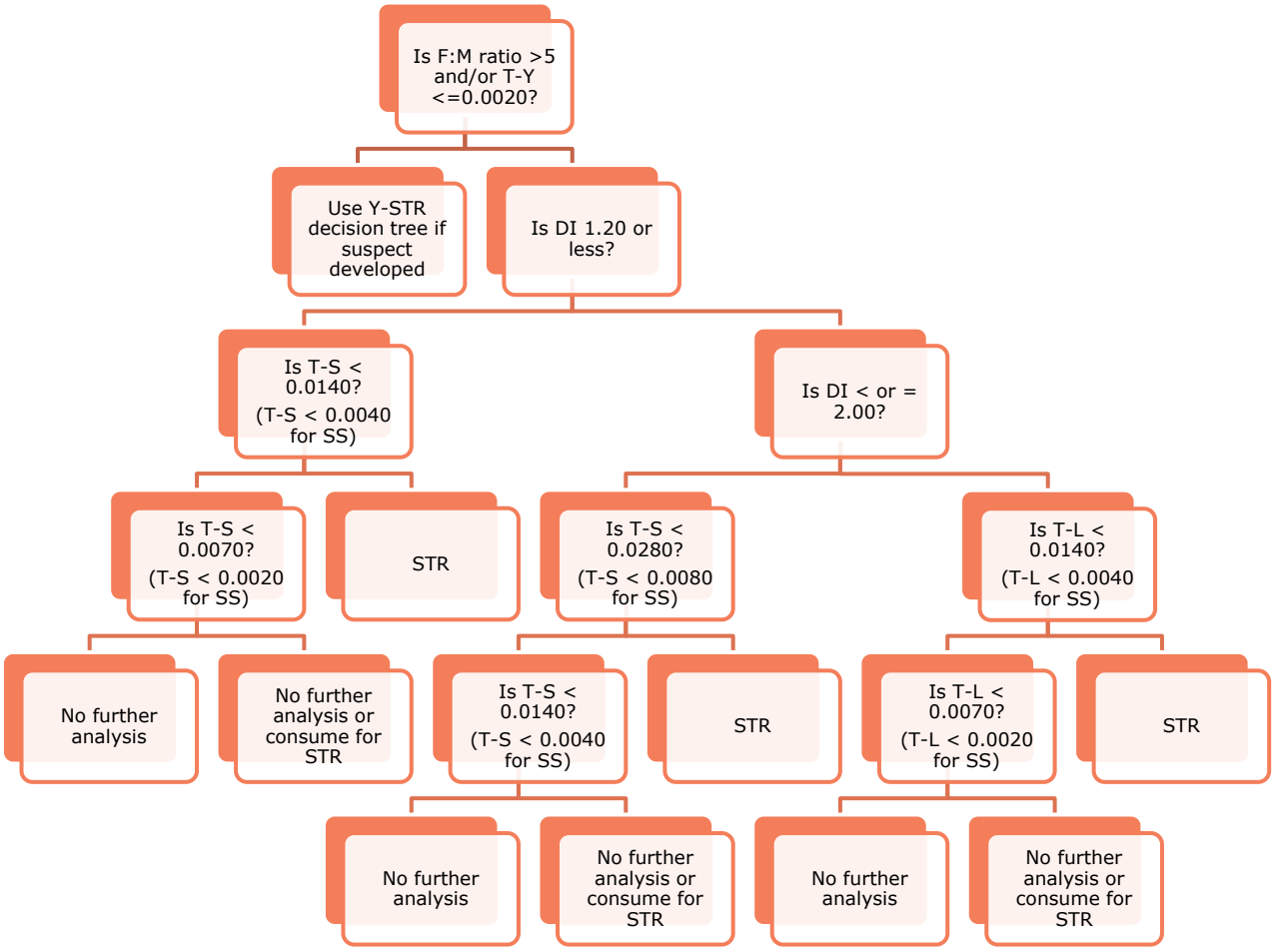
NON-CONSUMED questioned samples ROUTINE Y-STR Quant decision tree

- If T-Y < 0.0007 no further analysis.
- Y-STR analysis only performed in sexual assault and homicide cases.
- If STR analysis is a possibility, it should be attempted first.
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL.
- Cut-offs assume that lowest possible Y-STR amps are not being considered – see other flowcharts if they are.
- Yes = Left; No = Right



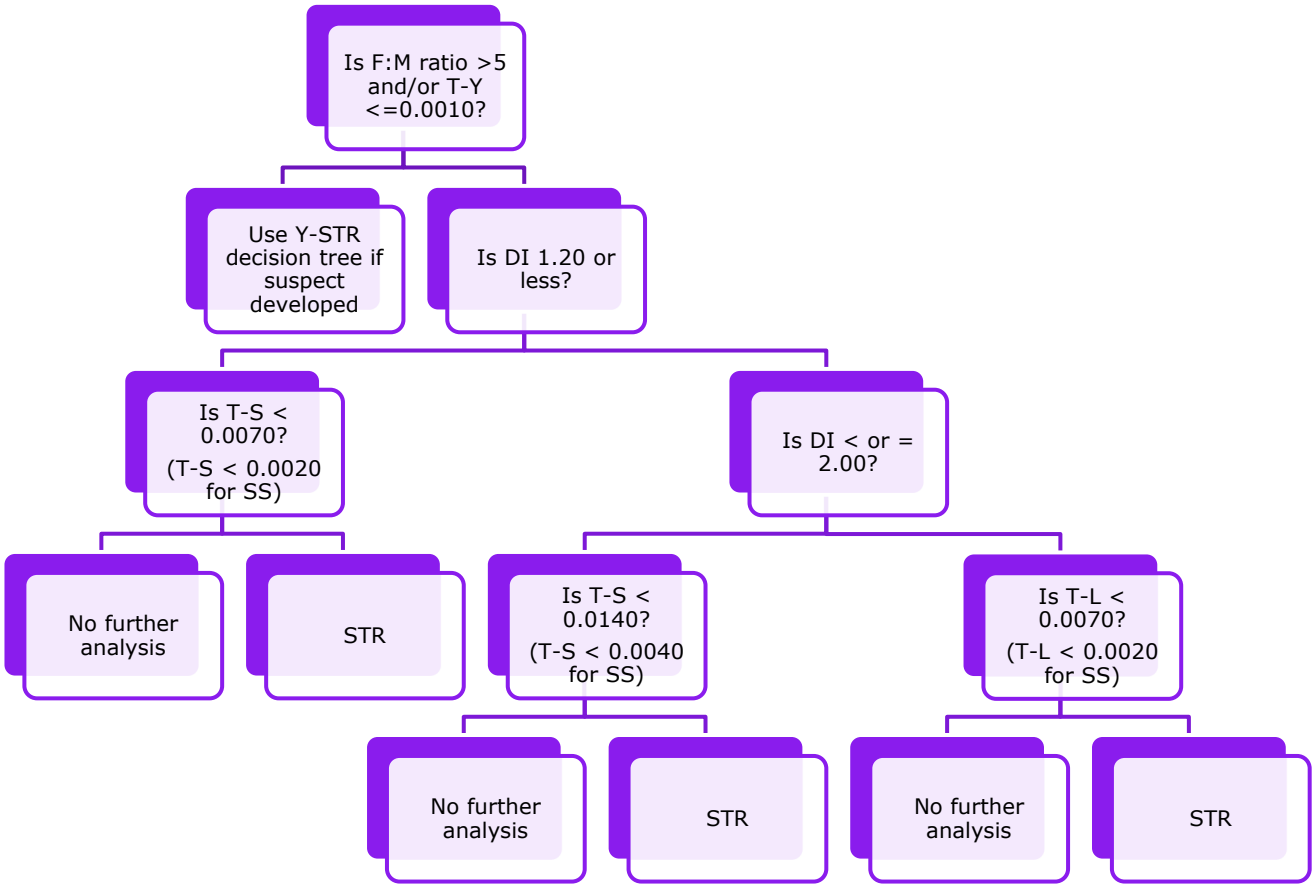
CONSUMED sample decision tree for <u>non-suspect</u> case extracts where male DNA is probative (or other cases when Y-STR not an option)

- Y-STR analysis only performed in sexual assault and homicide cases.
- If T-Y < 0.0007, no further analysis
- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS.
- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Yes = Left; No = Right



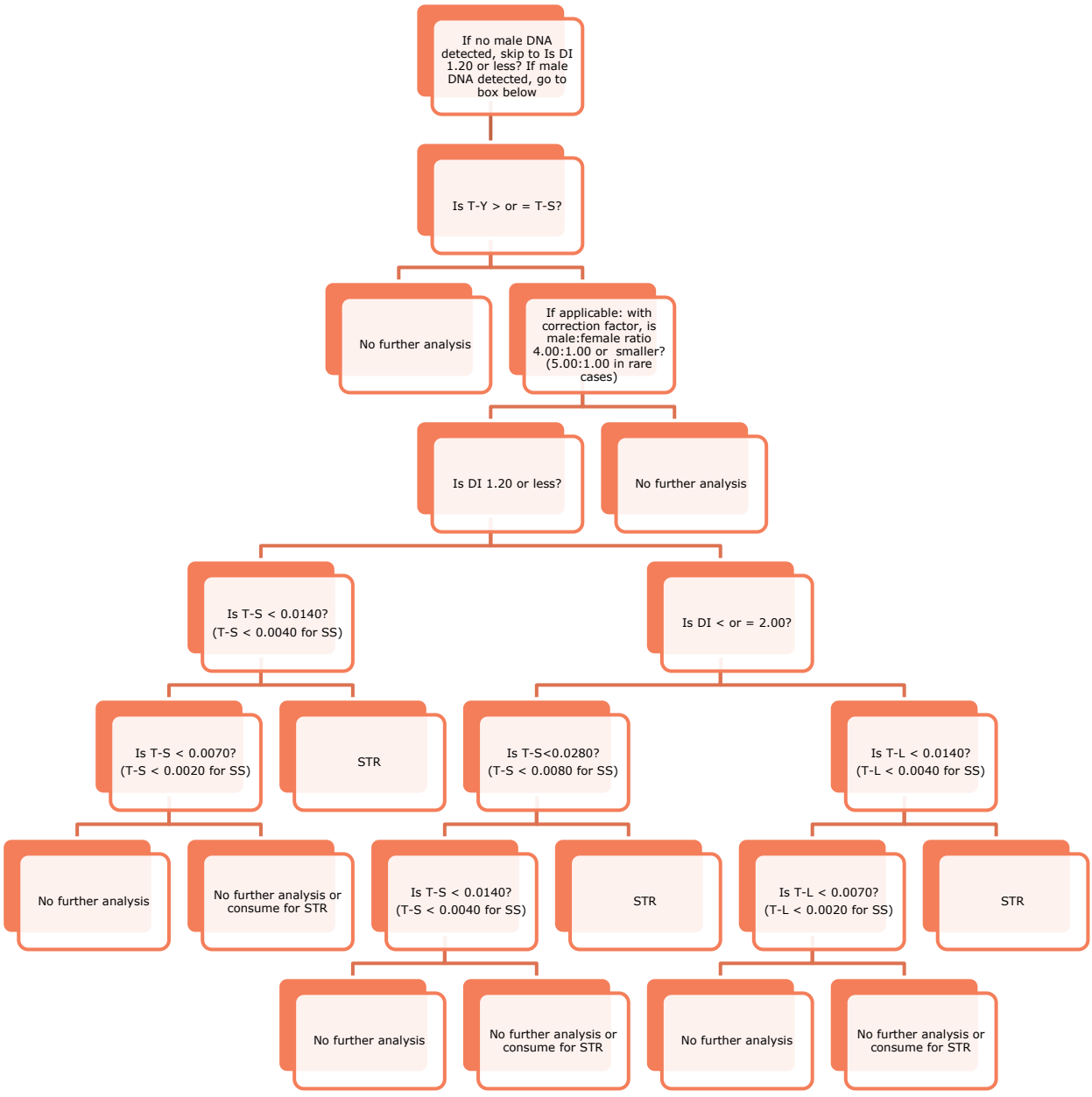
NON-CONSUMED sample decision tree for <u>non-suspect</u> case extracts where male DNA is probative (or other cases when Y-STR not an option)

- Y-STR analysis only performed in sexual assault and homicide cases
- If T-Y < 0.0007, no further analysis
- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL
- · Yes = Left; No = Right



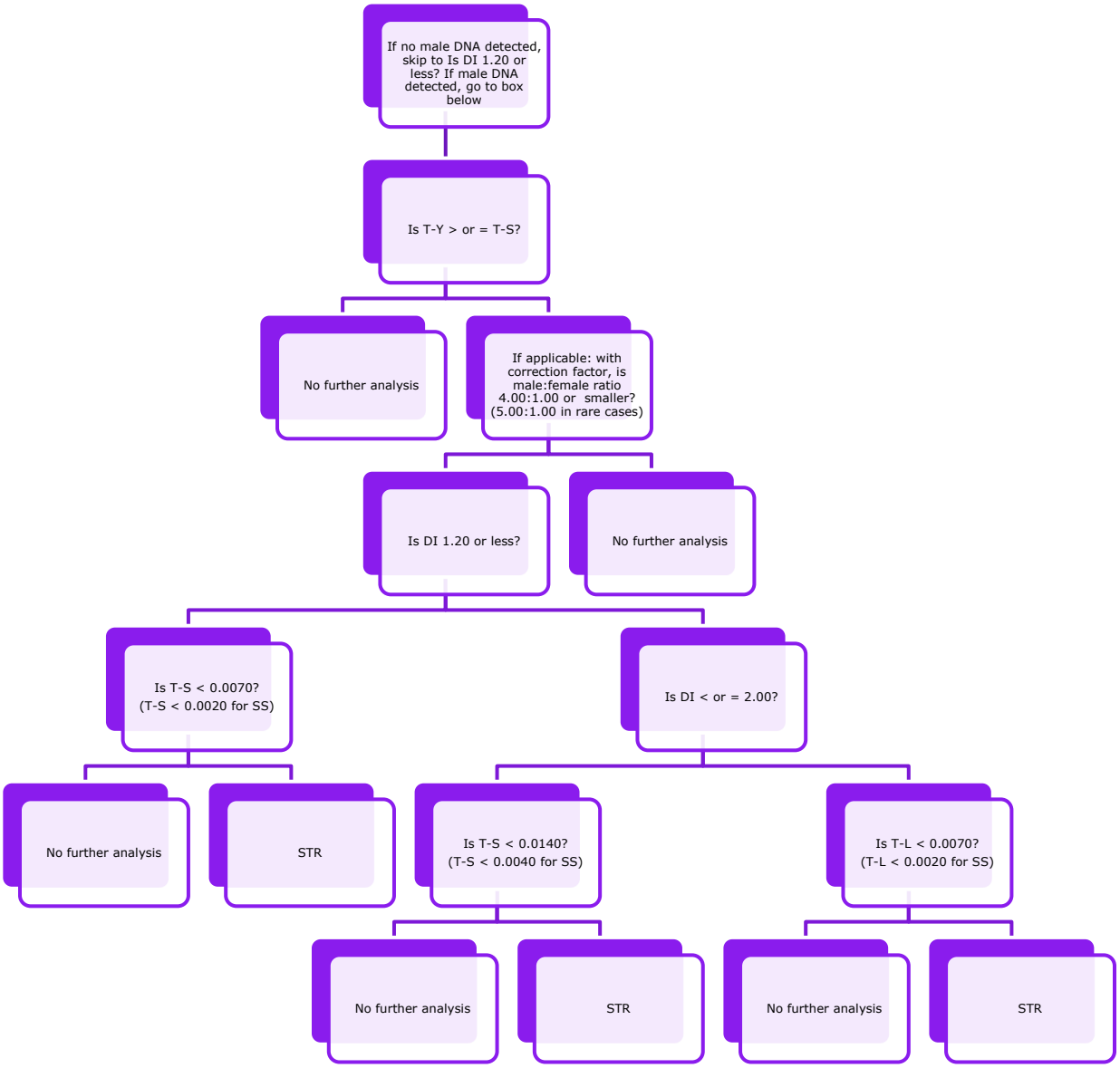
CONSUMED sample decision tree for extracts where female DNA is probative

- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Yes = Left; No = Right



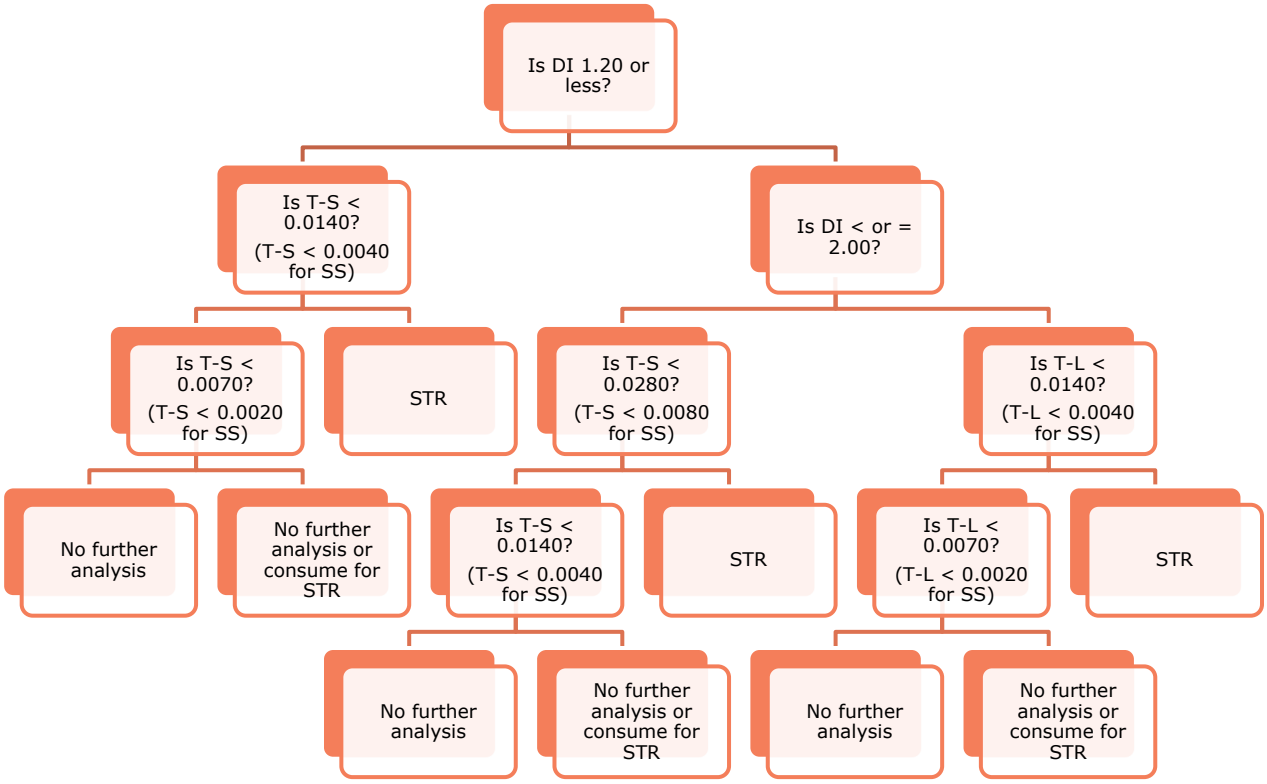
NON-CONSUMED decision tree for extracts where female DNA is probative

- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL
- Yes = Left; No = Right



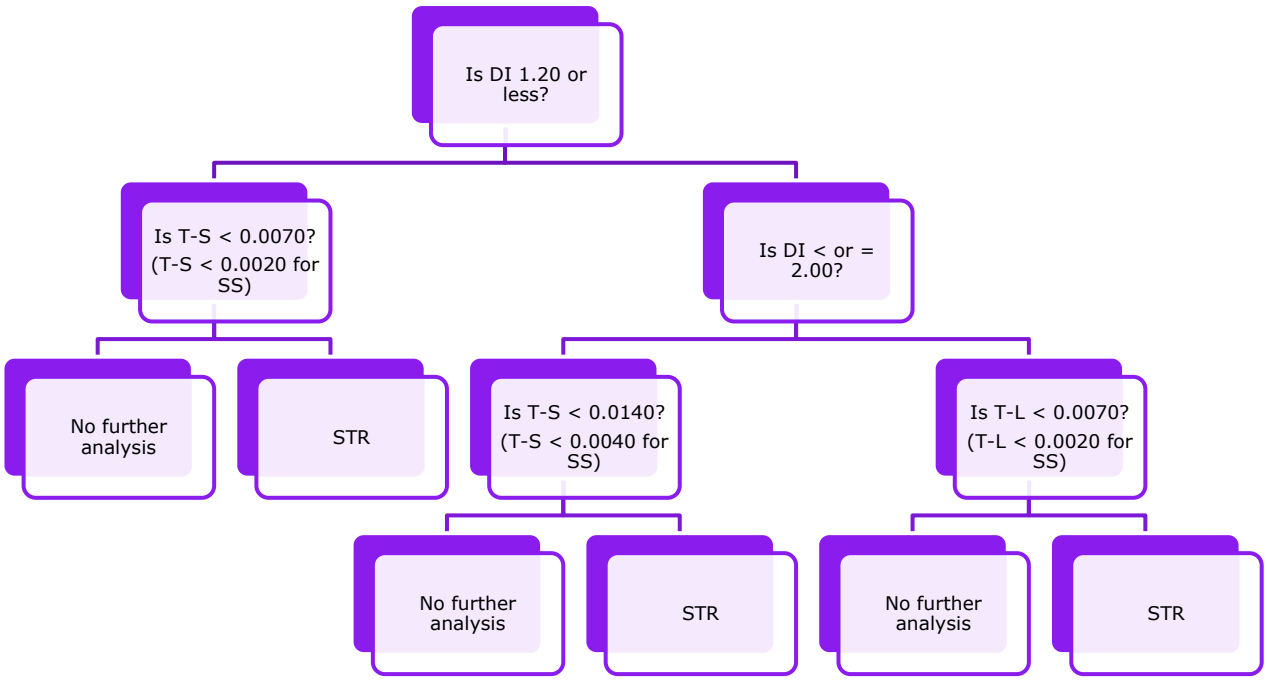
CONSUMED sample quant decision tree for extracts where any profile (male or female) would be probative

- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Yes = Left; No = Right



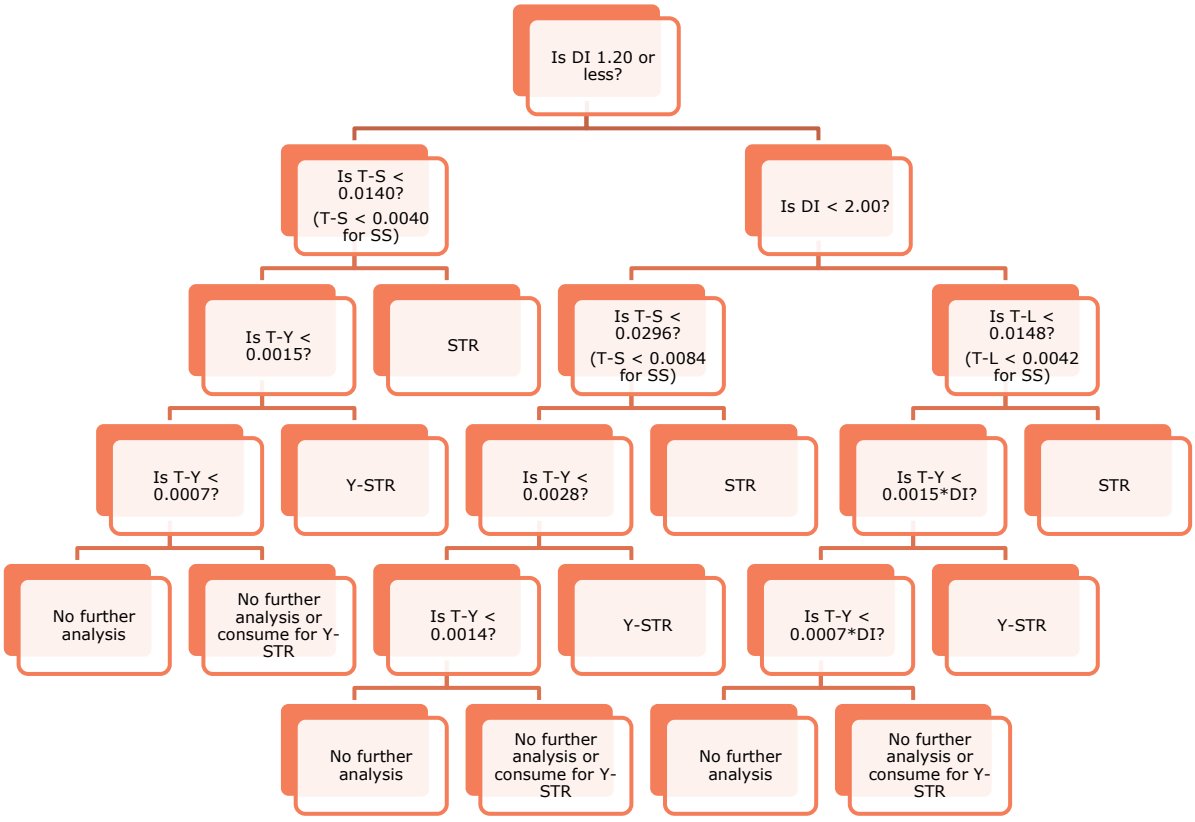
NON-CONSUMED sample quant decision tree for extracts where any profile (male or female) would be probative

- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL
- Yes = Left; No = Right



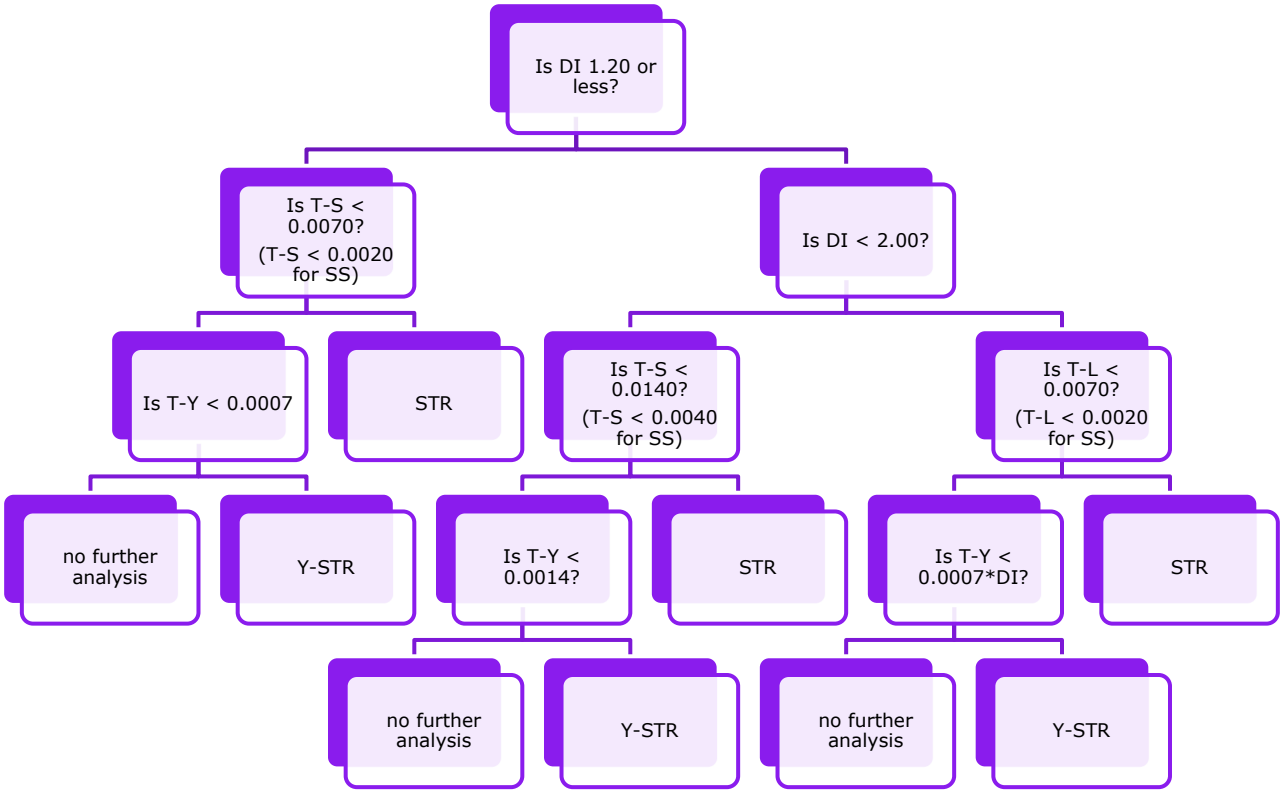
CONSUMED samples: Quant decision tree for suspect case extracts where male DNA is probative and LOWEST POSSIBLE Y-STRs are an option

- Y-STR analysis only performed in sexual assault and homicide cases
- Samples with a female: male ratio of 5.00:1.00 or greater are not eligible for STR analysis for a probative male – see Y-STR decision tree.
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Yes = Left; No = Right



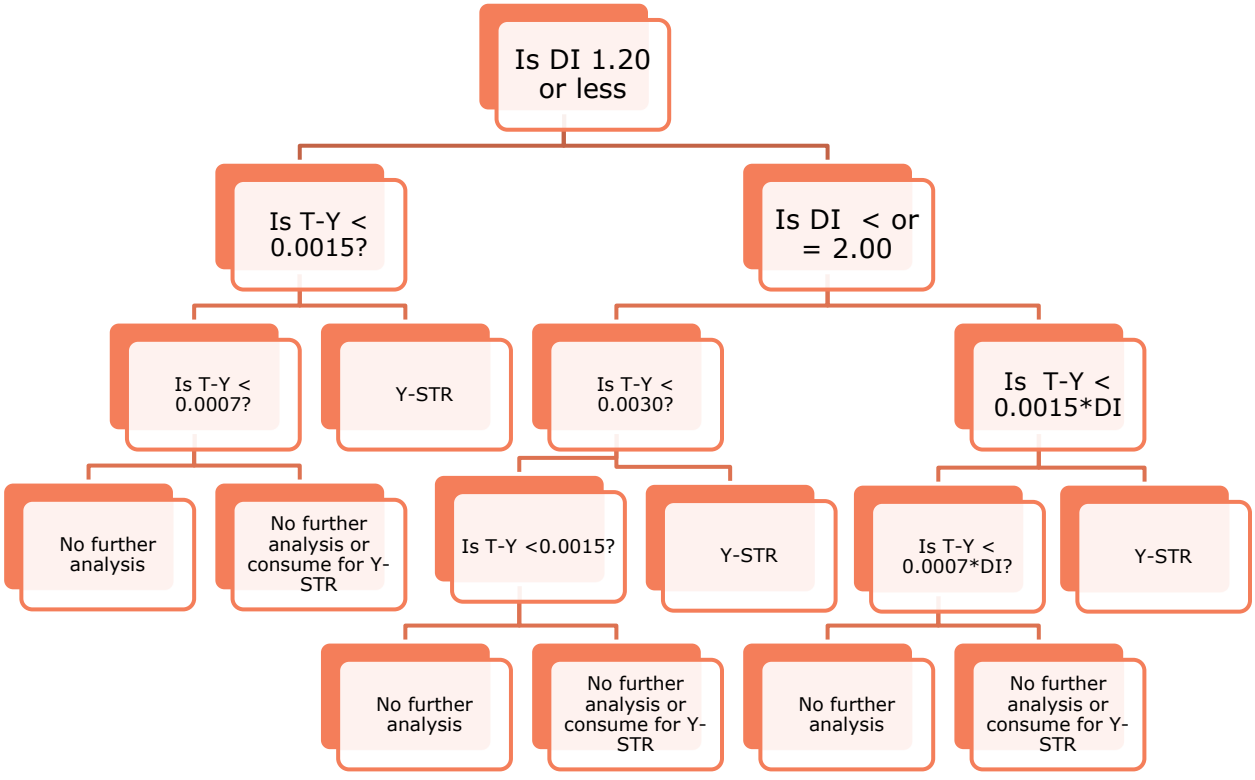
NON-CONSUMED samples: Quant decision tree for <u>suspect</u> case extracts where male DNA is probative and LOWEST POSSIBLE Y-STRs are an option

- Y-STR analysis only performed in sexual assault and homicide cases
- Cut-offs specific to STR are for likely mixtures; likely single source are noted as SS
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- Samples with a female: male ratio of 5.00:1.00 or greater are not eligible for STR analysis for a probative male – see Y-STR decision tree.
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL
- Yes = Left; No = Right



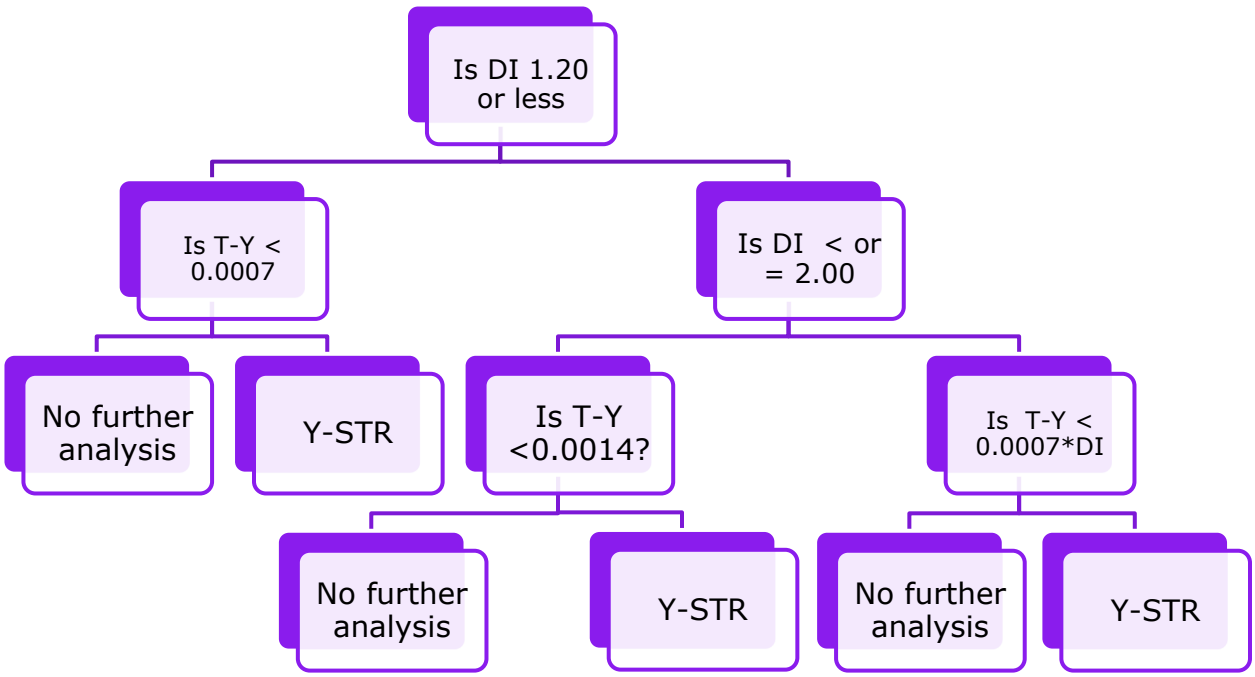
CONSUMED questioned samples: Y-STR Quant decision tree where LOWEST POSSIBLE Y-STRs are an option

- If T-Y < 0.0007 for a consumed sample extract, no further analysis.
- Y-STR analysis only performed in sexual assault and homicide cases
- If STR analysis is a possibility, it should be attempted first
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- Cut-offs assume that up to 18 µL of extract will be dried down and rehydrated to a volume of 15 µL; if permission is given to consume the extract, then all remaining extract will be used.
- Yes = Left; No = Right



NON-CONSUMED questioned samples Y-STR Quant decision tree where LOWEST POSSIBLE Y-STRs are an option

- If T-Y < 0.0007 no further analysis.
- Y-STR analysis only performed in sexual assault and homicide cases
- Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
- If STR analysis is a possibility, it should be attempted first
- T-S and T-L cutoffs assume that up to 38 µL of extract will be dried down and rehydrated to a volume of 15 µL
- Yes = Left; No = Right



4.5 Choosing appropriate samples for STR and Y-STR amplifications

The number of samples chosen for amplification, as well as which samples are chosen, will depend on a variety of factors. In this section, the selection process is described by general offense types, including sexual assault, other major crimes, and property crimes. This section provides general guidelines which may not be appropriate for every case.

ICS Amplification
Each extraction set must include at least one amplified ICS.

- If an extraction set will include only STRs, then only an STR amplification of an ICS needs to be included.
- If the extraction set could include Y-STR amplification, then a male ICS must be used amplified by Y-STR.

Any ICS must be amplified if:

- T-S is less than 0.0530 ng/µL,
- ICS is from a known female source with a T-Y reading above 0.0200 (Exception: epithelial fractions from differential extractions may reasonably be expected to include some male carryover and do not require amplification based on a detectable T-Y reading.)
- ICS sperm (or sperm/substrate) fraction with no detected male DNA

Criteria for assessing ICSs are included in the first Interpretation section.

Sample selection in sexual assault cases

How many samples to amplify in sex assault cases

Routine guidelines for number of amplifications per victim in a case:

- For cases where a female victim remembers events and only one (male) suspect is alleged, only one sample is amplified.
- Situations where it may be appropriate to amplify more than one sample:
    i. Victim is male
    ii. Multiple suspects
    iii. Recent prior consensual sex
    iv. Victim has no recollection of events
    v. Multiple allegations of penetration (e.g. both oral and vaginal penetration alleged)
    vi. Pre-pubescent or vulnerable victim who may not be able to accurately describe events
    vii. Underwear collected outside a sexual assault kit, where it is necessary to demonstrate both “wearer” and possible alleged assailant

How to prioritize samples in sex assault cases

Choice of which sample(s) to amplify is based on results of DNA quantification. Priorities are:

a. Likelihood of obtaining an STR profile from a victim SAK suitable for comparison to a suspect / entry in CODIS AND the item in question is consistent with penetration.
b. Likelihood of obtaining an STR profile from a suspect SAK suitable for comparison to a victim AND the item in question is consistent with penetration.
c. Likelihood of obtaining an STR profile from a victim SAK suitable for comparison to a suspect / entry in CODIS AND the item in question is NOT consistent with penetration.
d. Likelihood of obtaining an STR profile from a suspect SAK suitable for comparison to a victim AND the item in question is NOT consistent with penetration.
e. Likelihood of obtaining a Y-STR profile from a victim SAK suitable for comparison to a suspect AND the item in question is consistent with penetration.
f. Likelihood of obtaining a Y-STR profile from a victim SAK suitable for comparison to a suspect AND the item in question is NOT consistent with penetration.

STR amplification is generally preferable to Y-STR amplification based on its greater power of discrimination, as well as its ability to distinguish between patrilineal male relatives (except for identical twins). However, Y-STR amplification is preferred for sex assault cases when:

a. A high percentage of female DNA means that STRs are unlikely to yield a probative male profile, or
b. The amount of available DNA is so small that amplifying for STRs would require use of the entire extract, but there is enough present for Y-STR amplification.

All relevant submitted reference samples (victim, suspect, and/or elimination) are amplified in any case where questioned samples yield results suitable for comparison.

Sample selection in non-sex assault major crime cases

How many samples to select in non-sex assault major crime cases

In most non-sex assault major crimes cases, one or several samples might be extracted; but typically, two to four samples are amplified. However, given the complex nature of some cases in this category, that number could vary substantially. Examples of criteria which go into assessment of number of amplifications include:

- Number of possible contributors
- Number of items of potentially probative value

How to prioritize samples in non-sex assault major crime cases

- Highest priority is obtaining an STR profile suitable for comparison. (Note: Y-STRs are rarely performed in these cases unless sex assault is also suspected). This considers both quantity and quality of DNA present.
- Particularly for non-suspect cases, obtaining a profile suitable for CODIS entry is a high priority.
- For complex cases, when possible, confer with a case management supervisor, the submitting officer, and / or Department of Law to gather input for sample prioritization.

Sample selection in property crime cases

How many samples to select in property crime cases

In most property crime cases, one or several samples might be extracted, but typically only one is amplified. Exceptions are made for cases with more than one suspect, such as when:

- Submitting officer described a scenario with more than one suspect
- Quantification results indicate likely single source extracts including both female and male extracts
- Recovered evidence suggests more than one suspect (cigarette butts from two different brands, items in a stolen vehicle recovered from the driver and passenger sides)

How to prioritize samples in property crime cases

- Highest priority is obtaining an STR profile suitable for comparison. (Note: Y-STRs are never performed in property crime cases). This considers both quantity and quality of DNA present
- Higher priority is given to items closer to the crime scene (such as blood stain from inside the building rather than outside the building)
- Items such as steering wheels where the owner is very likely to be present are not processed unless an elimination sample from the owner is also submitted. Exceptions are made when the owner has not been in contact with an item for an extended period.

Range of analyst discretion in prioritizing samples for amplification

- No additional documentation is needed
    - Amplifications are chosen based on the criteria listed above, including situations where it may be appropriate to amplify more than one sample.
    - Decision of whether to perform Y-STR amplification prior to or after knowns are received does not require documentation.
- Permissible with documentation for the reason if future testing options are not impacted
    - Decisions do not follow guidelines above, but for a specific case-scenario reason
    - In some rare sex assault and/or major crimes cases, it is best to amplify using both STRs and Y-STRs:

  - Amplifying some samples with STRs and others with Y-STRs in order to address specific allegations: for example, it may be possible to get a full CODIS-eligible STR profile from a neck swab, but also useful to amplify a vaginal swab for Y-STR to address an allegation of penetration
  - Amplifying the same extract with both STRs and Y-STRs to address the number of male contributors in a complex mixture: For example, Y-STR amplification in addition to STR amplification may give a better idea of the minimum number of male contributors in a complex minor component.
- Require a Biology Deviation Request Form - Decisions that could potentially impact options for current or future testing. This does not apply to actions performed at specific instruction by Department of Law (e.g., permission to consume evidence is granted).

Section 5 Amplification and Genetic Analysis

References:
- *ProFlex® PCR System User Guide*
- *Global Filer PCR Amplification Kit User Guide*
- *PowerPlexY23 for Use on the Applied Biosystems Genetic Analyzers – Technical Manual*
- *GlobalFiler™ Express PCR Amplification Kit User Guide – Applied Biosystems*

This section covers the following topics:

5.1 Selection of amplification target values
- For any sample where the original material has been consumed, at least half the extract must be retained. For example, for 40 µL of extract from a consumed sample, 2 µL are used for quantification and at least 20 µL are to be retained, leaving up to 18 µL available for amplification.
- For any sample where only half or less of the original material was extracted, the entire extract may be consumed by drying it down and reconstituting with sterile dH_2O to the appropriate volume (15 µL for STR or 17.5 µL for Y-STR)
- No sample extract may be amplified at a greater concentration than its corresponding reagent blank. (QAS 9.5.1.2 and 9.5.1.3)
    - Questioned reagent blanks are routinely amplified at their maximum possible concentration (38 µL elution is dried down and rehydrated with 15 µL sterile water for STR or 17.5 µL sterile water for Y-STR analysis).
        - Exception: When a proficiency test is worked as a stand-alone, such that the questioned reagent blanks correspond ONLY to proficiency test extracts, and none of the proficiency test extracts require concentration, then the questioned reagent blank may be amplified at full volume without concentrating the extract. This exception does not require prior approval from the Technical Manager.
        - NOTE: For extraction sets which include any samples which may ultimately be amplified by Y-STR, both questioned reagent blanks are routinely dried down and amplified: one by STR and one by Y-STR. For extraction sets in which no Y-STR will ever happen (due to sample type, case type, and/or quantification results), it is not necessary to amplify a reagent blank for Y-STR; the second reagent blank is dried down and retained.
        - NOTE: Questioned sample extraction sets include a third reagent blank which is dried down and retained for future analysis if needed.

- Known reagent blank amplification volumes are routinely 15 µL for STR or 17.5 µL for Y-STR; these are only dried down and rehydrated if a corresponding reference sample has also been dried down and rehydrated.

- On occasion, extracts may be so concentrated that they require a 1:10 or 1:100 dilution before an amplification reaction can be set up. In such cases, a portion of the sample extract is diluted with TE^{-4} buffer, and the dilution is then used to set up the amplification reaction. Diluted samples are noted as such on the amplification worksheet.
- When the DI is greater than 1, it may indicate degraded DNA or the presence of PCR inhibitors; but a DI of less than 1.20 has minimal impact on amplification. Note that very low template DNA samples (T-S < 0.0050 ng/µL) may also have a DI greater than 1, in which case the DI is not necessarily an indicator of degradation or inhibition.

5.1.1 Adjusting amplification targets based on QT Y-intercepts slightly out of range

Changes in Y-intercept of the standard curve can affect the reported concentrations of unknown samples according to the formula:

Concentration difference = $2^{\Delta C_T}$

If the Y-intercept decreases in value, DNA quantity can be underestimated; if the Y-intercept increases in value, DNA quantity can be overestimated.

Guidance in this section applies to any QT standard curve where Y-intercepts are slightly out of validation range, defined as follows:

HIGHER:
T-Y values 26.31 – 27.05
T-L values 25.61 – 26.25
T-S values 27.31 – 28.05
LOWER:
T-Y values of 25.05 – 25.79
T-L values of 24.25 – 24.89
T-S values 26.05 – 26.79

<u>REQUIRED:</u> Minimum amplification thresholds are doubled for consumed-sample extracts with an elevated Y-intercept (e.g., if flowcharts indicate that the minimum T-Y value for amplification is 0.0007, but the standard curve T-Y is 26.70, then the elevated T-Y Y-intercept value means the new cut-off is 0.0014.)

<u>Documentation:</u> Note as a comment in LIMS-DNA – Min threshold for sample XXX raised due to elevated Y-intercept value.

<u>OPTIONAL - Y-int HIGH</u> (risk = under targeting): When amplification requires more than half the extract, or when samples are likely to be mixtures, the analyst may choose to manually adjust amplification targets (or amp volumes) in the override column in LIMS-DNA (see example).

Documentation: Manual calculations must be included in comments section in LIMS-DNA and must be technically reviewed. Note with calculations – Amp target adjusted due to elevated Y-intercept value.

OPTIONAL - Y-int HIGH (risk = under targeting): For low-level extracts made from half the original evidence, the analyst has the option to double the minimum amplification threshold (as is required for consumed sample extracts) **or** proceed to amplification using the existing minimum threshold. This decision will be made based on case-specific circumstances.

Documentation: No documentation is required if the extract is amplified. If the extract is not amplified because the higher threshold is applied, then note with quant documentation in the LIMS-DNA – Min threshold raised due to elevated Y-intercept value.

OPTIONAL - Y-int LOW (risk = over targeting): When amplification requires more than half the extract, the analyst may choose to manually adjust amplification targets or amp volumes (see example).

Documentation: Manual calculations must be included in comments section in LIMS-DNA and must be technically reviewed. Note with calculations – Amp target adjusted due to low Y-intercept value.

How to adjust amp values for Y-intercept values outside range:

1. **Find ΔC_T**

   For T-Y: Y-Intercept from your QT – 26.05

   For T-L: Y-intercept from your QT – 25.25

   For T-S: Y-intercept from your QT – 27.05

2. **Use that value to calculate concentration difference:**

   Concentration difference = $2^{\Delta C_T}$

3. **Use concentration difference to adjust amp target:**

   New amp target = previously calculated amp target * concentration difference

Example 1: Y-Intercept for T-Y in your quantification report is 26.700, which is HIGHER than accepted range (risk = under targeting)

The sample you wish to amplify has T-Y of 0.0200 (no degradation observed) and was made by cutting half the swab (so you can use all remaining 38 µL of extract).

Without adjustment, the calculated amount of extract for targeting 0.5 ng is **25 µL**.

ΔCT = 26.700 – 26.05 = 0.65

Concentration difference = 2^0.65 = 1.57

Adjusted amp volume = 25 µL * 1.57 = **39.25 µL** (In this example, that would mean using the entire extract.)

Example 2: Y-Intercept for T-S in your quantification report is 26.300, which is LOWER than the accepted range (risk = over targeting)

The sample you wish to amplify has a T-S of 0.0400 (no degradation observed) and was made by cutting half the swab (so you can use all remaining 38 µL of extract).

With no adjustment, the calculated amount of extract for targeting 1.0 ng is **25 µL**.

ΔCT = 26.300 – 27.05 = -0.75

Concentration difference = 2^-0.75 = 0.59

Adjusted amp target = 1.0 * 0.59 = 0.59

When you enter an override target of 0.59 ng, the amp volume becomes **14.75 µL** (In this example, that would mean you don't have to dry it down)

5.2 GlobalFiler (STR) Amplification

- Amplification volumes are selected based on the Quantifiler Trio Small Autosomal (T-S) results. Forensic casework amplifications should be targeted at 0.50 to 1.00 ng if sufficient extract is available. Exceeding an input of 1.00 ng can lead to increased artifacts, which can in turn complicate interpretation. See Selection of Amplification Target Values above for exceptions based on degradation.
- Add TE^{-4} buffer (manufacturer may label this as DNA Suspension Buffer) to bring the sample to a final volume of 15μL.
- Low-level samples appropriate to consume should be dried down with GenTegra-DNA and reconstituted with sterile water to make best use of the extract. This will typically be 15μL of sterile water but may be adjusted such that the target input is as high as possible. This includes:
    - Non-consumed original evidence - target value 0.5 ng - 0.076 ng for single source or 0.5 ng - 0.266 ng for mixture (T-S 0.0132 down to 0.0070 mix / 0.0020 SS, but values may be adjusted for DI), using all of the created extract
    - Consumed sample extracts – target value between 0.5 ng and 0.076 ng for single source or 0.266 ng for mixture (T-S 0.0625 to 0.0140 mix / 0.0040 SS, but values may be adjusted for DI), using up to 18μL of the created extract, OR
    - Consumed sample extracts where written permission has been granted by DOL to use all of the extract (T-S 0.0140 – 0.0070 mix / T-S 0.0040 – 0.0020 SS, but values may be adjusted for DI)
- Amplifying casework extracts created and retained in previous batches: When extracts previously dried down are reconstituted for amplification, the analyst must assess whether a corresponding reagent blank needs to be run.
    - If a corresponding reagent blank has previously been run at the same or higher concentration as the newly reconstituted casework extract, there is no need to amplify the remaining reagent blank replicate. LIMS-DNA documentation must indicate the batch in which the reagent blank was originally amplified so the analyst and technical reviewer can confirm its results.
    - If a corresponding reagent blank has not previously been run at the same or higher concentration as the newly reconstituted casework extract, a retained corresponding reagent blank replicate is rehydrated with 15 μL sterile water and amplified along with the casework extract.
        - On occasion, no corresponding reagent blank remains for analysis. In such cases, the analyst must consult with the Technical Manager to determine an appropriate course of action.
- *If sufficient extract is available*, the following amplification modifications are recommended:
    - For questioned extracts with a DI of 1.20 to 2.00: target 2.00 ng to 1.00 ng DNA, based on T-S.
    - For questioned and known extracts with a DI greater than 2.00: target 1.00 ng to 0.50 ng DNA based on T-L instead of T-S.
    - Note: for known extracts with a DI of 1.20 – 2.00, it is not necessary to increase the target DNA, since these are single source samples which are likely to yield full profiles even with some degradation present.

- Internal Control Specimen: At least one ICS per extraction set must be amplified, including GlobalFiler and/or PowerPlex Y23 as applicable to the set. The amplified ICS can be from either a questioned or known sample. See note below.
- Positive Amplification Control: Vortex Control DNA and spin the tube briefly. Add 10μL control DNA to 5μL of TE^{-4} buffer in a 0.5mL tube. See note below.
- Negative Amplification Control: Add 15μL of TE^{-4} buffer.

Note: A minimum of two DNA Control 007 positive amplification controls are routinely amplified and processed concurrently with each casework questioned amplification. A minimum of one DNA Control 007 positive amplification control is routinely amplified and processed concurrently with each casework known amplification. (QAS 9.5.3.1)

ProFlex thermal cycler heating is controlled by zones. A 96 well plate includes six zones, each comprised of two columns (i.e., columns 1 and 2 comprise zone 1, columns 3 and 4 comprise zone 2, and so on through zone 6). To ensure that each zone is performing appropriately, each zone used on an amplification plate must include at least one of the following as a means of ensuring that thermal cycling is performing correctly:

- A positive amplification control
- An ICS
- On a reference sample plate, a high-quality reference sample with a known profile – that is, one expected to yield a full profile with minimal degradation effects, from a previously typed individual such as a duplicate offender/staff sample, offender confirmation sample, casework known sample with a verified profile, or a buccal swab obtained from laboratory personnel whose genetic profile(s) was/were previously determined and documented

Each amplification plate must include DNA Control 007 as a positive amplification control (including two amplifications on questioned sample plates) – it is NOT necessary for each zone used on a questioned amplification plate to include two DNA Control 007 positive controls. When no DNA Control 007 amp was successful on a plate, use of an alternate positive control (as described in the bullet points above) must have documented approval of the Technical Manager so that potentially non-functioning tubes of DNA Control 007 can be promptly identified and taken offline.

Preparing a GlobalFiler amplification:

Amplification Master Mix Set Up

- If amplification reagents have been frozen, allow them to warm to room temperature, then vortex for 3 seconds and centrifuge briefly before opening tubes. Thawing is only required during the first use of a tube. After first use, reagents are stored at 2-8°C
- Transfer the amplification reagents to the designated PCR set-up area.

Note: Do not expose reagents to light for extended periods of time.
Note: Centrifugation of the primer mix should be minimal to avoid primer collecting at the bottom of the tube.

- Prepare a PCR master mix by adding the following volumes of reagents to an appropriately sized tube:
    - # of samples x 7.5µL Global Filer Master Mix
    - # of samples x 2.5µL Global Filer Primer Set

- Vortex the master mix and spin briefly.

Note: Prepare enough for a few extra reactions to allow for loss during pipetting. This is calculated automatically on the DNA amplification/electrophoresis worksheet (database) or in LIMS-DNA (casework).
Note: It is not essential to prepare a master mix for a small number of reactions.
Note: Preparation of the amplification reagents takes place in the PCR prep room. Once the reagents have been aliquoted into the plate, the plate, covered with Press and Seal, may be taken to the extraction lab for loading the samples or sample lysates.

- If casework extracts have been stored at 2-8°C, allow them to warm to room temperature, then vortex and spin briefly using a centrifuge.
- Prepare the samples to be amplified in individual tubes, using a total volume of **15µL.** Refer to Amplification and Genetic Analysis section for more details.
- Vortex the amplification master mix and spin briefly. Transfer **10µL** of master mix to each sample well of a 96-well amplification plate (optical skirted plate or non-skirted plate is acceptable).
- Cover the entire well plate with Glad® Press ‘n Seal (or equivalent, such as aluminum foil or kim wipes).
- Transfer the entire **15µL** of the prepared samples to the appropriate sample wells containing the PCR master mix. During sample addition, the pipette tip is inserted through the Press ’n Seal (or through aluminum foil, or kim wipes are shifted immediately before addition of sample).

Amplification on the thermal cycler

- Ensure thermal cycler has been turned on to allow the instrument time to properly warm up.
- Once all samples have been added to the amplification plate, remove the Press ’n Seal (or other plate covering) and cover the plate with a sheet of amplification tape.
- Transfer the plate to the PCR room and place directly into the thermal cycler.
- Close the heated cover.
- Double-check the selected program, ramp speed, and volume:
    - **program: gf-cswk**
    - **Max** ramping mode is used for amplification
    - Volume is **25 µL**
- Plate should ideally be removed from the thermal cycler less than 24 hours after completion of the run.
- On completion of the run, the amplified DNA is stored at 2 to 8°C. All amplified products will be disposed of upon completion of the technical and administrative reviews.

5.3 PowerPlex Y23 (Y-STR) Amplification

- Autosomal STR analysis is preferable to Y-STR analysis whenever possible, based on its much greater power of discrimination.
- In the absence of applicable reference samples, Y-STR profiles may have limited or no investigative value. When sufficient male DNA is present and at analyst discretion, Y-STR analysis may be either be performed prior to submittal of suspect reference sample(s) or may be performed after reference sample(s) are requested and submitted.
- Amplification volumes are selected based on the Quantifiler Trio Small Autosomal (T-S) results for reference samples, and for Male (T-Y) for questioned samples. Forensic casework amplifications should be targeted at 0.50 ng to 0.25 ng if sufficient extract is available. Exceeding an input of 0.50 ng can lead to increased artifacts, which can in turn complicate interpretation. See Selection of Amplification Target Values above for exceptions based on degradation.
- Low-level samples (concentration < 0.0130 ng/µL) appropriate to consume (suspect cases where written permission has been granted by DOL) should be dried down with GenTegra-DNA and reconstituted with sterile water to make best use of the extract. This will typically be 17.5µL of sterile water but may be adjusted such that the target input is as close to 0.50 ng as possible.
- Low-level samples appropriate to consume should be dried down with GenTegra-DNA and reconstituted with sterile water to make best use of the extract. This will typically be 17.5µL of sterile water but may be adjusted such that the target input is as high as possible. This includes:
    - Non-consumed original evidence - target value 0.25 ng - 0.027 ng (T-Y 0.0132 to 0.0007, but values may be adjusted for DI), using all of the created extract
    - Consumed sample extracts – target value between 0.25 ng and 0.027 ng (T-Y 0.0139 to 0.0015, but values may be adjusted for DI), using up to 18µL of the created extract, OR
    - Consumed sample extracts where written permission has been granted by DOL to use all the extract (T-Y 0.0015 – 0.0007, but values may be adjusted for DI)
- Amplifying casework extracts created and retained in previous batches: When extracts previously dried down are reconstituted for amplification, the analyst must assess whether a corresponding reagent blank has previously been run.
    - If a corresponding reagent blank has not previously been run for Y-STRs at the same or higher concentration as the newly reconstituted casework extract, the dried down corresponding questioned reagent blank replicate is rehydrated with 17.5 µL sterile water and amplified along with the casework extract.
        - On occasion, no corresponding reagent blank remains for analysis. In such cases, the analyst must consult with the Technical Manager to determine an appropriate course of action.
- *If sufficient extract is available*, the following amplification modifications are recommended:
    - For reference extracts with a DI greater than 2.00: target 0.50 ng to 0.25 ng based on T-L instead of T-S.
    - For questioned extracts with a DI of 1.20 – 2.00, target 1.00 ng to 0.050 based on T-Y

  - For questioned extracts with a DI greater than 2.00: target 0.25 ng to 0.50 ng * DI, based on T-Y (Example: for a questioned extract with a DI of 3.00, the template target would be 0.50 ng * 3.00 = 1.50 ng)
  - Note: for known extracts with a DI of 1.20 – 2.00, it is not necessary to increase the target DNA, since these are single source samples which are likely to yield full profiles even with some degradation present.
- Positive Amplification Control: Vortex Control DNA and spin the tube briefly. The 2800M control DNA is typically in 25 µL of 10 ng/µL solution. The first analyst to use the tube may dilute it to a 1.00 ng/µL solution by adding 225 µL of TE^{-4} buffer. Note on the tube the new concentration of the solution. Amplify 0.30 - 0.50 ng of the positive control (Ex: 0.5 µL of a 1:10 diluted 2800M standard + 17 µL TE^{-4} buffer). See note below.
- Negative Amplification Control: Add 17.5µL of TE^{-4} buffer.
- An ICS for each extraction set must be amplified by STRs, and it is necessary to additionally amplify using Y-STRs if Y-STR amplification could be considered for any samples in that extraction set type. (For example, a questioned extraction set which included only property crimes would NOT typically require Y-STR amplification, since Y-STRs are not typically performed on property crime casework.) Therefore, a male ICS is routinely used for this purpose. See note below.

  Note: A minimum of two DNA 2800M positive amplification controls are routinely amplified and processed concurrently with each casework questioned amplification. A minimum of one DNA 2800M positive amplification control is routinely amplified and processed concurrently with each casework known amplification. (QAS 9.5.3.1)

  ProFlex thermal cycler heating is controlled by zones. A 96 well plate includes six zones, each comprised of two columns (i.e., columns 1 and 2 comprise zone 1, columns 3 and 4 comprise zone 2, and so on through zone 6). To check that each zone is performing appropriately, each zone used on an amplification plate must include at least one of the following as a means of checking that thermal cycling is performing correctly:
- A positive amplification control
- An ICS
- On a reference sample plate, a high-quality reference sample with a known profile – that is, one expected to yield a full profile with minimal degradation effects, from a previously typed individual such as a duplicate offender/staff sample, offender confirmation sample, casework known sample with a verified profile, or a buccal swab obtained from laboratory personnel whose genetic profile(s) was/were previously determined and documented.

  Each amplification plate must be set up to include DNA 2800M as a positive control (including two DNA 2800M amplifications on questioned sample plates) – it is NOT necessary for each zone used on a questioned amplification plate to include two DNA 2800M positive controls. When no DNA 2800M amp was successful on a plate, use of an alternate positive control (as described in the bullet points above) must have documented approval of the Technical Manager so that potentially non-functioning tubes of DNA 2800M can be promptly identified and taken offline.

Preparing a PowerPlex Y23 amplification:

Amplification Master Mix Set Up

- If amplification reagents have been frozen, allow them to warm to room temperature, then vortex for 3 seconds and centrifuge briefly before opening tubes. Thawing is only required during the first use of a tube. After first use, reagents are stored at 2-8°C
- Transfer the amplification reagents to the designated PCR set-up area.

  Note: Do not expose reagents to light for extended periods of time.
  Note: Centrifugation of the primer mix should be minimal to avoid primer collecting at the bottom of the tube.

- Prepare a PCR master mix by adding the following volumes of reagents to an appropriately sized tube:

  - # of samples x 5µL PowerPlex Y23 5X Master Mix
  - # of samples x 2.5µL PowerPlex Y23 10X Primer Pair Mix

- Vortex the master mix and spin briefly.

  Note: Prepare enough for a few extra reactions to allow for loss during pipetting. This is calculated automatically in LIMS-DNA.
  Note: It is not essential to prepare a master mix for a small number of reactions.
  Note: Preparation of the amplification reagents takes place in the PCR prep room. Once the reagents have been aliquoted into the plate, the plate, covered with Press and Seal, may be taken to the extraction lab for loading the samples or sample lysates.

- If casework extracts have been stored at 2-8°C, allow them to warm to room temperature, then vortex and spin briefly using a centrifuge.
- Prepare the samples to be amplified in individual tubes, using a total volume of **17.5µL**. Refer to Amplification and Genetic Analysis section for more details.
- Vortex the amplification master mix and spin briefly. Transfer **7.5µL** of master mix to each sample well of a 96-well amplification plate (optical skirted plate or non-skirted plate is acceptable).
- Cover the entire well plate with Glad® Press ‘n Seal (or equivalent, such as aluminum foil or kim wipes).
  Transfer the entire **17.5µL** of the above prepared samples to the appropriate sample well, already containing the PCR master mix. During sample addition, the pipette tip is inserted through the Press ’n Seal (or through aluminum foil, or kim wipes are shifted immediately before addition of sample).

Amplification on the thermal cycler

- Ensure thermal cycler has been turned on to allow the instrument time to properly warm up.
- Once all samples have been added to the amplification plate, remove the Press 'n Seal (or other plate covering) and cover the plate with a sheet of amplification tape.
- Transfer the plate to the PCR room and place directly into the thermal cycler.
- Close the heated cover.
- Double-check the selected program, ramp speed, and volume:
    - **program: Y23-30cyc**
    - **Max** ramping mode is used for amplification
    - Volume is **25 µL**
- Plate should ideally be removed from the thermal cycler less than 24 hours after completion of the run.
- On completion of the run, the amplified DNA is stored at 2 to 8°C. All amplified products will be disposed of upon completion of the technical and administrative reviews.

5.4 Data Collection by 3500xl Genetic Analyzer

References: Applied Biosystems 3500/3500xL Genetic Analyzer User Guide

Start the Software and Instrument

- Turn the computer on and log into the computer
- Turn on the 3500xl Genetic Analyzer. Wait for the green status light to turn on.
- Ensure both the Daemon and Service Monitor have started by observing a green checkmark icon in the lower right-hand corner. This indicates that all 3500 services have loaded. This may take several minutes.
- Launch the Data Collection Software:
  Start > Programs > Applied Biosystems > 3500
- Log in to the 3500 Series Data Collection Software

Preparing the Instrument

- Navigate to the Dashboard of the software
- Check consumables by clicking **Refresh** to update consumable status.
- Refer to the Forensic Biology General Lab Maintenance manual if any maintenance or instrument preparations are required prior to running samples on the instrument.
- The expiration dates for the anode buffer and cathode buffer are “hard stops” – if these reagents are expired, they must be replaced for the run to proceed.
- The seven-days-on-instrument stop for the POP-4 polymer and the number of injections on a capillary are “soft stops” – as long as the resulting data for the ILS meets the quality standards described below, these stops may be overridden.
- Click **Start Pre-heat** to 60 °C to warm the oven.
- Check the pump assembly for bubbles and run the Remove Bubble wizard if necessary.

Sample Preparation for the 3500xl

- Prepare a loading master mix by adding the following volumes of reagents to an appropriately sized tube:

Amplification Kit	Amount of Size standard per sample	Amount Hi-Di Formamide per sample
GlobalFiler	0.4 µl GeneScan 600 LIZ	9.6µl
PowerPlex Y23	0.5µl WEN ILS 500 Y23	9.5µl
GlobalFiler Express	0.5 µl GeneScan 600 LIZ	9.5µl

Note: Prepare enough for a few extra reactions to allow for loss during pipetting.
Note: It is not necessary to prepare a master mix when only a few reactions are being prepared.

- Vortex the master mix and spin briefly. Transfer 10µl of master mix to appropriate sample wells of a 96 well plate.
- Add 10µl of Hi-Di Formamide to unused wells of a set of 24 (i.e. A1-H3).
- Add 1µl of allelic ladder and 1µl of each amplified sample to the appropriate wells. When all samples have been added, cover with plate septa.

- Briefly centrifuge the 96 well plate to remove any bubbles. Denature samples at 95˚C for 3 minutes and then snap chill for 3 minutes.
- Place the 96-well plate into the plate base provided with the instrument and snap the plate cover onto the plate, septa, and plate base.
- Verify that the holes of the plate retainer and the septa are aligned.
- Place the plate in the autosampler with the labels facing you and the notched corner of the plate in the notched corner of the autosampler. Close the instrument doors.

Creating a Plate Record

Create a Plate Record from the Data Collection Software

- In the dashboard click **Create New Plate**
- In the Define Plate Properties screen:
  - Enter a plate name (i.e. 10-1222KAL_Q_101224)
  - Select 96 for the number of wells
  - Select HID for plate type
  - Select 36cm for capillary length
  - Choose POP4 for polymer
  - Owner, barcode and description are optional fields.
- Click **Save Plate**
- Click **Assign Plate Contents**
- Using either plate view or table view enter sample names in the corresponding wells.
- Under the heading Assays, File Name Conventions, and Results Groups click **Add from Library** select the following Assay:
  - For GlobalFiler: AB GF_POP4_xl
  - For PowerPlex Y23: PPY23
  - For GlobalFiler Express: AB_J6OSR_LS_POP4_xl
- Select a naming convention, and a results group of your choice. Click **add to plate** and then click **close**.
- Select the wells for which to specify the assay file name conventions and results groups and enable the checkbox next to the name to assign it to the selected wells.
- Click **Save Plate** and save.

Create a Plate Record from an Export File

- Open a plate record template export file (i.e. .xls exported from instrument, or use export form LIMS-DNA).
- Copy and paste sample name info into the export file. (Rows not in use may be deleted but do not delete any columns) Save the record.
- Using the 3500xl Data Collection Software, click on **Create New Plate** on the dashboard.
- In the Define Plate Properties screen:
  - Enter a plate name (i.e. 10-1222KAL_Q_101224)
  - Select 96 for the number of wells
  - Select HID for plate type
  - Select 36cm for capillary length

  - Choose POP4 for polymer
  - Owner, barcode and description are optional fields.
- Click **Save Plate**.
- Click **Assign Plate Contents**.
- Click **Import** at the top of the screen and navigate to the saved plate record (make sure .xls files are searched).
- Click **Save Plate** and save.

Linking a Plate and Starting a Run

- Click **Link Plate for Run**. A message will appear saying Plate loaded successfully. Click **OK**.
- Ensure plate is linked to proper position on 3500xl.
- Select **Create Injection List**.
- Click **Start Run**.

Note: Ensure all prompts have cleared and the run begins before walking away.

Viewing Data During a Run

Refer to Applied Biosystems 3500/3500xl Genetic Analyzer User Guide

Storage of data files

After the run has finished, transfer a copy of the data from the 3500 computer to the designated location on the laboratory network. As of the effective date of this manual, CE data is stored at I: Discipline Shares/Forensic Biology/CE data. Copying the files to the laboratory network ensures that the data is regularly backed up.

Note: Location is dependent on network mapping and may vary slightly.

5.5 Data Analysis with GeneMapper™ ID-X

References: GeneMapper™ ID-X Software User Guide
GeneMapper™ ID-X Software Tutorial
GeneMapper™ ID-X Software User Bulletin

Logging in to GeneMapper™ ID-X

- Open GeneMapper™ ID-X
- Select the User Name and Database Host from the drop-down list and enter the appropriate password.
- Click OK.
- The main project window will open.

Creating a Project

- To add samples from the collection software at a workstation, go to the edit menu and select **Add Samples to Project**. Navigate to stored data as per current designation(s).

Note: Location is dependent on network mapping and may vary slightly.

- Select the raw data folder to be imported or select individual samples and click **Add to list**.
- When all samples have been selected, click **Add**.
- Select the appropriate Sample Type, Analysis Method, Panel and Size Standard for each sample and click the green arrow on the tool bar to analyze the samples.
    - For GlobalFiler:
        - Analysis method may be Global Filer Casework, Global Filer Blank, or Global Filer Troubleshooting (not used in routine casework)
        - Panel is GlobalFiler_Panel_v1.1.1X
        - Size Standard is GS600_LIZ(60-460)
    - For PowerPlex Y23:
        - Analysis Method is PowerPlex y23,
        - Panel is Alaska_PowerPlexY23_IDX_v2.0
        - Size Standard is WEN_ILS_500_IDX
    - For GlobalFiler Express:
        - Analysis Method is GFE
        - Panel is GlobalFiler_Express_v1.4.1X
        - Size Standard is GS600_LIZ_(60-460)

- The user will be prompted to name and save the project to the appropriate Security Group (GeneMapper ID-X Security Group). Projects are typically named with the Batch name, a Q or K, and the run date. Click OK.
    - Example: 15-0426CMD_Q_150428

- Analysis is complete when the green arrows in the Status column on the left are gone, and an Analysis Summary Screen appears.

Viewing the Data

- The ILS is viewed by highlighting all samples and clicking on the Size Match Editor, the icon with the red peaks on the toolbar.
- To view each sample, highlight the sample and click on the icon with the colored peaks to display plots. (This can also be done by View > display plots or Ctrl + L).
- Electropherograms can be printed from the sample's plot window. Various plots have been created for different sample types. Commonly used plot displays include:
    - GF Casework
    - GF and GFE Blank
    - GF and GFE Artifact View
    - PPY23 Casework
    - PPY23 Blank
    - Traditional Genotype Plot
    - Casework Blank
    - Casework artifacts
    - Casework zoom view
- To view information on a sample's injection time and other run information, highlight the sample of interest and click **View** on the toolbar > Sample Info (Ctrl + F1).
- To view raw data for a sample, click **View > Raw Data**.
- To view allele calls by sample and locus click the Genotypes tab from the main project window.

Section 5.6: Amplification and Analysis by RapidHIT

Modified Rapid DNA analysis, as performed with Rapid HIT instrumentation, is the semi-automated process of developing a CODIS acceptable (as applicable) STR profile from a casework or database reference sample. The "swab in – profile out" process consists of automated extraction, amplification with GlobalFiler Express chemistry, and separation and detection without human intervention, but requires an analyst to perform manual interpretation and technical review.

The RapidHIT™ ID System is approved for use with RapidHIT™ ID ACE GlobalFiler™ Express Sample Cartridge for single-source buccal swabs. The resulting profile is then analyzed using the RapidLINK™ Software.

Starting the instrument:

Press the power button on the front lower-right of the instrument. The button will change from green to blue. The startup screen will be displayed until the instrument finishes a system check and system prime (if needed). Once complete, the instrument will display the lock screen.

NOTE: Main power switch on back of instrument power should be kept on at all times to maintain the gel temperature.

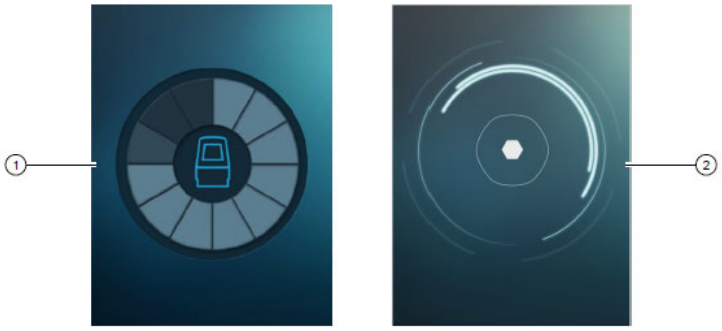
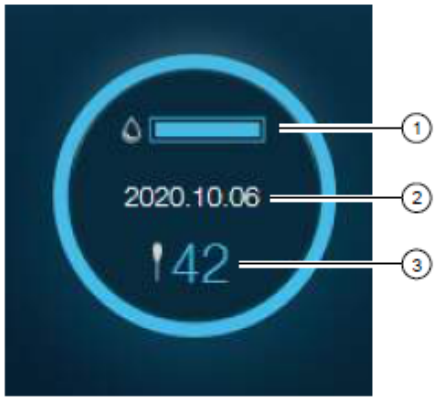


Figure 1 Startup and lock screens
① Startup screen
② Lock screen

On the sample identification screen, you can see the gel volume, expiration date, and number of runs that have been performed for the primary cartridge. The primary cartridge is good for ~150 sample runs.

Note: the expiration date listed here is the one set by the manufacturer for the reagent lot, and it may not account for length of time on the instrument. See FBGLM for details about setting an expiration date for the primary cartridge.



① Gel volume—Indicates the amount of gel remaining in the primary cartridge. When the amount of gel remaining is 30–16%, the outer ring turns yellow. When the amount of gel remaining is ≤15%, the outer ring turns red. The primary cartridge, including gel, must be changed when the outer ring is red.

② Expiration date—Indicates the expiration date of the gel or the primary cartridge, whichever is closest to expiration. The primary cartridge, including gel, must be changed when the expiration date is reached. Buffer expiration is considered in the primary cartridge expiration date.

③ Number of runs—The run count for the primary cartridge. The run count indicates the number of runs that have been performed, not the number of runs that are remaining (for example, "42" indicates that 42 runs have been performed).

Samples eligible for RapidHIT analysis using ACE GFE cartridges

The best candidates are those where one whole buccal swab, collected within the past five years, can be used. Older samples are less likely to yield a complete profile and are routinely extracted by EZ biorobot and quantified prior to amplification. RapidHIT analysis may be attempted on buccal swabs older than five years if abundant sample remains for re-testing, if needed. Partial buccal swabs, buccal swab cuttings, or any other sample types, are not acceptable for RapidHIT GFE ACE analysis.

Preparing a sample cartridge

Sample cartridges are stored at approximately 4ºC. They are individually packaged in resealable silver foil envelopes with purple labels. Control cartridges are included with shipments of sample cartridges and have different color labels.

Sample cartridge preparation should happen in an extraction laboratory space.

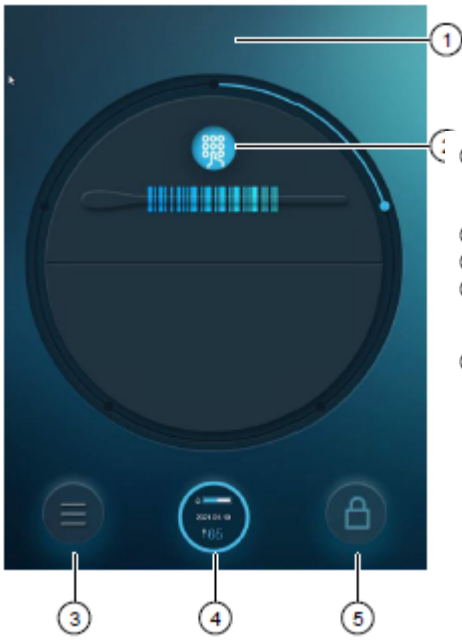
Snap a swab stick into the cartridge. NOTE: Snapping the swab stick so that it is just below the lid will ensure that the sample is held in place near the bottom of the sample well without blocking the bottom and will make it easier to remove the extracted swab if re-analysis is required.

Once the sample is loaded into the cartridge, keep the sample cartridge in its foil envelope until it is put on the instrument.

Casework and Database Samples

Running a sample:

1. Tap the lock screen to wake instrument up and sign in.
2. Tap the keypad button to enter the sample identification number.
    a. "ladder", "posctrl", and "negctrl" should not be included in the naming.



① No icon is displayed if the instrument is connected to the RapidLINK™ Software.
Indicates that the instrument is not connected to the RapidLINK™ Software
See "Check the RapidLINK™ Software connection" on page 46.
② Touch to display the keyboard to enter a sample identification number.
③ Touch to display the menu screen.
④ Displays the run count for the primary (reagent) cartridge. The run count indicates the number of runs that have been performed, not the number of runs that are remaining (for example, "65" indicates that 65 runs have been performed). For more information, see "View primary cartridge information" on page 36.
⑤ Touch to sign out and lock the touchscreen (displays the lock screen).

3. Place the cartridge into the instrument when prompted.
    a. NOTE: The run will start automatically.
    b. The run will take ~90-110 minutes to complete. The countdown timer starts at 110 minutes, but the typical run time is ~90 minutes. The timer stops when the run is complete and does not decrease to 0 minutes.
4. Once the run is complete, sign in and remove the sample cartridge from the instrument when instructed. Review the sample results.
    a. NOTE: The remove sample cartridge screen will only be displayed for ~30 seconds before the signed-in user is automatically signed out.
    b. NOTE: Do not remove the cartridge until you are prompted to do so.

Status	DNA profile is generated	Meaning	Action
Green 	Yes	The DNA profile does not contain quality score flags.	The DNA profile is ready for analysis by the RapidLINK™ Software. No further action is needed on the instrument.
Yellow 	Yes	• The DNA profile contains quality score flags, or • The run result contains only size standard peaks. It does not contain sample peaks.	The DNA profile is available for review in the RapidLINK™ Software. Proceed according to your standard operating protocol.
Red 	No	The DNA profile was not generated.	A DNA profile is *not* available for review in the RapidLINK™ Software. Proceed according to your standard operating protocol.

5. Tap **Done** to dismiss the result screen.
6. Review the sample data (see below). If no further analysis is needed, discard the sample cartridge in a designated biohazard bin. If re-extraction of the swab is needed, move the swab material from the cartridge into a sterile microfuge tube and discard the empty sample cartridge.
    a. Re-extraction is performed following the Questioned Direct protocol and must also include a corresponding ICS (if applicable) and reagent blank. NOTE: while this uses the questioned direct protocol, the re-extraction of a reference swab should NOT be performed within a set of questioned samples. However, it may be performed as part of a set of other reference samples.
7. Place a negative control cartridge, empty used sample cartridge, or utility cartridge in the Rapid instrument between runs. Note: Instrument must be in “Lock Screen” when inserting these types of cartridges between runs.

Reviewing Results/Electropherogram in the RapidLINK Software:

1. Open and log in to the RapidLINK Software.
2. Click on the Runs Screen tab to view instrument runs.
3. Click on the Sample Name of the sample you want to view. This will open the sample and its associated Allelic Ladder in GeneMarker.
4. Close out the view of the Allelic Ladder and double click on the sample you want to view from the sample tree on the left side of the screen.
    a. If you need to make edits/review the electropherogram, click the Browse By All Colors button at the top of the screen. Close this window when complete.
    b. NOTE: Any deleted peaks will be completely removed from the electropherogram.
5. Click the printer icon to print the sample electropherogram. (Note: do not click on the printer icon in the Browse By All Colors window.)
    a. Choose “EgramsOnlyExample Setting” in the dialog window.
    b. Under Dyes, ensure that orange is clicked on so that the ILS is included
    c. Click Preview and print to PDF onto a thumb drive.
    d. NOTE: The EgramsOnlyExample setting typically prints in such a way that the ILS peaks are clearly legible. If they are not, analysts may select another print

view in which the ILS calls are clearly legible. Also note that the EgramsOnlyExample view includes the related allelic ladder. The allelic ladder pages do not need to be included in the bench notes.

6. To export the Allele Report seen on the right side of the screen, click the Save icon and save to a thumb drive in .csv format.
7. Close out of GeneMarker.

Printing reagent and run documentation (Consolidated Audit Report):

1. Use the filter options in the Runs Screen tab of the RapidLINK Software to display the sample(s) ran for a specific day.
2. Click the PDF icon and save the document to a thumb drive.

Documentation:

- Casework only: Packaging and sampling information is documented in JT on an FB Analysis form.
- Electropherograms, including ILS
- Reagent and run documentation (Consolidated Audit Report) This page should be added to JT as an attachment to the request.
- Review of controls (positive, negative, and reagent blank): The front page of the verification documentation for the primary cartridge and the sample cartridge includes the technical review of control samples. Each of these front pages must be included as a JT attachment as well.

Raw data:

- Data from casework sample runs must be copied over from the RapidLink computer to be backed up on the I: drive prior to technical review.
- Data from CODIS analysis and CODIS confirmations will be backed up semi-annually and does not require transfer prior to technical review

Control samples

For more detailed directions on changing the RapidHIT primary cartridge, refer to the FBGLM and the RapidHIT System User Guide.

When a Running a control cartridge:

- Insert the control cartridge in the instrument. The instrument reads the label on the cartridge and automatically assigns the sample identification as “POSCTRL”, “NEGCTRL”, or “LADDER”.
- When the run is complete, remove the cartridge from the instrument.
- Review the status
    - Allelic ladder

        - Green check means no quality flags were tripped and expected alleles were called. (Passing allelic ladders are added to the allelic ladder library on the instrument.)
        - Red X means results were not as expected, or expected alleles were not called in the allelic ladder profile.
    - Positive Control
        - Green check means no quality flags were tripped and expected alleles were called.
        - Red X means results were not as expected, or expected alleles were not called in the allelic ladder profile
    - Negative Control
        - Green check means no quality flags were tripped, and no alleles were called in the negative control profile
        - Red X means alleles were called in the negative control
- Any failing control (red X) should be re-attempted with a new control cartridge. If additional control sample cartridges are not available, or if the second attempt also fails, consult the Technical Manager for a course of action.

Following successful status checks, electropherograms for each passing control must be manually interpreted by a qualified analyst. Manual comparisons are performed by checking the control data against the information listed below using the following procedures. Note: To ensure that all allele calls are legible in the allelic ladder documentation, in the Print window, select EgramsAndTableExample instead of PDF. This view will include allele calls in tabular form, split out by color. Since the ILS does not print in this view, the ILS can be printed separately in PDF view with only orange selected.

Documentation: Following technical review, verification paperwork is retained in SharePoint.

In addition, copies of the review page of the verification paperwork for the installed primary cartridge and the sample cartridge are included as JT attachments to the relevant request to capture the reviews performed on the controls as well as lot numbers and expiration dates.

Control samples are run on the RapidHIT instrument each time a new primary cartridge is installed. These include an allelic ladder, a positive control, and a negative control. An allelic ladder, positive control, and negative control must each be run, determined to be passing, and technically reviewed before the new primary cartridge can be used for casework or database samples. Passing controls are valid until the expiration or removal of the primary cartridge.

Section 6 Initial Interpretation in GMID-X / GeneMarker

Interpretation of controls and casework samples is based on the guidelines contained in this manual, as well as the qualified DNA analyst's training and experience, experimental results, and case specifics. In all cases, interpretations must be independently reviewed and verified based on established peer review procedures. The DNA Technical Manager may authorize the interpretation/reporting of data outside of the criteria defined herein and will document this approval with a Biology Deviation Request Form. Guidance specific to interpretation and report writing for proficiency tests can be found here.

This section covers the following topics:

	GlobalFiler	PowerPlex Y23	RapidHIT
Criteria for interpretation of batch controls	ILS	ILS	ILS
	Allelic ladder	Allelic ladder	Allelic Ladder
	ICS	ICS	
	Positive amp control	Positive amp control	Positive amp control
	Negative amp control and reagent blanks	Negative amp control and reagent blanks	Negative amp control
Criteria for interpretation of non-allelic peaks	Stutter	Stutter	Stutter
	Other artifacts	Other artifacts	Other artifacts
Criteria for interpretation of special cases	Off-ladder Tri-alleles	Off-ladder Duplication and null alleles	Out of Bin Tri-alleles

Note: These topics are addressed for GlobalFiler Express in the database protocol found here.

Section 6A Criteria for interpretation of controls, non-allelic peaks, and special case alleles - GlobalFiler

Criteria to evaluate internal size standards, allelic ladders, and analytical controls

Prior to assessment of casework data, the analyst must first examine the batch controls to ensure that the extraction, amplification, and genetic analysis processes are functioning correctly. These include: Internal Lane Standard (ILS), allelic ladder(s), internal control specimen (ICS), positive and negative amplification controls, and extraction reagent blanks. All or part of an extraction, amplification and/or run may need to be repeated depending on the results of the batch controls. Any issues raised by the performance of a batch control must be addressed prior to the release of affected casework samples. Unresolved failure of a batch control requires the Technical Manager, or designated individual, to approve a course of action.

Review of batch controls is routinely performed by viewing the project in GMID-X. The analyst performing the review visually examines each of the batch controls (without relying solely on software quality flags). Printouts of batch control documentation will be included as attachments in the Detection set in LIMS-DNA only when the control in question requires further investigation, such as contamination assessment in a reagent blank.

Interpretation of Batch Controls (QAS 9.6.1)

Internal Lane Standard (ILS) – GF – see image here

Global Filer is run with GeneScan 600 LIZ Internal Lane Standard (ILS). The analyst should verify that all peaks from 60-460 base pairs are present and labeled as shown. This verification may consist of confirming the sizing quality is greater than 0.5. If the sizing quality is 0.5 or lower, the analyst must confirm by visual inspection that each peak was correctly called. This check must be documented on the electropherogram for casework profiles, or in the LIMS-DNA packet for control samples.

The peak heights for the ILS peaks must be at least 160RFU (relative fluorescence units) to be identified and labeled by the GeneMapper™ ID-X software with a casework analysis method.

In case of ILS failure: Failure of an ILS (SQ = 0.5 or lower) can often be resolved by re-prepping and/or re-injecting the affected sample(s). If the ILS continues to fail after re-prepping and re-injecting, the analyst should consult the Technical manager for a course of action. Approval or approval with modification by the Technical Manager will be issued with a Biology Deviation Request Form. If approval is not issued, then the sample must be re-run and sample data from the plate with the failing ILS will not be used.

Refer to the GeneMapper™ID-X Software User Guide for details on manually labeling the size standard.

Allelic Ladder – GF – see image here
The GeneMapper™ ID-X software uses the allele calls of the ladder to assign allele calls to all the other samples in the project. The allele calls for the Global Filer allelic ladder are shown. The analyst should verify that all peaks from the allelic ladder(s) are present and labeled as shown.

The allelic ladder contains the most common alleles determined for each of the following loci: D3S1358, vWA, D16S539, CSF1PO, TPOX, Y-INDEL, Amelogenin, D8S1179, D21S11, D18S51, DYS391, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, and D2S1338. In addition, alleles not labeled in the allelic ladder (virtual alleles), may be detected and labeled in some of the samples analyzed.

At least one allelic ladder must be included in a GeneMapper™ ID-X project with the sample type designated as “allelic ladder”. All the peaks in the allelic ladder must be labeled correctly for the software to assign the correct allele calls to samples in the run. When more than one ladder in a project has the sample, type designated as “allelic ladder”, the software will average the allelic ladders to make the allele calls for the samples in the project.

Artifacts such as spikes or “pull-up” peaks may be present that are labeled as off- ladder (OL) peaks in the allelic ladder. If all the true allele peaks are labeled correctly, such OL peaks will not affect the sample allele calls. If a spurious peak (not a true allelic peak) is labeled as a true allele (i.e. it falls within an allele size range and is designated and labeled as an allele) at any particular locus, the remaining allele designations / labels at the locus shift and will impact the correct allele call / labeling of the samples in the project. Allelic ladders with such artifacts cannot be used to analyze data in the project. In such instances, re-injection is usually appropriate.

In case of allelic ladder failure: If a capillary electrophoresis run includes multiple allelic ladders,and at least one passing allelic ladder can be successfully applied to a given injection, then the non-passing ladder(s) can be changed from allelic ladder to sample in the GMID-X project and noted as failed injection in the LIMS-DNA packet. If there is no passing allelic ladder in the project which allows for successful analysis of samples, the the plate must be re-run and the corresponding sample data cannot be used.

Internal Control Specimen (ICS) – GF and PPY23

Definition of an Internal Control Specimen: An internal control specimen (ICS) is a designated, predetermined quality control sample whose expected profile is known based on prior amplification results. While reagent blanks are designed to assess the possibility of contamination in extraction reagents, the purpose of the ICS is to demonstrate that all analytical processes prior to quantification are working correctly to produce an extract with amplifiable DNA. The ICS is processed with an extraction batch and carried through at least quantification concurrently with the other samples. An ICS may include any known sample, including those obtained from laboratory staff, where the individual has been previously typed to generate a genetic profile.

Casework batches often contain multiple samples from previously typed individuals. Any one of these may be designated as the ICS for a set of known extractions. As good QA practice, the analyst confirms that all profiles from previously typed individuals yield expected profiles. Offender profiles verified in casework are documented by linking the cases in JT and filling out the appropriate offender evidence custom form.

ICS extracts are discarded following completion of the DNA technical review of the batch.

When are ICSs created: At least one ICS shall be created with each set of casework extractions.

How are ICSs processed: All ICS samples are quantified to ensure that they contain sufficient amplifiable DNA (T-S greater than or equal to 0.053 ng/µL), and that the presence or absence of male DNA, as seen in T-Y, is consistent with expected results based on known sample type.

When are ICSs amplified: A minimum of one ICS per extraction set must be amplified and must yield a correct profile.

When more than one ICS extract exists for an extraction set (such as multiple fractions of a differential extraction), an ICS extract must be amplified if:

- T-S is less than 0.053 ng/µL,
- ICS must be amplified by both GlobalFiler and PowerPlex Y23 if it is from a known female source with a T-Y reading above 0.02 (Exception: epithelial fractions from differential extractions may reasonably be expected to include some male carryover and do not require amplification based on a detectable T-Y reading.)
- ICS sperm (or sperm/substrate) fraction with no detected male DNA

Multiple ICS extracts from a differential extraction set may need to be amplified in batches where troubleshooting is required, e.g. assessing the possibility of sample switching.

Interpretation of ICSs: ICSs are expected to yield full profiles that are consistent with expected typing results. However, a partial profile for an ICS does not necessarily invalidate

the batch. If a minimum of fifteen complete CODIS core loci are detected for the ICS, with no inconsistent allele calls, the ICS is acceptable. The Technical Manager, or other designated individual, shall determine whether or not to approve the ICS when fewer than fifteen complete core loci are detected for the ICS. The data quality of other positive control samples in the batch and/or the quality of other concurrently extracted samples will be reviewed to determine the approval process.

The presence of artifacts in an ICS does not constitute a failed ICS, so long as the artifacts are not consistent with contamination. Artifacts are struck in the GMID-X project and checked during the electronic review of batch controls.

In case of ICS failure and uninterpretable data: If one ICS is amplified and does not yield a sufficiently complete profile, another ICS from the same batch must be amplified. All batch data is considered uninterpretable until and unless the issue is resolved.

In any of these situations (or similar situations), the analyst will initiate a Quality Assurance Review and attempt to determine the cause of the discrepancy:

- An incorrect STR profile is obtained for the ICS in a casework batch
- No designated ICS yielded a sufficiently complete profile
- An ICS was omitted from an extraction set

Positive Amplification Control – GF – see image here
A positive control is defined as a single source sample whose genetic profile was previously determined and from which a full profile was developed; it is used to evaluate the performance of the amplification and typing procedures. DNA Control 007 is used as a positive control for amplification with Global Filer Kit.

In case of positive control failure and uninterpretable data: A positive control sample which yields no profile, an incorrect profile, or an incomplete profile is considered a failed positive control. This includes DNA Control 007 as well as ICS or reference samples used to assess zone-wise performance of the thermal cycler. All data from any amplification zone which does not have at least one passing positive control is considered uninterpretable until and unless the issue is resolved.

The presence of artifacts in a positive control does not constitute a failed positive control, so long as they are not consistent with contamination. Artifacts are struck in the GMID-X project and checked during the electronic review of batch controls.

It may be necessary to re-prep and/or re-inject the positive control if the cause of failure appears to be a failed electrophoretic injection. If the re-prepped and/or re-injected positive control types successfully, the positive control is considered successful. If the re-prepped and/or re-injected positive control does NOT type successfully, consult with the TM to determine the appropriate course of action.

Negative Amplification Control and Extraction Reagent Blanks - GF
A reagent blank is carried through the entire analytical process as part of each extraction set. It contains all the reagents - except DNA template - used during extraction, amplification and typing. For questioned extracts, each reagent blank is dried down. Only one of the blanks (either replicate -1 or the blank with the higher observed relevant quantification reading) is rehydrated with 15 µL sterile water and amplified. Reagent blank replicates not amplified are created as items in the LIMS and retained.
Note: LIMS-DNA packet must include the rationale for choosing which blank was amplified by which chemistry when possible contamination was seen in more than one extract, based on qualification results. On occasion, duplicate reagent blanks may each have low level results. In that case, the analyst should assess which reagent blank is most relevant to STR and which is most relevant to Y-STR amplification. Typically, the blank with the higher T-Y quantification result would be considered more relevant for Y-STR analysis; but if only one blank is being amplified and one blank is showing markedly higher concentrations of DNA, then it may be more relevant to amplify the reagent blank with the higher overall quantification values by STR. If the analyst cannot readily determine which reagent blank is most appropriate for amplification, they should consult with the Technical Manager.

The negative amplification control contains only the PCR master mix (reagents used to prepare the PCR amplification mixture) for each batch of samples and the TE^{-4} buffer used to dilute the DNA samples.

The purpose of the negative controls is to detect systemic DNA contamination that might occur from the reagents, the laboratory environment, between the samples being processed, and/or due to improper handling of the samples by the analyst. The negative controls must be run at the most stringent set of conditions for the batch. (QAS 9.5.3.1)

Verification of the presence of amplicon in the negative controls is performed by viewing the presence of unincorporated primer peaks. The negative controls should not yield any true STR allelic peaks above the analytical threshold. When peaks greater than or equal to the reporting threshold are present in the range between 60-460 base pairs, the analyst will determine if the peaks are artifacts (e.g., spike, pull-up) or true allele peaks.

The presence of peaks above the AT will not invalidate the sample as long as the data is at levels too low for interpretation, or the peaks can be shown to be artifacts. Artifacts will be struck in the project and checked in the electronic review of the batch controls.

Although appropriate quality assurance practices are stringently applied and enforced, it is not unexpected that low levels of adventitious DNA (i.e., levels that result in peak heights for allelic data that are below the Laboratory's reporting threshold) attributable to staff scientists and/or consumable products directly involved in the analytical steps of such a highly sensitive technique may be detected. If a low-level profile does not have at least four complete loci detected at the Troubleshooting threshold, it is not possible to determine the source and no further investigation or documentation is required.

In case of negative control failure and uninterpretable data: The failure of a negative control may indicate a problem at the extraction or amplification level. When probable true allele peak(s) are detected above the analytical threshold, the analyst will reanalyze the profile using the Global Filer troubleshooting analysis parameters (60 RFU). If the low-level profile has at least four complete loci detected at the troubleshooting threshold, this constitutes a failed negative control. All data from any amplification which does not have a passing negative amplification control is considered uninterpretable until and unless the issue is resolved. All samples which correspond to a failed reagent blank are considered uninterpretable until or unless the issue is resolved.

To resolve a failed negative control, the analyst will investigate possible causes of contamination and, if possible, will determine the source of the contaminating DNA. Such electropherograms are printed and uploaded as attachments to the Detection set in LIMS-DNA, with analysis parameters clearly noted. In some cases, the contamination may be attributable to a known source, such as an adjacent sample or a lab worker who was in close proximity to the extract.

- If the contamination observed in the negative control is not observed in the corresponding casework samples, then the analyst will complete a Contamination Assessment Form. This form is submitted to the DNA Technical Manager for approval and resolution of the issue. Once signed by the DNA Technical Manager, it is documented in a Case Activity for each related case file. When appropriate, unidentified sources of contmaination will be kept in GMID-X for comparison purposes.
- If the level of contamination has the potential to interfere with casework interpretation, whether or not its source is identified, a Qualty Assurance Review must be initiated, and the DNA Technical Manager must be notified to determine an appropriate course of action.

Criteria for the interpretation of non-allelic peaks (QAS 9.6.2) - GF
The initial assessment of a casework sample electropherogram separates interpretable alleles from artifacts. Artifacts can occur in data and much of their nature and origin have been determined and documented. Peaks determined to be artifacts are struck on the full view electropherogram. Peaks struck manually are also initialed by the analyst. When it is not possible to distinguish between an artifact and a true allele, this is indicated on the electropherogram as Artifact/True Allele (A/TA). If the presence of a definite or possible artifact at a locus impacts the suitability of that locus for either comparisons or statistical analysis, that is documented on the electropherogram. Artifacts are often associated with an overabundance of template DNA. If the presence of many artifacts complicates data analysis, it may be appropriate to re-amplify the sample with less template DNA.

On questioned samples, binned peaks which are called as alleles but determined to be artifacts must include an explanation of why the peak was determined to be an artifact (such as poor morphology, baseline noise associated with large peaks, pull-up, etc.). This explanation is documented on the electropherogram. Binned artifacts documented as pull-up also require an artifact view to show corresponding base pairs between large peak and the artifact in question. While these artifacts may not line up to have identical base pair sizes, they must be close (within 0.5 base pairs) and must correspond to a large peak (often off scale, but not necessarily) in another color. Artifacts in questioned samples which do not bin as alleles and are called OL, OMR or spike, may be struck without a documented explanation and without an artifact view printout. All artifacts in reference samples may be struck without documented explanation and do not require artifact view printouts. Stutter does not require artifact view printouts.

Criteria for the interpretation of non-allelic peaks – stutter - GF
The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter (N-4), two bases shorter (N-2, infrequently observed), eight base pairs shorter (N-8) or four base pairs larger (N+4) than the corresponding base peak. This is the result of slippage of the Taq polymerase and is referred to as the stutter peak or stutter product.

The proportion of stutter product relative to the main allele peak is calculated by dividing the height of the stutter peak by the height of the main allele peak. The stutter percentage is fairly consistent for a particular locus. The following table lists the values of the maximum expected percentage of N-4 stutter for the loci in the Global Filer System when run on the Applied Biosystems 3500xl. The stutter values are based on data obtained by internal validation studies. These values, along with a universal N+4 stutter of 2%, are set as stutter filters used by the GeneMapper ID-X software. N-8 stutter has been observed rarely, typically in samples with more than 1 ng template DNA. As such, N-8 stutter is not included in the filters.

Maximum Expected N-4 Stutter Percentages for Global Filer loci on the 3500xl (in single source samples)

D3S1358 11.0%	vWA 12.0%	D16S539 10.0%	CSF1PO 10.0%	TPOX 6.0%	
Y-INDEL NA	Amelogenin NA	D8S1179 10.0%	D21S11 11.0%	D18S51 13.0%	DYS391 8.0%
D2S441 9.0%	D19S433 11.0%	TH01 5.0%	FGA 13.0%		
D22S1045 (see below)	D5S818 10.0%	D13S317 10.0%	D7S820 10.0%	SE33 15.0%	
D10S1248 12.0%	D1S1656 13.0%	D12S391 14.0%	D2S1338 12.0%		

Additional max stutter percentages:

- D22S1045 (-3 BP) 17.0%
- D22S1045 (+3 BP) 7.0%
- SE33 (-2BP) 5.0%
- D1S1656 (-2 BP) 3.0%

Peaks in the stutter position greater than the listed values may indicate the presence of DNA from more than one individual. However, elevated stutter is also associated with both excess template DNA and very low amounts of template DNA.

Elevated +4 / -4 stutter

NOTE: In situations where a minor component is not suitable for comparison to probative reference samples, and that minor component includes peaks which may be elevated +4/-4 stutter, it is not necessary to perform the calculations described below.

When two large peaks at a given locus are eight base pairs apart, elevated stutter is commonly observed halfway between the large peaks. (For example, when large 28 and 30 peaks are detected at D21S11, it is common to observe a 29 peak above the maximum expected stutter.) This is likely due to the additive effect of +4 stutter from the first peak and -4 stutter from the second. Elevated +4 / -4 stutter may be documented in one of two ways:

1. An additional 2.0% is added to the above maximum expected stutter percentages for alleles in +4 / -4 stutter positions. On the electropherogram, the analyst calculates the -4 stutter percentage and can document as follows: "~X % < Y% + 2.0% Elevated +/- stutter", where X is the calculated -4 stutter percentage and Y is the maximum expected stutter percentage for that locus.
2. Alternatively, the analyst may calculate and note the maximum expected +4 stutter (2.0% for all loci) and maximum expected -4 stutter (varies by locus; see previous table). This may be necessary when the peak contributing +4 stutter is significantly larger than the peak contributing -4 stutter. If the sum of these two calculations is greater than the peak height of the observed peak, then the observed peak can be noted as elevated +/- stutter.

   Example: At D21S11, 28 has a peak height of 9000 RFU, 29 has a peak height of 190 RFU, and 30 has a peak height of 800 RFU. The maximum expected +4 stutter for the 28 would be 9000 x 0.02 = 180. The maximum expected -4 stutter for the 30 would be 800 x 0.11 = 88. Thus, the maximum +4 / -4 expected stutter would be 180 + 88 = 268 RFU. Since the peak height of the 29 is 190 RFU, elevated stutter could be documented as follows: Max +4 = 180; Max -4 = 88; Total = 268; 190 < 268 Elevated +/- stutter

Note: In some cases, it may be useful to show this documentation as a way of confirming that a minor component is **not** attributable to elevated +/- stutter.

Assessment of stutter / true allele

NOTE: In situations where a minor component is not suitable for comparison to probative reference samples, and that minor component includes peaks which may be stutter or true alleles, it is not necessary to perform the calculations described below.

Striking a stutter peak is acceptable in the following situations:

- In questioned samples, when there is no indication of a mixture other than elevated stutter at four or fewer loci, the analyst will document the stutter as such on the electropherogram (including the calculated % and a notation that it was determined to be an elevated stutter peak).

- In questioned samples, when the +4 / -4 combined maximum expected stutter contribution is larger than the observed peak height in the stutter position, the analyst will document as described in the previous bullet.
- Reference samples are assumed to be single source. Therefore, any elevated stutter may be struck without further documentation.

Noting a stutter peak as indistinguishable artifact or true allele (A/TA) in questioned samples is acceptable in the following situations:

- When expected stutter contribution (either +4, -4, or +4 / -4) is subtracted from a peak in a stutter position and the resulting peak is below the analytical threshold (AT) of 160 RFU, the peak should be noted as A/TA.
- When possible elevated stutter peaks are observed at five or more loci in an otherwise apparent single-source questioned profile, and no minor peaks (or one minor peak) are observed outside of stutter positions, the elevated stutter peaks are documented as A/TA.

Subtracting stutter

In mixture interpretation, many instances arise where one peak is in a stutter position to another. In some circumstances, it is appropriate to subtract stutter contribution from a peak in a stutter position to a larger peak. Since this requires manual calculations that must be checked by a technical reviewer, this should be done judiciously. Examples of instances when maximum stutter should be subtracted from the smaller peak height are:

- Calculating contributor ratios in a two-source mixture If possible, it is better to use loci where stutter position is not a factor. If not possible, it is not necessary to subtract stutter from a sister allele if the sister peaks are adjacent (e.g., if the sister alleles for one contributor are 14 and 15, it is not necessary to subtract forward or backward stutter from either one)
- Assessing whether or not a peak is above IT
- Assigning major and minor contributors
- Subtracting stutter impacts a decision in a mixture deduction flowchart

Criteria for the interpretation of non-allelic peaks - Other Amplification and Injection Artifacts – GF, PPY23, and RapidHIT

Artifacts are data peaks resulting from the analytical process and are not attributable to an individual contributor to the sample. Peaks determined to be artifacts are either struck on the printed electropherogram for RapidHIT (not in GeneMarker) with a brief explanation for the strike (e.g., -A, pull-up, elevated stutter, etc.), or stuck in the GeneMapper ID-X project for control samples.

Baseline noise - GF
The analytical threshold (AT) for analysis of casework samples is 160 RFU. Data below the AT is considered indistinguishable from baseline noise and not suitable for interpretation.

Ideally, true allele peak heights should fall between 3000 and 12,000 RFU. Samples with peak heights near or above 12,000 RFU may include baseline noise that exceeds 160 RFU. Such baseline noise may be struck. However, if baseline noise is difficult to distinguish from possible low-level DNA, it may be more appropriate to re-amplify the sample with less template DNA.

Baseline noise – PPY23
The analytical threshold (AT) for Y-STR analysis of casework samples is 100 RFU. Data below the AT is considered indistinguishable from baseline noise and not suitable for interpretation. Ideally, true allele peak heights should fall between 2000 and 10,000 RFU. Samples with peak heights near or above 10,000 RFU may include baseline noise that exceeds 100 RFU. Such baseline noise may be struck. However, if baseline noise is difficult to distinguish from possible low-level DNA, it may be more appropriate to re-amplify the sample with less template DNA.

Baseline noise – RapidHIT
When artifacts are not pervasive throughout the sample and do not interfere with data interpretation, the sample does not require re-analysis. When an artifact is present within the size range of the alleles in a core locus and the presence of the artifact interferes with interpretation of the data in that locus, or when a sample exhibits artifacts in several loci, interpretation of the sample becomes more challenging, and it may be necessary to re-process the sample using a non-Rapid questioned direct or known direct protocol.

Injections with poor sizing quality – GF, PPY23, and RapidHIT
Poor injections can lead to broad peaks, which in turn can adversely impact interpretation by increasing the possibility of allelic drop out. These issues usually can be resolved by reinjection. Any sample with a sizing quality of 0.5 or below, or any sample which has loci with broad peaks, is only acceptable for interpretation if it is a single-source sample, and if all loci with broad peaks are heterozygous (with the exception of DYS391, which only has one allele in a single source male sample).
Note for RapidHIT: The instrument should flag ILS broad peaks as non-passing. However, manual review must confirm that ILS peaks sized correctly.

The following are examples of artifacts which may be struck and initialed on electropherograms. Re-prep and/or re-injection is not required if the artifact in question is clearly distinguishable and thus does not interfere with interpretation.

Pull-Up – GF, PPY23, and RapidHIT
Multi-component analysis is the process that separates the six different fluorescent dye colors into distinct spectral components. The dyes (for example, in GlobalFiler: 6-FAM, VIC, NED, TAZ, SID, and LIZ) emit their maximum fluorescence at different wavelengths, but there is some overlap of the emission spectra. A spectral calibration is performed for a specific dye set to create a matrix that corrects for the spectral overlap.

Pull-up is the result of incomplete separation of the emission spectra and is typically observed as a non-allelic peak at the same base size as a peak in another dye, often about 1.5% of the main peak. When 1 ng of DNA is targeted with GF, or 0.5 ng with PPY23, this is most likely to be present above AT associated with large peaks, typically homozygotes. Documentation of a peak as pull-up is required for questioned samples and includes a close up view of the locus in question as well as the corresponding color with the peak causing the pull-up, with base pairs included on the zoomed view electropherogram. Pull-up in reference sample profiles may be struck without the addition of zoomed view documentation.

Spikes – GF, PPY23, and RapidHIT
Spikes are non-allele peaks that may arise due to air bubbles, urea crystals or voltage spikes. Spikes are typically quite sharp and easy to distinguish from a true allele. Spikes usually appear in more than one color at the same base size and are not reproducible by re-injection.

Dye Blobs – GF, PPY23, and RapidHIT
Dye blobs, like spikes, are non-allele peaks that are easily distinguished from true alleles. Dye blobs may be the result from residual dye molecules left over from the synthesis of the primers or they may be dye molecules that fell off the primer during the amplification.

Incomplete 'A' nucleotide addition (-A or split peaks) – GF, PPY23, and RapidHIT
While –A peaks are a known artifact, none of these were observed in the 3500xl or RapidHIT validation studies. As such, any instances of –A require approval by the Technical Manager before the sample injection may be used for casework.

Persistent Kit Artifacts– GF, PPY23, and RapidHIT
Occasionally, one or more lots of PCR kits may exhibit persistent artifacts. These artifacts may or may not appear as true alleles. Sometimes, these artifacts are so common that they are observed in a high number of routine samples. Such artifacts will be noted in the validation or verification process and acknowledged by the Technical Manager. Alternatively, they may be documented in a bulletin from the kit manufacturer. Once documented, these artifacts should be struck and initialed, and do not require TM acknowledgement when observed in routine analysis.

Contamination – GF, PPY23, and RapidHIT
The verification of reagents prior to use in casework is conducted in part to detect contamination which permeates an entire lot of a given reagent or set of reagents. However, such checks will not necessarily detect contamination arising from a single event (for example, cross-contamination between two adjacent samples, or point contamination of a single consumable).

For casework samples, whenever data suitable for comparison is not attributable to any reference samples associated with its case, the possibility that the profile was introduced via contamination must be considered. As a part of the review process, both the analyst and the technical reviewer must rule out laboratory staff working in direct proximity to the open evidence as well as cross-contamination within the batch of samples. This comparison may be done manually or via the Profile Comparison tool in GeneMapper ID-X. The same principle applies to control samples where no DNA should be detected.

Furthermore, an assessment must be conducted to establish the source of the contamination. A Quality Assurance Review may be pursued depending on the results of the root cause analysis, or a Contamination Assessment Form must be completed if no further actions are deemed necessary (such as a profile attributable to the manufacturer).

Note for Y-STRs: Validation studies have shown that it is possible for some female contributors to show some low-level peaks when amplified with PowerPlex Y23. In situations where an unidentified low-level male contributor is observed, it may be necessary to rule out low-level detection of a female contributor.

Notes for RapidHIT: The presence of more than two peaks or severe PHR imbalance at one locus may be explained by a tri-allele, primer binding site mutation, variant allele, or other biological or procedural artifact. The presence of these at several loci is indicative of a mixed sample.

For reference samples, whenever four or more alleles indicative of contamination are called in a sample, that sample must be re-processed to obtain a single source profile.

The global 20% filter removes most low-level peaks from detection, but validation studies indicated that this did not necessarily apply at gender markers.

Criteria for the interpretation of tri-alleles and off-ladder alleles

Tri-alleles - GF
Occasionally, a single-source sample may be observed to have three alleles at one (rarely more) locus/loci. Tri-allelic patterns have been observed in single source samples and there are documented instances where different tissues from the same individual may or may not exhibit the tri-allelic pattern.

Samples exhibiting potential tri-alleles may be re-amplified to confirm the genotype observed at the locus (or loci). Statistical calculations, however, will not be performed at these loci.

If the potential tri-allele containing sample is re-amplified and found to be reproducible, the analyst may consider the tri-allele to be a reproducible observation without the A/TA designation. If the potential tri-allele is not reproducible, it will be labeled as an Artifact/True allele (A/TA).

If the same tri-allelic pattern is also observed in multiple samples (from the same case) but from different amplifications – for example, blood / semen / saliva / hair, with the same STR profile, the potential tri-allele may be considered a reproducible observation without the A/TA designation. No re-amplification of samples would be required in such instances.

Off-Ladder (OL) Alleles – GF and PPY23

The allelic ladder contains the most commonly observed alleles for the STR / Y-STR loci. Alleles which are not assigned a numeric designation in the standard ladder will be designated as off-ladder (OL) alleles. Peaks that are labeled as OL (off ladder) at a locus have a base size that is different from any of the alleles in the ladder and any of the virtual alleles. These peaks may be true alleles, but additional analysis is required to verify this.

Before determining whether an OL is a true allele or an artifact, the analyst must consider all other possible causes of OL occurrence.

When a true OL allele lies within the range of alleles for a particular locus, the allele will be reported as a variant of the integer (i.e. X.1, X.2, etc.). The appropriate allele call is determined by simultaneously examining the base sizes for the sample allele peak and the associated allelic ladder peaks in the relevant locus.

When the OL allele lies either above or below the largest or smallest allele in the ladder, the OL allele will be designated as either greater than (>) or less than (<) the allele from the ladder that is closest in proximity to the OL allele.

OL alleles considered to be true alleles may require re-injection to confirm, particularly when they are only observed in a mixture. If the OL allele is observed in multiple amplifications from a single item (such as two stains from the same item of clothing, or the sperm and epithelial fractions from a vaginal swab), further amplifications are not necessary to verify the OL allele. The DNA Technical Manager must approve reporting an OL allele seen only once in casework amplification and not confirmed by re-injection (e.g., limited amount of sample available for analysis when amplified product is not available for re-injection).

Documentation of the OL allele must include an electropherogram with both the sample and the associated allelic ladder for the locus, showing the OL allele(s) in the locus, with the bins and base pair sizes. The OL allele designation is to be written on the full view electropherogram.

Section 6B Criteria for interpretation of controls, non-allelic peaks, and special cases – PowerPlex Y23

Interpretation of Batch Controls (QAS 9.6.1)

Prior to assessment of casework data, the analyst must first examine the batch controls to ensure that the extraction, amplification and genetic analysis processes are functioning correctly. These include internal lane standard (ILS), allelic ladder(s), internal control specimen (ICS), positive and negative amplification controls, and extraction reagent blanks. All or part of an extraction, amplification and/or run may need to be repeated depending on the results of the batch controls. Any issues raised by the performance of a batch control must be addressed prior to the release of affected casework samples. Unresolved failure of a batch control requires the Technical Manager, or designated individual, to approve a course of action.

Review of batch controls is routinely performed by viewing the project in GMID-X. The analyst performing the review visually examines each of the batch controls (without relying solely on software quality flags). Printouts of batch control documentation will be included as an attachment in the Detection set in LIMS-DNA only when the control in question requires further investigation, such as contamination assessment in a reagent blank.

Internal Lane Standard (ILS) – PPY23 – see image here.
PowerPlex Y23 is run with WEN ILS 500 Internal Lane Standard. The analyst should verify that all peaks from 60-500 base pairs are present and labeled as shown. This verification may consist of confirming the sizing quality is greater than 0.5. If the sizing quality is 0.5 or lower, the analyst must confirm by visual inspection that each peak was correctly called. This check must be documented on the electropherogram by the analyst and initialed by the technical reviewer for casework profiles, or in the LIMS-DNA packet for control samples.

The peak heights for the ILS peaks must be at least 100RFU (relative fluorescence units) to be identified and labeled by the GeneMapper™ ID-X software with a casework analysis method.

Failure of an ILS can often be resolved by re-prepping and/or re-injecting the affected sample(s). If the ILS continues to fail after re-prepping and re-injecting, the analyst should consult the Technical manager for a course of action.

Refer to the GeneMapper™ID-X Software User Guide for details on manually labeling the size standard.

Allelic Ladder – PPY23 - see image here
The GeneMapper™ ID-X software uses the allele calls of the ladder to assign allele calls to all the other samples in the project. The analyst should verify that all peaks from the allelic ladder(s) are present and labeled as shown here.

The allelic ladder contains the most common alleles determined for each of the following loci: DYS576, DYS389 I, DYS448, DYS389 II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385, DYS456, and YGATAH4. In addition, alleles not labeled in the allelic ladder (virtual alleles), may be detected and labeled in some of the samples analyzed.

At least one allelic ladder must be included in a GeneMapper™ ID-X project with the sample type designated as “allelic ladder”. All the peaks in the allelic ladder must be labeled correctly for the software to assign the correct allele calls to samples in the run. When more than one ladder in a project has the sample type designated as “allelic ladder”, the software will average the allelic ladders to make the allele calls for the samples in the project.

Artifacts such as spikes or “pull-up” peaks may be present that are labeled as off- ladder (OL) peaks in the allelic ladder. If all the true allele peaks are labeled correctly, such OL peaks will not affect the sample allele calls. If a spurious peak (not a true allelic peak) is labeled as a true allele (i.e., it falls within an allele size range and is designated and labeled as an allele) at any particular locus, the remaining allele designations / labels at the locus shift and will impact the correct allele call / labeling of the samples in the project. Allelic ladders with such artifacts cannot be used to analyze data in the project. In such instances, re-injection is usually appropriate.

Internal Control Specimen (ICS) – PPY23

Refer to the GlobalFiler section on addressing ICS assessment and ICS failure.

Positive Amplification Control – PPY23 – see image here
A positive control is defined as a single source sample whose genetic profile was previously determined and from which a full profile was developed; it is used to evaluate the performance of the amplification and typing procedures. DNA 2800M is used as a positive control for amplification with PowerPlex Y23 Kit.

In case of positive control failure and uninterpretable data: A positive control sample which yields no profile, an incorrect profile, or an incomplete profile is considered a failed positive control. This includes DNA 2800M as well as ICS or reference samples used to assess zone-wise performance of the thermal cycler. All data from any amplification zone which does not have at least one passing positive control is considered uninterpretable until and unless the issue is resolved.

The presence of artifacts in a positive control does not constitute a failed positive control, so long as they are not consistent with contamination. Artifacts are struck in the GMID-X project and checked during the electronic review of batch controls.

It may be necessary to re-prep and/or re-inject the positive control if the cause of failure appears to be a failed electrophoretic injection. If the re-prepped and/or re-injected positive control types successfully, the positive control is considered successful. If the re-prepped and/or re-injected positive control does NOT type successfully, consult with the TM to determine the appropriate course of action.

Negative Amplification Control and Extraction Reagent Blanks – PPY23
A reagent blank is carried through the entire analytical process as part of each extraction or type of extraction. It contains all the reagents - except DNA template - used during extraction, amplification and typing. For questioned extracts, each reagent blank is dried down. Only one of the blanks (either replicate -1 or the blank with the higher observed quantification reading) is rehydrated with 17.5 µL sterile water and amplified. Reagent blank replicates not amplified are created as items in the LIMS and retained.

Note: LIMS-DNA packet documentation must include the rationale for choosing which blank was amplified by which chemistry when possible contamination was seen in both quantification results. On occasion, duplicate reagent blanks may each have low level results. In that case, the analyst should assess which reagent blank is most relevant to STR and which is most relevant to Y-STR amplification. Typically, the blank with the higher T-Y quantification result would be considered more relevant for Y-STR analysis; but if only one blank is being amplified and one blank is showing markedly higher concentrations of DNA, then it may be more relevant to amplify the reagent blank with the higher overall quantification values by STR. If the analyst cannot readily determine which reagent blank is most appropriate for amplification, they should consult with the Technical Manager.

The negative amplification control contains only the PCR master mix (reagents used to prepare the PCR amplification mixture) for each batch of samples and the TE^{-4} buffer used to dilute the DNA samples.

The purpose of the negative controls is to detect systemic DNA contamination that might occur from the reagents, the laboratory environment, between the samples being processed, and/or due to improper handling of the samples by the analyst. The negative controls must be run at the most stringent set of conditions for the batch. (QAS 9.5.3.1) Verification of the presence of amplicon in the negative controls is performed by viewing the presence of unincorporated primer peaks. The negative controls should not yield any true STR allelic peaks above the analytical threshold. When peaks greater than or equal to the reporting threshold are present in the range between 60-500 base pairs, the analyst will determine if the peaks are artifacts (e.g., spike, pull-up) or true allele peaks.

The presence of peaks above the AT will not invalidate the sample if the data is at levels too low for interpretation, or the peaks can be shown to be artifacts. Artifacts will be struck in the project and checked in the electronic review of the batch controls.

Although appropriate quality assurance practices are stringently applied and enforced, it is not unexpected that low levels of adventitious DNA (i.e., levels that result in peak heights for allelic data that are below the Laboratory's reporting threshold) attributable to staff scientists and/or consumable products directly involved in the analytical steps of such a highly sensitive technique may be detected.

Negative control failure and uninterpretable data: The failure of a negative control may indicate a problem at the extraction or amplification level. When probable true allele peak(s) are detected above the analytical threshold, the analyst will reanalyze the profile

using the PPY23 troubleshooting analysis parameters (60 RFU). If the low-level profile has at least four complete loci detected at the troubleshooting threshold, this constitutes a failed negative control. All data from any amplification which does not have a passing negative amplification control is considered uninterpretable until and unless the issue is resolved. All samples which correspond to a failed reagent blank are considered uninterpretable until or unless the issue is resolved.

To resolve a failed negative control, the analyst will investigate possible causes of contamination and, if possible, will determine the source of the contaminating DNA. Such electropherograms are printed and included as attachments to the Detection set in LIMS-DNA, with analysis parameters clearly noted. In some cases, the contamination may be attributable to a known source, such as an adjacent sample or a lab worker who was in close proximity to the extract.

- If the contamination observed in the negative control is not observed in the corresponding casework samples, then the analyst will complete a Contamination Assessment Form. This form is submitted to the DNA Technical Manager for approval and resolution of the issue. Once signed by the DNA Technical Manager, it is documented in a Case Activity for each related case file. When appropriate, unidentified sources of contamination will be kept in GMID-X for comparison purposes.
- If the level of contamination has the potential to interfere with casework interpretation, whether or not its source is identified, a Quality Assurance Review must be initiated, and the DNA Technical Manager must be notified to determine an appropriate course of action.

Criteria for interpretation of non-allelic peaks (QAS 9.6.2) – PPY23

Distinguishing Alleles from Artifacts
The initial assessment of a casework sample electropherogram separates interpretable alleles from artifacts. Artifacts can occur in data and much of their nature and origin have been determined and documented. Peaks determined to be artifacts are struck on the full view electropherogram. Peaks struck manually are also initialed by the analyst. When it is not possible to distinguish between an artifact and a true allele, this is indicated on the electropherogram as Artifact/True Allele (A/TA). If the presence of a definite or possible artifact at a locus impacts the suitability of that locus for either comparisons or statistical analysis, that is documented on the electropherogram. Artifacts are often associated with an overabundance of template DNA. If the presence of many artifacts complicates data analysis, it may be appropriate to re-amplify the sample with less template DNA.

On questioned samples, binned peaks which are called as alleles but determined to be artifacts must include an explanation of why the peak was determined to be an artifact (such as poor morphology, baseline noise associated with large peaks, pull-up, etc.). This explanation is documented on the electropherogram. Binned artifacts documented as pull-up also require an artifact view to show corresponding base pairs between large peak and the artifact in question. While these artifacts may not line up to have identical base pair sizes, they must be close (within 0.5 base pairs) and must correspond to a large peak (often off scale, but not necessarily) in another color. Artifacts in questioned samples which do not bin as alleles and are called OL, OMR or spike, may be struck without a documented explanation and without an artifact view printout. All artifacts in reference samples may be struck without documented explanation and do not require artifact view printouts. Stutter does not require artifact view printouts.

Stutter – PPY23

The PCR amplification of tetranucleotide Y-STR loci typically produces a minor product peak four bases shorter (N-4), two bases shorter (N-2, infrequently observed), eight base pairs shorter (N-8) or four base pairs larger (N+4) than the corresponding base peak. This is the result of slippage of the Taq polymerase and is referred to as the stutter peak or stutter product. Loci with repeating units of different lengths have corresponding stutter as well.

The proportion of stutter product relative to the main allele peak is calculated by dividing the height of the stutter peak by the height of the main allele peak. The stutter percentage is fairly reproducible for a particular locus. The following table lists the values of the maximum expected percentage of stutter for the loci in the PowerPlex Y23 System when run on the Applied Biosystems 3500xl. The stutter values are based on data obtained by internal validation studies as well as developmental validation studies. These values are set as stutter filters used by the GeneMapper ID-X software. N-(2 repeat units) stutter has been observed rarely, typically in samples in excess of 1 ng template DNA. As such, N-(2 repeat units) stutter is not included in the filters.

Max Expected Stutter Percentages for PowerPlex Y23 loci on the 3500xl (single source samples)

	REPEAT	N-1 UNIT	Stutter percentage	N+1 UNIT	Stutter percentage
DYS576	4	N-4	16.4	N+4	5.0
DYS389 I	4	N-4	8.2	N+4	5.0
DYS448	6	N-6	4.0	N+6	5.0
DYS389 II	4	N-4	15.2	N+4	5.0
DYS19	4	N-4	11.2	N+4	5.0
		N-2	10.2	N+2	3.8
DYS391	4	N-4	12.3	N+4	5.0
DYS481	3	N-3	29.8	N+3	5.0
DYS549	4	N-4	11.4	N+4	5.0
DYS533	4	N-4	10.7	N+4	5.0
DYS438	5	N-5	5.8	N+5	5.0
DYS437	4	N-4	8.4	N+4	5.0
DYS570	4	N-4	15.9	N+4	5.0
DYS635	4	N-4	13.0	N+4	5.0
DYS390	4	N-4	13.4	N+4	5.0
DYS439	4	N-4	10.7	N+4	5.0
DYS392	3	N-3	17.8	N+3	11.0
DYS643	5	N-5	3.9	N+5	5.0
DYS393	5	N-5	15.1	N+5	5.0
DYS458	4	N-4	16.3	N+4	5.0
DYS385	4	N-4	15.7	N+4	5.0
DYS456	4	N-4	15.0	N+4	5.0
YGATAH4	4	N-4	11.1	N+4	5.0

Peaks in the stutter position greater than the listed values may indicate the presence of DNA from more than one individual. However, elevated stutter is also associated with low amounts of template DNA. Many peaks which appear as elevated stutter are below the stochastic threshold, where increased variability in peak heights can be expected.

Criteria for the interpretation of non-allelic peaks - Other Amplification and Injection Artifacts – PPY23

Refer to the discussion in the GlobalFiler section for discussion of baseline noise, contamination, pull-up, spikes, dye blobs, -A, and persistent kit artifacts.

Criteria for interpretation of Y-STR special cases – PPY23

Null alleles
Primer binding site mutations may occasionally result in a null allele, where no allele is amplified at a given locus. A locus may be called as having a null allele without confirmation by re-amplification:

- only in single-source profiles,
- when all other alleles in the profile are greater than IT, and
- based on quantitation results, there is no indication that the proposed null allele could be the result of degradation or inhibition.

A null allele that meets these conditions is designated by writing “Null allele” on the electropherogram at the locus. The null allele may be included for purposes of population frequency Y-STR database searches.

Duplications
DYS385 a/b often has two alleles present in a single source profile. Occasionally, a single-source sample may be observed to have two alleles at one (rarely more) other locus/loci. In a single source profile, two alleles may be considered a true duplication without confirmation by re-amplification if both alleles are above IT and have a peak height ratio of 60.0% or greater.

It may be possible to search a locus with a duplication in population statistic Y-STR database searches.

Off-ladders
Refer to the discussion in the GlobalFiler section.

Section 6C Criteria for interpretation of controls, non-allelic peaks, and special case alleles – GlobalFiler Express on RapidHIT with GeneMarker

Criteria to manually evaluate and review internal size standards, allelic ladders, and analytical controls

Internal Lane Standard (ILS) – RapidHIT – see table here
RapidHIT GlobalFiler Express uses an internal lane standard. GeneMarker software uses cubic spline interpolation for sizing of fragments. This method does not compensate for standard fragments that deviate from the norm, which can lead to sizing inaccuracies. The analyst and technical reviewer must carefully verify that all 36 peaks from 80-505 base pairs are present and labeled as shown for each profile.

Pass: An ILS passes when all peaks are correctly called.

Fail: An ILS fails if any peaks are not called correctly.

Actions following a failed ILS: If the ILS fails, the sample must be re-analyzed. Rapid analysis may be repeated, or the swab may be re-analyzed through the questioned direct protocol. If re-analysis is not possible or not successful, please consult Technical Manager to determine course of action.

Allelic Ladder – RapidHIT – see table here
The GeneMarker™ software uses the allele calls of an allelic ladder to assign allele calls to all the other samples in the project. The allelic ladder contains the most common alleles determined for each of the 20 FBI STR core loci, Amelogenin, DYS391, SE33 and a Y indel. Alleles not labeled in the allelic ladder (virtual alleles), may be detected and labeled in some of the samples analyzed.

GeneMarker™ software selects a 'best fit' allelic ladder from the ladder library (or the ladder run during primary cartridge installation). One allelic ladder is included in each sample run folder. All the peaks in the allelic ladder must be labeled correctly for the software to assign the correct allele calls to samples in the run. The analyst must verify that all peaks from the allelic ladder(s) are present and labeled as shown. Additional low-level binned peaks may be present in the ladder; these do not cause the allelic ladder to fail unless they adversely impact the calling of the required alleles.

Pass: An allelic ladder passes if all required peaks are correctly called.

Fail: An allelic ladder fails if any of the required peaks are not correctly called.

Actions following a failed allelic ladder: If an allelic ladder run during primary cartridge installation does not pass, a second ladder must be run. If the second ladder also fails, consult the Technical Leader for a course of action. The instrument will be offline until the issue is successfully resolved.

Note: Rapid DNA allelic ladders are run during verification of a new primary cartridge, and an allelic ladder must pass before the primary cartridge can be used for casework or database analysis. Rapid DNA allelic ladders are not processed in parallel with casework or database samples.

Positive Amplification Control - RapidHIT– see profile here

A positive control sample is defined as a single source sample whose genetic profile was previously determined and from which a full profile was developed; it is used to evaluate the performance of the primary cartridge. The standard DNA template 007 is currently used as the positive amplification control for the RapidHIT ID GFE ACE cartridges. A positive control cartridge is a specifically labeled, sealed, pre-loaded cartridge whose RFID tag reads as a positive control. It is run following installation of a primary cartridge.

Pass: A positive control passes if a full and correct profile is obtained.

Fail: A positive control fails if a partial or incorrect STR profile is obtained.

Actions following failed positive control: When an incorrect or partial STR profile is obtained for the positive control run following installation of a new primary cartridge, a second positive control sample should be run. If the second positive control fails, please discuss with the Technical Manager to determine a course of action for troubleshooting. The instrument will be offline until the issue is successfully resolved.

Note: Rapid DNA positive controls are run during verification of a new primary cartridge, and a positive control must pass before the primary cartridge can be used for casework or database analysis. Rapid DNA positive control samples are not processed in parallel with casework or database samples.

Negative Amplification Control - RapidHIT
The purpose of a reagent blank or negative control is to detect DNA contamination that might occur from the reagents, the laboratory environment, between the samples being processed, and/or due to improper handling of the samples by the analyst.

A negative control sample is a specifically labeled, sealed, empty cartridge whose RFID tag reads as a negative control. It is run following installation of a primary cartridge.

A reagent blank is an empty sample cartridge run during the verification of a new lot of sample cartridges.

A reagent blank or negative control should not yield any true STR allelic peaks above the reporting threshold. When peaks greater than or equal to the reporting threshold are present in the range between 80-500 base pairs, the analyst will determine if the peaks are artifacts (e.g., spike, pull-up) or true allele peaks.

Pass: A negative control passes if no STR allelic peaks are above the reporting threshold. However, the presence of peaks above the reporting threshold will not invalidate the sample as long as the peaks can be shown to be artifacts. Artifacts will be labeled on the electropherogram.

Fail: A negative control fails if one or more true allele peaks are detected above the reporting threshold.

Actions following a failed negative control: When probable true allele peaks are detected above the reporting threshold, at several or all STR loci, the analyst will investigate possible causes. The Technical Manager will be consulted to determine the appropriate course of action.

Since the failure of a negative control may indicate a problem at the extraction or amplification level, a Quality Review Form may need to be completed based on the nature of the discrepancy. A second negative control must be run to assess whether the possible contamination was systemic or point contamination confined to a single consumable.The instrument (or lot of sample cartridges, for reagent blanks) will be offline until the issue is successfully resolved.

Note: Although appropriate quality assurance practices are stringently applied and enforced, it is not unexpected that low levels of adventitious DNA (i.e., levels that result in peak heights for allelic data that are below the Laboratory's reporting threshold) attributable to staff scientists and/or consumable products directly involved in the analytical steps of such a highly sensitive technique may be detected.

Note: Rapid DNA negative control samples are run during verification of a new lot of primary cartridge and new lot of sample cartridges, and a negative control must pass before the primary cartridge / sample cartridge lot can be used for casework or database analysis. Rapid DNA negative control samples are not processed in parallel with casework or database samples.

Criteria for interpretation of non-allelic peaks (QAS 9.6.2) – RapidHIT

Stutter - RapidHIT

The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter than the corresponding base peak. This artifact is the result of slippage of the Taq polymerase and is referred to as the stutter peak or stutter product. Stutter products four bases longer, or two bases shorter, than the corresponding base peak are less frequently observed. Two base pair stutter products are so rare that peaks in these positions are not filtered out in the analysis parameters.

The proportion of stutter product relative to the main allele peak is calculated by dividing the height of the stutter peak by the height of the main allele peak. The stutter percentage is fairly reproducible for a particular locus.

The GeneMarker™ ID-X database analysis methods have a global stutter filter of 20%. Peaks in the stutter position greater than 20% may indicate the presence of DNA from more than one individual. When there is no indication of a mixture, and the data is determined to be elevated stutter, the elevated stutter labels may be deleted in the project and the reason documented electronically.

It is unlikely that the presence of stutter peaks would interfere with the interpretation of database samples and therefore, re-amplification and/or re-injection of the sample is rarely required.

Criteria for the interpretation of non-allelic peaks - Other Amplification and Injection Artifacts – RapidHIT

Refer to the discussion in the GlobalFiler section for discussion of baseline noise, contamination, pull-up, spikes, dye blobs, -A, and persistent kit artifacts.

Criteria for interpretation of RapidHIT special cases

Tri-alleles

Tri-allelic patterns (three alleles at a single locus) are known to occur in single source samples and there are documented instances where different tissues from the same individual may or may not exhibit the tri-allelic pattern.

For forensic casework:

Confirming a tri-allele requires re-amplification. Since this is not possible with RapidHIT, the analyst may opt for one of the following courses of action:

- Mark the locus as not suitable for comparison. In this case, if a questioned sample profile was determined to match this reference profile, the corresponding question sample locus would not be eligible for comparisons or for population frequency statistics, if applicable.
- Re-analyze the RapidHIT digested swab material using the questioned direct protocol.
- Perform a new extraction using the known direct extraction protocol with fresh swab material from the same evidence.

For CODIS entry and CODIS confirmations:

Samples exhibiting tri-allelic patterns with all alleles of approximately equal intensity, or with the 3rd allele having a peak height greater than or equal to 50% of the second-largest peak, are entered as such in CODIS. When tri-allelic patterns are exhibited at two or more loci, the sample is entered as a multi-allelic offender.

For samples exhibiting possible tri-allelic patterns where the 3rd allele is less than 50% the height of the second-largest peak, only the two largest alleles will be entered into CODIS. The analyst will note that there is a possible tri-allele at the locus in question on the bench note documentation.

Out of Bin (OB) Alleles

The allelic ladder contains the most commonly observed alleles for the STR loci. True alleles are peaks that are detected and labeled by the GeneMarker software with a number and should lie within the range of the alleles in the ladder. Alleles which are not assigned a numeric designation in the standard ladder will be designated as off-ladder (OL) alleles. In GeneMarker software, this is labeled as out of bin (OB). Either abbreviation may be used.

Peaks that are labeled as OB (out of bin) at a particular locus have a base size that is different from any of the alleles in the ladder and any of the virtual alleles. These peaks may be true alleles, but additional analysis is required to verify this. Before determining whether an OB is a true allele or an artifact, the analyst must consider all other possible causes of OB occurrence. True OB alleles will exhibit peak morphology similar to the other alleles in the sample and the PHR should be appropriate relative to the other allelic peaks in the sample. Keep in mind that OB alleles exhibit correct morphology but may still indicate a problem with sizing.

<u>For forensic casework:</u>

Confirming an off-ladder allele requires re-injection. Since this is not possible with RapidHIT, the analyst may opt for one of the following courses of action:

- Mark the locus as not suitable for comparison. In this case, if a questioned sample profile was determined to match this reference profile, the corresponding question sample locus would not be eligible for comparisons or for population frequency statistics, if applicable.
- Re-analyze the RapidHIT digested swab material using the questioned direct protocol.
- Perform a new extraction using the known direct extraction protocol with fresh swab material from the same evidence.

<u>For CODIS entry and CODIS confirmations:</u>

When a true OB allele lies within the range of alleles for a particular locus, the allele will be reported as a variant of the integer (i.e., X.1, X.2, etc.). The appropriate allele call is determined by simultaneously examining the base sizes for the sample allele peak and the associated allelic ladder peaks in the relevant locus.

When the OB allele lies either above or below the largest or smallest allele in the ladder, the OB allele will be designated as either greater than (>) or less than (<) the allele from the ladder that is closest in proximity to the OL allele.

True OB alleles are documented with a comment on the electropherogram.

When the overall sample quality is poor, the analyst should consider re-analysis prior to designating an OB peak as a true allele.

Section 7 Interpretation of GlobalFiler and GlobalFiler Express by RapidHIT STR data

Under the guidelines included in this section, only single source and two source mixtures may be suitable for interpretation.

In this context, an interpretable profile may be:

- Single source profile (entire profile consistent with one person)
- Single source major component
    - Separated from a mixture of two or more individuals based solely on peak heights
    - Deduced from a two-source mixture where one source is known
    - (Could be achieved with a combination of these two approaches)
- Single source minor component from a mixture of two individuals
    - Separated from a mixture of two individuals based solely on peak heights
    - Deduced from a two-source mixture where one source is known
    - (Could be achieved with a combination of these two approaches)
- Two source mixture
    - Entire profile consistent with two sources where neither is known and cannot be separated as major and minor (indistinguishable)
    - Two source major component from a mixture of three or more individuals

This section covers the following topics:

STR - Single source profile summary table

Single source questioned sample		Rule	Exception(s) / Clarification	Documentation
Minimum for comparison/stat		Four complete loci eligible for stats	For non-probative, can compare if: * alleles < ST; * at least one full heterozygous locus; and * all alleles consistent with owner	PHRs < 60.0% Note loci unsuitable for stats/comparison as NS
Criteria for treating a profile as a single source (vs. mixture)		Possible minor peaks all < ST **and** may be artifacts	Report does not refer to additional contributor	A/TA on e-gram for possible artifacts
		Possible peaks < ST are not artifacts but at < 4 loci	Report language says DNA from more than one source may be present but can be treated as single source	Possible minor peaks in ( )
		Only 1 or 2 possible peaks are > ST at a single locus	Report language says DNA from more than one source may be present but can be treated as single source	Possible minor peaks in ( )
Criteria for comparison/stat eligibility	Homozygous locus	Allele > ST	Y-Indel, Amel, DYS391 suitable for comparison < ST	Loci not eligible marked as NS on e-gram
	Heterozygous locus	2 alleles > AT		

STR – Single source major / deduced component summary table

Single source major contributor		Rule	Exception(s) / Clarification	Documentation
Minimum for comparison/stat		Four complete loci eligible for stats		Loci not eligible marked as NS on e-gram
Criteria for separating major component based on peak heights	Homozygous locus	*Major allele > IT, AND *Minor allele(s) <= 20.0%		*PHRs where major not separated and [ ] NS locus *PHRs between 10.0-20.0%
	Heterozygous locus	*1 or 2 Major alleles > IT, major PHR >=60.0%, AND *Minor alleles <= 30.0%	Loci w/major PHR < 60.0% noted as unsuitable for stats	*PHRs where major not separated and [ ] NS locus *PHRs between 20.0-30.0%
Criteria for deducing based on obligate alleles		*Two source mixture where one source is known	Two obligate alleles > AT	*State assumptions (such as two sources, known contributor) *Note – 2 obligate alleles
Criteria for deducing a (major) contributor based on genotype assessment		*Two source mixture *One source known *Ratio of 1.00 unknown: 2.00 owner or high *Alleles > IT	Follow flowcharts and MIW	*State assumptions (such as two sources, known contributor) *Use MIW if deducing using flowcharts

STR - Single source minor / deduced component summary table

Probative/Non-probative	Rule		Exception/Clarification	Documentation
Probative – must assess whether minor component is complete at each locus	*Two source mixtures only *4 or more heterozygous loci are stat eligible *If fewer than 4 heterozygous minor loci, see Profiles for mixture stats table *Minor component loci not eligible for stat marked NS_{minor} individually or collectively	Separated by peak heights	*Major alleles>IT, AND *Minor alleles<30.0%	*PHRs where major not separated, [ ] NS locus *PHRs between 20.0-30.0%
		Separated by obligate alleles	Two obligate alleles >AT	*State assumptions (such as two sources, known contributor) *Note-2 obligate alleles
		Separated by genotype assessment	Follow flowcharts and MIW	*State assumptions (such as two sources, known contributor) *Use MIW if deducing using flowcharts
Non-probative –	*Two source mixtures only AND		*Includes all minor alleles	*A/TAs noted on e-gram

owner, elimination, or differential carryover	*4 or more loci fully detected, including at least one heterozygous locus AND *No DNA inconsistent with non-probative contributor	>AT *Low-level A/TA do not preclude this	

STR Indistinguishable two source / two source major mixture summary table

Mixture type	Rule	Exception/clarification	Documentation
Two-source indistinguishable mixture (~1.0:2.0 – 2.0:1.0 contributor ratio)	No more than four alleles per locus	A/TAs do not count	A/TAs noted on e-gram
	Peak heights above IT are logically consistent with two contributors	Degradation of one contributor may make this difficult to assess	If degradation impacts decisions, note on e-gram: Possible degradation
	For an indistinguishable mix, no alleles at locus below IT	Four allele loci (or loci with two obligate alleles) are considered complete. Exception described for 3 allele loci with one allele below IT	NA except for 3 allele loci with one allele below IT
Two source distinguishable mixture	To run a mix stat on combined major and minor, can only use loci where one can assume full detection	Calculate contributor ratio (CR) as (sum minor pk hts)/ (sum major pk hts) from two 4-allele loci. Document CR and assessment for all loci with less than two minor alleles	If heterozygous major, multiply smaller major peak by CR – if answer is above IT, can assume no drop out due masked by allele stacking
			If homozygous major, multiply major peak by half the CR – if above IT, can assume no drop out masked by allele stacking
			At each locus, rule out the possibility of drop out due to stutter filters. As applicable, note loci on e-gram as NS – possible stutter masking.
Two source major component	Major alleles > IT	If major has one or two alleles, (larger) minor peak <= 10.0% of (smaller) major peak	On e-gram, [ ] NS loci not separated. Note PHR if major/minor are separated.
		If major has three or four alleles, (larger) minor peak <= 20.0% of (smaller) major peak	

7.1 General STR interpretation guidance

Criteria for the interpretation of allelic peaks

Assessing interpretable alleles (QAS 9.6.3)
Alleles are peaks that are detected and labeled by the GeneMapper™ ID-X software with a number and should lie within the range of the alleles in the ladder, and they are clearly distinguishable from artifacts. All peaks are examined and evaluated in the process of data interpretation.

Three types of thresholds used for data analysis and interpretation (QAS 9.6.4)

- Analytical Threshold (AT) of 160 RFU: below this level, it is not possible to distinguish artifacts from true alleles. The detection limit set for data analysis is defined by the AT.
- Stochastic Threshold (ST) of 630 RFU: below the ST, there is a greater likelihood of drop-out of a sister allele at a heterozygous locus. When data is present below ST, allelic drop out must be a consideration.
- Interpretational Threshold (IT) of 1300 RFU: above the IT, peak height ratios are minimally affected by stochastic effects. Above the IT, sister alleles are expected to have a peak height ratio (PHR) of 60% or greater.

Alleles below the Stochastic Threshold
Stochastic effects result when limited DNA molecules are used to initiate PCR, and unequal sampling of the two alleles present from a heterozygous individual leads to peak height imbalance, elevated stutter, and/or allelic dropout. The ST of 630 RFU is based on validation studies and represents the RFU value below which stochastic effects can lead to drop out of a sister allele at a heterozygous locus. Alleles which fall between the AT and the ST are subjected to a higher degree of scrutiny due to the possibility of stochastic effects.

Note that when an allele is in a stutter position, the “stacking” of stutter with the peak can artificially inflate the peak height. Allele stacking must be considered when assessing whether a probative allele lies above or below the ST.

Reported refers to alleles whose peak heights are equal to or above the ST and are generally reproducible if re-amplified. Such alleles are routinely used in making comparisons between known and questioned sample genetic profiles under the interpretation guidelines stated in this manual. These alleles may also be described as **detected** in report language.

Observed refers to data (peaks) below the ST. Alleles below ST may be considered in these routine circumstances:

- reporting the presence of multiple sources of DNA in a sample
- reporting the presence of male DNA
- reporting the presence of an “owner” on a sample such as body swabs or clothing.

On occasion, depending on the quality of the data and the degraded or inhibited nature of the sample, such alleles may be used to generate a composite profile, with the documented approval of the DNA Technical Manager.

In most instances, allelic peaks below the ST are not suitable for use in comparisons and statistical calculations.

Alleles below the Interpretational Threshold and at or above the Stochastic Threshold
Alleles in this range may be subject to stochastic effects, but not likely to the extent of sister allele drop out. Probative alleles in this range may be suitable for comparison and for statistics. However, they cannot be relied upon to have predictable peak height ratios. Interpretational cutoffs for deduction of a single- source contributor that rely on assumptions about peak height ratios are not appropriate when the alleles in question fall below IT.

Note that when an allele is in a stutter position, the “stacking” of stutter with the peak can artificially inflate the peak height. Possible stutter contribution should be subtracted out when considering whether a probative allele falls above the IT.

Degradation and Inhibition

DNA degradation is the damage to DNA molecules caused by enzymatic or chemical activity. When DNA molecules are damaged in a primer binding site, or in the area between forward and reverse primers, then PCR amplification may be less efficient or not possible. Larger STR loci are more affected by degradation than smaller loci, resulting in profiles with a “ski-slope” shape, where peaks at the left of the profile are much larger than peaks on the right. Factors that lead to DNA degradation include heat, humidity, UV radiation, and the presence of nucleases. Degradation can be minimized by storage and handling practices that minimize exposure to degrading agents.

PCR inhibitors are substances not removed during the extraction process that impede or prevent DNA amplification. Inhibitors interfere with DNA amplification in several different ways, including preventing cell lysis, or preventing the polymerase activity essential to the PCR process. Some examples of potential PCR inhibitors include fabric treatments, leather, wood, soil, and leaf debris. Where possible and relevant, sample collection by swabbing a surface, instead of taking a cutting, can serve to minimize the presence of PCR inhibitors.

These effects are different from the stochastic effects which arise from low-template amplification. The stochastic effects of low template amplification are more likely to occur throughout a DNA profile and in less predictable patterns, rather than being more prevalent at larger loci.

Criteria for the interpretation of mixtures

Criteria for treating a profile as a single source vs. mixture

Indications of a DNA mixture sample can include:
(1) more than two alleles at two or more loci
(2) a peak in a stutter position that is unusually high
(3) significant peak height ratio imbalance for a heterozygous genotype
(4) observation of low-level DNA/data below ST, particularly outside of stutter positions

However, it can be difficult to determine whether or not an additional contributor is actually present when the peaks in question are low-level (that is, below ST) peaks in stutter positions and/or consistent with possible baseline artifacts. When all or most of the peaks that might suggest a possible contributor are consistent with possible elevated stutter and/or baseline artifacts, the profile cannot be definitely declared as a mixture (or, in some cases, as a mixture with more than two contributors).

Profile Interpretation: Single Source versus Mixture
Genetic profiles from questioned samples may be from a single individual (single source) or from multiple individuals (mixtures). Assessment of whether a profile is a single source or mixture, and whether a mixture can be separated out into single source components, impact subsequent interpretation. Documentation of interpretation, including assumptions, must be included in the report (either in the written body of the report or in the supporting bench note documentation).

For questioned samples that are assumed to be single source, it is not necessary to write out calculated percentages for peak height ratios when they are at or above 60%.

For questioned samples that are mixtures, it is not necessary to write out calculated percentages such as peak height ratios and stutter percentages when they are more than 10.0% above (or below) a minimum cutoff. For example, it is not necessary to note a peak height ratio of 80% (or 40%) if the minimum peak height ratio to call a major component is 60.0%. Documentation of calculations that are within 10.0% of a cutoff is very helpful during the review process for mixtures.

Because peak height ratios are of the greatest value in mixture assessments, it is not necessary to document peak height ratios in reference sample profiles unless the ratio is so extreme as to cast doubt as to whether the smaller peak is a true allele (20.0% or lower).

Mixture interpretation – assessment of number of contributors

The same principles used to assess whether a sample was single-source or a mixture can be applied to assessing number of possible contributors in a mixture.

The minimum number of contributors may be assessed by evaluating the loci that exhibit the most allelic peaks (i.e., if at most five alleles are detected at a locus, the DNA results are consistent with having arisen from at least three individuals, provided that none of those peaks are consistent with being elevated stutter). However, phenomena such as tri-alleles, primer binding site mutations, or allele sharing between close relatives can complicate the determination of number of contributors.

A mixture may be distinguishable, meaning major and minor contributors can be separated; or it may be indistinguishable. A major component may consist of either one or two contributors.

Indistinguishable mixtures consistent with being from two individuals suitable for comparison display the following properties:

- No more than four alleles at a given locus
- When peak heights are above IT, peak height ratios are logically consistent with being from two contributors. (For example, a locus with four peaks with RFU heights of 2000, 2000, 2000 and 10,000 is not logically consistent with being from two individuals.)
- Alleles below ST can be accounted for completely, either by the presence of exactly four alleles or by comparison to a known “owner” profile.

Indistinguishable mixtures consistent with being from more than two individuals are not suitable for any comparisons.

Additional caution must be used when analyzing mixtures which indicate degradation in the Degradation Index and/or by a “ski slope” profile where alleles at small loci amplify noticeably better than alleles at larger loci. Particularly in mixtures where one contributor is more degraded than another/others, degradation may result in results that require modified interpretation (such as only interpreting at small loci, or only interpreting based on the presence of obligate alleles), or mixtures that are not suitable for interpretation. Reasons for restricted interpretation for degraded mixtures must be documented on mixture interpretation worksheets or electropherograms.

Profile interpretation: Completeness of a locus
Assessment of a questioned profile includes the determination of which loci are suitable for comparison and/or for the calculation of population statistics.

Statistics-eligible loci considered complete – that is, with no indication of possible drop-out, either for the entire locus or just for a resolved component of a mixture – are suitable for exclusions as well as for population statistics (if corresponding population frequency data is available). Loci where possible drop-out is evident may not be suitable for comparison, depending on the number of sources and the extent of the possible drop-out. A locus with no alleles above the ST can only be considered complete, and therefore suitable for statistics, when genotype analysis indicates that drop-out is not a consideration (e.g., a heterozygous probative contributor).

A statistics-eligible locus where drop-out is a possibility is noted with NS on the electropherogram to indicate that the locus is not suitable for statistics. Examples of loci not suitable for statistics due to possible drop-out include:

- In a single-source profile, a locus with only one allele, and the allele is below ST
- In a two-source indistinguishable mixture profile, a locus where there are fewer than four alleles and at least one of the alleles is below ST
- In a two-source mixture profile with a minor component, a locus where a minor allele could be masked by the stutter filter adjacent to large major component peak

The NS at a given locus may apply to the locus as a whole, or only to the minor component. If the NS applies only to the minor component or mixture, this should also be noted on the electropherogram as NS_{min} or NS_{mix}.

NOTE: The reason a locus, component, or profile is not suitable for statistics must be documented on the electropherogram. Examples:

- For a locus: NS – major/minor not separable
- For a minor component: NS – insufficient DNA
- For a profile: NS – profile complexity

Profile interpretation: Degradation in Mixture Profiles

Poor sample quality can result in degraded DNA; and the extent and nature of the degradation can have a significant impact on the interpretation of a mixture. During the initial examination of a mixture and prior to comparison to references, the analyst should assess and document:

- observations indicating that the profile shows signs of degradation
- concerns raised by the nature and extent of the observed degradation, and
- descriptions of any necessary interpretation modifications

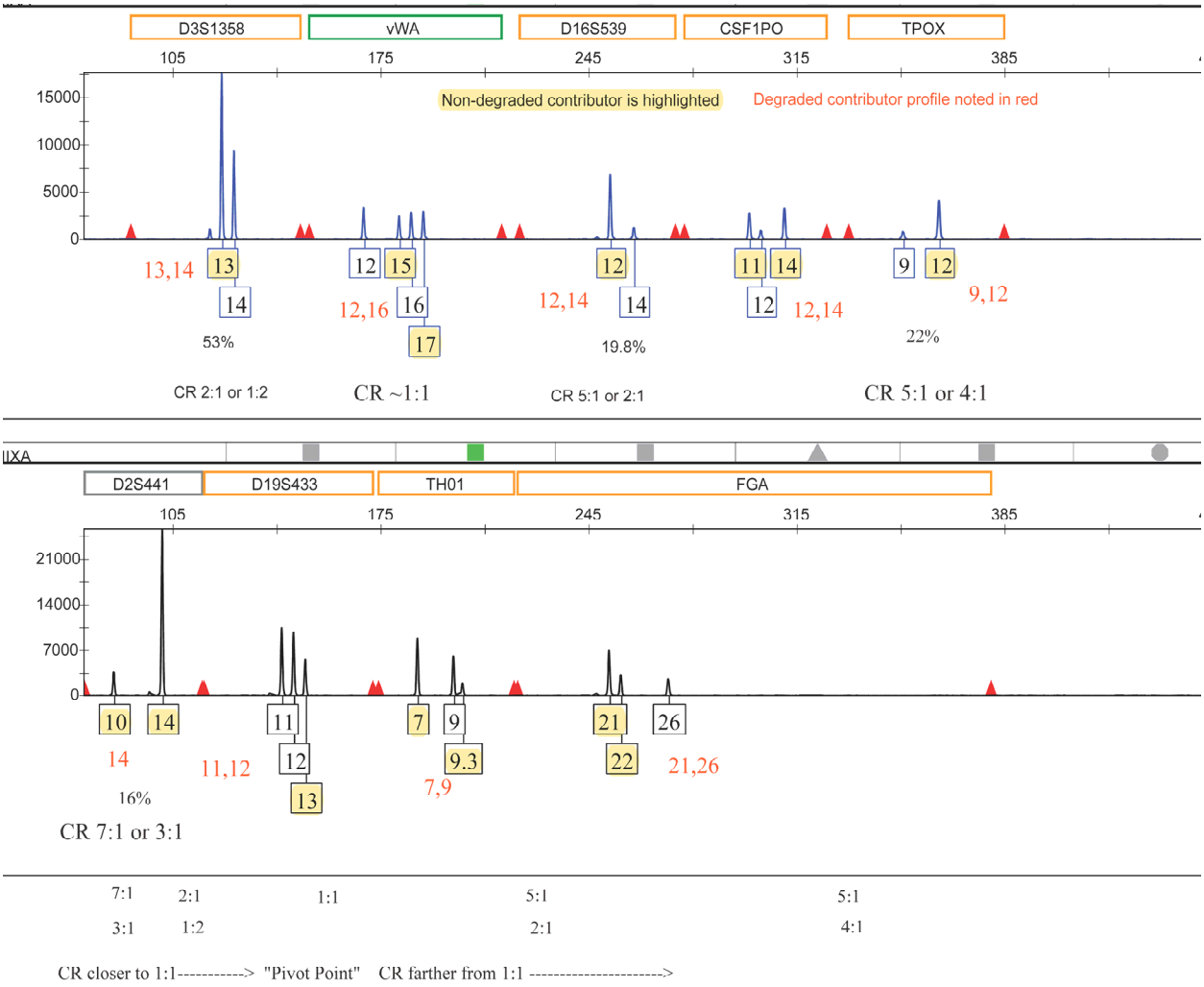
This documentation is recorded on the relevant electropherogram, typically at the bottom of a page where space permits. Selection of appropriate wording from the following suggestions will be useful for most degraded mixtures, but profile-specific circumstances may require unique statements.

1. Observations: The following examples of observations are typical of degraded mixtures, but modifications or other observations may be appropriate depending on the specific profile.
    a. DI = ____ (STR would typically note this for DI > 2)
    b. Downward slope in peak heights (would typically add this note for visually obvious difference in peak heights from small to large loci)
    c. Change in approximate CR (STR - would typically note this for profiles with visually obvious discrepancies - as an example, a four-allele locus with ~even peak heights and other loci with visibly obvious major and minor alleles)
    d. Change in approximate CR with male degraded (STR: would typically use this when visually obvious difference between peak height of Y-Indel and DYS391)

e. Change in approximate CR with apparent female degraded (would typically use this when peak height variability is observed between small and large loci to a greater extent than the difference between peak height of Y-Indel and DYS391)

2. Concerns: The following examples of concerns are typical for degraded mixtures, but other descriptions may be more appropriate depending on the specific profile.
    a. Number of contributors cannot be determined
    b. Unreliable CR – can't deduce / can't major-minor / can't assume complete (possible drop out)
    c. Due to differing degrees of degradation, major and minor contributors may be reversing within the profile (see example after 3.e.)
    d. Owner profile degraded / ~no impact on probative contributor
3. Modifications to interpretation: The following proposed suggestions for when and how to restrict interpretation based on observations and potential impacts are typical examples, but other suggestions may be appropriate depending on the specific profile.
        a. Interpretation at smaller loci only
        b. Interpretation based on obligates only
        c. Interpretation only loci with more info (e.g., not 2 allele loci in a two source mixture)
        d. No interpretation without elimination sample
        e. No interpretation due to sample quality

Example of reversing major and minor contributors as a result of degradation of one contributor:



Profile interpretation: Sequential unmasking

The assessment of a questioned sample profile takes place with 'sequential unmasking': initial assessment occurs before any probative reference samples are compared to that profile. However, an evidence item taken directly from an identified anatomical location (i.e., vaginal swab, penile swab, fingernail scrapings, etc.) and / or a piece of intimate apparel (i.e., undershirts, panties, bra, etc.) typically will yield DNA from the individual from which the evidence item was taken. In such circumstances, it is common to recover a DNA mixture which includes DNA consistent with that individual. In such cases it is useful to use the "owner's" (or any other non-probative) reference profile to assist in the interpretation DNA consistent with n of the questioned sample.

After completion of the initial interpretation of a questioned sample profile, additional DNA data may be used as a basis for re-interpretation (e.g., the use of a non-sperm fraction to inform a sperm fraction interpretation, learning that one of the known or possible contributors is tri-allelic or has a null allele at a locus, a moderate stringency CODIS hit which suggests likely incorrect separation of contributors in a mixture, some of the possible contributors are related and/or the extent of degradation of the DNA for one contributor). Any re-interpretation of the questioned profile that occurs shall be documented in the bench notes and must include the reasons for the re-interpretation.

Rules and Clarification for report language

- When all peaks in question are below ST and may be attributable to artifacts, a mixture cannot be assumed.
    - In report, conclusions are made as to the reportable DNA – that is, the DNA which is not attributable to possible artifacts.
- When peaks in question are below ST are *not* attributable to artifacts but are seen at fewer than four loci, DNA from more than one source (or more than two sources) may be present in the sample. Alternatively, if only one or two peaks are seen above ST at a single locus (if applicable, subtracting out the possible contribution of stutter), then DNA from more than one source (or more than two sources) may be present in the sample.
    - Report language regarding number of contributors should include “may” (as in, DNA from more than one individual may be present in this sample) to indicate that the presence of an additional contributor is a possibility but not a certainty. Because it is not certain that a minor contributor is present, it is not necessary to refer to a major component.
- Alternatively, if only one or two peaks are seen above ST at a single locus (if applicable, subtracting out the possible contribution of stutter), then DNA from more than one source (or more than two sources) may be present in the sample.
    - Report language regarding number of contributors should include “may” (as in, DNA from more than one individual may be present in this sample) to indicate that the presence of an additional contributor is a possibility but not a certainty. Because it is not certain that a minor contributor is present, it is not necessary to refer to a major component.

A profile with two or more alleles above ST (not attributable to artifacts) at two or more loci, or alleles below ST (not attributable to artifacts) at four or more loci, is deemed to be a mixture.

Documentation

- Possible artifact or true alleles are noted as A/TA on the electropherogram. Parentheses are not required for A/TAs on these profiles.

Possible peaks below ST which are not artifacts are marked with parentheses on the electropherogram

Distinguishing between multiple binned artifacts and a minor contributor

The amplification template target of 1 ng is used in part because minimal artifacts, including stutter, are detected at that target value. However, even when 1 ng is amplified, occasional instances of elevated stutter, particularly N-8 stutter, N+4 stutter, and elevated +4/-4 stutter, may be observed. If an electropherogram has 4 or fewer such peaks, all of which can be attributed to attributable artifacts (such as stutter or pull-up), the profile may be considered as not having a low-level minor contributor; provided all such artifacts are documented as such on the electropherogram.

Distinguishing a minor male contributor from possible artifact
In order to report that male DNA was detected, a minor contributor must have male DNA detected at two or three of the following:

- Y peak at Amelogenin
- 1 or 2 detected at Y-Indel
- Any binned allele called at DYS391

An indication of male DNA at only one of these loci could be attributable to an artifact, and no conclusion is reported as to whether male DNA may be present.

Incorrect interpretation of major/minor assessment or mixture deduction

Despite making best use of available information, an analyst may incorrectly deduce an unknown contributor from a mixture, or incorrectly separate major and minor contributors. When such discrepancies are discovered, they should be brought to the attention of the Technical Manager, who will determine the impact on interpretation and the necessary documentation.

7.2 Single Source STR Questioned Sample Interpretation

Typically, each locus is characterized by one or two labeled peaks or alleles. If two peaks/alleles (heterozygous locus) are detected at a locus, they should ideally be of equal intensity – that is, the peak height ratio (PHR) is approximately 1:1.

Peak height ratios are calculated by dividing the peak height (in RFUs) of the lower RFU allele by the peak height of the higher RFU allele, and then multiplying by 100 to express the PHR as a percentage. Based on validation studies, the minimum expected PHR for single-source samples, where there is no indication of a mixture and 1.00 ng of template DNA is used, is 60.0%. This ratio, however, may be lower with lower amounts of DNA. Peak-height-ratio imbalance may also result from degraded DNA or the presence of PCR inhibitors. Allelic dropout may occur in degraded/inhibited samples and the possibility of a second allele should be considered.

Minimum amount of data for a single source to be suitable for comparison

Criteria for interpretable versus uninterpretable data (QAS 9.6.5): A single source profile must have complete information for at least 4 statistic-eligible loci to be deemed interpretable and suitable for all comparisons. This applies to profiles with a single source of DNA as well as to deduced contributors and major or minor components from mixtures. A partial profile which does not meet this minimum is uninterpretable, unless otherwise excepted as described below.

Exception / Clarification: Using Alleles Below ST in non-probative comparisons

A single source profile may be consistent with an “owner” on an intimate sample or on an item “owned” by that person. A single source profile, partial profile, or minor component from any intimate sample which is solely attributable to an “owner” is not used for statistical analysis, but is addressed in the report with a qualitative statement.

These are situations in which a given profile might reasonably be expected to be present on an item. These conclusions are reported with a qualitative statement in the report, provided the following minimum criteria are met:

- Fully detected at a minimum of four loci (below ST and non-core loci are acceptable for this purpose)
- Fully detected at one or more heterozygous loci
- No DNA inconsistent with the expected profile (and/or artifacts) was detected in the partial profile.

If these minimum criteria are not met, no qualitative statement can be made regarding the source of the DNA.

Documentation:

On electropherogram, note peak height ratios < 60.0%, and note loci unsuitable for statistics or probative comparisons as NS.

Criteria for probative comparisons and eligibility for statistics

Rules
A locus must be deemed complete to be suitable for use in probative comparisons and population frequency calculations. Completeness for a locus in a single source profile is addressed as follows:

Homozygous loci: Detected allele must be above ST to be eligible for single source statistics and probative comparisons.

Heterozygous loci: Two detected alleles above AT and consistent with being sister alleles are eligible for single source statistics and probative comparisons.

In single source samples, the primary concern with using alleles below ST is the possibility of allelic dropout. An apparent homozygous allele below ST may in fact be a heterozygote with dropout of the sister allele. If both sister alleles of a heterozygote are present below the ST, dropout is not a possibility and therefore the alleles may be used for statistical calculations. Because this relies on the assumption of a single-source sample, the assumption must be documented in the report language (e.g., Assuming a single source profile…)

Any other instances of using alleles below ST without demonstrating reproducibility, including compositing alleles from multiple amplifications, require documented approval by the Technical Manager.

Exceptions / Clarification
- In a few specific instances (Amelogenin peaks, Y-Indel, and DYS391), single alleles below ST may be used for comparisons.
- Unlike in mixtures and possible mixtures, artifact/true alleles may be separated from the effective single-source profile when the single-source profile alleles are above ST.

Documentation

Loci which are not eligible for probative comparisons and statistics are noted as NS on the electropherogram.

7.3 Interpretation of STR Single source major / deduced component

Minimum amount of data for a single source to be suitable for comparison

Criteria for interpretable versus uninterpretable data (QAS 9.6.5): A single source profile must have complete information for at least 4 statistic-eligible loci to be deemed interpretable and suitable for all comparisons. This applies to profiles with a single source of DNA as well as to deduced contributors and major or minor components from mixtures. If a minimum of four loci cannot be separated, then separation should not be attempted. Note: while the profile may not be interpretable with respect to the single source contributor, it may be interpretable as an indistinguishable mixture – see indistinguishable mixture interpretation section

Documentation

Loci which are not eligible for probative comparisons and statistics are noted as NS, and the reason for ineligibility is also noted on the electropherogram.

Criteria for separating major component based on peak heights

Separating a mixture into major and minor components is easier when the number of loci exhibiting four alleles increases and the ratio of the major contributor to the minor contributor increases above a 4:1 ratio. Distinguishable mixtures of this type will result in a single source major component suitable for probative comparisons.

Notes:

- Since peak height ratios as low as 60.0% have been observed in single source samples with ideal concentrations of DNA, and even lower peak height ratios have been observed with lower concentrations of DNA, peak height ratio information can be used most effectively when alleles are above the IT.
- It may be necessary to factor out stutter when determining the ratios described below.
- The presence of more than two contributors means that greater caution must be used with respect to alleles sharing and peak height ratios.
- Whether the minor component is suitable for probative comparisons depends on the number of contributors. Refer to the section on interpretation of single source minor/deduced component
- Some mixtures where major and minor contributors could be separated are nonetheless treated as a whole. This is often the case for the non-probative fraction of a differential extraction. In those instances, the minor component does not need to be noted on the electropherogram.

Rules

Proposed major component has one allele

- Major component allele must be over IT
- Minor allele(s) peak height must be 20.0% or less of the major allele

- If these conditions are not met, locus cannot be separated

Proposed major component has two alleles
- At least one of the major alleles must be over IT and PHR >60.0%
- Minor alleles must be 30.0% or less of the smaller major allele
- If both conditions are not met, locus cannot be separated

Documentation
- If a mixture can be successfully separated into major and minor components, and the minor component will be interpreted separately, the minor component alleles are designated on the electropherogram with parentheses.
- The loci where an unambiguous major or minor component is not distinguishable are marked in brackets (e.g. [10,11,12]) on the electropherogram.
- Peak height ratios are typically documented on the electropherogram or mixture interpretation worksheet (MIW) when they are within ~10.0% of the cutoff value.
- Peak height ratios may be noted on the electropherogram or mixture interpretation worksheet to document possible genotypes, if appropriate.

Criteria for deducing potential contributors based on obligate alleles

For some two source mixtures where one source is assumed, it may only be possible to separate based on the presence of two obligate alleles. Some examples of this are:
- a mixture with a contributor ratio less than 1.00:2.00 (unknown: owner)
- a mixture where one or both contributors are highly degraded
- a mixture with many or all peaks below IT

Rule:
For two source mixtures where one source is known, an “owner” profile may be used to deduce the profile of the foreign contributor based solely on the presence of two obligate alleles.

Clarification

Because this assumes the presence of two sources where one source is known, the two detected obligate alleles need to be >AT and consistent with being sister alleles.

Documentation

Because this kind of profile assessment relies on assumptions:
- report language refers to component as deduced (or deduced major, as applicable)
- report language must include assumptions relied upon (two source mixture, identity of one source is known)

Electropherogram must be noted to clearly indicate which loci are eligible for statistics:
- Loci are noted as 2 OBL on the electropherogram, with the two obligate alleles indicated.
- Loci which are not eligible for a single source statistic are noted on the electropherogram as ND (not deduced) or NS (not statistic-eligible), along with the reason for ineligibility.

Criteria for deducing a (major) contributor based on genotype assessment
Refer to flowcharts of deductions at 2-allele and 3-allele loci.
NOTE: The deduction flowcharts are designed for interpretation of most loci where all alleles are above IT. It may be possible to deduce a foreign contributor profile at a locus where one or both of the owner alleles is below IT but above ST, or a locus where all alleles are above IT but where the flowcharts lead to not deducing. To deduce such a locus, the analyst may use the MIW to document their assessment of all possible genotypes for the locus for each possible combination by using the contributor ratio to calculate PHRs for the possible genotypes. When this documented assessment clearly indicates only one genotype combination that is reasonable, given the contributor ratio for the profile as a whole, then the locus can be deduced. It is recommended that this process be used only when it has the potential to impact whether or not a mixture is distinguishable.

Rule
Mixtures which contain DNA from two individuals in a proportion of ~1.00 / 2.00 to 2.00 / 1.00 cannot readily be separated into major and minor contributors, due to the fairly even balance between contributors. In these cases, it may be possible to use an "owner's" profile to deduce the genetic profile for an unknown contributor. For body swabs, the "owner" is the person from whose body the sample was collected. For non-intimate samples, the "owner" is a person whose DNA might reasonably be expected to be present on an item, based on information from the submitting agency. Examples of this would be a person's own DNA detected on his/her clothing or property, described as belonging to that person on a Request for Laboratory Service form or evidence packaging. Assumptions relied upon for deduction must be documented in the case report.

Explanations/Clarification

For mixtures with a contributor ratio less than 1.00: 2.00 (unknown: owner), an elimination sample can be used to deduce only based on alleles detected, not on peak height ratios.

For profiles which contain contamination: if the source of contamination can be identified, it may be possible to use its known profile to deduce a profile that is suitable for comparisons.

It may not be possible to deduce an unknown contributor at all loci; those loci which cannot be deduced must be labeled as such. The unknown contributor profile must be deduced at a minimum of four statistic-eligible loci in order to be deemed suitable for comparison. A deduced profile (or partial profile) is treated as a single source profile for the purposes of drawing conclusions and issuing statistics.

Several criteria should be taken into consideration when deducing an unknown contributor using peak height ratio information:

- Deduction must include a genotype assessment based on the presence of obligate allele(s). All genotypes incorporating the obligate allele(s) must be considered in determining the deduced profile.

- In samples with high quantities of DNA, relative amounts of DNA from each contributor can be approximated by examining loci with four alleles. However, these approximations are only reliable when:
    - At least two loci with no allele sharing between contributors (preferably four-allele loci, but three allele loci will work if the known contributor is homozygous) are averaged together
    - No alleles below ST are present
    - SE33 is not included in the calculation
    - There is no minor, low-level third source in the sample
- Peak height ratios may be used to compare different genotype possibilities when alleles are above the IT. When deducing based on allele calls (e.g., a locus with two alleles foreign to the owner's profile), alleles do not need to be above IT or ST.
- Based on observations of casework data and validation studies, the following loci do not exhibit sufficient reliability of peak height ratios to support deduction which relies upon peak height ratios: D22S1045, SE33, and D2S1338. However, these loci can be deduced based on the presence of obligate alleles, if appropriate.
- As a general rule, based on validation studies, heterozygous alleles from a single contributor should have a peak height ratio (PHR) of 60% or higher when the peak heights of both alleles are above the IT. When peak heights are below the IT, greater variability may be observed.
- When any alleles at a locus are below the ST, the possibility of allelic drop-out must be considered. Therefore, such loci are only suitable for deduction if two obligate alleles are above the AT.

Exceptions:

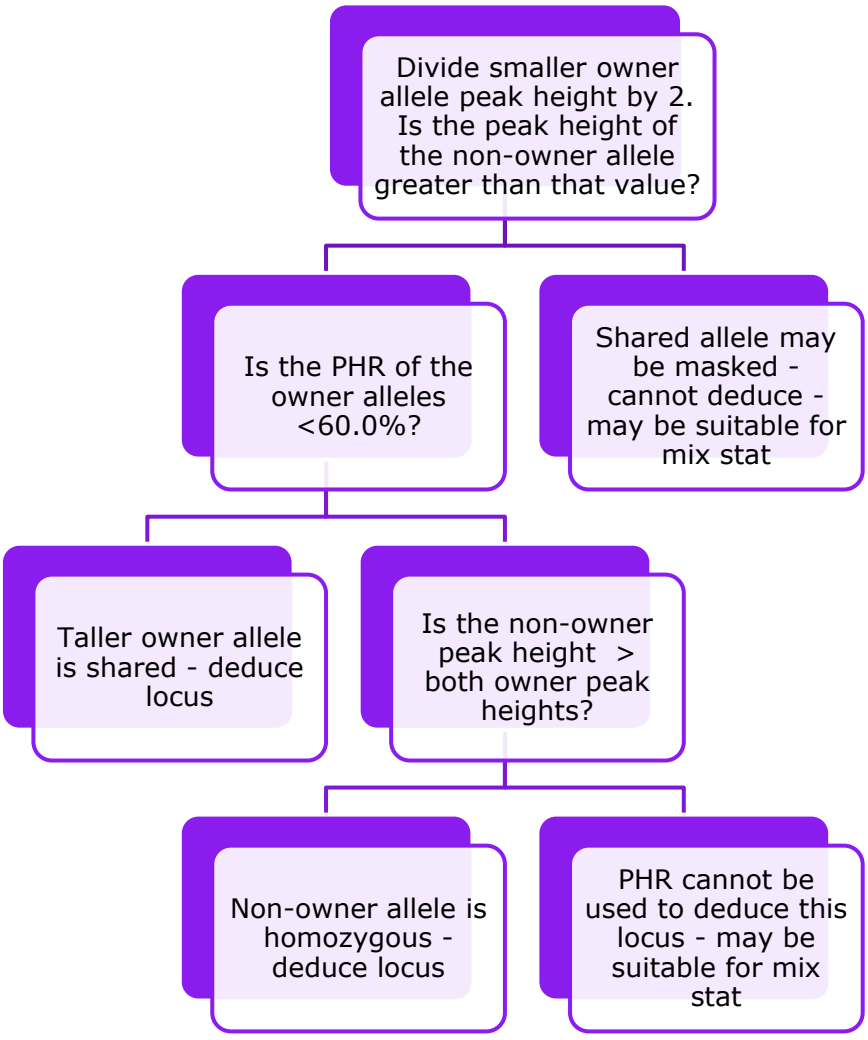
- The flowcharts were designed based on validation studies. However, on occasion, the flowcharts may lead to a conclusion which does not make sense in conjunction with the calculated contributor ratio. When an analyst believes the deduction protocol leads to a potentially incorrect conclusion in light of the contributor ratio, the analyst should not deduce the locus, and document the reason for that decision on the Mixture Interpretation Worksheet.

Documentation

- The rationale for deduction of the genetic profile for an unknown contributor when deduction is performed based on the following flowcharts must be documented on a Mixture Interpretation Worksheet, which is included in the bench notes.
- Report language must state assumptions, such as two source mixture and identity of the known contributor.

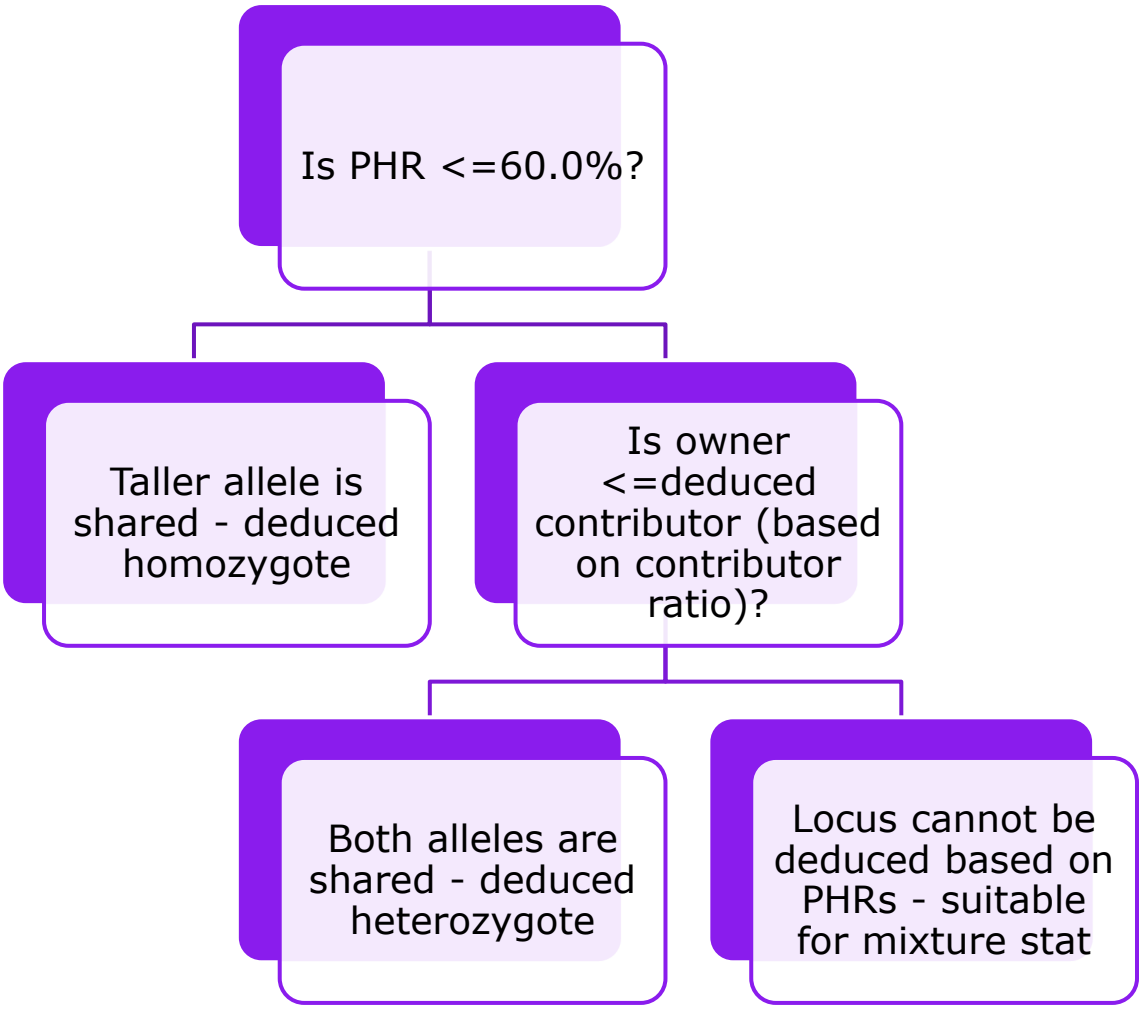
Flowchart: Deduction at 3-Allele Locus with a heterozygous elimination sample

- Max stutter must be subtracted from peaks if it has the potential to impact flowchart decision points
- All alleles at two-or three-allele deduced locus must be at or above IT
- Before beginning, assess possible drop out of the second contributor:
  - Use at least two four-allele loci with all alleles above ST (not SE33) to calculate an approximate contributor ratio. Ratio must be no less than 1.00:2.00 unknown to owner in order to deduce.
  - Given this ratio, confirm that a minor contributor peak would be above ST
- Yes = Left; No = Right
- ***Interpret with caution near specific cutoff values. If you think the deduction protocol leads to a potentially incorrect conclusion, mark the locus as Not Deduced on the MIW and document the reason for that decision.***



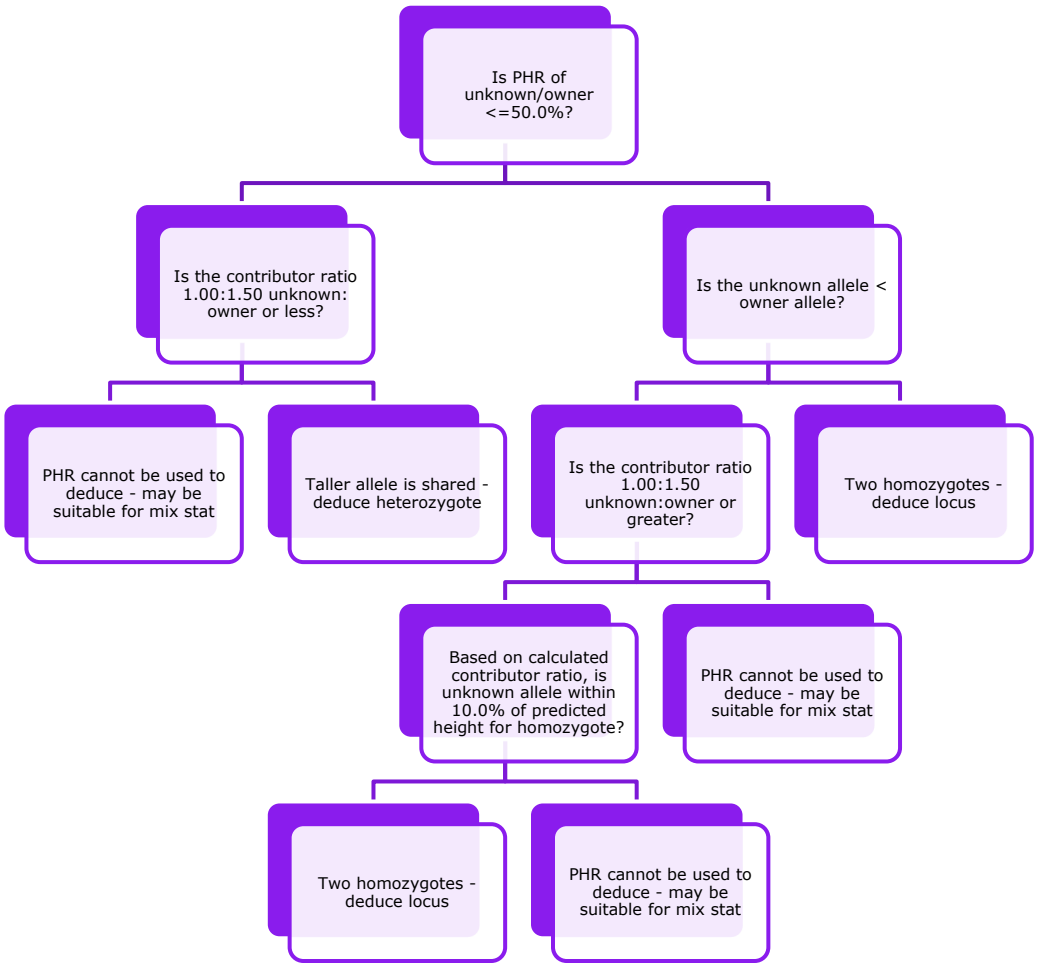
Flowchart: Deduction at 2-Allele Locus with a heterozygous elimination sample

- Max stutter must be subtracted from peaks if it has the potential to impact flowchart decision points
- All alleles at two-or three-allele deduced locus must be at or above IT
- Before beginning, assess possible drop out of the second contributor:
    - Use at least two four-allele loci with all alleles above ST (not SE33) to calculate an approximate contributor ratio. Ratio must be no less than 1.00:2.00 unknown to owner in order to deduce.
    - Given this ratio, confirm that a minor contributor peak would be above ST
- Yes = Left; No = Right
- ***Interpret with caution near specific cutoff values. If you think the deduction protocol leads to a potentially incorrect conclusion, mark the locus as Not Deduced on the MIW and document the reason for that decision.***



Flowchart: Deduction at 2-Allele Locus with a homozygous elimination sample

- Max stutter must be subtracted from peaks if it has the potential to impact flowchart decision points
- All alleles at two-or three-allele deduced locus must be at or above IT
- Before beginning, assess possible drop out of the second contributor:
  - Use at least two four-allele loci with all alleles above ST (not SE33) to calculate an approximate contributor ratio. Ratio must be no less than 1.00:2.00 unknown to owner in order to deduce.
  - Given this ratio, confirm that a minor contributor peak would be above ST
- Yes = Left; No = Right
- To calculate predicted unknown homozygote peak height (HPH):
  - owner HPH * unknown/owner contributor ratio = predicted unknown HPH
- ***Interpret with caution near specific cutoff values. If you think the deduction protocol leads to a potentially incorrect conclusion, mark the locus as Not Deduced on the MIW and document the reason for that decision.***



7.4 Interpretation of STR single source minor / deduced component interpretation

Probative comparisons must assess whether minor component is complete at each locus

Rules

- If a two-source mixture can be successfully separated into major and minor components, the minor component can be considered complete single source at heterozygous loci only. A minimum of four heterozygous minor loci are required for interpretation, probative comparisons, and statistics.
- If the profile has fewer than 4 heterozygous minor loci, it may be interpretable as an indistinguishable mixture.

Exception / Clarification and Documentation

Refer to the clarification provided for separating a major component, including separation by peak heights, separation by obligate alleles, and separation by genotype assessment. Bear in mind that, with respect to minor components, separation based on peak heights can only lead to an interpretable minor component with a two-source mixture.

Non-probative comparisons for a minor component

Rules

Certain special cases are routinely encountered in forensic casework which lead to minor components for which probative comparisons and statistical analysis are not a necessary consideration. Two common examples of these situations are minor components arising from differential carryover and minor components consistent with an “owner” on an intimate sample. These are situations in which a given profile might reasonably be expected to be present on an item. These conclusions are reported with a qualitative statement in the report, provided the following minimum criteria are met:

- Two source mixtures only
- Non-probative profile is fully detected at a minimum of four loci (homozygous or heterozygous, below ST, and non-core locus SE33 are acceptable for this purpose)
- Non-probative profile is fully detected at one or more heterozygous loci
- No DNA inconsistent with the expected non-probative profile (and/or artifacts) is detected in the profile/partial profile/minor component.

If these minimum criteria are not met, no qualitative statement can be made regarding the source of the DNA.

Explanation / Clarification

Minor components arising from differential carryover
The differential extraction protocol is designed to optimize the separation of spermatozoa DNA from epithelial cell DNA. However, this separation process is rarely perfect. As a result, carryover is often observed, where the major contributor to one fraction is observed as a minor contributor in a different fraction – for example, from a vaginal swab which was differentially extracted, the major contributor to the sperm fraction may be detected as a minor contributor in the epithelial fraction. Because more than one profile is associated with the sample, some additional interpretational concerns apply specifically to instances of low-level differential carryover.

- When all minor component alleles are attributable to differential carryover, the analyst will report that DNA consistent with differential carryover was observed in the minor component. This is reported if applicable for all sperm and sperm/substrate fractions, as well as epithelial fractions from non-internal cavity samples. Epithelial fractions from internal cavities, such as oral, rectal, or vaginal swabs, are run as a quality assurance check only and are not routinely included in reported conclusions. For epithelial fractions from internal cavities, the checking for differential carryover is a part of the review process and does not require further documentation.
- When all minor component alleles are attributable to a combination of differential carryover and artifact/true alleles, the analyst will describe results in the reported conclusions as described above.
- Minor components which are attributable to differential carryover do not require a separate statistic, if a statistic is being issued for its primary fraction. Example: A swab from a condom is differentially extracted. The sperm fraction yields a single-source male profile. The epithelial fraction yields a major component consistent with being from a single source female, and the minor component is consistent with carryover from the sperm fraction. In this case, a single source statistic would be issued for the sperm fraction and for the major component from the epithelial fraction. It is not necessary to run a statistic on the minor component of the epithelial fraction since the minor is attributable to the sperm fraction from the same swab.

Minor components consistent with an "owner" on an intimate sample
When a sample is collected directly from a person's body, it is not unusual to detect DNA consistent with that person in the sample. When the minor component of an intimate sample is attributable to the owner, or the owner and artifact/true alleles, it is described in the reported conclusions (except for internal/cavity samples). Underwear and clothing collected directly from a person can be considered as intimate samples and may be reported with a qualitative conclusion instead of a statistic.

7.5 Interpretation of STR Indistinguishable two source / two source major mixture

Two source indistinguishable mixture (~1:2 to ~2:1 contributor ratio)

Interpretable data: A mixture must have sufficient information at a minimum of 15 statistics-eligible loci (or 75% of detected statistics-eligible loci, if profile is partial) to perform comparisons to probative reference samples. In a two-source mixture, the locus can be considered completely detected, interpretable, and suitable for mixture statistics when

- Four alleles (or a heterozygous minor component) are detected, or all detected alleles are above the IT AND it is reasonable to assume all alleles are fully detected
- Peak heights above IT are logically consistent with two contributors
- Note: some three allele loci in two source indistinguishable mixtures may be deemed complete – see details under Clarification / Explanation

In a two-source mixture, if a locus cannot be considered complete, it should be noted as not suitable for mixture statistics (NS_{mix}) on the electropherogram.

Uninterpretable data (QAS 9.6.5): An indistinguishable mixture is uninterpretable when:

- Three or more sources are present
- Two or more sources are present in an indistinguishable minor component
- Less than 15 of the detected loci in a two-source mixture can be considered complete
    - In a severely degraded mixture, a minimum of four complete loci (out of six or fewer detected loci) are required for interpretation

Clarification / Explanation

To be suitable for statistical analysis, a locus must be deemed complete. As the quantity of DNA present becomes smaller, more concerns about possible drop out arise. Specific concerns include:

- Stochastic drop-out: when one allele is detected above AT but below ST, it is possible that a heterozygous sister allele has dropped out.
- Stutter masking: an allele may be effectively filtered out by the stutter filter associated with a large peak.
- Allele stacking: an allele shared by contributors may appear to be above the ST, when the portion of the allele contributed by the minor contributor is actually below the ST. In this case, drop out of a sister allele could happen even when the called sister allele is above ST. Therefore, the ST alone cannot predict the possibility of sister allele drop out.
- Complete drop-out: in instances where the major component is much larger than the minor component, the minor component may drop out entirely.

These concerns must be considered when assessing whether alleles at a given locus are completely detected. Given that the presence of multiple sources of DNA in a sample can make peak height ratio information potentially less reliable, the more conservative IT is applied here.

Degradation of one or both contributors may complicate the assessment of indistinguishable two source mixtures. If degradation impacts decisions made during interpretation, a note must be added to the electropherogram explaining the reasoning.

Special case: Three-allele loci in two source indistinguishable mixtures where two alleles are >IT and one allele falls below IT but above ST require further assessment as described below. This case does NOT apply to two-source major components of three or more source mixtures, because these calculations do not account for the possibility of allele stacking from minor component alleles. Also, these loci will not be suitable for deduction by peak heights (deduction by obligate alleles is permissible). Documentation of this assessment is required at each applicable locus.

- First, rule out the potential impact of degradation. These special case exceptions only apply for profiles which fall within the range of indistinguishable (1:2 to 2:1) across the entire profile. If the locus is ineligible for consideration due to potential degradation effects, add note on electropherogram: "NS_{mix} – potential degradation effects."

- Next, subtract all applicable stutter. If any alleles are below ST after stutter is removed, add note on electropherogram: "allele <ST after stutter removed – NS_{mix}"

- For the case where two of the three alleles are >IT AND their PHR is < 60.0%, the PHR indicates that the taller peak must be shared. This locus may be deemed complete if the smallest peak is >ST. Add note on electropherogram: "No alleles < ST w/o stutter. 2 alleles >IT with PHR of XX.X%. Locus fully detected"

- For the case where two of the three alleles are >IT and their PHR is >= 60.0%, the possibility of stutter masking must be considered. If the tallest of the three peaks has no peak in its minus stutter position, then stutter masking is a possibility, and the locus cannot be considered complete. For example, at a locus with alleles 14, 15, and 16, if the 14 is the tallest allele, stutter masking is possible; if the 15 or the 16 is the tallest allele, then stutter masking can be ruled out. Add appropriate documentation to electropherogram:

    - Stutter masking ruled out: ""No alleles < ST w/o stutter. 2 alleles >IT with PHR of XX.X%. Stutter masking ruled out for tallest allele – locus fully detected"

    - Stutter masking NOT ruled out: ""No alleles < ST w/o stutter. 2 alleles >IT with PHR of XX.X%. Stutter masking not ruled out for tallest allele – NS_{mix}"

Two source distinguishable mixture

In order to assess when it is reasonable to assume complete detection in a distinguishable mixture:

1. Calculate the relative contributor ratio, based on at least two loci with four alleles above ST and not including SE33. Document this calculation on the electropherogram (Ex. Based on the ratios observed at D3 and D5, the relative contributor ratio is ~4.00/ 1.00) Note: if there are not at least two four-allele loci with all four alleles above ST, then it is only reasonable to assume full detection at loci with two heterozygous minor alleles.
2. Based on the peak heights of the alleles detected at the locus, would a *heterozygous minor* contributor have peaks below the IT? If one minor allele is present and above the IT, this is less of a concern, since complete drop out would not be expected when the sister allele is above IT. However, when no minor alleles are detected separately from the major component alleles, it may be due to either allele sharing with the major contributor or allelic drop out. When no minor alleles are detected separately from the major alleles, the contributor ratio can be used to calculate the predicted height of minor alleles at the locus, and thus to assess the possibility of drop out. It is important to keep in mind that the calculated contributor ratio assumes that either both the major and the minor are heterozygous, or both the major and the minor are homozygous.
    a. If the major is heterozygous, multiply the smaller major peak by the contributor ratio. If the resulting hypothetical peak height is below IT, then the locus cannot be considered complete due to the possibility of stochastic drop out.
    b. If the major is homozygous, multiply the major peak height by half the contributor ratio. This is necessary to compare a homozygous major with a heterozygous minor. For example, if the ratio of a homozygous minor allele to a homozygous major allele is 1/4, then the ratio of each heterozygous minor allele to the homozygous major allele would be 1/8. If the calculated comparison is below the IT, the locus cannot be considered completely detected due to possible drop out masked by allele stacking.
3. Based on the peak heights of the alleles detected, could a lower-level heterozygous contributor (calculated as in step 2) have peaks that could be filtered out by a stutter filter on one of the detected alleles? If so, the locus cannot be considered completely detected due to possible stutter masking.

Note: If close biological relatives potentially involved, four-allele loci may not be available to calculate contributor ratios, and an alternative means of determining a relative contributor ratio should be discussed with the Technical Manager.

Two source major component

Distinguishable mixtures of this type will result in a two-source major component suitable for probative comparisons.

Separating a two-source major component will usually only be possible when the major mixture is well-balanced (~2.00:1.00 to 1.00:2.00) and when 1.00 ng of DNA is amplified. Separating out a minor third contributor is often further complicated by allele sharing between the major contributors as well as with the minor contributor. The minor component will not be suitable for comparisons based on the number of contributors, likelihood of allele sharing, and possible drop out.

- Major component alleles must be above IT
- At loci where the major component has one or two alleles, minor component allele(s) peak height must be below 10.0% of the smallest major allele
- At loci where the major component has three or four alleles, minor component allele(s) peak height must be below 20.0% of the smallest major allele.
- If these conditions are not met, the locus cannot be separated.
- Minimum number of distinguishable loci required for calling a two-source major component:

Data detected at this number of stat-eligible loci (Y-Indel, Amelogenin, and DYS391 do not count toward this number)	Minimum # loci where two source major can be distinguished
21	15
20	15
19	14
18	14
17	13
16	12
Data at 15 or fewer loci requires a Biology Deviation Request Form	

7.6 Interpretation of reference sample profiles run by modified RapidHIT

Interpretation of RapidHIT DNA reference profiles is based on the guidelines contained in this section, as well as the qualified DNA analyst's training and experience. In all cases, interpretations must be independently reviewed and verified based on established peer review procedures. The DNA Technical Manager may authorize the interpretation/reporting of data outside of the criteria defined herein and will document this approval with an approved deviation form.

Typically, in a reference profile, each locus is characterized by one or two labeled peaks or alleles of approximately equal intensity. Interpretation of a reference profile includes assessing for correct and complete information at each locus. Filters, including the stutter filter, locus-specific filter, global filter, and minimum off-ladder intensity filter, are in place to remove artifacts.

Check profile for completeness:
Peak height ratio imbalance:
The GeneMarker software is set to flag samples (IMB flag) with a PHR below the expected minimum of 40%. PHR imbalance (in a single source sample) may occur with low quantities of template DNA, degraded template DNA or in the presence of PCR inhibitors. Severe imbalance may result in allelic dropout, where one or more alleles occur below the detection threshold or are not visible at all. A sample where at least one locus shows peak height imbalance will be flagged with a yellow X, indicating this is a locus that requires particular attention from the analyst and reviewer. The analyst must examine the totality of the profile to rule out the possibility of a mixture caused by contamination or an unusual artifact.

Possible drop out:
Stochastic effects, including extreme peak height imbalance, are typical of samples with very low levels of DNA. One risk associated with such samples is the possibility that a true heterozygote will appear to be a homozygote due to a sister allele below the analytical threshold. To minimize this risk, RapidHIT uses locus-specific homozygote threshold values (IHO, or Inconclusive Homozygote flag). The inconclusive homozygote threshold is analogous to the GlobalFiler stochastic threshold employed in casework analysis or the homozygote threshold used in database analysis, but specifically addresses the concern of partial drop out in a low-level RapidHIT reference sample.

Other than gender markers, any homozygous locus with IHO flag must be considered as potentially a partial profile at that locus.

Forensic casework:
Since re-amplification with more DNA is not possible with RapidHIT, the analyst may opt for one of the following courses of action:

- If few loci are affected by full or partial drop-out, the analyst can mark the locus/loci as not suitable for comparison. In this case, if a questioned sample profile was determined to match this reference profile, the corresponding question sample locus/loci would not

be eligible for comparisons or for population frequency statistics, if applicable. NOTE: The determination of exactly how many loci are acceptable to drop without pursuing further analysis is case-dependent, but at a minimum the profile should have sufficient complete loci for the profile to be suitable for comparison to the relevant questioned sample(s).
- Re-analyze the RapidHIT digested swab material using the questioned direct protocol.
- Perform a new extraction using the known direct extraction protocol with fresh swab material from the same evidence.

CODIS entry:
If allelic dropout, or possible drop-out based on the homozygous threshold, is observed in one or more core STR loci, the analyst will make a "good faith effort" to re-process the sample in order to generate a complete genetic profile. "Good faith efforts" for RapidHIT include:
- Re-analyze the RapidHIT digested swab material using the questioned direct protocol.
- Perform a new extraction using the known direct extraction protocol with fresh swab material from the same evidence.

When incomplete typing results are possible at one or more STR loci, indicate this by selecting "Yes" in the Partial Profile field in CODIS for the applicable loci.

Composite profiles of offender samples, created by combining the typing results from separate extracts, are acceptable for entry and upload to NDIS. Prior to creating this type of composite profile, the biographical information and overlapping loci shall be verified as being concordant.

CODIS confirmations:
These do not require that a full profile is obtained, and they do not need to be uploaded to CODIS. A CODIS Administrator and/or DNA Technical Manager must sign off on a profile confirmation when less than 50% of the original core alleles are confirmed.

If a profile could not be confirmed by RapidHIT, the analyst should follow up by either:
- Re-analyzing the RapidHIT digested swab material using the questioned direct protocol.
- Performing a new extraction using the known direct extraction protocol with fresh swab material from the same evidence.

If all confirmation attempts are ultimately unsuccessful, the CODIS match can be reported without profile confirmation; the CODIS communication must include a statement that the profile was not verified.

Special case: Profile comparisons with one mismatched allele
On occasion, the RapidHIT instrument has produced a passing profile with one incorrectly called allele, commonly at the small end of the allele calling range. Analysts can optimize their chance of catching possible mis-calls by carefully reviewing the ILS standards. If ILS peak mis-calls or omissions are noted, it may be possible to resolve the issue by manually adjusting ILS size standard calls, but that requires a Biology Deviation Request Form. Even with these options, the possibility of an undetected mis-call remains.

CODIS confirmations: The main purpose of a CODIS profile confirmation is to ensure that no sample switch occurred. Given the awareness of the potential for RapidHIT to mis-call one allele in a passing profile, the presence of one mismatched allele does not negate the profile confirmation if all other called alleles are concordant and at least 50% of the original core alleles are correctly confirmed.

Casework comparisons to a non-probative reference (no statistics): In most situations, comparison to a non-probative reference sample does not require calculation of statistics. A non-probative reference sample may be called as matching to a RapidHIT reference profile with one mismatched allele when no statistics are calculated based on that inclusion, but the mismatch must be cited in the reporting language and noted in the bench note documentation for the reference profile.

Casework comparisons to a reference sample (statistics calculated): If a comparison between a RapidHIT reference profile and a questioned profile indicates a possible mis-call in the reference profile and statistics are required, the analyst must attempt re-analysis of the reference sample via EZ biorobot extraction. The re-analysis may be of the RapidHIT-processed swab or of a fresh portion of the reference sample. If no reference sample is available for re-analysis, the analyst must consult the TM for a course of action. The RapidHIT reference profile is included in the bench notes with documentation for not using it (i.e., Profile data not used due to possible mis-called 8.3 allele at D2S441.)

7.7 Comparison of STR DNA Results

Criteria used for the formulation of conclusions:

When an analyst assesses a questioned sample profile to determine its suitability for making comparisons to reference samples and calculating population frequency statistics, there are several considerations to make for the profile as a whole:

- Probative versus non-probative: thresholds for comparison differ for some non-probative samples, such as intimate samples. These are described in more detail in the preceding sections. Also, probative comparisons require statistics, while non-probative comparisons do not.
- Degradation / inhibition: when extreme inhibitory or degradation effects are present, or when degradation impacts contributors in a mixture to differing degrees, the analyst must consider the impact on the interpretation of the whole profile.
- Degree of relatedness: some case scenarios which indicate the close relatedness of individuals in the case may prompt the analyst to interpret with greater caution and/or include report language to address the possibility of relatedness.
- Completeness of a locus: for probative comparisons, only complete loci are suitable for determining inclusions and exclusions
- Assumptions: when assumptions are used for deducing foreign contributors, they must be documented in the case file. These typically include
    - Two source mixtures
    - Owner / known contributor
- Minimum thresholds for number of complete loci required for comparison, as well as the criteria for assessing completeness of each locus, are described previously in this section.

The interpretation process generally results in one or more of the following conclusions:

- Exclude
- Cannot exclude
- Foreign DNA detected
- Inconclusive
- Insufficient DNA / Data
- No genetic profile
- Complex mixture

Note: Comparisons and conclusions for Forensic Parentage cases are addressed specifically in the Forensic Parentage section.

Exclusion:

An exclusion is declared when the known sample has alleles that are not observed in the questioned sample, and there is no scientific explanation for the absence.

Scientific explanations may include, but are not limited to, factors such as inhibition, degradation, low template DNA and/or primer binding site variations or mutations.

Cannot Exclude:
For single source samples (including resolved mixtures): The donor of the known sample cannot be excluded as a source of the questioned sample because there are no significant differences between the allele designations obtained from these samples.

For unresolved 2-source mixtures: When alleles detected in a known/reference sample are also detected in at least 75% of the detected core STR loci in a DNA mixture, and when there is a scientific explanation for any inconsistencies, an individual may be considered a potential contributor to the sample.

Foreign DNA Detected:
If alleles that could not have been contributed by the individuals for whom known/ reference sample profiles were submitted for comparison with the questioned samples, the conclusions for this sample may include a statement indicating that DNA that could not have been contributed by X/Y/Z was also detected in the sample.

No Genetic Profile:
No genetic profile is declared when there is reportable data at fewer than four loci. This includes fewer than four complete core loci in probative samples, as well as those which do not meet minimum requirements for intimate swabs consistent with owner or differential carryover.

Inconclusive:
If the STR results support neither inclusion nor exclusion, the comparison will be deemed inconclusive and reported as such. When a profile is deemed inconclusive, an explanation must be included for the reason. Common reasons for inconclusive results are insufficient DNA/data or genetic complexity of a mixture.

Insufficient DNA/Data:
Low amounts of DNA can lead to stochastic effects such as allelic drop out, elevated stutter, and peak height ratio variability. In many instances, incomplete loci cannot be appropriately used to make exclusions or inclusions. When fewer than four loci can be considered complete, this profile will be considered insufficient and will not be utilized for comparison.

Complex Mixture:
A complex mixture may be declared when DNA from multiple sources is detected in a sample, there is evidence of stochastic effects (i.e., drop out) at several or all loci (from limited DNA), and/or a clear major component cannot be deduced. Accordingly, the sample will be deemed not suitable for comparison. This is the case for indistinguishable mixtures with three or more contributors.

Close Biological Relatives:
In a mixture where two close biological relatives (parent-child or full siblings) cannot be excluded as contributors, a comparison can still be made, and a mixture statistic issued if appropriate. However, the report must include an additional statement to clarify that a modified statistic that takes into account relatedness may be more appropriate. At this time, the Alaska Crime Laboratory does not perform such statistics; but upon request, the DNA Technical Manager can refer agencies to outside resources.

Non-probative associations

Many situations arise in forensic casework where a positive association between a questioned sample and a known sample (inclusion) does not have probative value for the criminal investigation. Examples of non-probative comparisons would be:

- Owner on a body swab
- Owner on an item of clothing or piece of property where the attribution to the owner is not in question (e.g., owner on an item of clothing collected from the owner, or steering wheel swab from the owner's car)
- Victim's consent partner on victim's body swabs

In these cases, a qualitative statement describing the association is sufficient for reporting purposes; statistical analysis is not required for these associations. Examples of qualitative statements for non-probative associations are listed in the DNA reporting guidelines.

These associations rely on the assumption that the individual's DNA may reasonably be expected to be present. This assumption can be documented in the report in one of the following ways:

- Swabs collected from a person's body are identified as such in the Item Description section of the report.
- Clothing or property collected from a person are identified as such in the Item Description section of the report. Alternatively, a statement is added to the conclusion for that item, indicating that the item has been previously identified as belonging to the person.
- Elimination samples and consent partners are identified as such in the Item Description section of the report. Alternatively, a positive association between a questioned body swab or clothing and a known consent partner includes a statement in the conclusion that this individual has been previously identified as a consensual partner of the person in question.

Probative associations

A positive association between a questioned sample and an individual whose DNA is not reasonably expected requires population statistics. If a profile is not of sufficient quality or quantity to calculate population statistics, then it is not suitable for performing comparisons to probative reference samples.

A single statistic may be calculated for multiple identical profiles. (Example: two blood stains on a shirt yield identical profiles, with a positive association to a probative reference sample).

If profiles from multiple items are positive associations to the same individual but are not fully identical profiles (typically due to partial drop out in at least one profile), statistics must be calculated for each of the items.

It may be appropriate to issue multiple statistics for a single item, if those statistics reflect different associations made to that item. (Example: a swab from a boot yields a single source major component as well as a minor component which is suitable for comparisons as a mixture. If this profile has positive associations to two different people, it is appropriate to run both a single source statistic for the major component, and a mixture statistic for the minor component. If only one positive association is made, it is only necessary to calculate the relevant statistic for that association.)

Note: A sample which has undergone differential extraction yields sperm, substrate and/or epithelial profiles from the same item. Differential carryover is frequently observed in such samples. It is not necessary to calculate a separate statistic for the minor component of a fraction that is attributable to differential carryover if the probative contributor has been addressed in a different fraction. Likewise, if multiple fractions from one differential extraction yield profiles consistent with being from the same individual, it is only necessary to issue a statistic on one of those profiles, since all fractions originate from the same single sample.

Special case: Using a biological parent or child for a reference sample in lieu of the person of interest.

Best practice is always to collect a reference sample directly from a person of interest. However, under unusual circumstances, it may not be feasible to collect a reference sample from a person of interest (e.g., when they are no longer in the country, have died, etc.), and a reference from a biological parent or biological child of the person if interest is collected instead. Typically, this option is reserved for cold case homicides, time-sensitive cases with a threat to public safety, or similarly extreme situations.

Comparison to a single-source profile:

In theory, in a biological parent/child relationship, the individuals should have one allele in common at each locus. However, mutations between generations are a possibility.

- Four or more loci with non-consistent genetic markers – the biological parent/child of the reference individual is excluded. It is not necessary to calculate zero-parent Combined Parentage Index (CPI) in such cases.
- Two or three loci with non-consistent genetic markers – these results are inconclusive. The CPI will be calculated and reported, but further testing with additional markers will be recommended to the submitting agency.

- One locus with an inconsistent genetic marker within one repeat unit of the obligate allele – given the possibility of a genetic mutation, this will not be deemed an exclusion. In such a case, the biological parent/child of the reference individual cannot be excluded, and CPI will be calculated and reported.
- All loci have consistent genetic markers – the biological parent/child of the reference individual cannot be excluded, and the CPI will be calculated and reported.

Comparison to a two-source mixture:

When compared against a biological parent/child of the person of interest, two-source questioned sample profiles will be **suitable for exclusions only.**

- Four or more complete loci with no consistent alleles – the biological parent/child of the reference individual is excluded.
- Three or fewer loci with no consistent alleles – these results are inconclusive.

Sample report language:

Exclusions:

An individual inherits their DNA alleles from their parents. Barring mutations, one of the DNA alleles found in an offspring must be present in the biological mother and the other allele in the biological father. Assuming XXX is the biological father/mother/child of YYY, XXX is excluded as a source of DNA detected in the sample(s) above.

Inconclusive:

An individual inherits their DNA alleles from their parents. Barring mutations, one of the DNA alleles found in an offspring must be present in the biological mother and the other allele in the biological father. Assuming XXX is the biological father/mother/child of YYY, no conclusions are reported as to whether XXX could be the source of DNA detected in the sample(s) above. (For single source only: It is recommended that further testing be performed to gain additional information from more genetic loci.)

Inclusion:
An individual inherits their DNA alleles from their parents. Barring mutations, one of the DNA alleles found in an offspring must be present in the biological mother and the other allele in the biological father.

XXX [submitted known contributor] is assumed to be a biological parent/child of YYY.

Based on the questioned DNA profile, a biological parent/child of XXX cannot be excluded as possible contributor to this profile.

[If applicable: A single genetic inconsistency is observed for the obligate allele at locus___. This is indicative of a mutation or recombination event in which the allele inherited from the alleged parent by the child has been altered.]

The observed genetic evidence is at least ZZZ [most common CPI among all calculated populations] times more likely if XXX is the true biological mother/father/child of the contributor to the questioned profile rather than if an unrelated individual is a biological mother/father/child of the contributor.

It is recommended that a reference sample from YYY be submitted for analysis and direct comparison to the questioned DNA profile.

7.8 Performing STR Population Statistical Frequency Calculations

References: *Moretti, T. et.al, Population data on the expanded CODIS core STR loci for eleven populations of significance for forensic DNA analysis in the United States (2016) Forensic Science International: Genetics 25: 175 - 181.*

Budowle, B. et al., Population studies on three Native Alaska population groups using STR loci. (2002) Forensic Science International 129: 51-57.

Expanded Loci Genotyping and Statistical Evaluation of Three Alaskan Native Populations (FBI correspondence, posted on lab website)

Statistical analyses will be performed when the genetic typing results fail to exclude an individual(s) as a source of the DNA in a questioned sample, and the positive association is to a probative reference sample. The statistic is calculated from the questioned profile and includes the statistics-eligible loci which are completely detected and eligible for population statistics. (Note: in the rare case that a known sample does not yield a full profile at all statistics-eligible loci, then the questioned profile loci which could not be compared to the reference sample are not included in the statistic.)

For differential samples, a separate statistical analysis is not required for differential carryover in a minor component, if statistics are calculated from the relevant individual(s) in a different fraction from the same item.

Statistical analyses are calculated with the U.S. Department of Justice, FBI Popstats program, using either the Random Match Probability Formula for single source samples or the Mixture Formula for mixed samples. Combined Parentage Index calculations are performed as applicable for forensic paternity cases. The resultant values may be truncated for reporting but should never be rounded up (i.e. 34,675,000 may be reported as 34 million or 34.6 million, but not 34.7 million). For frequencies more rare than 1 in 330 billion (approximately 1000 times the population of the United States), a ceiling value of 1 in 330 billion is reported.

The Alaska Scientific Crime Detection Laboratory routinely calculates the frequency/probability and reports the most common from among the following populations: Caucasian, African American, Athabaskan, Inupiat and Yupik.

Caucasian and African-American population frequency statistics are calculated using the Expanded FBI STR 2015 frequency database. Theta correction factor for these populations is 0.01.

Athabaskan, Inupiat, and Yupik population frequency statistics are calculated using the Alaska Expanded Core 2017 frequency database. Theta correction factor for these populations is 0.03.

Random Match Probability Formula – for single source profiles

Note: Only data from complete, single source loci are used to calculate statistics with this method. Uninterpretable loci (those with possible allelic dropout), or loci where a single source contributor could not be determined, are not to be used in this calculation.

To calculate the frequency of a locus genotype, the following formulae are used:

Heterozygotes 2pq
Homozygotes $p^2 + p(1-p)q$

The p and q represent the frequencies of two different alleles. Theta (q) is an empirical measure of population subdivision/substructure or "relatedness".

For the Caucasian and African American populations, q=0.01
For the Alaskan Native populations, q=0.03

The multi-locus genotype frequency is estimated by multiplying together the genotype frequencies from the different loci. The expected frequency of a DNA profile in a population is the inverse of the multiple locus genotype frequency.

Combined Probability of Inclusion (CPI; Mixture Formula)

Note: Only data from complete, interpretable loci are used to calculate statistics with this method. Uninterpretable loci (those with possible allelic dropout or loci where a two-source major component could not be determined), are not to be used in this calculation.

To calculate the probability of a random individual in the population being a contributor to a mixture for a locus, the following formula is used:

$(p_1 + p_2 + \ldots p_n)^2 = P_{LOCUS}$

The p_1, p_2, and p_n are the frequencies of occurrence of the alleles at the locus.

The combined mixture profile probability is calculated by taking the product of the individual locus probabilities. To estimate the number of individuals in a population that could have contributed to the mixture, the inverse of the combined mixture profile frequency is calculated.

Using Popstats to calculate population frequency statistics

***Minimum allele frequencies*:** Popstats calculates minimum allele frequency as 5/2N where N is the size of the database.

Calculating Frequencies/Probabilities Using Popstats

- Open Popstats (in the CODIS software) and choose either Forensic Single Sample or Forensic Mixture from the menu.
- In the configuration summary, verify that the database is the Expanded FBI STR 2015 database, and the theta value is set to 0.01 (see image below).
- All populations except African American and Caucasian can be unchecked as these data are not reported.
- In the Specimen ID field, enter the lab #_Item#

Note: When entering a Forensic Mixture, be sure to enter the profile in the left most available fields (labeled "Mixture" in the image below). The middle and right columns are for use only in calculating likelihood ratios.

- In the Comments field, add any additional info (i.e., sperm fraction, major profile)
- Enter the alleles deemed appropriate for statistical analysis (see STR Data Interpretation section).

Note: It is only appropriate to enter alleles for loci where statistics can be calculated for all required populations.

- Click the Calculate button or select Calculate from the Popstats pull-down menu.
- Forensic Single Source
    - Click the 1/f button.
    - Click the printer icon or select Print from the File pull-down menu. Select Broward Report and print.
- Forensic Mixture
    - The result should default to the Inclusion probability
    - Click the printer icon or select Print from the File pull-down menu. Select Probability of Inclusion (Short) and print.

Note: The above steps must be performed for both the Alaska Expanded Core 2017 database and the Expanded FBI STR 2015 database. The theta correction factor (0.01 for the STR database and 0.03 for the Alaska database) must be specifically selected for single-source statistic calculations when toggling between databases. Theta values are not used for calculation of mixture statistics. Ensure the check box is unmarked in the configuration summary.

- To change to the Alaska Expanded Core 2017 database, click Browse (in the configuration summary tab) to navigate to the Alaska Expanded Core 2017 folder and click OK (see image below).

Note: It may be necessary to expand the configuration summary window to reveal the Browse button when calculating Forensic Mixture statistics (see image below).

- For single source statistics, change the theta value to 0.03 using the drop-down menu in the configuration summary tab.

Note: The database and theta value must be changed in this order or the theta value will revert back.

- Click the Calculate button and repeat the above steps for printing the resulting statistics

Protocols for performing statistical calculations for forensic parentage are in the appendices.

Section 8: Y-STR Data Interpretation

Interpretation of casework samples is based on the guidelines contained in this section, as well as the qualified DNA analyst's training and experience, experimental results, and case specifics. In all cases, interpretations must be independently reviewed and verified based on established peer review procedures. The DNA Technical Manager may authorize the interpretation/reporting of data outside of the criteria defined herein and will document this approval by initialing and dating comments on the relevant paperwork.

This section covers the following topics:

Y-STR Interpretation Summary Table

Note: when a peak in a two-source mixture is determined to be a shared peak, that locus "counts" toward minimum distinguished loci.

Note: Contributor ratio (CR) calculated using 4 loci (both alleles > IT is possible)

Comparison	Rule	Minimum (distinguished) loci	Exception/Clarification
Low-level profiles (3 or less alleles >IT)	Single-source profile	6 loci	Suitable for exclusions and/or non-probative inclusions only (see clarifications below)
	Any indication of possible 2nd contributor = not suitable for any comparisons	NA	NA
Probative inclusions	Single source profile	12 loci	Only DYS385 needs to be >ST to be considered complete
	Single source deduced contributor	12 loci	2 allele locus: probative allele > ST
			1 allele locus: probative allele > IT and CR used to rule out stutter masking
			DYS385: only separate if 2 obligate alleles
	Single source major or minor from a two-source mixture (or at least two sources)	12 loci	Refer to Y-STR major/minor flowchart for detailed separation rules
	Single source major from a 3 or more-source mixture	16 loci	Major > IT and PHR 60% or less
Non-probative	Single source profile or	6 loci	All alleles accounted for by

inclusions	component		elimination
Exclusions	Single source or two source mixture has at least one obligate non-matching allele >ST	6 complete loci	* Completeness in a **mix** locus means alleles(s)>ST and alleles in stutter position >ST after max stutter removed
			* Completeness for **single source** profile – some alleles can be <ST if at least one non-matching allele is >ST

8.1 General Y-STR Interpretation Guidance

Criteria for the interpretation of allelic peaks

Assessing interpretable alleles (QAS 9.6.3)
Alleles are peaks that are detected and labeled by the GeneMapper™ ID-X software with a number and should lie within the range of the alleles in the ladder, and they are clearly distinguishable from artifacts. All peaks are examined and evaluated in the process of data interpretation.

Three types of thresholds used for data analysis and interpretation (QAS 9.6.4)

- Analytical Threshold (AT) of 100 RFU: below this level, it is not possible to distinguish artifacts from true alleles. The detection limit set for data analysis is defined by the AT.
- Stochastic Threshold (ST) of 600 RFU: below the ST, there is a greater likelihood of drop-out of a sister allele at a heterozygous locus. When data is present below ST, allelic drop out must be a consideration. Additionally, data below ST may require extra caution in determining whether a peak is a true allele.
- Interpretational Threshold (IT) of 1500 RFU: above the IT, peak height ratios are minimally affected by stochastic effects. Below IT, and to a greater extent below ST, peak height ratios can vary significantly from predicted values.

Alleles below the Interpretational Threshold and at or above the Stochastic Threshold
Alleles in this range may be subject to stochastic effects that impact the reliability of peak height ratios between major and minor contributor alleles. As such, data below IT requires extra caution when separating into major and minor components.

Alleles below the Stochastic Threshold
Stochastic effects result when limited DNA molecules are used to initiate PCR, and unequal sampling of the two alleles present from a heterozygous individual leads to peak height imbalance, elevated stutter, and/or allelic dropout. The ST of 600 RFU is based on validation studies and represents the RFU value below which stochastic effects can lead to drop out of a sister allele at a duplication locus, typically DYS385. Peaks which fall between

the AT and the ST are subjected to a higher degree of scrutiny due to the possibility of stochastic effects.

Note that when an allele is in a stutter position, the “stacking” of stutter with the peak can artificially inflate the peak height. Allele stacking must be considered when assessing whether a probative allele lies above or below the ST.

Reported refers to alleles whose peak heights are equal to or above the ST and are generally reproducible if re-amplified. Such alleles are routinely used in making comparisons between known and questioned sample genetic profiles under the interpretation guidelines stated in this manual. These alleles may also be described as **detected** in report language.

Observed refers to data (peaks) below the ST. Alleles below ST may be considered when reporting the presence of an elimination reference on a sample such as body swabs or clothing.

On occasion, depending on the quality of the data and the degraded or inhibited nature of the sample, such alleles may be used to generate a composite profile, with the documented approval of the DNA Technical Manager.

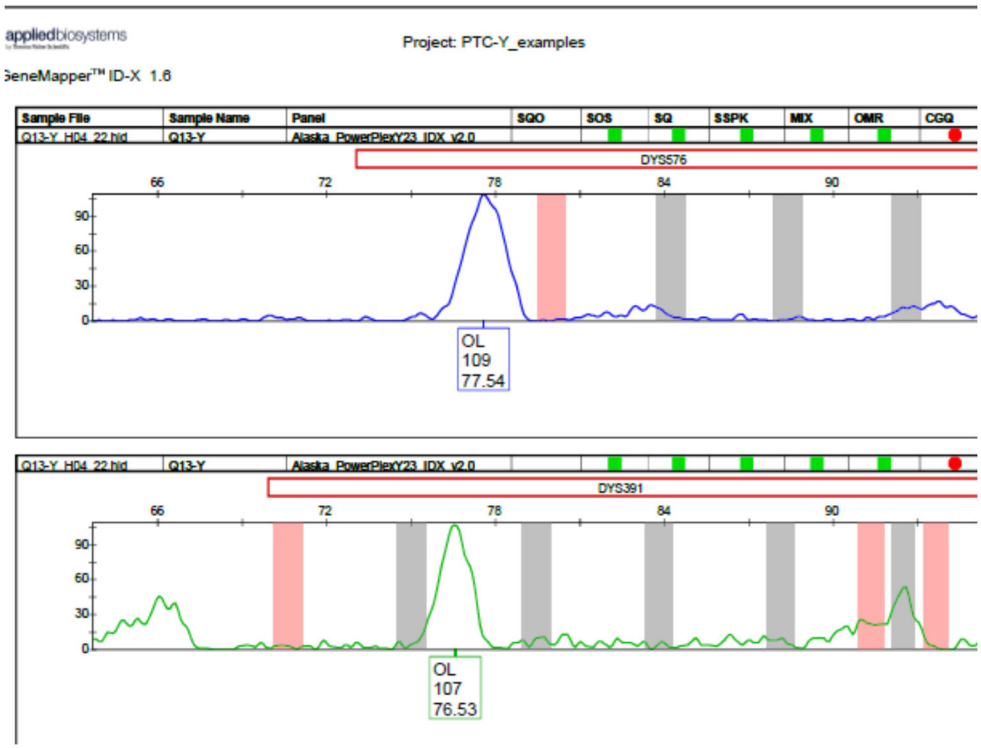
Common low-level artifacts in Y-STR profiles

Several artifacts are routinely observed in Y-STR profiles when the extract has been dried down with Gentegra LD and a low amount of template DNA has been amplified (typically less than 0.25 ng). Low-level profiles are characterized by most peaks having peak heights near or below the stochastic threshold. Some of the commonly observed artifacts with the potential to impact interpretation are described below. Analysts may reference manufacturer publications as well as internal validation data for other observed peaks which may be struck as artifacts.

<u>Artifacts commonly seen in-house resulting from Gentegra LD:</u>

Fluorescein (blue dye channel): ~78 bp artifact, usually a broad peak or “haystack” morphology

JOE (green dye channel): ~77 bp artifact, usually a broad peak or “haystack” morphology; occasionally bins as a ‘5’ at DYS391



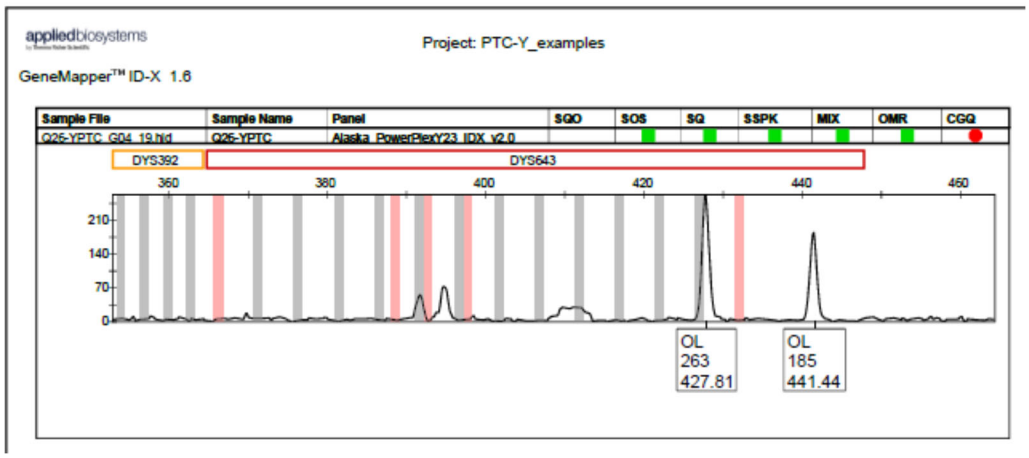
Low-level or baseline OL artifacts can be struck without comment. However, some of the low-level Y-STR artifacts can bin and potentially be mistaken for true alleles.

A binned GTD artifact at DYS391 should include a zoom view with base pairs labeled. It can be noted as GTD artifact and struck if the base pair and peak morphology are approximately consistent with the image above (base pairs are not exact and some shifting can be expected). If zoom view is not included in the bench notes, the base pairs must be checked by the technical reviewer in the GMID-X project.

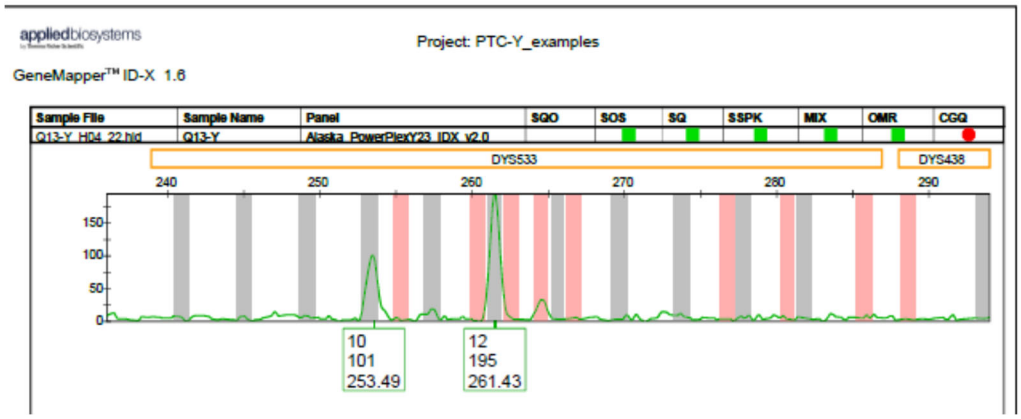
<u>Artifacts commonly seen in-house and associated with a very high female to male ratio:</u>

The artifacts in the images below are from a sample with a female to male ratio of ~41 000 to 1.

TMR-ET (yellow dye channel): ~428 and 441 bp artifacts, often called as OLs at DYS643



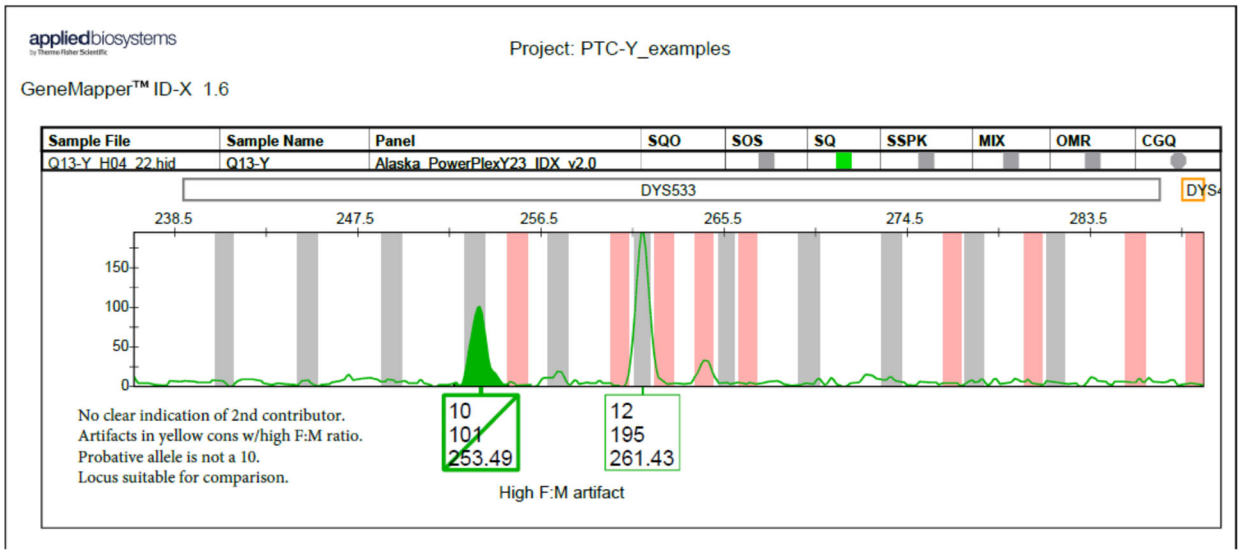
JOE (green dye channel): ~253 bp artifact, often bins as a ‘10’ at DYS533, might be shifted in bin. Peak height for this artifact have been seen above ST; peak height cannot be used to evaluate whether a ‘10’ at this locus is a true allele.



Due to the peak height and morphology of the green dye 253 bp artifact, it can be difficult or not possible to tell if a ‘10’ is arising from a true contributor or is an artifact. The presence of the 428 and 441 bp artifacts in the yellow dye channel is a strong indicator of interference due to a high female: male ratio. However, extreme caution must be used when interpreting DYS533 in low-level / consumed extract amplifications; and a ‘10’ at this locus is NEVER suitable for comparisons in low-level samples.

- The most conservative and preferred approach is to note DYS533 as not suitable for interpretation in all low-level profiles, regardless of allele calls.
- To be deemed suitable for interpretation in low-level profiles, regardless of allele calls, DYS533 must be printed in a zoom view that includes base pairs, then assessed and documented as follows:
    - No clear indication of a second contributor at other loci

- Artifacts in yellow cons. w/high F: M ratio
- Probative allele cannot be attributed to the ~253 bp artifact
  - Alternative wording – Probative allele is not a '10' so artifact ruled out
- Locus suitable for comparison
- [If applicable, strike the '10' and note as High F: M artifact]



Criteria for interpretation of all Y-STR profiles: Sequential Unmasking

The assessment of a questioned sample profile takes place with 'sequential unmasking': initial assessment occurs before any probative reference samples are compared to that profile. However, in some cases an elimination sample (such as from a consent partner) is available for comparison. In such cases the profile from the elimination sample may be used to assist in interpretation of the questioned sample.

After completion of the initial interpretation of a questioned sample profile, additional DNA data may be used as a basis for re-interpretation (e.g., learning that one of the known or possible contributors has a duplication or has a null allele at a locus, and/or the extent of degradation of the DNA for one contributor). Any re-interpretation of the questioned profile that occurs shall be documented in the bench notes and must include the reasons for the re-interpretation.

Criteria for the interpretation of low-level Y-STR profiles

Low-level Y-STR profiles are defined as those where three or fewer loci have an allele at or above IT. Low-level Y-STR profiles may arise from amplification of a low quantity or quality of DNA. Several factors can complicate the interpretation of low-level Y-STR profiles, such as:

- Increased stochastic effects
- Artifacts with peak heights similar to true alleles
- Difficulty in distinguishing mixtures from single-source profiles
- Inability to assess for potential preferential degradation

For these reasons, low-level Y-STR profiles can fall into one of two interpretational categories:

1. Low-level single source Y-STR profiles with a minimum of six alleles are not suitable for probative inclusions but may be suitable for exclusions only for probative samples, as well as non-probative inclusions.
2. Low-level Y-STR profiles which cannot be determined to be single source, or which do not have at least six alleles, are not suitable for comparisons, typically due to insufficient DNA.

<u>Assessing whether a low-level Y-STR profile is single-source:</u>

In low-level Y-STR profiles, any indication of a possible second contributor means that a single source profile cannot be assumed, and the profile is not suitable for comparisons. These include:

- Any locus besides DYS385 with any two binned peaks (At DYS385, the presence of three or more peaks)
- Visually concerning peak(s) with good morphology which may indicate a true allele falling slightly below AT.

Assessing number of contributors in low-level profiles that are not suitable for comparison.

Low-level Y-STR profiles can be particularly challenging for determination of number of contributors. Artifacts, elevated forward and reverse stutter, as well as the possibility of low-level contamination all potentially complicate the determination. In instances when a low-level profile is not suitable for inclusions and/or exclusions, *it is not required for a number of possible contributors to be reported*. In some instances, it might be helpful to include, such as to support a statement that the reason a profile is not suitable for comparison is profile complexity, if the determination is readily supportable – i.e., several loci with more than one peak that could not be elevated stutter or artifacts. However, a reason of complexity does not require assignment of a specific number of contributors.

Alternatively, the report may include a statement that the number of contributors could not be determined due to insufficient DNA.

Criteria for the interpretation of (not low-level) Y-STR profiles

NOTE: Null alleles and duplications
The mixture interpretation guidelines for all Y-STR loci except DYS385 are predicated on the assumption that each contributor has one and only one allele per Y-STR locus. If one or more contributors have either a null allele or a locus with allele duplication, mixture interpretation may not be appropriate at that locus or loci. This determination may only be apparent after the reference sample profiles are generated. If a relevant reference sample is found to have a null allele or a duplication which has the potential to impact mixture interpretation of a corresponding questioned locus:

- At that locus/loci, the questioned profile should not be separated (major/minor or deduction)
- If interpretation changed, a comment must be added to the bench notes to explain why the questioned profile locus interpretation changed after examination of the reference profile(s)
- Questioned profiles at the non-separated locus/loci should still be compared for possible exclusions
- Unseparated loci are not eligible for calculation of population frequency statistics.

Criteria for distinguishing between multiple binned artifacts and a minor contributor
The amplification template target of 0.50 ng is used in part because minimal artifacts, including stutter, are detected at that target value. However, even when 0.50 ng is amplified, occasional instances of elevated stutter, particularly +4/-4 stutter, may be observed. In addition, pull-up between dye colors and instances of elevated baseline noise or "drop-in" have been observed, especially when peak heights exceed 10,000 RFU. Elevated stutter, both +4 and -4, is more common with low template DNA.

Validation studies indicate that most binned artifacts occur at peak heights below 600 RFU, including elevated stutter, pull-up, and baseline noise. As such, all peaks below 600 RFU that suggest a possible mixture / additional contributor must be interpreted with caution, and possible reasons that could lead to an artifact being called as a true allele must be ruled out.

When low level peaks are assessed as not an additional contributor:
If an electropherogram has 4 or fewer peaks below ST, all of which can be attributed to artifacts, the profile should be considered as not having a low-level minor contributor, provided all such artifacts are documented as such on the electropherogram.

Peaks that could be attributed to artifacts include:
- peaks designated as artifact or true allele after stutter is removed
- peaks that are above AT (after stutter is removed, if applicable) but could logically be the result of a known cause of artifacts, such as pull-up or elevated stutter.

For interpretation purposes, the remaining profile is treated like a single-source profile, and it is not described as a major component.

Documentation:
  - Each low-level artifact peak is marked on the electropherogram with parentheses.
  - If the peak is less than AT after stutter is removed, note with A/TA to indicate artifact or true allele.
  - If the profile will be treated as single source, add a note to the electropherogram such as: No other sign(s) of second source – treat as SS.

Note: A high-quality sample with abundant male DNA should result in a Y-STR profile with most or all alleles above IT. However, in degraded or lower template single-source profiles, alleles at some loci may fall below IT or below ST.

- Loci where the main peak is above IT:
  - Artifact peaks at loci with main peaks >IT are typically <10% of the height of the main peak. As such, they are readily identifiable and may not require further consideration.
- Loci where the main peak is below IT but above ST:
  - Artifact peaks at loci with main peaks >ST are very unlikely to 'reverse', where the artifact peak height is higher than the true peak. In an abundance of caution,

loci where the peak height ratio is >30% (after stutter is subtracted) should be bracketed and marked as NS-artifact, and not used for comparison.
    - Artifacts where the main peak is <IT but above ST AND the PHR is 30% or less can be noted as described above and the main peak can be used for comparisons.
    - A locus with a single peak below IT but above ST in an assumed single source profile can be used for comparisons.
- Loci where the main peak is at or below ST:
    - At the low end of this range, 'reversal', in which an artifact peak height appears at the same or higher peak height as the main allele, is a possibility, due to the stochastic behavior of low-level peaks. Loci where two adjacent peaks are present at or below ST are not suitable for comparison; these loci should be bracketed and marked as NS-artifact.
    - A locus with a single peak below ST in an assumed single source profile can be used for comparisons.

<u>When low level peaks are assessed as an additional contributor:</u>
A profile is generally considered a mixture when two or more alleles above ST are present at two or more single-copy loci. In these cases, the low-level alleles are marked in parentheses, and the report references that a minor component was detected.

<u>When low level peaks are assessed as a possible contributor:</u>
In between the situations described above are situations where a minor contributor may or may not be present. In these instances, the report language must reflect the uncertainty of whether there is a true minor component (e.g., DNA from an additional male source may be present)

Reminder: this guidance does not apply to low-level profiles – see separate section specific for interpretation of low-level Y-STR profiles.

Some common interpretation scenarios are described in the table below:

IF profile includes a main contributor plus…	**AND the low-level peak(s)**	**THEN Report language for the main contributor**	**AND Report language describing number of contributors**
4 or fewer additional peaks below ST	**All** could be attributable to artifacts	Treat like a single source profile	Not mentioned
5 or more additional peaks below ST	(how many could be artifacts doesn't matter)	Describe/treat as a major component	Male DNA from more than one source was detected
4 or fewer additional peaks below ST	**At least one cannot** be attributable to an artifact	Describe/treat as a major component	…more than one contributor may be present…
1 or more low-level peaks	One is above ST <u>and</u> not attributable to an artifact	Describe/treat as a major component	…more than one contributor may be present…
2 – 5 peaks >ST,	(Artifacts are not routinely	Describe/treat as a	Male DNA from more than

at different loci	observed above ST, but the possibility should be ruled out)	major component	one source was detected

Mixture interpretation – assessment of number of contributors
Note: It is not possible to declare the number of contributors with absolute certainty, since more than one person could have the same Y-STR profile. The Y-STR interpretation guidelines in this manual were written for situations in which the case specifics, including both case scenario and the observed reference profiles, do not suggest the presence of close male relatives within the pool of possible contributors. When case specifics suggest the possibility of close male relatives, interpretation must consider the potential for a very high degree of allele sharing. Please consult with the Technical Manager in such cases to determine an appropriate course of action.

Genetic profiles from questioned samples may be from a single individual (single source) or from multiple individuals (mixtures). Assessment of whether a profile is a single source or mixture, a minimum number of contributors, and whether a mixture can be separated out into single source component(s), determines subsequent interpretation. Documentation of interpretation, including assumptions, must be included in the bench notes. Typically, this documentation is written on the electropherogram or included in the written report language.

Unlike autosomal STR analysis, Y-STR mixtures are not used for inclusions. However, in some circumstances, Y-STR mixtures may be used for exclusions. The guidelines for interpretation of a Y-STR profile are highly dependent on whether a single source profile can be drawn from the data.

Indistinguishable mixtures may be suitable for exclusionary purposes. Only single-source components isolated from mixtures are suitable for probative inclusions as well as exclusions. Mixtures might be distinguishable by deduction, major/minor separation, or occasionally by a combination of the two methods.

Y-STR Single Source Samples
In a typical single source sample, loci are characterized by one labeled peak or allele. If two peaks/alleles are detected at DYS385, they should ideally be of equal intensity – that is, the peak height ratio (PHR) is approximately 1:1.

In a typical single source profile, DYS385 is the only locus where two peaks might be observed. Peak height ratios are calculated by dividing the peak height (in RFUs) of the lower RFU allele by the peak height of the higher RFU allele, and then multiplying by 100 to express the PHR as a percentage. Based on validation studies, the minimum expected PHR for a heterozygous locus, where there is no indication of a mixture and 0.50 ng of template DNA is used, is 60.0%. This ratio, however, may be lower with lower amounts of DNA. Peak-height-ratio imbalance may

also result from degraded DNA or the presence of PCR inhibitors. Allelic dropout may occur in degraded/inhibited samples and the possibility of a second allele should be considered at DYS385. If only one allele is detected at DYS385, and it is below ST, the locus cannot be considered as complete, and the locus may be suitable for exclusions only.

Note: Because DYS385 is the only locus where heterozygous alleles are routinely encountered, it is not necessary for alleles at other loci to be greater than ST in a single source sample to be considered complete and therefore suitable for inclusions and exclusions.

Y-STR mixtures

A profile is interpreted as consistent with being from two individuals when at least six loci have two alleles above ST, and no loci other than DYS385 have more than two alleles.

- Peaks which have been determined to be artifacts do not count toward the two alleles per locus. These are peaks which are struck. Examples include:
  - Pull-up peaks
  - Peaks determined to be artifacts associated with Gentegra LD.
  - Peaks that fall below 100 RFU when forward and reverse stutter are both subtracted.
- Peaks which could be artifacts or true alleles do count and must be considered as possible indications of another contributor.

Note: When peaks are above IT but some loci have peak height ratios that vary widely (such as of 90% and 20%), it suggests the possibility of a third, likely closely related, contributor. Alternatively, large variance in peak height ratios when peaks are below ST could indicate degradation or low-level template DNA. Regardless of the cause, if no single copy loci have more than two alleles but the peak height ratios are not largely consistent, the profile should be reported as having at least two contributors.

Relying on the profile being a mixture from exactly two individuals, it is possible to assume the minor component is complete under the conditions specified in the following sections.

Above two contributors, profiles are reported as being from at least three individuals, at least four individuals, and so on. For each minimum number of contributors, there must be at least two loci with that number or more alleles above ST. For example, if least two single-copy loci have four alleles above ST, then DNA from at least four individuals was detected in the sample.

Profile interpretation: Degradation in Mixture Profiles

Poor sample quality can result in degraded DNA; and the extent and nature of the degradation can have a significant impact on the interpretation of a mixture. During the initial

examination of a mixture and prior to comparison to reference samples, the analyst should assess and document:

- observations indicating that the profile shows signs of degradation
- concerns raised by the nature and extent of the observed degradation, and
- descriptions of any necessary interpretation modifications

This documentation is recorded on the relevant electropherogram, typically at the bottom of a page where space permits. Selection of appropriate wording from the following suggestions will be useful for most degraded mixtures, but profile-specific circumstances may require unique statements.

Observations: The following examples of observations are typical of degraded mixtures, but modifications or other observations may be appropriate depending on the specific profile.

A. DI = ____ (Y-STR may or may not use, depending on relevance)

b. Downward slope in peak heights (would typically add this note for visually obvious difference in peak heights from small to large loci)
c. Change in approximate CR (Y-STR would typically note this for profiles with visually obvious discrepancies – as an example, a two-allele locus with ~even peak heights and other loci with low PHRs)

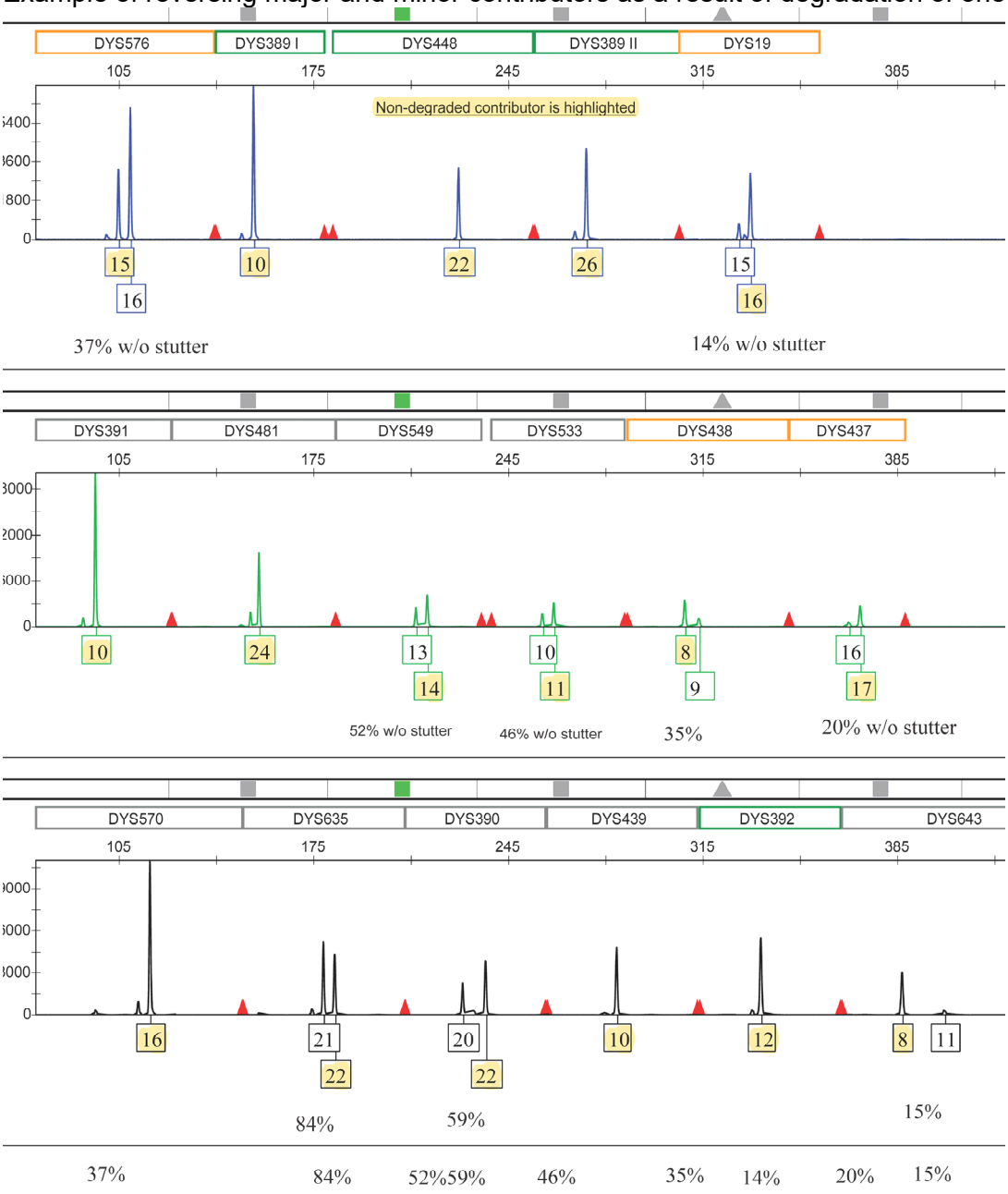
Concerns: The following examples of concerns are typical for degraded mixtures, but other descriptions may be more appropriate depending on the specific profile.

a. Number of contributors cannot be determined
b. Unreliable CR – can't deduce / can't major-minor / can't assume complete (possible drop out)
c. Due to differing degrees of degradation, major and minor contributors may be reversing within the profile (see example after Modifications to interpretation.)
d. Owner profile degraded / ~no impact on probative contributor

Modifications to interpretation: The following proposed suggestions for when and how to restrict interpretation based on observations and potential impacts are typical examples, but other suggestions may be appropriate depending on the specific profile.

a. Interpretation at smaller loci only
b. Interpretation based on obligates only
c. Interpretation only loci with more info
d. No interpretation without elimination sample
e. No interpretation due to sample quality

Example of reversing major and minor contributors as a result of degradation of one contributor:



8.2 Interpretation of Y-STR Questioned Profiles

Criteria for interpretable versus uninterpretable data (QAS 9.6.5)

Fully interpretable (suitable for probative inclusions): A single source profile, or a single-source component of a mixture, must be suitable for comparison at a minimum of 12 loci to be deemed suitable for probative inclusions. Requirements for each mixture component type are described in detail later in this section.

Examples of fully interpretable profiles include:
- profiles with a single source of DNA
- deduced contributors
- single-source major component from a mixture
- single-source minor component from an apparent two-source mixture

Interpretable with limitations: Profiles which are not suitable for probative inclusions may be suitable for non-probative inclusions or for exclusions only.

Non-probative inclusions: A single source profile, partial profile, low-level profile, or minor component from any sample which is solely attributable to an elimination sample is not used for statistical analysis but is addressed in the report with a qualitative statement.

Requirements:
- Alleles are suitable for comparison at a minimum of 6 loci are detected and
- The elimination sample profile can account for all the alleles in question.

Exclusions only**:** A single source or mixture profile may be suitable for exclusions, even if it is not suitable for inclusions.

Exclusions are based on the presence of at least one obligate allele above ST which does not match the reference profile. Exclusions in minor components or low-level samples may not be based on the absence of an allele, because low-level contributors may have data which falls below the detection threshold due to stochastic effects.

Requirements:

- Locus (or component) can be considered complete
- For profiles with a mixture: allele(s) at locus (or component of locus) are above ST. For alleles in stutter positions, max stutter is subtracted, and the remaining peak height must be above ST to be considered for exclusions.
- At least six loci meet these criteria

Examples of profiles that would be suitable for exclusions only include:

- partial single source profile with fewer than 12 loci detected
- indistinguishable two-source mixture with no indication of drop-out
- distinguishable minor component in an apparent two source mixture, detected at 6-11 loci
- low-level single source profile with at least six alleles detected

Not interpretable: Profiles may be uninterpretable because of insufficient data, profile complexity, or the inability to distinguish artifacts from true alleles.

Examples of profiles that are not interpretable or suitable for comparisons:

- Low-level profiles or minor components where more than one contributor may be present
- Indistinguishable mixtures of 3 or more sources
- Low-level single source profiles in which all obligate non-matching allele(s) are below ST.

Criteria for Y-STR Mixture Interpretation

Deduced contributors

To be suitable for deduction, these conditions must be true:

- Both an elimination sample and a probative reference sample(s) have been submitted for analysis
- The questioned profile is consistent with being from two individuals. A profile is interpreted as consistent with being from two individuals when at least six loci have two alleles above ST, and no loci other than DYS385 have more than two alleles. Peaks which have been determined to be artifacts do not apply. Peaks which could be artifacts or true alleles do apply.
- If deduction is not possible at a minimum of 12 loci, the deduction should not be performed.

Deduction can allow for more complete interpretation of a two-source profile when the questioned profile cannot be fully separated into major and minor components. Little or no degradation effects present in the questioned profile will maximize the chance of successful deduction, but deduction based on obligate alleles only may be possible even when degradation is observed.

Rules for deduction

- Begin by calculating a contributor ratio by averaging the peak heights of elimination and probative alleles from at least four two-allele loci, using alleles above IT (above ST is acceptable when there are not four loci with both alleles above IT).

For loci with two alleles:

- The probative allele must be above ST. If the probative allele is in a stutter position, max stutter is subtracted before determining if the peak is above ST.
- If the locus is suitable, deduce the non-elimination allele as the probative allele.

For loci with one allele:

- The allele in question must be above IT.
- Based on the contributor ratio, determine whether the probative allele would be below ST or potentially masked by stutter filter. If the probative allele would be less than ST or potentially masked, the locus cannot be deduced.
- If the locus is suitable, deduce one allele shared by elimination and probative contributor

For DYS385:

- This locus can only be deduced when two obligate probative alleles are present.

Documentation of deduction is noted on the electropherogram.

- Assumptions are that the mixture is 2 sources only, and the elimination reference is one contributor. These must be stated in the report and do not need to be written on the electropherogram.
- Contributor ratio calculation is included on the electropherogram
- Loci not deduced are noted as ND with the reason the locus could not be deduced.
- Elimination alleles are noted with an “E”
- Deduced probative alleles are noted with a “P”
- A deduced shared peak is noted with “SP”

Rules for major/minor separation when DNA from more than one contributor may be present AND/OR more than one contributor was detected

- A profile is described as 'DNA from more than one contributor may be present' when the profile includes a main contributor plus either of the following situations (disregarding DYS3385):
  - Four or fewer additional peaks below ST, where at least one peak cannot be attributable to an artifact
  - One or more low level peaks, where at least one is above ST and cannot be attributable to an artifact
- A profile is described as 'Male DNA from more than one source was detected' when the profile includes a main contributor plus either of the following situations (disregarding DYS3385):
  - Five or more additional peaks below ST (how many are artifacts doesn't matter)
  - Two to five additional peaks above ST, at different loci
- A major component must be distinguishable at a minimum of 12 loci according to these rules, or major/minor should not be separated
- Major/minor separation of the profile when the contributor ratio is between ~2: 1 and 1: 2 is inappropriate. That is unlikely to be the case for profiles in this category, and there will likely not be sufficient loci to accurately calculate a contributor ratio. If a contributor ratio in this range is suspected, document Possible High Contributor Ratio as the reason the profile was not separated.
- DYS385 cannot be separated into major and minor components if more than one allele is present. If only one allele is present and it is above IT, it may be included in the major component.
- Two-allele locus - proposed major component allele is above IT
  - Peak height ratio must be 60.0% or less to separate into major and minor components
- Two-allele locus - proposed major component allele is below IT but above ST
  - When major component allele is above ST, peak height ratio must be 30.0% or less.
  - Degradation, low template, or shared alleles may mean that separation of major and minor alleles is not appropriate when the major peak is above ST.
- One-allele locus – major component only
  - A single peak above ST or above IT at a locus can be assumed to be in the major component

Rules for major/minor separation in mixture consistent with being from two sources

- These rules apply when the questioned profile is consistent with being from exactly two individuals. A profile is interpreted as consistent with being from two individuals when at least six loci have two alleles above ST, and no loci other than DYS385 have more than two alleles. Peaks which have been determined to be artifacts do not apply.
- A major component must be separated at a minimum of 12 loci according to these rules, or major/minor should not be separated
- Major/minor separation when the contributor ratio for the profile is between ~2: 1 and 1: 2 is inappropriate. A contributor ratio can be calculated using four two-peak loci where both the major and minor alleles are above IT (ensures the consistency in the peak height ratios). It is only necessary to document the calculated contributor ratio when that is the reason that major/minor separation did not occur.
- DYS385 cannot be separated into major and minor components if more than one allele is present. If only one allele is present and it is above IT, it may be included in the major component.
- Two-allele locus - proposed major component allele is above IT
    - Peak height ratio must be 60.0% or less to separate into major and minor components
- Two-allele locus - proposed major component allele is below IT but above ST
    - When major component allele is above ST, peak height ratio must be 30.0% or less.
    - Degradation, low template, or shared alleles may mean that separation of major and minor alleles is not appropriate when the major peak is above ST.
- One-allele locus – major component only
    - A single peak above ST or above IT at a locus can be assumed to be in the major component
- One allele locus – shared major and minor component allele
    - Under these conditions, a single peak locus may be inferred to be a shared peak that includes both the major and minor contributor:
        - A contributor ratio can be calculated as described above
        - The minor: major contributor ratio is greater than 1.00:5.00 (ensures that the minor component will be above stutter percentages at all loci)
        - The peak at the single-allele locus is above IT
        - A single peak allele which is determined to be a shared peak is noted on the electropherogram as “SP”.
- The assumption of the mixture being consistent with being from two individuals must be included in the report language.
- If at least 12 distinguishable minor alleles (not shared) are above ST, the minor component and the major component are each suitable for inclusions and exclusions.
- If at least 6 minor alleles (not shared) are above ST, the minor component is suitable for exclusions only and the major component is suitable for inclusions and exclusions.
- If fewer than 6 minor alleles (not shared) are above ST, the minor component is not suitable for comparisons and the major component is suitable for inclusions and exclusions.

Rules for major/minor separation in a mixture of at least two sources

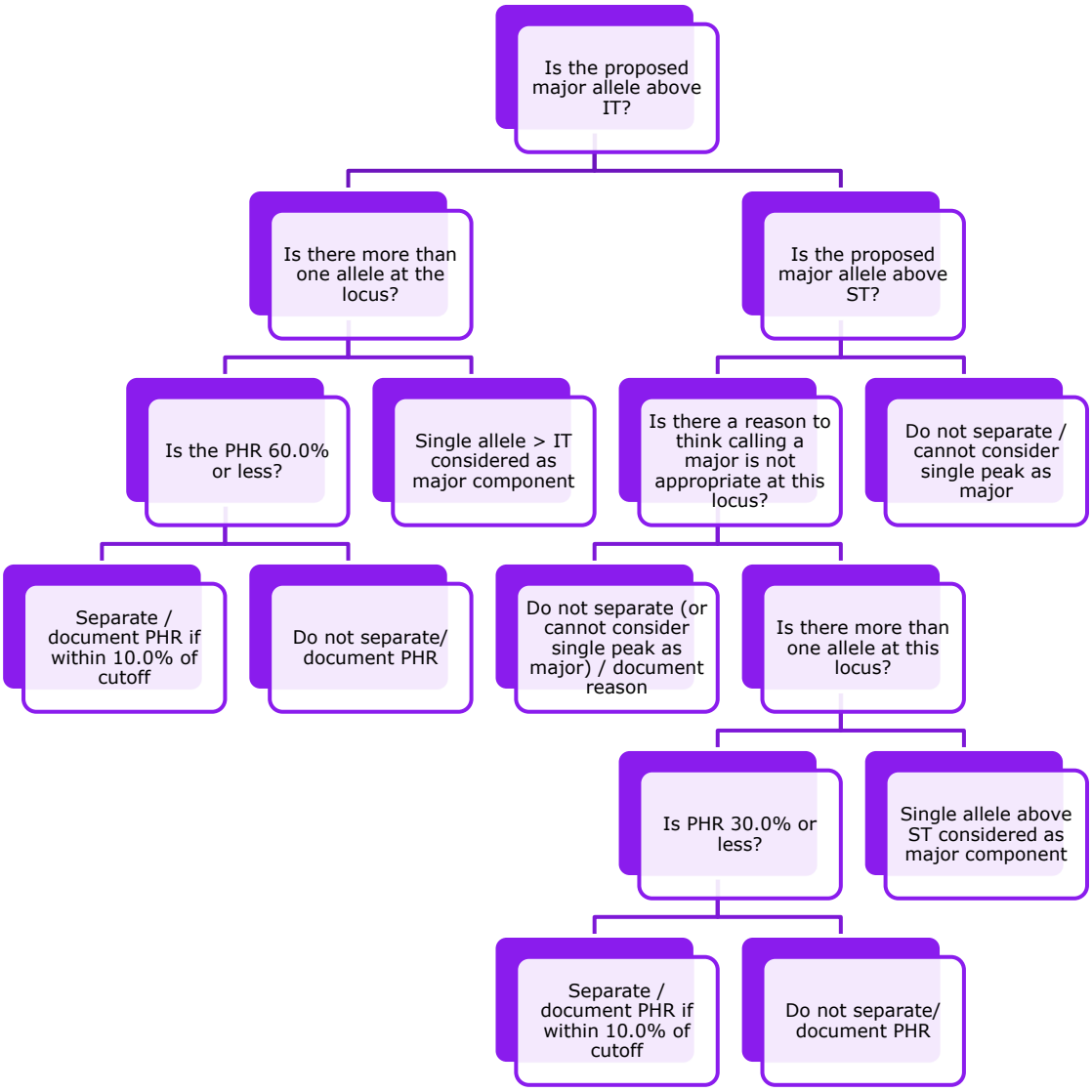
- Mixtures of this type include two contributors plus a possible low-level third.
- A major component must be distinguishable at a minimum of 12 loci according to these rules, or major/minor should not be separated
- DYS385 cannot be separated into major and minor components if more than one allele is present. If only one allele is present and it is above IT, it may be included in the major component.
- Two-allele (or more) locus - proposed major component allele is above IT
    - Peak height ratio must be 60.0% or less to separate into major and minor components
- Two-allele (or more) locus - proposed major component allele is below IT but above ST
    - When major component allele is above ST, peak height ratio must be 30.0% or less.
    - Degradation, low template, or shared alleles may mean that separation of major and minor alleles is not appropriate when the major peak is above ST.
- One-allele locus – major component only
    - A single peak above ST or above IT at a locus can be assumed to be in the major component
- Under these conditions, the major component is suitable for inclusions and exclusions, but the minor component is not suitable for comparisons due to the possibility of drop-out or multiple contributors (insufficient DNA or genetic complexity).

Rules for major/minor separation in a mixture with at least three or more contributors

- Major component allele must be above IT
- Locus with more than one allele: peak height ratio must be 60.0% or less to separate a major component
- Locus with one allele: can be assumed major component if above IT
- A major component must be separated at a minimum of 16 loci according to these rules, or major/minor should not be separated
- DYS385 cannot be separated into major and minor components if more than one allele is present. If only one allele is present and it is above IT, it may be included in the major component.
- Under these conditions, the major component is suitable for inclusions and exclusions, but the minor component is not suitable for comparisons due to its genetic complexity.

Major/minor separation in a Y-STR mixture – more than one / two sources / at least two sources

- **If a profile cannot be separated at 12 or more loci, separation should not be performed.** Given the less informative nature of Y-STR profiles, separation should be attempted at as many loci as feasible to maximize chance of successful interpretation.
- DYS385 cannot be separated into major and minor components if more than one allele is present. If only one allele is present and it is above IT, it may be included in the major component.
- **Examples of reasons that separation might not be appropriate** at a given locus or for a profile as a whole include degradation, allele sharing, low-template DNA concerns, and/or contributor ratio in the range of 1:2 to 2: 1. These issues could potentially affect loci with alleles over IT but are more of a concern when peak heights are between IT and ST because of increased peak height variability in that range.
- PHR = peak height ratio
- Yes = Left; No = Right



8.3 Comparison of Y-STR Results

Refer to STR interpretation section for discussion of types of result comparisons for probative and non-probative samples.

In a case where Y-STR profiles suggest a close biological relationship between potential contributors (parent-child, siblings, or any individuals with Y-STR profiles that differ at no or very few loci), the ability to draw conclusions may be impacted. Reported conclusions must reflect any impact caused by potential relatedness among submitted references.

8.4 Probative associations and statistical analysis

Statistical analyses will be performed when the genetic typing results fail to exclude an individual(s) as a source of the DNA in a questioned sample, and the positive association is to a probative reference sample. The statistic is calculated from the questioned profile and includes the statistics-eligible loci which are completely detected (or have a declared null allele) and are eligible for population statistics. (Note: in the rare case that a known sample does not yield a full profile at all statistics-eligible loci, then the questioned profile loci which could not be compared to the reference sample are not included in the statistic.)

Statistical analyses are performed by searching the profile on the YHRD website.

- From the home page, select Search the database from the top menu
- Select Manually enter the haplotype/haplotypes to search for
- In the top row labeled Dataset, select Y17
- In the rows labeled Kit, select PowerPlex Y23.
- Enter data for all loci suitable for inclusions, then click Search
- In the blue bar, click Add features to this report
- From the dropdown menu, choose National Database (with subpopulations, 2014 SWGDAM-compliant)
- Once again, in the blue bar, click Add features to this report
- From the dropdown menu, choose Metapopulation
- In the result box that appears under National Database (with subpopulations, 2014 SWGDAM-compliant) result box, click to change, then choose Eskimo Aleut from the dropdown menu.

The most common frequency among the Caucasian, African American, Native American, and Eskimo Aleut population groups is reported, using the Observed profile probability with 95% confidence interval.

Section 9 Report Writing

This section covers the required content for Forensic Biology reports.

9.1 Required Content

Forensic Biology report required content

All Forensic Biology reports will contain the following (as applicable to the analysis performed):

- Date report was issued (automatically populates)
- Laboratory and agency case numbers (automatically populates)
- Name of submitting agency and case officer (automatically populates)
- List of all items for which analysis was requested in the assignment
    - Includes identification of which samples (such as stains, swabs, or other sub-items) were tested. In this context, tested means that an attempt was made to isolate DNA from the sample.
    - include the agency item # in ( ) if the item was re-numbered at the laboratory
    - this is not necessary if the laboratory number contains the original agency item #
    - elimination reference samples are noted as such in parentheses, such as (previously identified as a consent partner of Jane Smith)
    - items which belong to a specified individual are noted as such in parentheses, such as (previously identified as belonging to Jane Smith)
- When DNA analysis is reported for samples retained by another discipline in the laboratory, or comparisons are made to profiles reported by another laboratory, the report should reference the previously issued report via the request custom form.
    - Include the issuing laboratory, if other than the AK SCDL.
- Results of presumptive/confirmatory tests (in body of report) if not previously reported in a biological screening report
- Results, conclusions, and opinions, for all tested items, based on the DNA typing results where appropriate (guidelines for reporting are provided in section 9.2 and may be modified, as necessary, on a case-by-case basis). Note that results are given for each differential fraction generated.
- Results of any statistical analyses performed
- Explanation of why testing was stopped, if appropriate
- Statement of which profiles have been entered (or removed) in CODIS, if appropriate
- Description of analyses performed, including technology used and a list of STR loci amplified (this is contained in the Methods section of the report
- Description of statistical analyses performed including the methods and loci used in the calculations (this is contained in the Methods section of the report)
- The disposition of the evidence (this is usually contained in the standard report template)
    - Consumed evidence is noted either in the body of the report or in the items analyzed section.

    - Retained sub items may be noted in the items analyzed section. For example, if two isolated stains were retained from item 123, then the 123 item description would also include “123-1 and 123-2 were retained as 123JLF”
- Any known samples that are required for (further) DNA analysis, when applicable
- Signatures of the reporting analyst and the technical reviewer (the analyst will electronically sign the report by setting the milestone to Draft Complete prior to submitting for technical review; only the analyst is required to scan their barcode and enter a pin when signing the report in LIMS)

9.2 Common report terminology

The JT / LIMS-DNA workflow is pre-populated with appropriate report terminology. In the rare instance of needing to write a report manually, please refer to previously issued report language and/or consult with the Technical Manager.

Section 10 Review

All case reports issued by the Forensic Biology discipline will be subjected to a technical review and an administrative review. All supporting documentation that is part of the case record or the LIMS-DNA packet will be subjected to a technical review. Refer to LIMS-DNA Review Work Instructions for more details on how reviews are performed.

Technical reviews of casework will be conducted by a second qualified analyst, in accordance with the laboratory Quality Assurance Manual (current version) and the FBI QAS Guidelines (current version) as applicable.

Administrative reviews of casework will be conducted by an analyst qualified for casework within the Forensic Biology discipline. Any report which includes DNA profile(s) must be administratively reviewed by an analyst qualified in DNA analysis. Note: technical and administrative review for a case may be performed by the same analyst.

The analyst and the reviewer may consult a third qualified analyst, if necessary, when there is a disagreement on how to report a result. If the analysts are unable to come to an agreement, the DNA Technical Manager will be consulted to make the final decision.

The completion of the final laboratory report, technical review and administrative review are tracked electronically in LIMS.

When a case has been through technical and administrative review, the report will be issued in accordance with laboratory policies and procedures.

This section covers the following topics:

10.1 Compiling documentation for casework review

Refer to JT / LIMS-DNA Work Instructions for details of how to record casework and related batch control documentation.

10.2 Review of Casework Analyzed In-House

Technical review of Forensic Biology casework includes the following, at a minimum and as applicable, to ensure compliance with the interpretation guidelines contained within this document:

- Review of all worksheets contained within the bench notes and LIMS-DNA packet
- Review (usually electronic) of the ILS for all passing samples

- Review (usually electronic) of all allelic ladders designated as such
- Review (usually electronic) of at least one passing amplification positive control per zone
- Review (usually electronic) of all reagent blanks and negative amplification controls
- Review of DNA typing data (used to draw conclusions) for questioned and known samples
- Verification that all results/conclusions in report are supported by data
- Review of all statistical analyses
- Verification of CODIS eligibility and review of entry for all entered profiles
    - Eligibility review must occur prior to entry
    - Eligibility review of a Forensic Unknown profile is based on the electropherogram
    - Eligibility review of a Forensic Mixture or Forensic Partial profile is based on the Match Estimation report provided by the analyst along with the report and bench notes. The Match Estimation report should include notes of any loci to be marked as partial, as well as any notations of obligate alleles. Since this content is reflected in the profile that is ultimately uploaded, it is not necessary to retain the Match Estimation report once the review process has been completed.

Administrative review of Forensic Biology casework will be conducted by a second qualified analyst, in accordance with the FBI QAS Guidelines (current version) and includes the following, at a minimum, to ensure compliance with the interpretation guidelines contained within this document:

- Review of case file and final report
- Review of chain of custody and evidence disposition
    - The chain of custody review will typically include checking that all retained evidence and dried extracts have been repackaged and returned to their appropriate locations.
    - In extreme rush cases, it may not be expedient to wait for extracts to be dried down and repackaged before distributing the final report. In such cases, it is okay for the administrative review to not include a chain of custody review of final evidence returns. However, in such cases, this must be clearly noted using the Reviewer field of the request.

The completion of the full technical review is documented by the technical reviewer milestone in the LIMS. All documentation (including bench notes, forensic histories, checklists, CODIS specimen detail reports, etc. as case appropriate) must be in JT for final completion of the administrative review. The completion of the full administrative review, including a check of all scanned documentation, is documented by the administrative reviewer milestone in the LIMS.

When a case has been through technical and administrative review, the report will be issued in accordance with laboratory policies and procedures. Genetic profiles suitable for CODIS will be uploaded in accordance with CODIS policies and procedures.

Note: Following completion of all reviews, both LIMS-DNA Supplemental Workbooks and raw data files from 3500 analysis are ultimately retained in SharePoint. For ease of future discovery requests, it is recommended that the Supplemental workbook and raw data files are zipped together by batch for long term retention in SharePoint. Review checklists may be used to assist in the review process but are not retained with the case file.

10.3 Review of RapidHIT data

Genetic profiles and all supporting documentation generated during analysis will be subjected to a technical review.

RapidHIT Profiles in Forensic casework:

In addition to all the review elements discussed in the Review section, technical review of RapidHIT reference profiles in Forensic Biology casework specifically includes the following, at a minimum and as applicable, to ensure compliance with the interpretation guidelines contained within this document:

- Review of all relevant documentation contained within the LIMS-DNA packet
- Review of the ILS for all passing samples
- Run data (Consolidated audit document) page is included
- Review of allelic ladder run in-house (performed during verification of the primary cartridge; documentation of the review is included as a scanned JT attachment) Note: technical review of systemic library allelic ladders was completed and documented as a part of the validation of RapidHIT.
- Review of all amplification positive controls (performed during verification of the primary cartridge; documentation of the review is included as a scanned JT attachment)
- Review of all reagent blank/negative amplification controls (performed during verification of the primary cartridge and/or sample cartridge lot; documentation of the review is included as a scanned JT attachment)
- Ensure that the lot numbers for the Sample Cartridge (SC) and Primary Cartridge (PC) are consistent between the run data page and the verification pages.
- Review of DNA typing data for known sample

Rapid HIT profiles in batched CODIS samples and CODIS confirmations:
The DNA analyst will submit a packet containing a STR Offender Batch Review Checklist and sample electropherogram(s), and control sample and run documentation to another qualified analyst for technical review. Unlike in batched database analysis, profiles generated by RapidHIT are manually entered into CODIS. This review will be conducted in accordance with the FBI QAS Guidelines and includes the following, at a minimum, to ensure compliance with the interpretation guidelines contained within this document:

- Review of the ILS for all passing samples
- Reagent and run documentation (Consolidated Audit Report) is included
- Review of allelic ladder run in-house (performed during verification of the primary cartridge; documentation of the review is included with central log) as well as the allelic ladder used for analysis of the sample.
- Review of all amplification positive controls (performed during verification of the primary cartridge; documentation of the review is included with sample bench notes or central log)
- Review of all reagent blank/negative amplification controls (performed during verification of the primary cartridge and/or sample cartridge lot; documentation of the review is included with sample bench notes or central log)
- Review of all DNA typing data for all passing samples
- Ensure that the lot numbers for the Sample Cartridge (SC) and Primary Cartridge (PC) are consistent between the run data page and the verification pages.
- Verification of eligibility for all profiles uploaded by analyst, to include selection of an appropriate specimen category
- Concordance with previous results for re-processed samples

The analyst and the reviewer may consult a third qualified DNA analyst, if necessary, when there is a disagreement on how to report a result. If the analysts are unable to come to an agreement, the DNA Technical Manager will be consulted to make the final decision.

A STR Offender Batch Review Checklist is used to document completion of the individual components of the technical and administrative review. Upon completion of the technical review, the analyst may import the passing samples to SDIS. Another qualified analyst or a CODIS Administrator will then complete an administrative review, to include reconciling the results of the offender duplicate and casework autosearches.

Upon completion of the technical and administrative reviews, NDIS eligible samples are uploaded by a CODIS Administrator. Additional information on CODIS procedures is contained in the CODIS Administrative Manual. Upon completion of the upload, the following paperwork is archived as a .pdf in SharePoint:

- Central log documentation
- Electropherograms of DNA profiles
- STR Offender Batch Review Checklist

Rapid HIT profiles for CODIS confirmations worked individually:
Since CODIS confirmations cannot currently be included in the LIMS-DNA workflow, the most efficient way for a casework analyst to confirm an offender profile is by using RapidHIT analysis. This process does not require a retained checklist. The technical reviewer must check the following elements in JT:

* Offender number(s) and analyst initials are on each page (should be automatically added to JT attachments)
* Offender number(s) on documentation is consistent with LIMS
* Start and end dates of examination/analysis are documented
* Run data (consolidated audit document), verification of sample cartridge, verification of primary cartridge, electropherogram of confirmation sample (includes ILS) are retained in JT
* SOPs are linked to request in LIMS
* ILS on confirmation profile is passing
* Review of Allelic ladder documented on verification of primary cartridge
* Review of positive and negative controls are documented on verification of primary and/or sample cartridge
* Lot numbers on sample and primary cartridge lot verifications match lot numbers on run data
* Results are consistent with previous typing results
* Technical review of all electropherograms: marked as reviewed in JT
* Results (e.g. profile verified; or insufficient data for verification) given for the tested item
* Profile verification status updated in evidence custom form, as applicable

Appendix A: EZ2 Recovery Protocols

These protocols include directions for recovery of DNA samples when an EZ2 run is stopped mid-process, either intentionally (stopped by the user) or unintentionally (power failure, instrument error, etc.).

- Because the recovery protocols are rarely used, best practice would be to have a second qualified analyst witness the recovery processing. Witnessing is documented in the Verifications/Performance Monitoring/Hair tab in the FB Analysis form.
- The reagent cartridges should be labeled and retained in a DNA evidence refrigerator until quantification results indicate that DNA was successfully recovered.

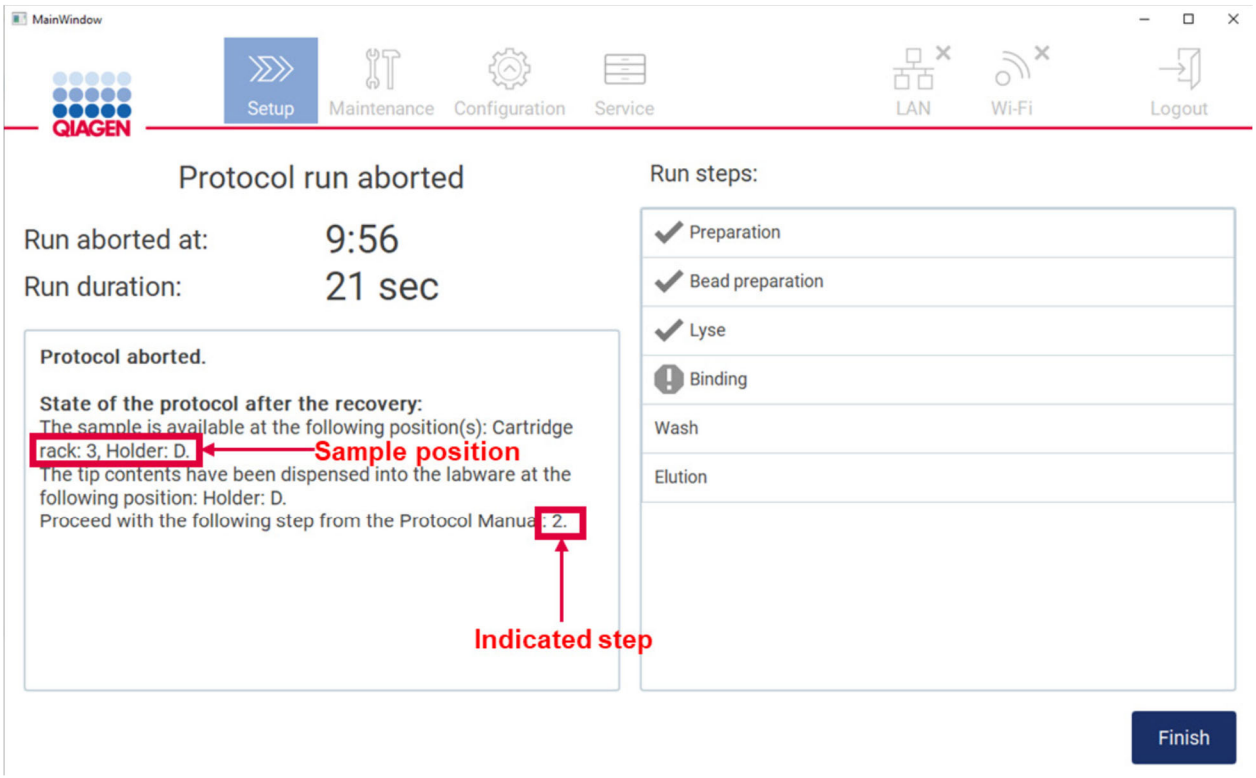
Documentation:

- If an EZ2 run is aborted by the user, it is not necessary to add documentation to the instrument log.
- If an EZ2 run unintentionally stops, the event must be noted in the instrument log book, along with a suspected cause (e.g., power outage, tip holder was picked up along with pipette tip, etc.). The Technical Manager must also be notified.
- If a recovery protocol is used, the LIMS-DNA packet must note which EZ2 run(s) failed and which recovery protocol was used.
- If the recovery protocol is demonstrably successful, as shown by robust recovery of at least one ICS or casework sample (T-S or T-L value of 1.0 or higher), and no casework samples or reagent blanks are adversely impacted, no further documentation is required.
- If the recovery protocol cannot be demonstrably deemed successful, or if downstream amplification of reagent blanks or casework are impacted, consult with the Technical Manager for the appropriate course of action and/or documentation.

Note: if the instrument was switched off unintentionally, re-start the device. The recovery screen should appear.

Note: if the sample remains in the pipette tip after the instrument switched off, place a tube under the tip and remove the tip from the pipette head. The liquid should now run out of the tip into the tube.

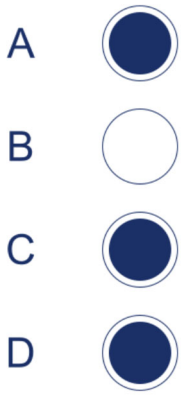
Example of a recovery screen:



This screen gives necessary information for sample recovery, highlighted in red in the above diagram.

Cartridge rack: This identifies the well within the cartridge. In the image below, numbering starts from the bottom (the well closest to the door when the cartridge is loaded on an instrument).

Rack (Holder): If DNA is in one of the tubes in the rack in front of the cartridge rack, the letter corresponds to these locations, where D is the position nearest the door of the instrument:



Indicated step: refer to the chart below to determine which recovery step is needed:

Indicated Step	Process status	Corresponding run step(s) on recovery screen
1	Sample untouched	Preparation, Bead preparation
2	Buffer MTL added to the sample, beads may or may not have been added	Lyse / Binding
3	During wash 1	Wash
4	During wash 2	Wash
5	During wash 3	Wash
6	During rinse step	Wash (Current step = rinse)
7	During elution step	Elution

Recovery Protocol for Step 1: If the run fails before the sample lysate has been touched, replace the consumables and restart the run.

Recovery protocol for step 2 – Lyse / Binding:

- Recover the lysate and all beads, if already added. Expected volume is ~900 µL (trace protocol) – 1600 µL (large volume protocol).

- If initial run was Trace protocol, the lysate and beads are recovered in a single tube and run with the in a Large Volume protocol.
- If the initial run was Large volume, the lysate and beads must be recovered in two tubes, with a maximum of 900 µL per tube.
  - Run with Large volume protocol, elution in **water** (not TE).
  - Combine the elute from the two tubes and add Gentegra to dry down.
  - Reconstitute with TE buffer.
  - Quantification may be performed either after the eluates are initially combined or after the combined eluate is reconstituted. Note that the FBPM quantification decision trees assume a volume of 40 µL, so triage decisions will need to take into account the different volume, if applicable.

Recovery protocol for steps 3 through 5 – Washing

- Resuspend the beads in the cartridge rack by pipetting up and down.
- Transfer the beads to the elution tube (Rack D).
- To get a bead pellet, centrifuge to the elution tube at 6000 x g for 1 minute.
- Remove and discard supernatant, leaving a total volume of ~200 µL in the tube.
- Run the recovered sample in a Trace protocol (elution in 40 µL for questioned extracts, 40 µL or 200 µL for knowns).

Recovery protocol for step 6 – Rinse

- Recover all beads and all water used for the rinse.
- Split into two fractions of about 500 µL each Run with Large volume protocol, elution in **water** (not TE).
- Combine the elute from the two tubes and add Gentegra to dry down.
- Reconstitute with TE buffer.
- Quantification may be performed either after the eluates are initially combined or after the combined eluate is reconstituted. Note that the FBPM quantification decision trees assume a volume of 40 µL, so triage decisions will need to take into account the different volume, if applicable.

Recovery protocol for step 7 – Elution

- Place sample on a thermomixer (set to room temperature) for 5 minutes at 900 rpm.
- To get a bead pellet, centrifuge to the elution tube at 6000 x g for 1 minute.
- Transfer the eluate (supernatant) to a new tube.

Back to Prioritizing Analysis, Sampling, and Extraction.

Appendix B Long-Term Storage of DNA Extracts by GenTegra-DNA

Note: All references in this manual to samples being dried down are referring to extracts dried with GenTegra-DNA (or, in the case of previously dried down extracts, dried with DNA Stable LD).

1. All questioned extracts (except from proficiency tests) are routinely retained. Retained DNA extracts are dried down when the analyst has determined that no further work is required on the extract. GenTegra DNA (GTD) may be added to extracts before or after amplification.
2. Prepare the GTD 5X stock solution:
    a. Add 0.55 mL sterile water to the GenTegra-DNA tube
    b. Dissolve with occasional gentle vortexing for 5-10 minutes.
    c. Note the date when water was added on the tube. The expiration date for the rehydrated GTD stock is 3 months after the date of hydration. (Example: GTD tube hydrated on November 2, 2021 would have an expiration date of February 2, 2022. For convenience of documentation, a GTD tube hydrated on November 30, 2021 would have an expiration date of February 28, 2022.)
3. Spin down the DNA extract by centrifuging at approximately 14,500 rpm for 5 minutes.
4. For DNA extract volumes up to 245 µl, add 5 µl of GTD 5X stock directly to the DNA extract. Mix the sample thoroughly with gentle pipetting. Avoid dispensing bubbles into the sample.
    a. NOTE: For samples with extract volumes below 10 µl, add 10 µl of sterile water to the extract (as well as to its corresponding reagent blank) prior to adding the GTD. This ensures that DNA in small volumes of liquid have adequate contact with the GTD solution.
5. Dry the DNA extract solution by placing the uncapped tube in a rack on the lab bench or in a laminar flow ventilation hood (recommended). The uncapped tube may be covered with a Kimwipe ®. Ideally, drying times are 24 hours for 38 µl volumes. Samples must be dried completely for optimal protection and stability when stored at room temperature.
6. Once dry, cap the tube and store in either (a) a dry storage cabinet at room temperature or (b) in a foil-lined, moisture barrier envelope with a silica gel desiccant packet added (also at room temperature). Typically, one extract is packaged per foil envelope. More than one extract derived from a single sample (such as sperm and epithelial fractions) may be packed in a single foil envelope if the envelope contains one desiccant packet per extract. Dried extracts are retained long-term in the original evidence packaging. Alternatively, if the foil envelope does not fit in the original packaging, or if the original item is to be returned to the submitting agency, a new item is created in the LIMS.
7. To recover GTD dried samples, add sterile water. The maximum volume of added water can be as large as the volume of the sample prior to drying down. Up to 38 µL of extract eluted in TE buffer may be reconstituted in as little as 15 µL dH2O. However, if a larger volume of TE-eluted extract is dried down, the minimal volume for reconstitution must

also be proportionally larger. For example, if 80 µL of extract eluted in TE is dried down, it must be reconstituted in no less than 30 µL dH2O. This is specifically the case for extracts eluted in TE buffer, not in water. Incubate the sample at room temperature for 15 minutes to allow complete rehydration. Mix the sample thoroughly with vortexing or by using a Thermomixer at 900 rpm without added heat. The use of the mixer is specifically recommended for samples rehydrated with less than 20 µL dH2O; however, additional vortexing may be necessary to ensure the sample is thoroughly homogenized. Store unused rehydrated samples at room temperature or at 4°C for up to 10 days. After ten days, or sooner if work is completed, unused samples can be redried as in step 5 without appreciable DNA loss.

Note: This recovery procedure is also used for extracts dried with DNAstable ® LD

8. When a questioned sample is recovered and amplified, the corresponding reagent blank previously unworked (usually labeled as reagent blank replicate -2) is brought up in the same volume of sterile water and amplified as well.
9. Extracts may be dried down and rehydrated multiple times, until a maximum of 75% of the original sample is removed.

Some common scenarios for drying down by Gentegra-DNA:

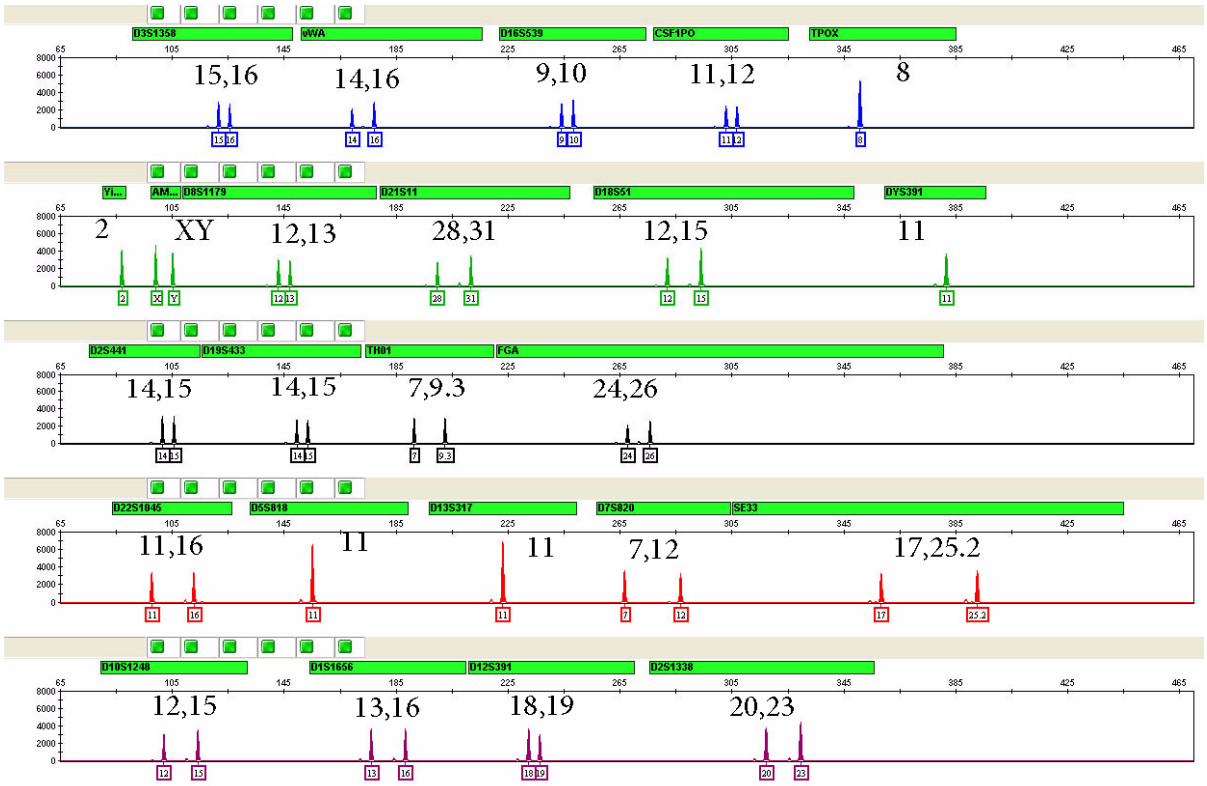
**The scenarios below assume GTD either added before quant then dried down and brought back to selected volume, or added after amplification.*

Purpose	Volume dried down	Volume dH_2O to reconstitute	Reagent blank concerns	Limitaitons and considerations
Long-term storage of casework extract without amplification	~38 µL (or full volume of extract after quantification for most questioned extracts)	N/A	Typically reagent blanks are amped at the time of creation to rule out (or troubleshoot) contamination.	N/A
Full consumption of extract of sample – 40 µL elution volume	~38 µL (or full volume of extract after quantification)	Either 15 µL for GlobalFiler or 17.5 µL for PowerPlex Y23 amplification	Corresponding reagent blank treated the same	For extracts from casework items which were fully consumed for extraction (e.g., a single swab, all of a blood stain, etc.), written permission to consume must be obtained prior to amplification.
Amplification of the "full half" of available extract (while retaining half for future testing) Two options for processing are shown.	The retained half of the original extract (20 µL for a 40 µL elution) stays in its tube with 5 µL GTD added and dried down. All remaining extract (18 µL if one quant was done) removed to an amp setup tube with 5 µL GTD added and dried down.	Either 15 µL for GlobalFiler or 17.5 µL for PowerPlex Y23 amplification, added to the amp tube only	Typically reagent blanks are fully concentrated and amplified.	For a 40 µL elution, the half available to the analyst is 20 µL to be used for quant and amp. If two quants are run, then only 16 µL are available for amp.
	~38 µL (full volume of extract after quantification)	At least 15 µL and up to 38 µL. Volume can vary, but analyst can use no more than 47.4% of the rehydrated extract. Remainder is dried down again and retained.	Typically reagent blanks are fully concentrated and amplified.	47.4% allows for full use of analyst's half of the extract and assumes 2 µL were used for quant. (18 µL / 38 µL = 47.4%) If two quants were done, the analyst can use 44.4% (16 µL / 36 µL).

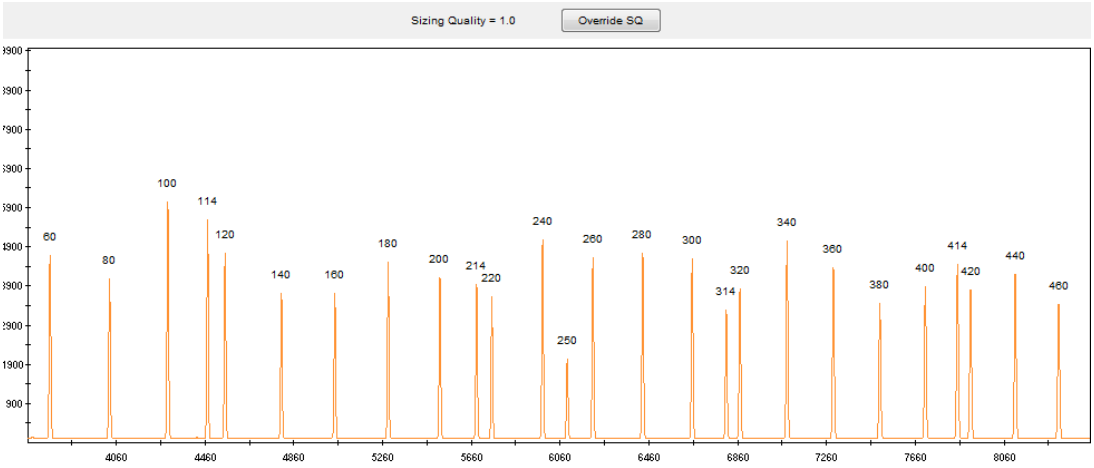
Purpose	Volume dried down	Volume dH_2O to reconstitute	Reagent blank concerns	Limitaitons and considerations
Amplification of more than half but less than all the extract Two options for processing are shown.	All extract to be amped removed to an amp setup tube with 5 µL GTD added and dried down. The remainder of the original extract (less than 20 µL for a 40 µL elution) stays in its tube. If extract volume is <10 µL then add 10 µL water. 5 µL GTD added and dried down.	Either 15 µL for GlobalFiler or 17.5 µL for PowerPlex Y23 amplification, added to the amp tube only	Typically reagent blanks are fully concentrated and amplified.	Requires permission to consume. Consider slightly overtargetting (by no more than ~20%) and using the whole extract, especially for likely mixtures and/or degraded samples.
	~38 µL (full volume of extract after quantification)	At least 15 µL. After amp volume removed: If 10 uL or more extract remains, dry down as is. If <10 µL of extract remains, add 10 uL water and 5 µL additional GTD before drying down.	Typically reagent blanks are fully concentrated and amplified.	
Full consumption of extract – 80 µL total TE elution volume (such as 2 x 40 µL TE elutions combined	~78 µL (full volume of extract after quantification)	Either 30 µL for GlobalFiler or 35 µL for PowerPlex Y23 amplification	As long as the reagent blank is at the same concentration as the casework sample, not necessary to run and combine two blanks	If TE buffer is concentrated beyond ~40 µL down to ~15 µL, it will inhibit amplification. This rule can be applied for greater numbers of combined elutions as well.
Full consumption of extract – 40 µL TE elution + 40 µL water elution	~78 µL (full volume of extract after quantification)	Either 15 µL for GlobalFiler or 17.5 µL for PowerPlex Y23 amplification	Reagent blank must be created the same way	Use when remaining evidence is fully extracted and combined with an original extract – can only do this when original blank is also available.

Appendix C: Allelic Ladder, ILS, and Positive Control Profiles

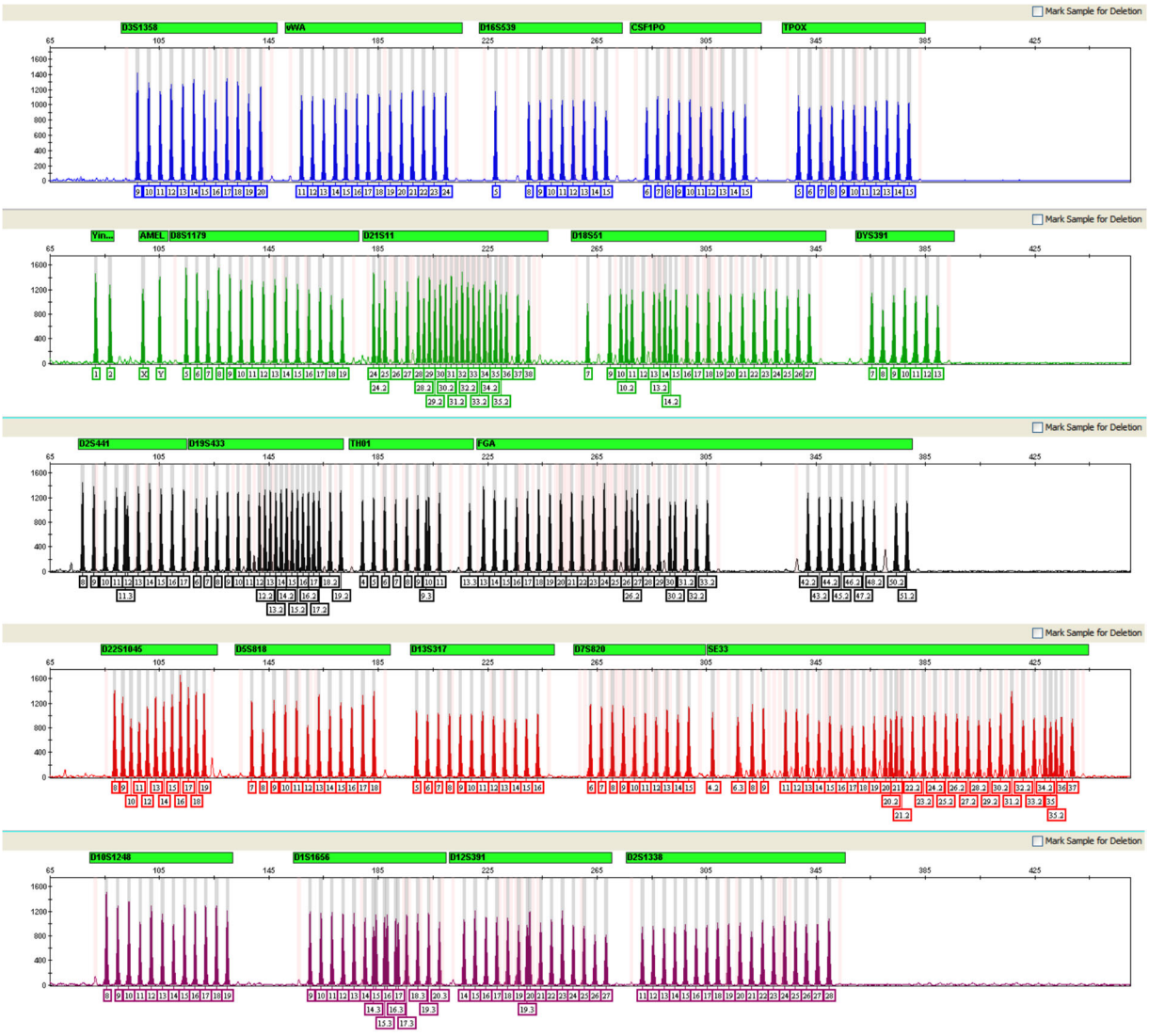
GlobalFiler Positive Control 007 Profile (also GlobalFiler Express,including RapidHIT)



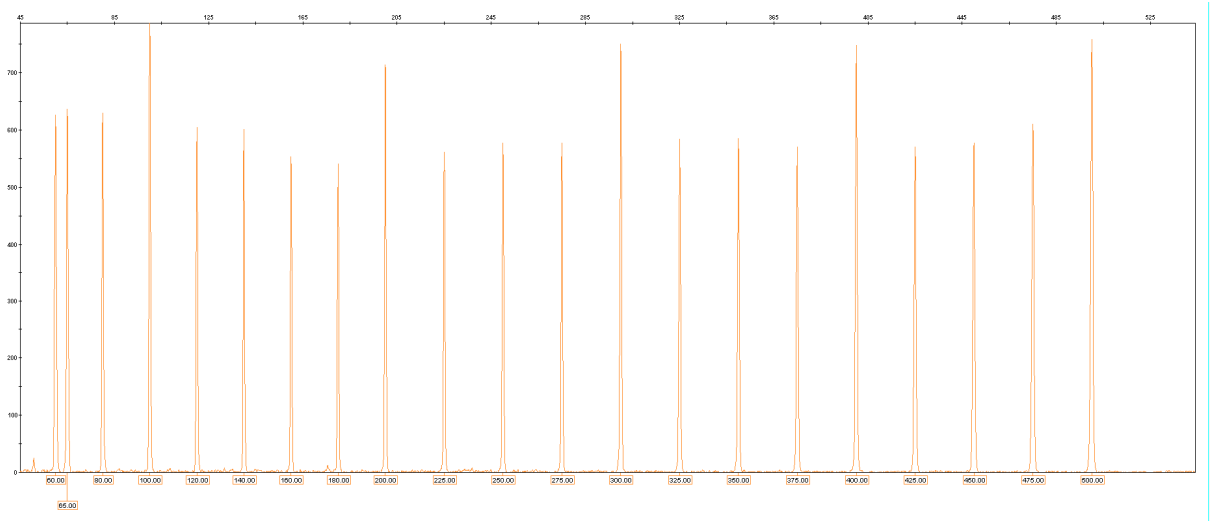
GlobalFiler ILS



GlobalFiler Allelic Ladder:



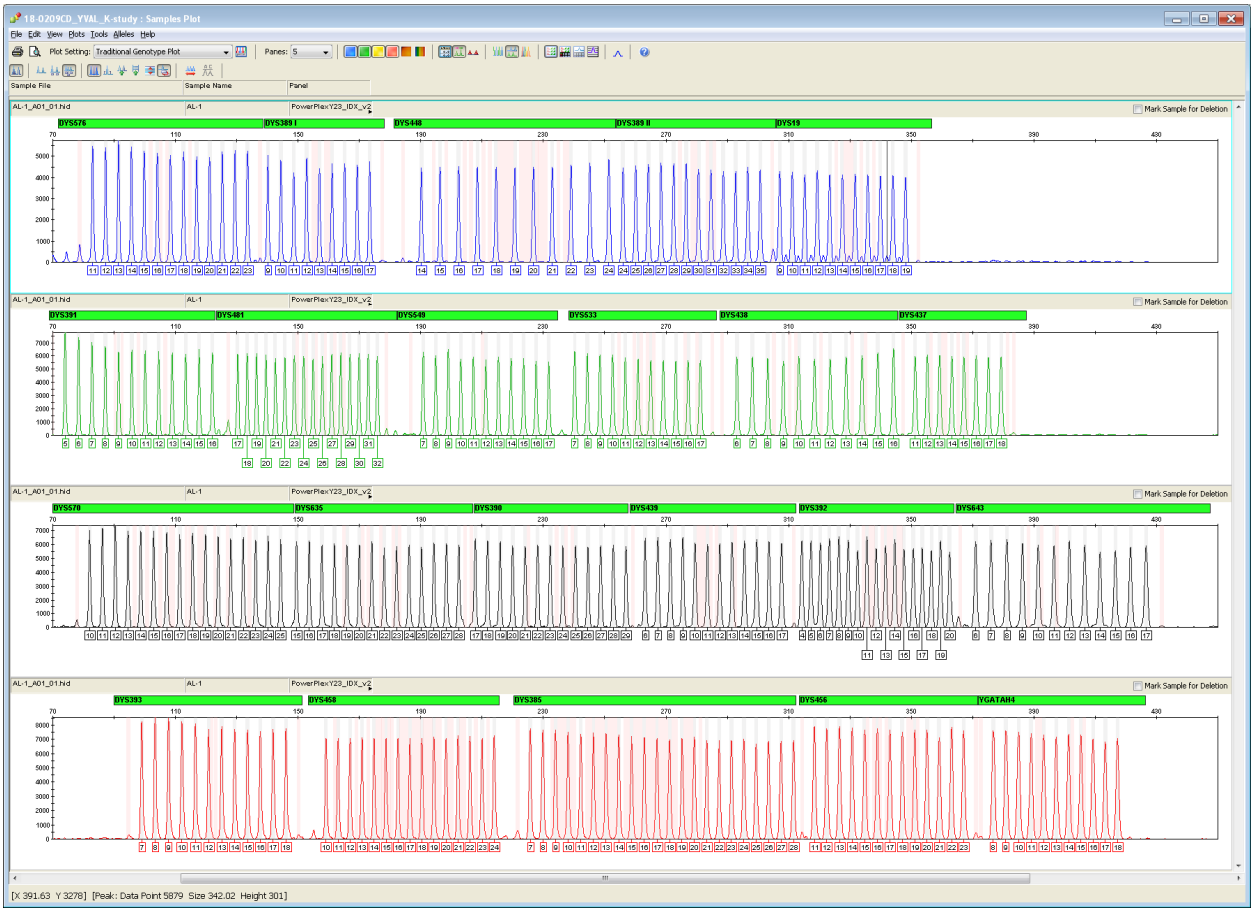
PowerPlex Y23 ILS



The PowerPlex® Y23 System Allele Determinations for 2800M Control DNA.

DYS576	18
DYS389I	14
DYS448	19
DYS389II	31
DYS19	14
DYS391	10
DYS481	22
DYS549	13
DYS533	12
DYS438	9
DYS437	14
DYS570	17
DYS635	21
DYS390	24
DYS439	12
DYS392	13
DYS643	10
DYS393	13
DYS458	17
DYS385a/b	13, 16
DYS456	17
Y-GATA-H4	11

PowerPlex Y23 Allelic Ladder



RapidHIT ILS:

80.0	**147.8**	**260.0**	**410.0**
90.0	**156.0**	**280.0**	**420.0**
100.0	**160.0**	**300.0**	**425.0**
120.0	**170.0**	**320.0**	**430.0**
129.8	**180.0**	**340.0**	**440.0**
135.2	**190.0**	**360.0**	**450.0**
139.3	**200.0**	**380.0**	**475.0**
144.0	**220.0**	**390.0**	**490.0**
151.8	**240.0**	**400.0**	**500.0**

RapidHIT Allelic Ladder

RapidHIT GFE Allelic Ladder Calls

Blue					Green					
D3S1358	vWA	D16S539	CSF1PO	TPOX	Y-Indel	Amel	D8S1179	D21S11	D18S51	DYS391
9	11	5	6	5	1	X	5	24	7	7
10	12	8	7	6	2	Y	6	24.2	9	8
11	13	9	8	7			7	25	10	9
12	14	10	9	8			8	26	10.2	10
13	15	11	10	9			9	27	11	11
14	16	12	11	10			10	28	12	12
15	17	13	12	11			11	28.2	13	13
16	18	14	13	12			12	29	13.2	
17	19	15	14	13			13	29.2	14	
18	20		15	14			14	30	14.2	
19	21			15			15	30.2	15	
20	22						16	31	16	
	23						17	31.2	17	
	24						18	32	18	
							19	32.2	19	
								33	20	
								33.2	21	
								34	22	
								34.2	23	
								35	24	
								35.2	25	
								36	26	
								37	27	
								38		

RapidHIT GFE Allelic Ladder Calls (continued)

Yellow			
D2S441	D19S433	TH01	FGA
8	6	4	13
9	7	5	14
10	8	6	15
11	9	7	16
11.3	10	8	17
12	11	9	18
13	12	9.3	19
14	12.2	10	20
15	13	11	21
16	13.2	13.3	22
17	14		23
	14.2		24
	15		25
	15.2		26
	16		26.2
	16.2		27
	17		28
	17.2		29
	18.2		30
	19.2		30.2
			31.2
			32.2
			33.2
			42.2
			43.2
			44.2
			45.2
			46.2
			47.2
			48.2
			50.2
			51.2

Red				
D22S1045	D5S818	D13S317	D7S820	SE33
8	7	5	6	4.2
9	8	6	7	6.3
10	9	7	8	8
11	10	8	9	9
12	11	9	10	11
13	12	10	11	12
14	13	11	12	13
15	14	12	13	14
16	15	13	14	15
17	16	14	15	16
18	17	15		17
19	18	16		18
				19
				20
				20.2
				21
				21.2
				22.2
				23.2
				24.2
				25.2
				26.2
				27.2
				28.2
				29.2
				30.2
				31.2
				32.2
				33.2
				34.2
				35
				35.2
				36
				37

Purple			
D10S1248	D1S1656	D12S391	D2S1338
8	9	14	11
9	10	15	12
10	11	16	13
11	12	17	14
12	13	18	15
13	14	19	16
14	14.3	19.3	17
15	15	20	18
16	15.3	21	19
17	16	22	20
18	16.3	23	21
19	17	24	22
	17.3	25	23
	18.3	26	24
	19.3	27	25
	20.3		26
			27
			28

Appendix D: Protocols for STR Forensic Parentage Casework

Relationship Testing Terminology

These are standard definitions, included here for ease of access. For purposes of convenience, many of these definitions are phrased in terms of an alleged father, since that is the most common scenario encountered in forensic paternity work.

- **Likelihood Ratio**: the ratio of two probabilities of the same event under different hypotheses. For example, in the case of one-parent paternity testing, the likelihood ratio compares the support of the genetic evidence for the hypothesis that the alleged man is the true biological father, against the support of the biological evidence that a random and unrelated man is the true biological father.

- **Paternity Index (PI)**: A likelihood ratio at a single genetic locus that compares the probability of the observed genotypes (DNA profiles) if the tested man is the true biological father, to the probability of the observed genotypes (DNA profiles) if a random untested man is the true biological father.

- **Combined Paternity Index (CPI)**: Because the genetic information at each of the loci is inherited independently, paternity indexes can be multiplied together to get a combined paternity index. The CPI is a measure of the strength of the genetic information from several loci. It indicates whether the hypothesis that the tested man is the father or the hypothesis that a random untested man is the father is more supported by the genetic evidence.

- **Combined Paternity Index Ranges**: In theory, for a CPI less than one, the genetic evidence is more consistent with non-paternity than paternity. In theory, for a CPI greater than one, the genetic evidence supports the hypothesis that the tested man is the father: “It is XXX times more likely to see the genetic results if the tested man was the true biological father than if an untested random man was the father.”

- **Probability of Paternity**: A calculation based on both the paternity index and the prior odds. This probability should, in theory, include all evidence in the case, including both the non-genetic information and the genetic information from the DNA paternity test; as such, it is a measure of the weight of all the evidence. In practice, it is common to assume a prior probability of 0.5, or 1:1, when calculating and reporting probability of paternity.

- **Mutation rate**: the rate at which a genetic marker mutates or changes over time. It is the number of mutations per hundreds of generations expressed as a decimal value or a percentage. It indicates how often, on average, one expects a random man in the population, unrelated to the child whose parentage is in question, to appear as if he is the biological father based on that marker.

- **Mean power of exclusion**: the average probability that a random person would have a pattern of genetic information inconsistent with paternity at a particular locus. It indicates how often, on average, one expects a random person in the population, who is unrelated to the child whose parentage is in question, to be correctly excluded as a biological parent.

- **One-parent Paternity**: A scenario that includes biological reference samples from a child, one known parent and one alleged parent.

- **Zero-parent Paternity**: A scenario that includes biological reference samples from a child and one alleged parent (with no reference from a known biological parent).

- **Exclusion**: The obligate parental alleles in the child do not match the alleles in the alleged father in at least four loci. The alleged father is excluded from being the biological father of the child being tested.

- **Inclusion (Cannot Exclude)**: The obligate parental alleles in the child match the alleles in the alleged father at all (or all but one) of the loci. The alleged father cannot be excluded from (or is consistent with being) the biological father of the child being tested.

- **Reverse parentage**: A scenario in which a missing person's reference sample is compared to samples from a pair of alleged biological parents.

- **Parentage**: Refers to either paternity or maternity; paternity and parentage are often used interchangeably in genetic testing terminology.

Significance Estimations in Relationship Testing

As with ASCDL casework, calculations will be performed and reported for the following populations: Caucasian, African American, Athabaskan, Inupiat, and Yupik. Calculations are performed using Popstats in the current version of CODIS. The resultant values may be truncated for reporting but should never be rounded up.

Some assumptions underlying the statistical calculations include:

- Hardy-Weinberg equilibrium
- In a false trio - the biological father is from the same population
- In a false trio – the biological father is unrelated to the mother and child
- In a false trio – the biological father is the same genotype as the tested father
- PI for a mutation is not allele dependent

For one-parent forward calculations, 0.5 is used for the prior probability.

For all calculations at a locus with possible mutation, Paternity Index = mutation rate for that locus / mean power of exclusion for that locus. Calculations will use mutation rates by locus as reported by NIST, and mean powers of exclusion from the published articles for the respective databases refer to Table 9 Combined Mutation Rates for STR loci located here: 2019 AABB Relationship Testing Technical Report.

Paternity Index (PI) is calculated for each locus, according to the formulas listed in the tables below. In those tables, capital letters refer to the allele(s) present in each individual tested, and the small letters refer to the frequency of the allele(s).

After PI is calculated (as below) for each locus, the Combined Paternity Index (CPI) is calculated by multiplying together all the individual PIs.

Assuming a prior probability of 0.5, probability of paternity (expressed as a percentage) is calculated as (CPI/(CPI+1)) x 100 and reported to three decimal places (XX.xxx %).

Forward one-parent testing:

Known Parent	Child	Alleged Parent	Paternity Index
BD	AB	AC	1/2a
BC	AB	AC	1/2a
BC	AB	AB	1/2a
BC	AB	A	1/a
B	AB	AC	1/2a
B	AB	AB	1/2a
B	AB	A	1/a
AB	AB	AC	1/[2(a+b)]
AB	AB	AB	1/(a+b)
AB	AB	A	1/(a+b)
AB	A	AC	1/2a
AB	A	AB	1/2a
AB	A	A	1/a
A	A	AC	1/2a
A	A	A	1/a

Forward zero-parent testing:

Child	Alleged Parent	Paternity Index
AB	AC	1/4a
AB	AB	(a+b)/4ab
AB	A	1/2a
A	AC	1/2a
A	A	1/a

Reverse parentage testing:

Alleged Parent - 1	Child	Alleged Parent -2	Paternity Index
BD	AB	AC	1/8ab
BC	AB	AC	1/8ab
BC	AB	AB	1/8ab
BC	AB	A	1/4ab
B	AB	AC	1/4ab
B	AB	AB	1/4ab
B	AB	A	1/2ab
AB	AB	AC	1/8ab
AB	AB	AB	1/4ab
AB	AB	A	1/4ab
AB	A	AC	$1/4a^2$
AB	A	AB	$1/4a^2$
AB	A	A	$1/2a^2$
A	A	AC	$1/2a^2$
A	A	A	$1/a^2$

Data Interpretation for forensic parentage

On occasion, reference samples may be of poor quality or degraded by environmental conditions. For such samples, loci not deemed complete (due to the possibility of partial or complete allelic drop-out) will not be considered exclusionary solely on the basis of incomplete information. All complete loci will be considered in assessing consistency between reference samples.

- Four or more loci with non-consistent genetic markers – the alleged parent(s) is/are excluded. It is not necessary to calculate Combined Parentage Index (CPI) in such cases.
- Two or three loci with non-consistent genetic markers – these results are inconclusive. The CPI will be calculated and reported, but further testing with additional markers will be recommended to the submitting agency.
- One locus with an inconsistent genetic marker within one repeat unit of the obligate allele – given the possibility of a genetic mutation, this will not be deemed an exclusion. In such a case, the alleged parent(s) cannot be excluded, and CPI will be calculated and reported.
- All loci have consistent genetic markers – the alleged parent cannot be excluded, and the CPI will be calculated and reported.

Calculating Frequencies/Probabilities Using Popstats – Paternity

[**Note**: On occasion, especially when entering mutation rates or toggling between databases, the screen may not automatically refresh to show updated changes. If you do a Print Preview, you can confirm that changes have been applied.]

A. One-parent forward paternity (trio):

1. Open Popstats and choose Parentage from the menu on the left side of the screen.
2. Choose the Parentage tab at the top of the screen.
3. Use the Specimen ID field to enter the lab number at least, item number space permitting. If the item numbers do not fit in the typed file they may be hand-written on the printout.
4. Under Subtype, select the Trio button
5. Enter STR information from all complete loci (If a locus has partial information, it will not be used for statistics – this includes not only the reference with the partial information, but the same locus in the other references as well).
6. When the correct information has been entered, select the Calculate button (upper left).
7. If any loci have mutations (mismatched), you will be prompted to enter the mutation rate and the mean power of exclusion (see following image).
8. Enter the locus-specific mutation rate from Table 9 Combined Mutation Rates for STR loci located here: 2019 AABB Relationship Testing Technical Report.
9. Enter the locus-specific mean probability of exclusion (PE) from the published database references listed at the end of this section of the manual. Please note that these values are NOT the default values included in the Popstats software.
10. Once these values are entered, click OK, then click Calculate.

11. Printing: Under the File tab, select Print, then select Parentage Trio Calculations, then select Print.
12. Print out statistic reports for the Caucasian and African American databases (from the Expanded FBI STR 2015 database file) and the Athabaskan, Inupiat, and Yupik databases (from the Alaska Expanded Core 2017 database file). In order to toggle between databases, click on the Configuration Summary tab on the left side. Choose Browse to switch between the Alaska and FBI databases. Once the new database has been selected, the software will return to the Target Profile tab, where you will again choose Calculate.

Note: It may be necessary to expand the configuration summary window to reveal the Browse button when changing the population database.

13. As before, if necessary, you will be prompted to enter mutation rate and mean power of exclusion. When finished, choose OK. Print report as described previously.

B. Reverse parentage (trio):
   1. Open Popstats and choose Parentage from the menu on the left side of the screen.
   2. Choose the Parentage tab at the top of the screen.
   3. Use the Specimen ID field to enter the lab number at least, item number space permitting. If the item numbers do not fit in the typed file they may be hand-written on the printout.
   4. Under Subtype, select the Reverse button
   5. Repeat steps 5-13 from Section A.
   6. On reverse parentage trios, Popstats does not allow for the use of a locus with a mutation. In cases with a mutation, omit the locus with the mutation from the Popstats calculation. On the Popstats printout, manually calculate the PI for the locus with the mutation. Use this manual calculation to adjust the CPI and Probability of Paternity calculations as well. Show work clearly.

C. Zero-parent forward:
   1. Open Popstats and choose Kinship from the menu on the left side of the screen.
   2. Choose the Kinship tab at the top of the screen.
   3. Use the Specimen ID field to enter the lab number at least, item number space permitting. If the item numbers do not fit in the typed file they may be hand-written on the printout.
   4. On the right side of the screen, make sure that only the PO (Parent-Offspring) box is checked (see following image)
   5. Repeat steps 5-10 from section A.
   6. Printing: Under the File tab, select Print, then select Popstats Single Parentage Statistics, then select Print (see following image).

Print out statistic reports for the Caucasian and African American databases (from the Expanded FBI STR 2015 database file) and the Athabaskan, Inupiat, and Yupik databases (from the Alaska Expanded Core 2017 database file).

Appendix E Database Procedures

This section includes the following topics:

- **Database set-up and management**
- **GlobalFiler Express Amplification Set-up**
- **Database Interpretation – Controls and Non-allelic Peaks**
- **Database Interpretation – Samples**
- **Good faith efforts**
- **Database Review**
- **Upload to CODIS**

In addition to the GlobalFiler Express and GlobalFiler procedures described in this appendix, the use of modified RapidHIT is also permissible for rush analysis of individual database samples, or for CODIS profile confirmations. Directions can be found via the links below:

- **RapidHIT amplification and profile analysis**
- **RapidHIT interpretation of controls and non-allelic peaks**
- **RapidHIT profile interpretation**
- **RapidHIT review**

[Ctl+Click to follow link; Alt+Left arrow to return here]

Database Batch Set-Up and Management

Batch Set-up for Initial Processing
In LIMS, view the DNA Database requests available for analysis.

To assign a batch of samples for initial processing, select DB-Start as the reason and search. The desired number of samples can be assigned by populating the analyst name in the appropriate field and selecting the down arrow(s). Samples may be assigned individually or in groups.

NOTE: Once samples are assigned, they cannot be unassigned without the assistance of the LIMS administrator.

Create the database batch worksheet

Exporting LIMS sample list to create an Excel spreadsheet

- Open Microsoft Excel
- In JTRAX select “Search”
    - Ad Hoc Query
        - Load Query
        - DNA new database assignments (public, not in alpha/numeric order)

- Select yourself as individual
- Select the date service assigned is between ___ and ___ for the newly assigned samples
- (A private query may be created. Use the output fields as listed below)

- Select output fields
  - Case Number
  - Individual Type
  - Individual Gender
  - Sort by ascending case number
  - Select "OK"
- Run Query

- Chose "Export to XML" option and save query to your chosen location.

- Open Microsoft Excel
  - Go to "Data"
    - "From Other Sources"
      - "From XML Data Import" – now navigate to the location where you saved your list (select "all files types" – to the right of file name to see your file). Double click on file
      - This will open a "New Web Query" box
        - Click on "Options" – click on the "Disable Date Recognition" within the Web Query Options. Leave others as is. Select "OK"
        - The above bullet may no longer be applicable. Historically imports had trouble with 08-xxx and 09-xxx samples regarding dates.

- Select Import. You will be prompted "Where do you want to put the data" – Leave it as default (existing worksheet A1)

From this master list, groups of sample IDs and offender types can be copied and pasted into the database batch worksheet.

Review the assigned samples to see if any lab number has been duplicated. This often indicates that a -2a or -3a sample exists. These can be used as part of the 5% quality control samples for a batch but are not easily copied/pasted because they should not be positioned directly next to their duplicate. Additionally, duplicates do not have to be in the same batch as their corresponding sample.

Adding samples to the database batch worksheet

- Open the current GFEWS
- Batch name is typically DBYY-MMDD followed by the analyst's initials. If doing multiple batches in a single day, add an additional identifier. For example DB16-0720RT_A.
- Enter the sample names (lab case number) and codes (ex. A1, A2 …..)
    - This can be done by copying from the spreadsheet created in previous section
    - Remember that wells A1, A4, A7 and A10 (of a full 96 well amplification plate) will be for the allelic ladder. It is only necessary to run one ladder per injection when running a partial plate. The last two wells (G12 and H12 on a full plate) are for the reagent blank/negative and positive amplification controls.
    - Make sure the worksheet contains 5 duplicate samples – 1 per injection, 1 injection will have 2 duplicates but don't place them right next to each other.
- To assist with QC checks, check the appropriate box on the worksheet for female samples.
- Save the worksheet

In LIMS, transfer the necessary boxes to your custody.

- Evidence Transfer
    - From: (leave blank)
    - To: scan your badge; enter your PIN
    - Evidence to Transfer: scan your box(es). **Important:** say "NO" when prompted if you want to empty your container(s). If you click yes, you will need to re-containerize all of the samples in the box.
    - Click Apply – say "yes" to "record evidence transfer" prompt.

GlobalFiler Express Amplification Set-up

Amplification Master Mix Set Up

- If amplification reagents have been frozen, allow them to warm to room temperature, then vortex for 3 seconds and centrifuge briefly before opening tubes. If Master Mix is in a bottle instead of a tube, swirl to mix**.** Thawing is only required during the first use of a tube. After first use, reagents are stored at 2-8°C
    - For the first use of the GlobalFiler Express kit only: Add the following volumes of Master Mix Additive to the master mix tube:
        - 200 reaction kit – 80µL Master Mix Additive
        - 1000 reaction kit – 390µL Master Mix Additive
    - Gently invert the Master Mix tube 10 times and centrifuge briefly. If Master Mix is in a bottle instead of a tube, swirl to mix.
    - Mark the cap of the Master Mix tube with a (+) to indicate that Master Mix Additive has been added.
    - Discard the Master Mix Additive tube.
    - Note the thaw date on the Master Mix and Primer tubes – the expiration date for the reagents is now the earlier date of the manufacturer's expiration date or six months from the date of the first thaw.
- Transfer the amplification reagents to the designated PCR set-up area.

Note: Do not expose reagents to light for extended periods of time.
Note: Centrifugation of the primer mix should be minimal to avoid primer collecting at the bottom of the tube.

- Prepare a PCR master mix by adding the following volumes of reagents to an appropriately sized tube:
    - # of samples x 6.0µL Master Mix
    - # of samples x 6.0µL Primer Set

- Vortex the master mix and spin briefly.

Note: Prepare enough for a few extra reactions to allow for loss during pipetting. This is calculated automatically on the DNA amplification/electrophoresis worksheet.
Note: It is not essential to prepare a master mix for a small number of reactions.
Note: Preparation of the amplification reagents takes place in the PCR prep room. Once the reagents have been aliquoted into the plate, the plate, covered with Press and Seal, may be taken to the extraction lab for loading the samples or sample lysates.

Preparing a GlobalFiler Express direct amplification:

- Prepare the samples to be amplified in individual tubes, as follows:
  - Label the appropriate number of 1.5mL tubes.
  - One entire swab tip is snapped off (or expelled) into the sample tube. It is not necessary to retain the swab stick.
    - For database samples, if only one swab was present, transfer the sample to the "Consumed in Analysis" location in the LIMS. The empty envelope may now be disposed of once any comments on the card [from the collecting agency] are added to the note field on the evidence item in the LIMS.
    - For database samples, each batch of extractions must include randomly placed internal control samples, comprising approximately 5% of the batch. This is typically five samples for a full batch. These may be previously typed samples, staff duplicates, or offender duplicates for samples in the current run.
  - Each batch of extractions must include a reagent blank/negative amplification control consisting of Prep-n-Go buffer.
  - Add 400µL of Prep-n-Go buffer to each sample (and reagent blank/negative amplification control tube) and let stand for at least 20 minutes but ideally no more than 60 minutes at room temperature (about 20 to 25°C). NOTE: Incubation time can have a significant impact on the profile ultimately obtained for the sample.
    - Alternative: Instead of a room-temperature digest, samples may be incubated on a Thermo-mixer at 90ºC with shaking at 900 rpm for approximately 20 minutes. While this method variation should lead to a higher first-time pass rate, it will also change preferred options for Good Faith Efforts.
  - Note: Following amplification set-up, transfer the tubes with lysates to short-term (less than 2 weeks) storage at 2 to 8°C. Amplification past two weeks is possible but not recommended since it may result in lower peak heights.
- Pipet 12µL of master mix into each well of a 96-well VWR amplification reaction plate. NOTE: on a full database plate, wells A1, A4, A7, A10 and any unused wells do not require master mix.
- Cover the plate with Glad Press and Seal.
- After sample incubation, transfer 3µL of lysate to the prepared amplification plate.
- To the reagent blank/negative amplification control well (typically well H12 on a full database plate): add 3µL of Prep-n-Go buffer from the reagent blank tube.
- To the positive amplification control well (typically well G12 on a full database plate): add 3µL of DNA Control 007.

Amplification on the thermal cycler

- Once all samples have been added to the amplification plate, remove the Press 'n Seal (or other plate covering) and cover the plate with a sheet of amplification tape.
- Transfer the plate to the PCR room and place directly into the thermal cycler.
- Close the heated cover.
- Double-check the selected program, ramp speed, and volume:
    - For GlobalFiler Express:
        - **program: gfe26c**
        - **Max** ramping mode is used for amplification
        - Volume is **15 µL**
- Plate should ideally be removed from the thermal cycler less than 24 hours after completion of the run.
- On completion of the run, the amplified DNA is stored at 2 to 8°C. All amplified products will be disposed of upon completion of the technical and administrative reviews.

Data Collection by 3500xl Genetic Analyzer

References: Applied Biosystems 3500/3500xL Genetic Analyzer User Guide

Start the Software and Instrument

- Turn the computer on and log into the computer
- Turn on the 3500xl Genetic Analyzer. Wait for the green status light to turn on.
- Ensure both the Daemon and Service Monitor have started by observing a green checkmark icon in the lower right-hand corner. This indicates that all 3500 services have loaded. This may take several minutes.
- Launch the Data Collection Software:
  Start > Programs > Applied Biosystems > 3500
- Log in to the 3500 Series Data Collection Software

Preparing the Instrument

- Navigate to the Dashboard of the software
- Check consumables by clicking **Refresh** to update consumable status.
- Refer to the Forensic Biology General Lab Maintenance manual if any maintenance or instrument preparations are required prior to running samples on the instrument.
- The expiration dates for the anode buffer and cathode buffer are "hard stops" – if these reagents are expired, they must be replaced for the run to proceed.
- The seven-days-on-instrument stop for the POP-4 polymer and the number of injections on a capillary are "soft stops" – as long as the resulting data for the ILS meets the quality standards described below, these stops may be overridden.
- Click **Start Pre-heat** to 60 °C to warm the oven.
- Check the pump assembly for bubbles and run the Remove Bubble wizard if necessary.

Sample Preparation for the 3500xl

- Prepare a loading master mix by adding the following volumes of reagents to an appropriately sized tube:

Amplification Kit	Amount of Size standard per sample	Amount Hi-Di Formamide per sample
GlobalFiler	0.4 µl GeneScan 600 LIZ	9.6µl
GlobalFiler Express	0.5 µl GeneScan 600 LIZ	9.5µl

Note: Prepare enough for a few extra reactions to allow for loss during pipetting.
Note: It is not necessary to prepare a master mix when only a few reactions are being prepared.

- Vortex the master mix and spin briefly. Transfer 10µl of master mix to appropriate sample wells of a 96 well plate.
- Add 10µl of Hi-Di Formamide to unused wells of a set of 24 (i.e. A1-H3).
- Add 1µl of allelic ladder and 1µl of each amplified sample to the appropriate wells. When all samples have been added, cover with plate septa.
- Briefly centrifuge the 96 well plate to remove any bubbles. Denature samples at 95˚C for 3 minutes and then snap chill for 3 minutes.
- Place the 96-well plate into the plate base provided with the instrument and snap the plate cover onto the plate, septa, and plate base.
- Verify that the holes of the plate retainer and the septa are aligned.
- Place the plate in the autosampler with the labels facing you and the notched corner of the plate in the notched corner of the autosampler. Close the instrument doors.

Creating a Plate Record
Create a Plate Record from the Data Collection Software

- In the dashboard click **Create New Plate**
- In the Define Plate Properties screen:
  - Enter a plate name (i.e. 10-1222KAL_Q_101224)
  - Select 96 for the number of wells
  - Select HID for plate type
  - Select 36cm for capillary length
  - Choose POP4 for polymer
  - Owner, barcode and description are optional fields.
- Click **Save Plate**
- Click **Assign Plate Contents**
- Using either plate view or table view enter sample names in the corresponding wells.
- Under the heading Assays, File Name Conventions, and Results Groups click **Add from Library** select the following Assay:
  - For GlobalFiler: AB GF_POP4_xl
  - For GlobalFiler Express: AB_J6OSR_LS_POP4_xl
- Select a naming convention, and a results group of your choice. Click **add to plate** and then click **close**.
- Select the wells for which to specify the assay file name conventions and results groups and enable the checkbox next to the name to assign it to the selected wells.
- Click **Save Plate** and save.

Create a Plate Record from an Export File

- Open a plate record template export file (i.e. .xls exported from instrument).
- Copy and paste sample name info into the export file. (Rows not in use may be deleted but do not delete any columns) Save the record.
- Using the 3500xl Data Collection Software, click on **Create New Plate** on the dashboard.
- In the Define Plate Properties screen:
    - Enter a plate name (i.e. 10-1222KAL_Q_101224)
    - Select 96 for the number of wells
    - Select HID for plate type
    - Select 36cm for capillary length
    - Choose POP4 for polymer
    - Owner, barcode and description are optional fields.
- Click **Save Plate**.
- Click **Assign Plate Contents**.
- Click **Import** at the top of the screen and navigate to the saved plate record (make sure .xls files are searched).
- Click **Save Plate** and save.

Linking a Plate and Starting a Run

- Click **Link Plate for Run**. A message will appear saying Plate loaded successfully. Click **OK**.
- Ensure plate is linked to proper position on 3500xl.
- Select **Create Injection List**.
- Click **Start Run**.

Note: Ensure all prompts have cleared and the run begins before walking away.

Viewing Data During a Run

Refer to Applied Biosystems 3500/3500xl Genetic Analyzer User Guide

Storage of data files

After the run has finished, transfer a copy of the data from the 3500 computer to the designated location on the laboratory network. As of the effective date of this manual, CE data is stored at I: Discipline Shares/Forensic Biology/CE data for as long as the GMID-X project is in use. Ultimately, raw data files are retained in SharePoint.

Note: Location is dependent on network mapping and may vary slightly.

Data Analysis with GeneMapper™ ID-X

References: GeneMapper™ ID-X Software User Guide
GeneMapper™ ID-X Software Tutorial
GeneMapper™ ID-X Software User Bulletin

Logging in to GeneMapper™ ID-X

- Open GeneMapper™ ID-X
- Select the User Name and Database Host from the drop-down list and enter the appropriate password.
- Click OK.
- The main project window will open.

Creating a Project

- To add samples from the collection software at a workstation, go to the edit menu and select **Add Samples to Project**. Navigate to stored data as per current designation(s).

Note: Location is dependent on network mapping and may vary slightly.

- Select the raw data folder to be imported or select individual samples and click **Add to list**.
- When all samples have been selected, click **Add**.
- Select the appropriate Sample Type, Analysis Method, Panel and Size Standard for each sample and click the green arrow on the tool bar to analyze the samples.
    - For GlobalFiler:
        - Analysis method may be Global Filer Casework, Global Filer Blank, or Global Filer Troubleshooting (not used in routine casework)
        - Panel is GlobalFiler_Panel_v1.1.1X
        - Size Standard is GS600_LIZ(60-460)
    - For GlobalFiler Express:
        - Analysis Method is GFE
        - Panel is GlobalFiler_Express_v1.4.1X
        - Size Standard is GS600_LIZ_(60-460)

- The user will be prompted to name and save the project to the appropriate Security Group (GeneMapper ID-X Security Group). Projects are typically named with the Batch name, a Q or K, and the run date. Click OK.
    - Example: 15-0426CMD_Q_150428

- Analysis is complete when the green arrows in the Status column on the left are gone, and an Analysis Summary Screen appears.

Viewing the Data

- The ILS is viewed by highlighting all samples and clicking on the Size Match Editor, the icon with the red peaks on the toolbar.
- To view each sample, highlight the sample and click on the icon with the colored peaks to display plots. (This can also be done by View > display plots or Ctrl + L).
- Electropherograms can be printed from the sample's plot window. Various plots have been created for different sample types. Commonly used plot displays include:
    - GF Casework
    - GF and GFE Blank
    - GF and GFE Artifact View
    - Traditional Genotype Plot
    - Casework Blank
    - Casework artifacts
    - Casework zoom view
- To view information on a sample's injection time and other run information, highlight the sample of interest and click **View** on the toolbar > Sample Info (Ctrl + F1).
- To view raw data for a sample, click **View > Raw Data**.
- To view allele calls by sample and locus click the Genotypes tab from the main project window.

Database Interpretation – Controls and Non-Allelic Peaks

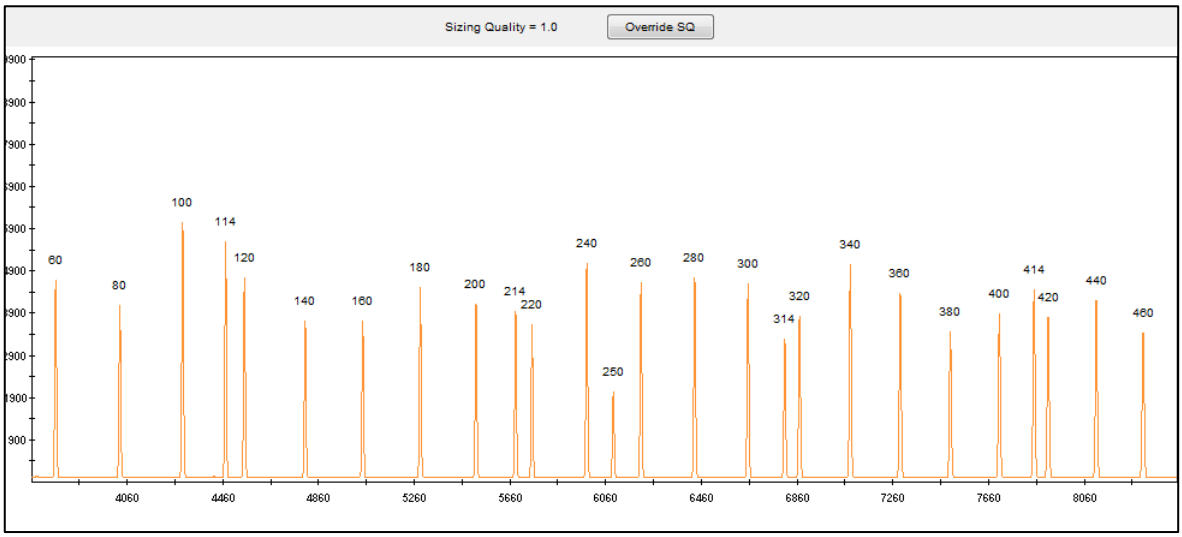
Interpretation of database samples is based on the guidelines contained in this section, as well as the qualified DNA analyst's training and experience. In all cases, interpretations must be independently reviewed and verified based on established peer review procedures. The DNA Technical Manager may authorize the interpretation/reporting of data outside of the criteria defined herein and will document this approval by initialing and dating comments on the relevant paperwork.

The minimum peak height acceptable for database sample STR loci alleles is 175RFU. Lower threshold limits for hit confirmations or ILS (Internal Lane Standard) may be authorized by the DNA Technical Manager.

Interpretation of Batch Controls

Prior to assessment of data, the analyst must first examine the batch controls to ensure that the extraction, amplification, and genetic analysis processes are functioning correctly. These include: Internal Lane Standard (ILS), allelic ladder(s), internal control specimen (ICS), positive amplification control, and negative amplification control (also serves as reagent blank). All or part of an extraction, amplification and/or run may need to be repeated depending on the results of the batch controls. Any issues raised by the performance of a batch control must be addressed prior to the release of affected database samples. Unresolved failure of a batch control requires the Technical Manager, or designated individual, to approve a course of action.

Internal Lane Standard (ILS)
GlobalFiler Express uses GeneScan 600 LIZ as an internal lane standard. The analyst should verify that all peaks from 60-460 base pairs are present and labeled as shown.
This verification consists of confirming that the sizing quality is greater than 0.5. If the sizing quality is 0.5 or lower, the analyst must confirm by visual inspection that each peak was correctly called. Once verified, the analyst should override the sizing quality to reflect that the ILS has been manually reviewed. In addition, samples with broad peaks and a Sizing Quality of 0.5 or less are more susceptible to allelic drop out, therefore any profiles with broad peaks at homozygous loci must be re-injected.



The peak heights for the ILS peaks must be at least 175RFU (relative fluorescence units) to be identified and labeled by the GeneMapper™ ID-X software with the database method.

Failure of an ILS can often be resolved by re-prepping and/or re-injecting the affected sample(s). If the ILS continues to fail after re-prepping and re-injecting, the analyst should consult the Technical manager for a course of action.

Allelic Ladder

The GeneMapper™ ID-X software uses the allele calls of the ladder to assign allele calls to all the other samples in the project. The analyst should verify that all peaks from the allelic ladder(s) are present and labeled as shown.

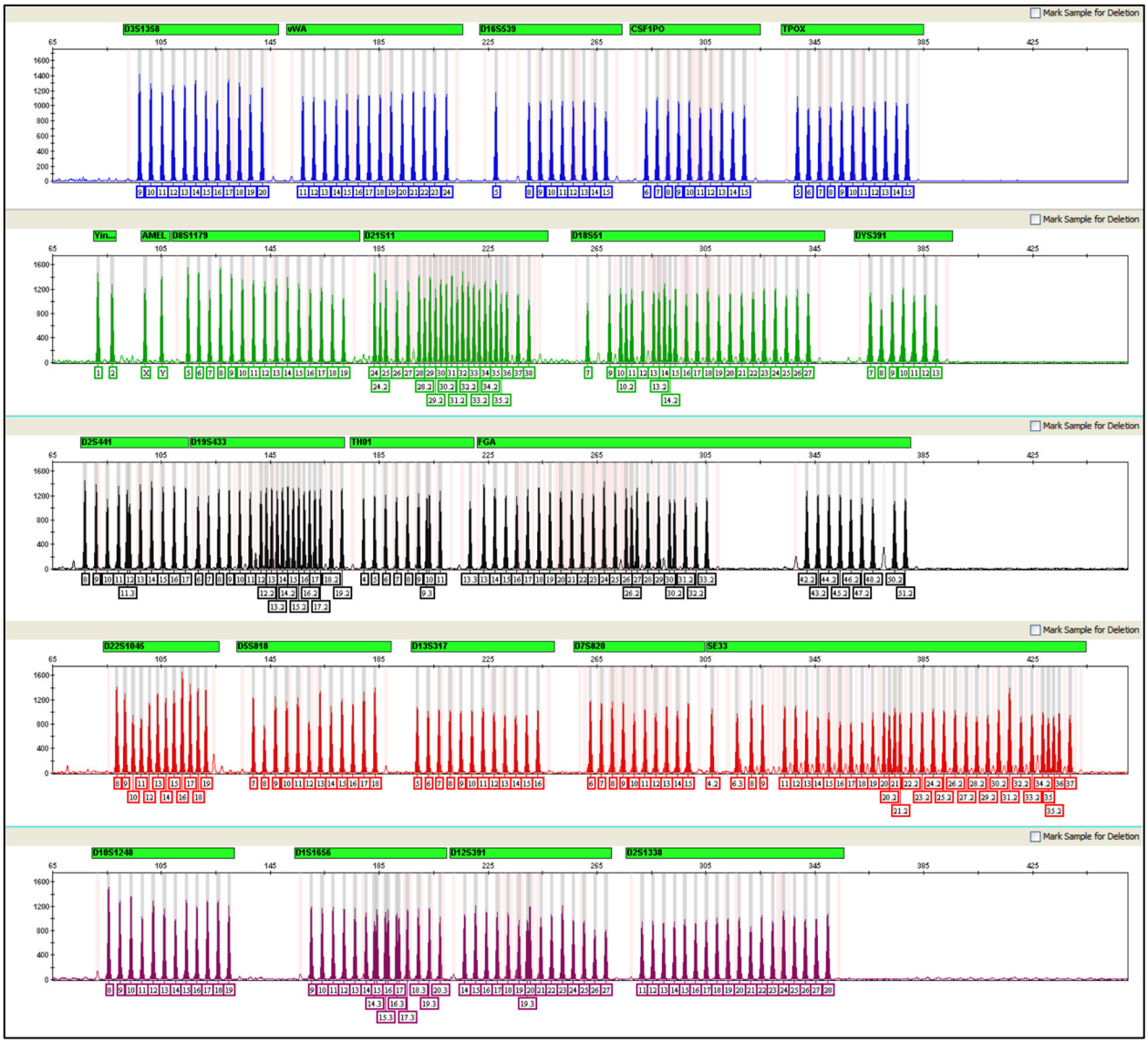
The allelic ladder contains the most common alleles determined for each of the 20 FBI STR core loci, Amelogenin, DYS391,SE33 and a Y indel. In addition, alleles not labeled in the allelic ladder (virtual alleles), may be detected and labeled in some of the samples analyzed.

At least one allelic ladder must be included in a GeneMapper™ ID-X project with the sample type designated as “allelic ladder”. All the peaks in the allelic ladder must be labeled correctly for the software to assign the correct allele calls to samples in the run.
When more than one ladder in a project has the sample type designated as “allelic ladder”, the software will average the allelic ladders to make the allele calls for the samples in the project.

Artifacts such as spikes or “pull-up” peaks may be present that are labeled as off- ladder (OL) peaks in the allelic ladder. If all the true allele peaks are labeled correctly, such OL peaks will not affect the sample allele calls. If a spurious peak (not a true allelic peak) is labeled as a true allele (i.e., it falls within an allele size range and is designated and labeled as an allele) at any particular locus, the remaining allele designations / labels at the locus shift and will impact the correct allele call / labeling of the samples in the project. Allelic ladders with such artifacts cannot be used to analyze data in the project. If other allelic ladders in the run are successful, it may be possible to change the sample type for the failed ladder from Allelic Ladder to Sample. In that case, the failed ladder is not averaged in with the other ladders. If omitting the failed allelic ladder does not lead to successful analysis, re-injection is usually appropriate.

Additional information about the allelic ladder can be found in the GlobalFiler Express PCR Amplification Kit User Guide.

GlobalFiler Express Allelic Ladder:



Positive Amplification Control

A positive control is defined as a single source sample whose genetic profile was previously determined and from which a full profile was developed; it is used to evaluate the performance of the amplification and typing procedures. The standard DNA template 007 is currently used as the positive amplification control for the GlobalFiler Express Amplification Kit.

Note: ProFlex thermal cycler heating is controlled by zones. A 96 well plate includes six zones, each comprised of two columns (i.e., columns 1 and 2 comprise zone 1, columns 3 and 4 comprise zone 2, and so on through zone 6). To ensure that each zone is performing appropriately, each zone used on an amplification plate must include at least one of the following as a means of ensuring that thermal cycling is performing correctly:

- A DNA Control 007 positive amplification control
- An ICS, such as a duplicate offender sample or a staff duplicate sample

Generally, approximately 5% of the samples in a database batch will be ICS quality control samples. Amplification plates must be set up so that each thermal cycler zone includes at least one positive control (as defined in the bullet points above). Failure to obtain the expected profile for at least one positive control sample in a zone requires re-amplification of the all the samples in that zone.

Another option for a failed positive control is to re-prep and/or re-inject the positive control if the cause of failure appears to be a failed electrophoretic injection. As long as the re-prepped and/or re-injected positive control types successfully, the positive control is considered successful.

When an incorrect STR profile is obtained for one or more of the positive control samples in a batch, other profiles in the batch will not be entered into CODIS until the issue is resolved. The CODIS Administrator, or a designated individual, may approve the entry of select profiles on a case by case basis.

DNA Control 007 (image can be found here)

D3S1358	**15, 16**
VWA	**14, 16**
D16S539	**9, 10**
CSF1PO	**11, 12**
TPOX	**8**
Y-INDEL	**2**
Amelogenin	**X, Y**
D8S1179	**12, 13**
D21S11	**28, 31**
D18S51	**12, 15**
DYS391	**11**
D2S441	**14, 15**
D19S433	**14, 15**
TH01	**7, 9.3**
FGA	**24, 26**
D22S1045	**11, 16**
D5S818	**11**
D13S317	**11**
D7S820	**7, 12**
SE33	**17, 25.2**
D10S1248	**12, 15**
D1S1656	**13, 16**
D12S391	**18, 19**
D2S1338	**20, 23**

Negative Controls
A reagent blank/negative amplification control is carried through the entire analytical process as part of each extraction or type of extraction. It contains all the reagents - except DNA template - used during extraction, amplification and typing. For GlobalFiler Express, the control consists of Prep-n-Go buffer and the PCR master mix (reagents used to prepare the PCR amplification mixture).

The purpose of the negative control is to detect DNA contamination that might occur from the reagents, the laboratory environment, between the samples being processed, and/or due to improper handling of the samples by the analyst. The negative control must be run at the most stringent set of conditions for the batch.

Verification of the presence of amplicon in the negative control is performed by viewing the presence of unincorporated primer peaks.

The negative control should not yield any true STR allelic peaks above the reporting threshold. When peaks greater than or equal to the reporting threshold are present in the range between 60-460 base pairs, the analyst will determine if the peaks are artifacts (e.g. spike, pull-up) or true allele peaks.

The presence of peaks above the reporting threshold will not invalidate the sample as long as the peaks can be shown to be artifacts. Artifacts will be documented in the GeneMapper ID-X project.

When probable true allele peaks are detected above the reporting threshold, at several or all STR loci, the analyst will investigate possible causes. The Technical Manager will be consulted to determine the appropriate course of action.

Since the failure of a negative control may indicate a problem at the extraction or amplification level, a Quality Review Form may need to be completed based on the nature of the discrepancy.

Note: Although appropriate quality assurance practices are stringently applied and enforced, it is not unexpected that low levels of adventitious DNA (i.e., levels that result in peak heights for allelic data that are below the Laboratory's reporting threshold) attributable to staff scientists and/or consumable products directly involved in the analytical steps of such a highly sensitive technique may be detected.

Quality Control Check
Offender profiles are compared against the profiles of relevant staff members to ensure that contamination by laboratory staff is not included in uploaded profiles. This can be accomplished using the Profile Comparison tool in GeneMapper ID-X or by manual

comparison. Staff member buccal swabs may be used intentionally as quality control samples; and these are expected to yield profiles concordant with the profiles on record. However, the unexpected presence of a laboratory staff profile indicates a problem at the extraction or amplification stage and requires a Quality Review Form.

Artifacts
Artifacts are data peaks resulting from the analytical process and are not attributable to an individual contributor to the sample.

When artifacts are not pervasive throughout the sample and do not interfere with data interpretation, the sample does not require re-analysis. When an artifact is present within the size range of the alleles in a core locus and the presence of the artifact interferes with interpretation of the data in that locus, or when a sample exhibits artifacts in several loci, interpretation of the sample becomes more challenging and it may be necessary to re-process the sample. Artifacts may be resolved merely by re-injecting a sample; or they may require re-prepping with less amplification product, re-amplification or re-extraction to resolve. If the interfering artifact is located in one of the non-CODIS-core loci, the analyst may choose to enter the profile, omitting all data from the affected locus.

Samples uploaded into CODIS must not contain any labeled artifacts or OL alleles. The analyst may remove the artifact label. Artifact or OL peaks are either deleted or re-labeled by right clicking on the peak and adding a comment '(microvariant allele', 'artifact' etc.). Any labeled artifacts deemed true alleles should be noted on the batch worksheet.

Incomplete 'A' nucleotide addition (-A or split peaks)
Platinum Taq™, like many other DNA polymerases, catalyzes the addition of a single nucleotide (predominantly adenosine) to the 3' ends of double-stranded PCR products. This non-template addition results in a PCR product that is one base longer than the actual template, and the PCR product with the extra nucleotide is referred to as the "+A" (base peak) form. The final step of the STR amplification process is a terminal extension step to promote complete non-template 'A' nucleotide addition. The "-A" form is the peak that represents the actual template length. The "+A" peak will be the predominant form, with the "-A" peak typically not being detected.

Failure to attain complete terminal nucleotide addition (typically due to an excess of template DNA) results in "split peaks", two peaks above threshold that are one base apart. If the labeled "-A" peaks are not pervasive throughout the sample and the sample appears to otherwise be a single source sample, the labels may be deleted in the project and the reason documented electronically.

Pull-up
Multi-component analysis is the process that separates the six different fluorescent dye colors into distinct spectral components. The six dyes (6-FAM, VIC, NED, TAZ, SID, and

LIZ) emit their maximum fluorescence at different wavelengths, but there is some overlap of the emission spectra. A spectral calibration is performed for a specific dye set to create a matrix that corrects for the spectral overlap.

Pull-up is the result of incomplete separation of the emission spectra and is typically observed as a non-allelic peak at the same base size as a peak in another dye. This typically occurs at high RFU values, when an excess of template DNA has been amplified.

Pull-up may also occur when the matrix file is not current. A new spectral calibration is performed whenever a new capillary array is placed on the instrument or whenever any part of the optics have been realigned and/ or replaced. A new spectral calibration may also need to be performed when an overall decrease in spectral separation is observed.

If the pull-up is prevalent enough to interfere with data interpretation, the sample may need to be re-injected. Otherwise, the labels may be deleted in the project and the reason documented electronically.

Stutter
The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter than the corresponding base peak. This artifact is the result of slippage of the Taq polymerase and is referred to as the stutter peak or stutter product. Stutter products four bases longer, or two bases shorter, than the corresponding base peak are less frequently observed. Two base pair stutter products are so rare that peaks in these positions are not filtered out in the analysis parameters.

The proportion of stutter product relative to the main allele peak is calculated by dividing the height of the stutter peak by the height of the main allele peak. The stutter percentage is fairly reproducible for a particular locus.

The GeneMapper™ ID-X database analysis methods have a global stutter filter of 20%. Peaks in the stutter position greater than 20% may indicate the presence of DNA from more than one individual. When there is no indication of a mixture, and the data is determined to be elevated stutter, the elevated stutter labels may be deleted in the project and the reason documented electronically.

It is unlikely that the presence of stutter peaks would interfere with the interpretation of database samples and therefore, re-amplification and/or re-injection of the sample may not be required.

Spikes
Spikes are non-allele peaks that may arise due to air bubbles, urea crystals or voltage spikes. Spikes are typically quite sharp and easy to distinguish from a true allele, usually

appearing in more than one color at the same base size, and not reproducible by re-injection.

Peaks labeled as such by the GeneMapper™ ID-X software do not interfere with upload to CODIS and no re-analysis is required if the spike does not interfere with interpretation of the sample.

Dye Blobs
Dye blobs, like spikes, are non-allele peaks that are easily distinguished from true alleles. Dye blobs may be the result from residual dye molecules left over from the synthesis of the primers or they may be dye molecules that fell off the primer during the amplification.

If the artifact is labeled but does not interfere with data interpretation, the dye blob label(s) may be deleted in the project and the reason documented electronically.

Persistent Kit Artifacts
Occasionally, PCR kits may exhibit persistent artifacts that may or may not appear as true alleles. Typically, these artifacts are observed and documented during kit verification process. Alternatively, they may be documented in a manufacturer bulletin. As with other artifacts, if the artifact does not interfere with data interpretation, the labels may be deleted in the project and the reason documented electronically.

Contamination
The verification of reagents prior to use in the analysis of database batches is conducted in part to detect contamination which permeates an entire lot of a given reagent or set of reagents. However, such checks will not necessarily detect contamination arising from a single event (for example, cross-contamination between two adjacent samples).

For database samples, whenever data suitable for comparison indicates that DNA from more than one individual is present in a sample, the sample must be re-processed in an effort to obtain a single source profile. In the meanwhile, no samples from the affected batch are to be uploaded without approval from the DNA Technical Manager.

Furthermore, an assessment must be conducted (using the form provided in FBCP) in an attempt to establish the source of the contamination. Corrective Action may be pursued depending on the results of the root cause analysis.

Database Interpretation - Samples

Typically, each locus is characterized by one or two labeled peaks or alleles of approximately equal intensity.

Allele peak heights should typically fall between 1000 and 6000RFU. Peak heights outside this range may be acceptable; however, it is possible that these samples will require careful interpretation. Alleles with peak heights less than 350 RFU may occasionally exhibit peak height imbalance due to stochastic effects caused by low template copy number.

Peak height ratios (PHR) are calculated by dividing the peak height (in RFUs) of the lower RFU allele by the peak height of the higher RFU allele, and then multiplying this dividend by 100 to express the PHR as a percentage. The GeneMapper™ ID-X software is set to flag samples with a PHR below the expected minimum of 50%. PHR imbalance (in a single source sample) may occur with low quantities of template DNA, degraded template DNA or in the presence of PCR inhibitors. Severe imbalance may result in allelic dropout, where one or more alleles occur below the detection threshold or are not visible at all.

The presence of more than two peaks or severe PHR imbalance at a locus may be explained by a tri-allele, primer binding site mutation, variant allele, or other biological or procedural artifact. The presence of these at several loci is indicative of a mixed sample.

Tri-alleles

Tri-allelic patterns (three alleles at a single locus) are known to occur in single source samples and there are documented instances where different tissues from the same individual may or may not exhibit the tri-allelic pattern.

Samples exhibiting tri-allelic patterns with all alleles of approximately equal intensity, or with the 3rd allele having a peak height greater than or equal to 50% of the second-largest peak, are entered as such in CODIS. When tri-allelic patterns are exhibited at two or more loci, the sample is entered as a multi-allelic offender.

For samples exhibiting possible tri-allelic patterns where the 3rd allele is less than 50% the height of the second-largest peak, only the two largest alleles will be entered into CODIS. The analyst will note that there is a possible tri-allele at the locus in question on both the batch worksheet and the comments section in the CODIS entry.

Off-Ladder (OL) Alleles

The allelic ladder contains the most commonly observed alleles for the STR loci. True alleles are peaks that are detected and labeled by the GeneMapper™ ID-X software with a number and should lie within the range of the alleles in the ladder. Alleles which are not assigned a numeric designation in the standard ladder will be designated as off-ladder (OL) alleles.

Peaks that are labeled as OL (off ladder) at a particular locus have a base size that is different from any of the alleles in the ladder and any of the virtual alleles. These peaks may be true alleles but additional analysis is required to verify this. Before determining whether an OL is a true allele or an artifact, the analyst must consider all other possible causes of OL occurrence.

True OL alleles will exhibit peak morphology similar to the other alleles in the sample and the PHR should be appropriate relative to the other allelic peaks in the sample. When the overall sample quality is poor, the analyst should consider re-amplification prior to designating an OL peak as a true allele.

When a true OL allele lies within the range of alleles for a particular locus, the allele will be reported as a variant of the integer (i.e., X.1, X.2, etc.). The appropriate allele call is determined by simultaneously examining the base sizes for the sample allele peak and the associated allelic ladder peaks in the relevant locus.

When the OL allele lies either above or below the largest or smallest allele in the ladder, the OL allele will be designated as either greater than (>) or less than (<) the allele from the ladder that is closest in proximity to the OL allele.

True OL alleles are documented on the batch worksheet.

Low-Level DNA Samples
Stochastic effects, including extreme peak height imbalance, are typical of samples with very low levels of DNA. One risk associated with such samples is the possibility that a true heterozygote will appear to be a homozygote due to a sister allele below the analytical threshold. To minimize this risk, a homozygote threshold (HT) of 350 RFU must be used for interpretation of database samples analyzed by GlobalFiler Express. The homozygote threshold is analogous to the GlobalFiler 630 RFU stochastic threshold employed in casework analysis, but specifically addresses the concern of partial drop out in a low-level database sample. A homozygous locus may only be considered complete if the peak height of the allele is equal to or greater than 350 RFU.

Any homozygous locus with an allele peak height below 350 RFU must be considered as potentially a partial profile at that locus.

NOTE: The homozygote threshold of 350 RFU does not apply to profiles with broad peaks and sizing quality of 0.5 or less. Injections with broad peaks and sizing quality of 0.5 or less are only acceptable when it can be demonstrated that drop out could not have occurred.

- Samples which have been previously typed, such as QA samples and positive controls, and which can be shown to be complete by their previous results, are acceptable with broad peaks.

- Database profiles where all loci with broad peaks are heterozygous, which confirms that drop out could not have occurred at the locus, are acceptable.
- All other profiles with broad peaks must be re-injected and/or re-analyzed until a profile without broad peaks is achieved.

If allelic dropout, or possible drop-out based on the homozygous threshold, is observed in one or more core STR loci, the analyst will make a "good faith effort" to re-process the sample in order to generate a complete genetic profile.

- It is not necessary to proceed with "good faith efforts" on QC samples if at least four of them yield complete data for at least ½ of the core loci. If not, attempt re-analysis on all QC samples that were not complete at the 20 core loci.

If the "good faith effort" also fails to generate a complete genetic profile, several possibilities exist:

- A profile with data (partial or complete) at all core loci and no more than three core loci with possible dropout (i.e. a homozygous peak below the HT) is eligible for upload to NDIS.
- A profile with complete information for at least 10 STR core loci is acceptable for upload to SDIS.
- Consult the DNA Technical Manager or a CODIS Administrator for any profile which does not meet the above criteria after "good faith" efforts.

When incomplete typing results are possible at one or more STR loci, indicate this by selecting "Yes" in the Partial Profile field in CODIS for the applicable loci.

Data may be composited from multiple amplifications/injections of the same lysate/extract of a database sample in order to obtain a more complete profile at the core loci.

Composite profiles of offender samples, created by combining the typing results from separate extracts, are acceptable for entry and upload to NDIS. Prior to creating this type of composite profile, the biographical information and overlapping loci shall be verified as being concordant.

"Good Faith Efforts" at Recovery of Low-level Direct Amplification Known Samples

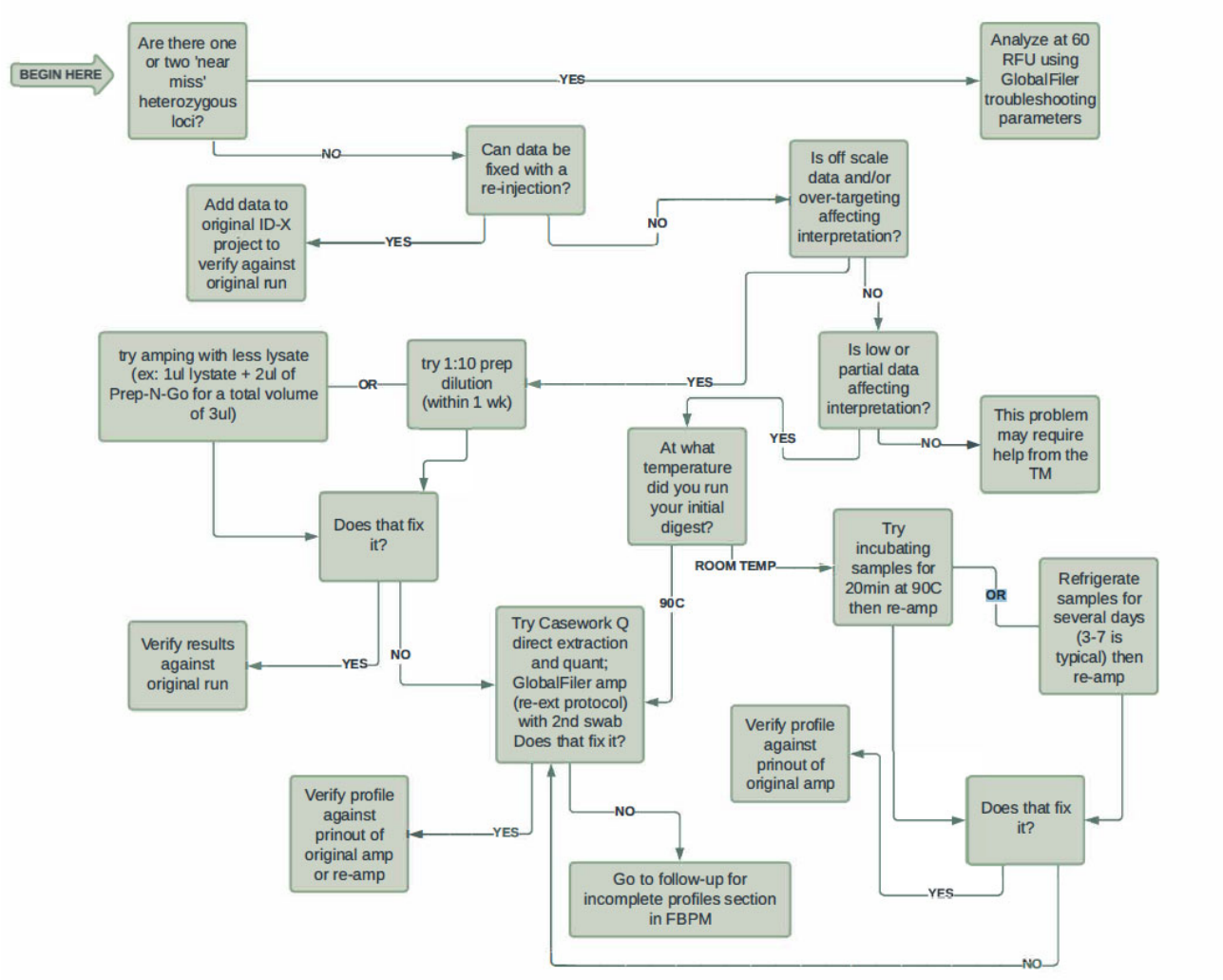
If allelic dropout, or possible drop-out based on the homozygous threshold, is observed in one or more core STR loci, as per the NDIS Operation Procedure, the analyst will make a "good faith effort" to obtain a complete genetic profile. ***A "good faith effort" may include any appropriate combination of the following options used to obtain a full interpretable profile at all CODIS core loci.*** Good faith efforts described in this section must be completed for all samples within a batch before any portion of the batch is submitted for technical review. Alternatively, a LIMS request may be created for any samples requiring re-extraction, and the batch may be submitted after all re-injections, re-preparations and re-amplifications are completed.

If good faith efforts are unsuccessful and a duplicate sample is available, it shall be processed in a new batch of database samples using GlobalFiler Express.

Good faith efforts always require a comparison between the original profile (if any is obtained) and the follow-up profile to ensure that no incorrect transfer(s) occurred and that results are consistent.

- For samples that are re-injected, re-prepped with diluted amplification product, or re-amplified, the new data may be added to the original GMID-X project for a verification comparison within the project. If the differences between allelic ladders are significant enough to cause sizing issues in the combined project, then it will be necessary to keep the runs in separate projects.
- Re-extracted samples are always analyzed in separate projects. For re-extracted samples where the original GFE analysis yielded interpretable/complete data for at least 50% of the core loci, the analyst shall submit a printout of the original amplification to the technical reviewer for a concordance check.

Overview flowchart of Good Faith Efforts:



One or two 'near-miss' loci
GlobalFiler Troubleshooting parameters, which uses an analytical threshold of 60 RFU, may be used to analyze low-level samples (or larger loci in highly degraded samples) with no more than two loci where:

- There are two alleles at the locus
- One or both alleles fall slightly below the homozygote threshold.

Samples analyzed this way must be described in the central log documentation (e.g., 60 RFU for TH01). This approach may not be appropriate for all low level samples, especially those with baseline noise or possible low-level artifacts. Analysts should pay particular attention to peak morphology of peaks between 60 RFU and the usual analytical threshold. Note that this approach has no effect on the homozygote threshold.

Re-prepping Samples Over Targeted with GlobalFiler™ Express
Samples that produce DNA profiles with an extensive amount of pull up may be diluted at a 1:10 ratio on the original amplification plate and re-prepped for CE analysis. This must be done within one week of the original amplification date.

To dilute the samples:

- Replace the amp tape with Glad Press n Seal and circle the affected well(s).
- Add a sufficient amount of deionized formamide (dF) to the affected well(s) and gently pipette up and down five times to mix the contents. Typically, the well will contain 14µL of amplified product after the initial prep, and 126µL dF will be added.
- Place an additional layer of Glad Press n Seal over the top of the amplification plate
- From this point, prepare a CE plate as usual.
- Re-seal the amplification plate with adhesive tape and store at 2-8°C.

Amplification or re-amplification to address over-targeting
One option available for dealing with samples known or likely to be over targeted is to prepare the amplification reaction with less lysate added per reaction (e.g., adding 1 µL lysate + 2 µL Prep-N-Go per well, for a total volume of 3 µL). Samples amplified using less lysate must be noted as such in the central log documentation, such as '1 µL lysate amp'.

Re-amplification to address partial drop-out or drop-out
The incubation time of less than 60 minutes at room temperature is optimized for a majority of whole-swab samples. Samples containing less DNA may benefit from prolonged incubation time at 2 – 8 °C. As a second attempt to gain a full profile for CODIS entry, the lysate may be incubated at 2 – 8 °C for up to two weeks and amplification re-attempted. In this case, the only change to the amplification protocol is the length of the incubation time. Amplification past two weeks is possible but not recommended since it may result in lower peak heights.

Note: Once a digest has been incubated at 90ºC, re-amplification is unlikely to yield higher peak heights. Samples with (possible) partial profiles after a 90ºC digest should proceed to extraction by EZ Biorobot.

Extraction by EZ Biorobot and Amplification by GlobalFiler
If re-amplification is not successful and additional untested swab material remains (i.e. a second swab within the original item), extraction shall be attempted. The remaining swab may be extracted using either the protocol for Direct (non-differential) Extraction for Questioned samples or the Known Direct Extraction protocol. The resulting extracts will then be quantified and amplified using GlobalFiler:

Amplification Master Mix Set Up

- If amplification reagents have been frozen, allow them to warm to room temperature, then vortex for 3 seconds and centrifuge briefly before opening tubes. Thawing is only required during the first use of a tube. After first use, reagents are stored at 2-8°C
    - Note the thaw date on the Master Mix and Primer tubes – the expiration date for the reagents is now the earlier date of the manufacturer's expiration date or six months from the date of the first thaw.
- Transfer the amplification reagents to the designated PCR set-up area.

Note: Do not expose reagents to light for extended periods of time.
Note: Centrifugation of the primer mix should be minimal to avoid primer collecting at the bottom of the tube.

- Prepare a PCR master mix by adding the following volumes of reagents to an appropriately sized tube:
    - # of samples x 7.5µL Global Filer Master Mix
    - # of samples x 2.5µL Global Filer Primer Set

- Vortex the master mix and spin briefly.

Note: Prepare enough for a few extra reactions to allow for loss during pipetting. This is calculated automatically on the DNA amplification/electrophoresis worksheet.
Note: It is not essential to prepare a master mix for a small number of reactions.
Note: Preparation of the amplification reagents takes place in the PCR prep room. Once the reagents have been aliquoted into the plate, the plate, covered with Press and Seal, may be taken to the extraction lab for loading the samples or sample lysates.

Preparing a GlobalFiler amplification:

- If casework extracts have been stored at 2-8°C, allow them to warm to room temperature, then vortex and spin briefly using a centrifuge.
- Prepare the samples to be amplified in individual tubes, using a total volume of **15µL.** Refer to Amplification and Genetic Analysis section for more details.
- Vortex the amplification master mix and spin briefly. Transfer **10µL** of master mix to each sample well of a 96-well amplification plate (optical skirted plate or non-skirted plate is acceptable).
- Cover the entire well plate with Glad® Press 'n Seal (or equivalent, such as aluminum foil or kim wipes).
- Transfer the entire **15µL** of the prepared samples to the appropriate sample wells containing the PCR master mix. During sample addition, the pipette tip is inserted through the Press 'n Seal (or through aluminum foil, or kim wipes are shifted immediately before addition of sample).

Amplification on the thermal cycler

- Once all samples have been added to the amplification plate, remove the Press 'n Seal (or other plate covering) and cover the plate with a sheet of amplification tape.
- Transfer the plate to the PCR room and place directly into the thermal cycler.
- Close the heated cover.
- Double-check the selected program, ramp speed, and volume:
    - For GlobalFiler:
        - **program: gf-cswk**
        - **Max** ramping mode is used for amplification
        - Volume is **25 µL**
- Plate should ideally be removed from the thermal cycler less than 24 hours after completion of the run.
- On completion of the run, the amplified DNA is stored at 2 to 8°C. All amplified products will be disposed of upon completion of the technical and administrative reviews.

For data collection and data analysis, refer to directions listed earlier in this section.

Controls and offender reference samples will be analyzed according to the parameters set forth for casework reference samples. When multiple offenders are analyzed as a batch, the analyst may document the analysis of the samples electronically and upload the profiles as a .xml file (as described elsewhere in this manual). When batch uploading offenders, the offender samples are documented using the sample list page of GFEWS (current version).

CODIS confirmations may be uploaded if they meet eligibility requirements. One of the specimens will later be changed to offender duplicate as described in this manual. Under current protocols, CODIS confirmations cannot be performed and documented in the LIMS-DNA workflow.

If the database analyst is performing extraction and amplification as part of the database batch, analysis should be conducted as soon as is practicable.

If the "good faith effort" also fails to generate a complete genetic profile, several possibilities exist:

- A profile with data (partial or complete) at all core loci and no more than three core loci with possible dropout (i.e., a homozygous peak below the HT) is eligible for upload to NDIS.
- A profile with complete information for at least 10 STR core loci is acceptable for upload to SDIS.
- Consult the DNA Technical Manager or a CODIS Administrator for any profile which does not meet the above criteria after "good faith" efforts.

Request Management after Completion of Initial Analysis

Every new database sample will automatically have two requests created. The parent request (with a Reason of DB-Start) will clear upon assignment, prior to analysis. The child requests are managed upon completion of technical review, prior to being submitted for administrative review.

Samples that did not generate a complete profile will need to be managed individually, at the case level and should be addressed first. Samples that yielded a complete profile and require no further analysis will be managed via a worklist.

Management of Samples with Incomplete Profiles (no profile or partial at one or more core loci)

- If there is not another swab available for testing, the following actions are taken:
  - Select one of the following reasons on the child request: DB-No Profile, DB-Partial
  - Assign the child request to yourself to clear it.
  - Send an e-mail to the "DNA Notify" group to advise that the flag needs to be reset in APSIN and the individual needs to be re-collected. Retain e-mail in case activities in LIMS.
- If there is another swab available (either a second swab in the current item *or* a duplicate sample, the following actions are taken:
  NOTE: For a duplicate sample, if prints are available on both samples they must first be verified to the same SID. If there is a discrepancy, alert a CODIS Administrator.
  - Add the Complexity of DB-ReExtract to the child request

- Upon completion of the analysis of the second sample, follow the procedure below if a complete profile is obtained. Alternatively, follow the procedure above if a complete profile is still not obtained.

On occasion, it may be appropriate to modify an offender entry based on subsequent typing of another sample for the same individual. For example, an offender sample may yield only a partial profile, but a sample submitted later in casework yields a complete profile. The offender entry may be updated with data obtained from the subsequent sample. Typically, this will be done only when the initial entry was incomplete at the original 13 core CODIS loci.

Management of Samples with Complete Profiles

- Create a worklist
  - Filter by *Unassigned* DNA Database requests
  - Sort the list of available requests by Req # to show the child requests at the top of the list in the upper window. The columns can be sorted by clicking on the headers at the top.
  - Move the child requests for passing samples to the lower window. It is possible to move a set and then move individual back to the upper window.
  - Once only passing samples for the current batch remain in the lower window, select your name from the Assigned To drop-down in the upper right corner of the display.
  - Click Save to clear the child requests for passing samples.

Batch Set-up for Re-Extract Processing

In LIMS, view the DNA Database and CODIS Confirmation requests available for analysis.
To assign a batch of samples for processing, select DB-ReExtract as the complexity and search. Leaving the Service field blank will return both DNA Database and CODIS Confirmations at the same time.

NOTE: Once samples are assigned, they cannot be unassigned without the assistance of the LIMS administrator.

Post-analysis, management of DNA Database child requests is as previously described.

Profile verification status is captured using the custom form on the evidence item in LIMS. Work instructions can be found in the CODIS Administrative Manual, Appendix J. For CODIS Confirmation requests, findings (profile verified or similar) must be entered for the Draft Complete and Technical Review milestones to be rolled.

Review of Database Projects

Database projects are routinely reviewed electronically, with the technical reviewer looking at the controls/samples in GeneMapper ID-X rather than printed electropherograms. The analyst may choose to submit printed electropherograms to facilitate documentation and review of artifacts, true OL alleles, tri-alleles, concordance checks on re-analyzed samples, etc.

Batches of database samples are uploaded into SDIS by creating an Export Table for CODIS. Prior to creating this table, the analyst must confirm that only eligible profiles are marked for export (as Convicted Offender, CO Duplicate, Arrestee, Staff Duplicate). This is done by selecting the appropriate Specimen Category while in GeneMapper ID-X. Samples not exported to CODIS are marked “no export”.

Review of Offender Database Samples Analyzed In-House

Genetic profiles and all supporting documentation generated in the course of analysis will be subjected to a technical review. The DNA analyst will submit a packet containing the database batch worksheet and any printed electropherograms to another qualified database analyst for technical review. This review will be conducted in accordance with the FBI QAS Guidelines and includes the following, at a minimum, to ensure compliance with the interpretation guidelines contained within this document:

- Review of the ILS for all passing samples
- Review of all allelic ladders designated as such
- Review of all amplification positive controls and quality control samples
- Review of all reagent blank/negative amplification controls
- Review of all DNA typing data for all passing samples
- Verification of eligibility for all profiles marked for export, to include selection of an appropriate specimen category
- Concordance with previous results for re-processed samples

The analyst and the reviewer may consult a third qualified DNA analyst, if necessary, when there is a disagreement on how to report a result. If the analysts are unable to come to an agreement, the DNA Technical Manager will be consulted to make the final decision.

A review checklist is used to document completion of the individual components of the technical and administrative review. Upon completion of the technical review, the analyst may import the passing samples to SDIS. Another qualified analyst or a CODIS Administrator will then complete an administrative review, to include reconciling the results of the offender duplicate and casework autosearches.

Upon completion of the technical review, the GMID-X project will be exported (using GeneMapper Manager) to SharePoint.

Upon completion of the technical and administrative reviews, NDIS eligible samples are uploaded by a CODIS Administrator. Additional information on CODIS procedures is contained in the CODIS Administrative Manual. Upon completion of the upload, the following paperwork is archived as a .pdf in SharePoint:

- Database batch worksheets
- STR Offender Batch Review Checklist
- SDIS Import Reconciliation Report

Review of Outsourced Offender Database Samples

The laboratory does not currently use a vendor laboratory for DNA analysis. Should the laboratory enter into a contract for DNA analysis of database samples with a vendor laboratory, a procedure for the in-house review of outsourced samples will be created before analysis begins.

Upload of projects to CODIS

Incomplete profiles

a. If SDIS only – designate as such on the batch worksheet and mark for upload to SDIS with the project. The sample must be unmarked at SDIS to prevent it from going to NDIS. If a subsequent sample yields a more complete profile, the initial sample will be changed to a duplicate.

b. NDIS eligible – designate the appropriate specimen category on the batch worksheet and mark for upload to SDIS/NDIS with the initial project. If a subsequent sample yields a more complete profile, the initial sample may be changed to a duplicate. If the original sample was already sent to NDIS, it will automatically come back out once the specimen category is changed.

c. If the initial sample does not yield a complete profile at the 20 core loci and a second swab or duplicate sample exists, it will be processed in a subsequent batch of samples.

Export batch from ID-X to CODIS

Batches of offender samples analyzed in-house are exported from GeneMapper ID-X as CMF 3.2 (.xml) files and then imported into SDIS as follows. When exporting, AK0020200 should be entered in the fields for both the source and destination labs.

1. Open Specimen Manager from the Analyst Workbench
2. Select Import Specimens under the Specimen Manager pull down menu or click on the Import icon on the toolbar.
3. Select the appropriate CMF file (.xml) and click Open. When prompted, the CODIS User importing the file should select Data Import and their User ID and click OK.

4. A message will appear indicating that the import file was successfully imported; click OK.
5. Open the Message Center and select the Import STR Files tab; double click on the bold file to validate the file.
6. Select the Import Reports tab; double click on the bold file.
7. The reconciliation report details the samples that will be imported. The CODIS User should check to see that all profiles can be successfully uploaded and identify any problem samples.
8. Once any problems are corrected, the analyst can select the file within the Import STR Files tab and execute using the icon in the toolbar. The reconciliation report (for the import) will be printed and retained with the offender batch paperwork.
9. Edit any samples with (possible) partial locus dropout to set the partial profile flag to yes at the applicable loci.

   Note: Marking a locus as partial will automatically trip the flag for the sample. A sample for which there is only complete locus dropout does not require a partial flag to be marked yes.

10. Un-mark any profiles not suitable for upload to NDIS.
11. A CODIS Administrator or another qualified analyst may be consulted to troubleshoot problems with importing offender batches.
12. Administrative review is completed by a CODIS Administrator or qualified database analyst and includes reconciliation of the matches resulting from the duplicate offender and AK new searches. These searches are scheduled to occur automatically Monday- Friday at 7pm. Data must be searched prior to an NDIS upload. Additional information on CODIS searches is contained in section 3.3 of the CODIS Administrative manual.
13. Upload of NDIS eligible profiles is completed by a CODIS Administrator.
14. Once the samples have been successfully uploaded to NDIS, the paperwork will be returned to the analyst to archived in SharePoint

Appendix F Proficiency Tests

This section provides additional guidance on handling of proficiency tests, where it varies from the handling/procedures of routine casework.

Casework Proficiency Test

Analysis

- All questioned items in the test case, including both epithelial and sperm (or sperm/substrate) fractions if differentially extracted, must be carried all the way through amplification and reported with the test results.
- Initial analysis of test items should use no more than half the sample.
- For Y-STR qualified analysts, Y-STR analysis is required in at least one proficiency test per year and must be performed on all male items in the proficiency test. For differentially extracted items, only one fraction per item needs to be Y-STR amplified.
    - There are two situations where it is acceptable to defer Y-STR analysis to the second proficiency test in a calendar year:
        - The first proficiency test does not have any detectable male DNA in either questioned sample, OR
        - Verified Y-STR reagents are not available at the time of the first proficiency test
        - Any other exception requires prior approval by DNA Technical Manager or supervisor.
    - If Y-STR analysis was successfully completed in the first proficiency test of the calendar year, it is not necessary to perform Y-STR analysis on the second proficiency test.
- Items will be reported as per the test provider's instructions when these vary from normal reporting procedures. However, they must be examined as per lab protocol.
- Test takers may consult with a similarly qualified peer during their examinations if that person is not assigned the same test.
    - Consulting another qualified examiner is required when specified as per the laboratory procedure.
- When a scenario suggests a possible sexual assault, **all** questioned samples (including swabs) must be tested by ALS, AP Spot test, and p30 card. This rule does not apply for proficiency tests where the screening results are provided.
- If necessary, extracts and/or evidence may be consumed without requesting permission to do so. Whenever possible, original material should be retained.
- Prior experience with Collaborative Testing Services (CTS) has shown that some fabric substrates interfere with EZ1 extraction. Specifically, questioned blood samples might yield better results if the blood is swabbed off the substrate instead of using a direct cutting of the fabric. While this handling method is not required for a first attempt, it will be required for a second attempt if the first attempt does not yield a full profile.
- At least one quantification plate, including standard curve and NTC wells, must be set up by the analyst performing the proficiency test. (It is okay to use a standard

curve prepared by another analyst, and to include casework samples from another analyst.)
- Every effort should be made to obtain a full profile at all loci. This may entail re-amplification or re-extraction of samples.

Documentation

- The applicable DNA conclusion page provided by the internal/external test provider (that is, the ones filled out with lab results and conclusions) will become part of the DNA analyst's bench notes and is typically the first numbered page. The page must include the lab number, date, and analyst initials.
- The LIMS report will be auto generated. Custom forms need to be completed to capture results and generate bench notes. Calculation of population frequency statistics is only required when specifically requested by the test provider.
- STR tables from the external provider will be filled out according to manufacturer's directions.
    - All data suitable for comparison is included on the table, including carryover from differential extraction.
    - Because the test provider assumes that all data reported in the table is suitable for comparison, any data which is not suitable for comparison under current FBCP guidelines must be clearly indicated as such in the test provider's comment section.
    - Any findings that have the potential to impact the interpretation (such as possible drop-out, artifact or true allele, etc.) should be noted in the test provider's comment section. Comments are not required for data determined to be artifacts and not included on the table (such as elevated stutter).
    - Because no contributors are assumed on proficiency test samples, mixtures are not deduced.
    - Major and minor contributors should be separated, as appropriate. Follow the test manufacturer's directions for noting minor alleles.
- Upon completion of the test, return to FBOUTGOING. Hold in Evidence Vault Shelf B. Post-test provider results, PT samples are destroyed or brought to discipline to be used for training.

Review and submission

- For DNA online data entry, review of the electronic submission form is completed by the administrative reviewer.
- After completion and electronic submission of the test, the test taker scans a printout from the test portal which includes submission date and time, as well as submitted results, into the LIMS case file.
- Submission of external test results
    - FTS (Forensic Testing Services)
        - The laboratory quality manager will provide the analyst with the FTS test information and the laboratory case #

  - The analyst logs in to the FTS site to retrieve the test result form, enter results and save to the FTS system
  - The analyst provides the technical reviewer with a printed copy of the completed results form for review; corrections are made as required
  - The analyst notifies the laboratory quality manager when the test is completed, report released in LIMS and notes complete in case images
- CTS (Collaborative Testing Services) – the procedure for CTS tests is provided in the laboratory quality manual
  - If the test taker is not able to submit the results to CTS, this task must be performed by a reviewer.
- FA (Forensic Assurance) – the procedure for FA tests is provided in the laboratory quality manual

Casework Proficiency Test – Interpretation Only CTS Test

- Tests are assigned to the analyst by the Evidence section, and a virtual test item is created in the LIMS. Once assigned, the analyst should start the chain of custody:
  - The agency the item is transferred from is *SCDL*, and the agency representative is *PT, Collaborative Testing Servces*.
  - Item is transferred to the analyst *via CTS portal*.
- The test consists of a zipped file that the analyst downloads from the CTS portal. Within the zipped file are data for multiple testing kits, in both PDFand HID (data file suitable to analyze in GMID-X).
  - CE data and PDFs from amplification chemistries not used by the lab can be deleted immediately.
  - CE Data from amplification chemistries to be interpretated in the current test can be transferred to the CE storage area on the lab network or kept in the analyst's lab network folder. Data files will only need to be be retained until notification from the test creator indicates the test was completed successfully.
  - NOTE: PDFs and CE data files tend to take a very long time to load. PDFs may be password protected. To save the PDF in compiled bench notes, it may be necessary to open each PDF and then print to PDF to create a file that is not password protected.
- Analysts relying solely on the digital interpretation test to maintain proficiency must take at least one test per year interpreting STRs (GlobalFiler), and at least one per year interpreting Y-STRs (PowerPlex Y23). It is highly recommended that the first test of the year be interpretated for Y-STRs at a minimum, in case the second test of the year does not include profiles containing male DNA.

- As a part of the proficiency test, analysts must review documentation of control samples, including allelic ladders, positive and negative amplification controls, and reagent blanks.
- Because the PDFs of negative control samples do not include the primer peak, the negative control data must be imported into GMID-X for analysis along with their corresponding allelic ladders. Positive controls may be checked either in GMID-X or in PDF format, although it is probably simpler to check them in GMID-X.

Documentation

- Retained documentation includes, as applicable: conclusion pages from the CTS form, electropherograms of test items used for interpretation, and statistics printouts. Controls must be checked by the analyst and the technical reviewer, but they are not included in the LIMS-DNA packet. Names of GMID-X projects are included in the LIMS-DNA packet.
- The analyst chooses one electropherogram in the selected amplification kit for interpretation of each test item. This can either be chosen from the PDFs, or the data can be imported into GMID-X and printed from there. NOTE: If the provided electropherogram PDFs are used, the analyst will need to add the lab number and the print date to each page.
- Each questioned sample profile must be entered into the test form. In addition, the analyst must select the most appropriate interpretation statement from the available options. Profiles suitable for exclusions only should be described as not suitable for comparisons.
- Assessment of whether low level peaks are artifacts or true alleles must be performed, regardless of whether the profile is suitable for comparison. Peaks determined to be A/TA are not included on the allele table but are described in the comments section of the test.
- If a questioned profile is determined to match a reference sample, the analyst must calculate the appropriate population frequency statistics, following FBPM protocol.
- Profile frequencies are given using the report language described in FBPM.
- As applicable, the following language is recommended for the field labeled as *Please list any databases used in the statistical analysis of item XX below:*

  STR population frequency statistics calculated in Popstats using Expanded FBI STR 2015 Caucasian and African American populations as well as Alaska expanded core 2017 (includes Athabaskan, Inupiat, and Yupik populations). Only the most common frequency among the five populations or 1 in 330 billion is reported, whichever is more common. Y-STR population frequency statistics calculated in YHRD.org using the Y17 dataset with the National Database (with Subpopulations, 2014 SWGDAM compliant) - United States and the Eskimo Aleut metapopulation. Only the most common frequency is reported, using the 95% confidence interval.
- The applicable DNA conclusion page provided by the internal/external test provider

(that is, the ones filled out with lab results and conclusions) will become part of the DNA analyst's bench notes and is typically the first numbered page. The page must include the lab number, date, and analyst initials.

- The LIMS report need only state that the case is a proficiency test and no report is being issued. Calculation of population frequency statistics is only required when specifically requested by the test provider.
- STR tables from the external provider will be filled out according to manufacturer's directions.
    - All data suitable for comparison is included on the table.
    - Because the test provider assumes that all data reported in the table is suitable for comparison, any data which is not suitable for comparison under current FBPM guidelines must be clearly indicated as such in the test provider's comment section.
    - Any findings that have the potential to impact the interpretation (such as possible drop-out, artifact or true allele, etc.) should be noted in the test provider's comment section. Comments are not required for data determined to be artifacts and not included on the table (such as elevated stutter).
    - Because no contributors are assumed on proficiency test samples, mixtures are not deduced.
    - Major and minor contributors should be separated, as appropriate. Follow the test manufacturer's directions for noting major/minor alleles.
    - When the test is completed, the item is transferred back to SCDL, again via CTS portal. At that point the item is considered to be returned to the submitting agency. However, data should be retained until notification is received that the test was successfully completed.

Review and submission

- For DNA online data entry, review of the electronic submission form is completed by the administrative reviewer.
- After completion and electronic submission of the test, the test taker scans a printout from the test portal which includes submission date and time, as well as submitted results, into the LIMS case file.
- Submission of external test results from CTS (Collaborative Testing Services) is provided in the laboratory quality manual
    - If the test taker is not able to submit the results to CTS, this task must be performed by a reviewer.

Database proficiency tests

Analysis

- Good faith efforts extend to all loci, not just core loci.
- Analysts who perform database analysis exclusively must include at least one sample per proficiency test which is analyzed both with direct amplification and with extraction, quantification, and GlobalFiler amplification.
- Analysts qualified in Rapid DNA analysis must analyze at least one swab by RapidDNA per year. For database analysts, this should be attempted in the first PT of the calendar year.
- For convenience of review, printouts of the database sample electropherograms may be included with the paperwork submitted for technical review. However, these pages are not included with the completed documentation, with the exception of Rapid DNA electropherograms.

Documentation

- Batch paperwork and review checklist are scanned to the appropriate folder in the Forensic Biology folder on the lab network.

Review and submission

- Technical and administrative review may be performed together by the same reviewing analyst.
- Review includes confirming that the electronic data forms are filled in completely and correctly.
- The procedure for CTS tests is provided in the laboratory quality manual
    - If the test taker is not able to submit the results to CTS, this task must be performed by a reviewer.
- After completion and electronic submission of the test, the test taker archives proof of submission from the test portal which includes submission date and time, as well as submitted results, into the LIMS case file.

Effective: 9/23/2024 Version 14.0

Version 13.0 was revised to version 14.0 prior to the effective date of 9/23/2024. Version 13.0 was never an active version. BMB 9/26/2024

Appendix G Revision History

Location	Revision made
throughout	**Spelling, typographic errors, page numbering, and formatting issues corrected**
throughout	References to PSA changed to p30
throughout	References to casework central logs, bench notes, and their documentation procedures have been updated to reflect LIMS-DNA workflow and documentation
Section 2.4	Removed directions for old model of p30 cards
Throughout Section 3	Removed option to put EZ1/2 digests on the instrument in a 1.5 mL tube.
Section 3.1	Changed requirement to create three reagent blanks in questioned extraction sets (was previously two). Changed requirement to include amplification of one ICS extract per extraction set (not per batch).
Section 3.2	Under Fingernails or fingernail clippings, added more specific instructions based on case-specific objectives.
Section 3.7	Replaced procedure for combining previously generated extract with new extraction of remaining sample with policy update regarding samples previously reported as requiring permission to consume
Section 4.2	Under Range of analyst discretion with documentation, added option to make adjustments based on a kit-specific verification. Under section In case of NTC failure, removed "or if the quantity is 0.001 or greater in either NTC well"
Section 4.3	Under Determining suitability for future testing for probative male, removed option for combining existing extract with a newly created extract. Added clarification that Y-STR amplification is routinely no lower than 0.1 ng but allowed for case-specific exceptions down to 0.02 ng.
Section 4.4	In flowchart *CONSUMED sample quant decision tree for extracts where any profile (male or female) would be probative,* 4th box in 4th row has been updated. All flowcharts incorporating Y-STR amplification have been updated to reflect routine and non-routine amplification thresholds.
Section 5.1	Added Note that third reagent blanks in questioned sample extraction sets are dried down and retained.
Section 5.1.1	Added guidance for adjusting amplification targets based on Quantifiler Trio Y-intercept values
Section 5.6	Added clarification that negative/empty/utility cartridges must be loaded when the instrument is in "Lock Screen"
Section 7.1	Added a section to describe Degradation and Inhibition. Under Profile interpretation: Completeness of a locus – added requirement to document reason for NS on electropherogram.
Section 7.5	Under Two source major component, added clarification regarding minimum number of distinguishable loci.
Section 8	Major changes to Y-STR interpretation throughout this section.
Section 9.1	Added clarification that all differential fractions created will need reported conclusions.
Appendix E: Batch Set-up for Re-Extract Processing	Updated to reflect that DB-ReExtract should now be captured as a complexity, rather than a reason. Removed lines in 1st paragraph for assigning to analyst prior to re-extract processing. Updated reference at bottom of page, regarding the custom form for capturing profile verification, and added hyperlink.
Section 3.2	All timeframes previously described in days (such as 2 days) are now described in hours (such as 48 hours)

Section 4.3	Added clarification: Routine Y-STR amplification of ~0.1 ng or more often yields a full or partial profile suitable for inclusions and exclusions. Y-STR amplification as low as 0.02 ng may be appropriate on a case-specific basis, such as when no other items are suitable for amplification and/or when a Y-STR profile suitable for exclusions only would have specific probative value to the case.
Section 4.4	Outdated flowcharts removed. Clarification added to section 4.3 was also added to headers of relevant flowcharts. Color-coding and its key were added.
Sections 5.2, 5.3, and 6A	Updated to new ICS policy