WASHINGTON STATE PATROL

CASEWORK

STR ANALYSIS PROCEDURES

CRIME LABORATORY DIVISION

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2. INTRODUCTION

It is important to note that procedure development is an ongoing process. The DNA Technical Leader should be consulted for significant changes to any of the following procedures or for developing additional procedures. These changes shall be discussed at a functional area meeting (or through e-mail or phone conference calls) prior to being incorporated into this manual. Allowances (non-significant changes) can be made for adapting procedures (e.g. volume, tube size/type, spin times, incubation times) to accommodate unusual case samples and situations. Allowances to adapt a standard procedure are a recognized part of casework, as these may be necessary to meet the requirements of certain cases or samples. These deviations must be scientifically sound and should be documented in the casework notes.

The standard method of analysis of DNA conducted by the Washington State Patrol Crime Laboratory (WSPCL) for criminal cases and for the convicted offender database is by the analysis of Short Tandem Repeat (STR) regions by Polymerase Chain Reaction (PCR). The WSPCL examines 27 loci: the original Combined DNA Index System (CODIS) core 13 loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, vWA, TPOX, D18S51, D5S818, and FGA); the additional newly adopted 7 CODIS core loci (D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433, and D22S1045); Amelogenin and DYS391 (for sex discrimination); Penta D, Penta E and SE33 for increased discrimination; and rapidly mutating YSTR loci DYS570 and DYS576.

PCR is a method used for the amplification of a specific DNA segment whereby two oligonucleotides (primers) anneal to opposite strands and flank a DNA region to be copied. The synthesis reaction is repeated for a number of cycles and results in the exponential accumulation of the specified DNA segment, the termini of which are defined by the 5' ends of the primers used in the reaction.

Y-Screening

Casework Direct Kit

The Casework Direct Kit is used for rapid screening of sexual assault samples for male DNA. The kit is capable of lysing sperm and non-sperm cells in one step using 1-thioglycerol as the reducing agent for effective sperm cell lysis. The lysate produced is considered a “dirty” lysate since cell debris, proteins, and other insoluble materials are present along with the DNA of interest. The Casework Direct lysate is suitable for human and male DNA quantification without any further DNA purification step required. If further validation studies are completed, Casework Direct lysates may also be determined suitable for amplification but have not been authorized for such use at this time.

Note: Samples from sexual assaults involving both a male victim and male perpetrator are expected to contain male DNA. Therefore, these sample types may not be suitable for Y-screening.

Extraction

DNA suitable for PCR-based typing is obtained by either an automated approach like those used by the QIAGEN BioRobots or a manual approach like the organic solvent extraction method. The organic method can be done in combination with other purification methods such as EZ1 or EZ2 Connect DNA purification.

A differential lysis procedure is used to isolate sperm cell DNA from other cellular sources of DNA, such as vaginal epithelial cell DNA, via separation of the initial sample into two different fractions. To accomplish this, advantage is taken of a particular characteristic of sperm cell membranes: sperm cells are resistant to lysis unless a reducing agent, such as dithiothreitol (DTT), is present. Therefore, other cells in the presence of sperm cells can be preferentially lysed with Proteinase K, the mixture spun, and the supernatant containing the non-sperm cell DNA removed. This supernatant sample is designated as

Fraction 1. The pelleted sperm cells can then be lysed using Proteinase K and DTT; this sample is designated as Fraction 2. It should be noted that the differential lysis procedure is often not completely effective in separating sperm cell DNA from other cellular sources of DNA. The analyst has the discretion to choose a procedure that is appropriate for a particular sample.

Human DNA Quantification

PowerQuant™ System

The PowerQuant™ System is a five-dye, four-target hydrolysis probe-based qPCR multiplex that amplifies multicopy targets to quantify the total human and human male DNA present in a sample. The human autosomal primers amplify an 84-base pair sequence while the Y chromosome primers amplify two multicopy loci (81bp and 136bp sequences). The use of two multicopy loci minimizes the effect that variation in copy number of any given marker can have on [Auto]/[Y] ratios and increases the sensitivity for male DNA. The system also detects degradation by targeting a longer amplicon (294bp) derived from a different region of the same locus as the autosomal target. Due to its greater length, the degradation amplicon is more susceptible to degradation and the presence of inhibitors. The ratio of DNA concentrations determined with the autosomal and degradation targets ([Auto]/[D] ratio) can be used to evaluate the degree of degradation. Additionally, the PowerQuant™ System includes an internal PCR control (IPC) to detect inhibitors in an amplification reaction. The IPC primers produce an amplified product that is 435bp and is the longest target in the PowerQuant™ System. This makes it more susceptible to inhibitors than the other targets in the multiplex.

Data generated using the PowerQuant™ System can help determine whether an unknown DNA sample is suitable for short tandem repeat (STR) analysis, what is the appropriate STR system to use (e.g., autosomal or Y-STR) and whether the DNA is degraded, or PCR inhibitors are present. This information may be used to guide decisions regarding sample processing including resampling, optimizing template volume to add to an STR amplification, adjusting for degradation or inhibition in samples, or halting analysis.

A standard curve is required to determine the DNA concentration of unknown DNA samples. This standard curve is generated using the amplification results from a dilution series of a male DNA standard of known concentration.

Amplification

STR PCR amplification is performed using the ABI GeneAmp® PCR System 9700 thermal cycler instrument or the Applied Biosystems ProFlex PCR System.

The polymerase used requires heat activation. This has the advantage that the enzyme is not active during the setup of the samples. The formation of "primer dimers" is avoided and there is no time constraint placed upon the setup procedure. To activate the enzyme, the amplification cycle includes a hot start enzyme activation at the beginning of the amplification process.

Three steps are involved in each amplification cycle. First, the template DNA is made single-stranded by heat denaturation. In the second step, the temperature is lowered so that annealing of the oligonucleotide primers to the template may occur. This interaction is favored because, initially, the ratio of primer to template is large. Annealing of the PCR primers to the template DNA is responsible for the technique's high level of specificity. Under optimized conditions, annealing of complimentary DNA strands (primers) should only occur at those sites on the template flanking the region to be amplified. In this way, a sequence of several hundred bases can be selected from a background of, in the case of human DNA,3 x 10^9 base pairs.

In the third step, the temperature is raised to a point that favors both specific annealing of the primers and extension by the DNA polymerase. These three steps are repeated over and over again to achieve a

sufficient quantity of amplified product. The target DNA is amplified at an exponential rate if one assumes a 100% efficient PCR reaction. Even when carefully controlled, a PCR reaction is rarely, if ever, 100% efficient.

Some amplification polymerases add an extra nucleotide (usually adenosine) to the end of a strand of DNA. If this reaction only occurs partially, then DNA of two lengths, differing by one base pair, will be produced from the same template and primers, causing the appearance of a split peak. Having amplified product of uniform size is important in STR analysis, as it is the length of the final product that is detected. A length variance of one base pair is detectable by the system, and therefore undesirable. To avoid this, the addition can either be prevented or conditions chosen so virtually all the amplification product is converted. Since the latter is easier, a 60ºC soak is included at the end of polymerization cycle to drive the addition reaction to completion.

Detection

STR Typing Kits

Kits that contain the necessary components to amplify and detect Amelogenin and the 20 core STR loci used by CODIS are commercially available. It is possible to detect and distinguish the products of several loci amplified together. This is referred to as multi-component analysis. The Fusion 6C kit allows for co-amplification and fluorescent detection of the 20 CODIS core loci as well as Amelogenin and DYS391 for sex determination and Penta D, Penta E, SE33, DYS570, and DYS576 for higher discrimination.

Fusion 6C kit loci

Locus	**Size Range (bp)**[1]	**Dye Color**[2]
Amelogenin	80 – 89	Blue
D3S1358	90 – 151	Blue
D1S1656	152 – 209.50	Blue
D2S441	211 – 252	Blue
D10S1248	254 – 302.50	Blue
D13S317	304.50 – 357	Blue
Penta E	362 – 482	Blue
D16S539	74 – 129.4	Green
D18S51	131 – 217.5	Green
D2S1338	221.5 – 304	Green
CSF1PO	313 – 366.5	Green
Penta D	373.5 – 470	Green
TH01	65 – 118	Yellow
vWA	121 – 192	Yellow
D21S11	197 – 266.5	Yellow
D7S820	268 – 315.5	Yellow
D5S818	317.50 – 380	Yellow
TPOX	390 – 448	Yellow
D8S1179	66 – 129.8	Red
D12S391	130.10 – 190.5	Red
D19S433	192 – 255	Red
SE33	270 – 429	Red
D22S1045	430 – 478	Red
DYS391	79.5 – 131	Purple
FGA	134 – 299	Purple
DYS576	302 – 370	Purple

[1] Values obtained from the GMID-X panels; the estimated size generated by the CE unit will be different.
[2] The dyes used are FL-6C (blue), JOE-6C (green), TMR-6C (yellow), CXR-6C (red), TOM-6C (purple), and WEN-6C (orange). For more information on the loci and dyes, see the Fusion 6C user's manual.

DYS570	380 – 464	Purple

As a supplemental DNA analysis method, the WSPCL also employs the PowerPlex® Y23 System amplification kit for the detection of male haplotypes.

The PowerPlex® Y23 System PCR Amplification Kit is a Short Tandem Repeat (STR) multiplex that amplifies 23 Y-STR loci. These 23 loci are found on the non-recombining region of the Y chromosome, allowing the amplification of only human male DNA. The alleles at each locus are inherited as one linked block of genetic information that is passed down through a paternal lineage. This technology allows for the analysis of forensic casework samples that contain small amounts of male DNA in the presence of large amounts of female DNA. Both DY570 and DY576 are classified as rapidly mutating loci.

Y23 STR Locus	Size Range (bp)[3]	Dye/Color
DYS576	97–145	Fluorescein/Blue
DYS389 I	147–179	Fluorescein/Blue
DYS448	196–256	Fluorescein/Blue
DYS389 II	259–303	Fluorescein/Blue
DYS19	312–352	Fluorescein/Blue
DYS391	86–130	JOE/Green
DYS481	139–184	JOE/Green
DYS549	198–238	JOE/Green
DYS533	245–285	JOE/Green
DYS438	293–343	JOE/Green
DYS437	344–380	JOE/Green
DYS570	90–150	TMR-ET/Yellow
DYS635	150–202	TMR-ET/Yellow
DYS390	207–255	TMR-ET/Yellow
DYS439	263–307	TMR-ET/Yellow
DYS392	314–362	TMR-ET/Yellow
DYS643	368–423	TMR-ET/Yellow
DYS393	101–145	CXR-ET/Red
DYS458	159–215	CXR-ET/Red
DYS385 a/b[4]	223–307	CXR-ET/Red
DYS456	316–364	CXR-ET/Red
Y-GATA-H4	374–404	CXR-ET/Red

Multicomponent Analysis

Multicomponent analysis uses two separate properties to distinguish between loci: length variance and fluorescent labels.

Length Variance

The amplified product from each locus will vary in length, dependent upon the number of repeats found in a particular STR polymorphism. By careful selection of primers, it is possible to produce amplified products for several loci, all of which fall into discrete and separate size ranges. Thus, a particular locus can be identified by the size of its alleles.

Fluorescent Label

Different fluorescent labels can be attached to the primers used in the amplification process. The emission spectra of these labels are sufficiently different such that the contribution made by each label in

[3] Size in actual base pairs; the estimated size generated by the CE unit will be different
[4] DYS385 a/b is a duplicated locus on the Y chromosome and is counted as 2 loci when describing.

a mixture can be calculated both quantitatively and qualitatively. Thus, even if two separate DNA fragments co-migrate they can be differentiated by their fluorescent labels.

By careful selection of both primers and fluorescent dyes, several different co-amplified loci can be distinguished based both upon their fluorescence and the size of the fragment lengths. Theoretically, it is possible to detect a large number of co-amplified loci by a combination of these two detection methods.

Each of the dyes employed detects loci in discrete size ranges. This allows for the detection of multiple loci at one time. Each amplification kit dedicates one dye to label the internal size standard.

Genetic Analyzer (Capillary Electrophoresis [CE] Unit)

The genetic analyzer (CE unit) separates DNA fragments based upon their size and also upon their attached fluorescent labels using capillary electrophoresis. A window in the capillary is continually being illuminated by a laser. As fragments of DNA with fluorescent labels pass by the window, they are excited, and the resulting emitted light collected and analyzed.

The Applied Biosystems 3500 Genetic Analyzers is currently being used by the WSPCL for the analysis of DNA fragments with fluorescent labels.

Internal Size Standards

An appropriate internal size standard is added to each sample prior to its electrophoresis. The internal size standard consists of several amplified fragments of DNA, all of which have been sequenced and are of known length. The migration time of each fragment is recorded, and these data are used to calculate an estimated length for the other DNA fragments that are detected.

Interpretation of Results

The data generated is analyzed using software programs available from Applied Biosystems GeneMapper® *ID-X* software. The software converts the raw data from the CE instrument into discrete peaks, assigns a size to those peaks based upon their estimated length in base pairs, and converts the estimated fragment sizes in base pairs to designated alleles by comparing the fragment sizes to the known alleles in a standard called an allelic ladder. The program displays that data in the form of an electropherogram.

3. Y-SCREENING FOR SEXUAL ASSAULT EVIDENCE

1. Evaluate the sexual assault kit contents and case information to determine the most appropriate screening strategy.
    a. In general, all body swabs to include orifice and skin swabs should be sampled for Y-screening.
    b. Analyst discretion may be used in selecting an appropriate set of swabs for Y-screening. Documentation shall be retained in the casefile to include justification when a swab set is not examined (e.g. oral swabs from a conscious victim where no alleged oral penetration occurred, or skin swabs from "touched" areas where casual consensual contact is alleged).
2. Place a small portion of the sample (1/8 to 1/4 of the swab material or similar sized cutting from other materials) in an appropriate microcentrifuge tube or tube/basket.
3. Prepare the components of the Casework Direct kit:
    a. Dilute 1-thioglycerol tenfold in amplification grade water. Undiluted 1-thioglycerol is viscous. To facilitate accurate pipetting, warm to room temperature, pipette slowly, avoid pipetting small volumes, and ensure 1-thioglycerol is not on the outside of the pipette tip.
    b. Vortex to sufficiently mix (e.g. 10–15 seconds).
    c. The diluted 1-Thioglycerol is stable at 4 °C for 6 months.
4. Determine the number of samples for Y-screening to include a reagent blank(s) (# of samples = n) and prepare the needed amount of Casework Direct Solution using the table below. Vortex the prepared Casework Direct Solution to sufficiently mix.

Casework Direct Solution*	
Casework Direct Reagent	400 µL × (n+1)
Diluted 1-Thioglycerol	2 µL × (n+1)

*may be used at half volume (200 µL CWD reagent/1 µL diluted 1-Thioglycerol)

5. Add between 200 µL and 400 µL of Casework Direct Solution to each sample, ensuring adequate reagent to cover the sample.
6. Briefly vortex, and incubate the samples for 30 minutes at 70 °C.
7. Substrates do not need to be removed prior to manual DNA quantification set-up. Substrate removal is required prior to automated DNA quantification set-up. To remove the substrate:
    d. Transfer the substrate to an appropriate spin basket and spin at maximum speed in a microcentrifuge for 3 to 5 minutes. Ensure no liquid remains in the spin basket then discard the spin basket and substrate.
    e. Alternatively, substrate removal may be accomplished using an Investigator Lyse&Spin assembly (or similar) followed by centrifugation at 10,000–20,000×*g* for 3–5 minutes. Ensure no liquid remains in the spin basket then discard the spin basket and substrate. If liquid remains, repeat centrifuging at a higher speed (up to 20,000×*g*) and/or transfer the substrate and liquid into a new basket.
8. The samples are now ready for DNA quantification. The samples should be quantified as soon as practical but may be stored refrigerated or frozen (≈4 °C or less) for up to one month.

4. LYSIS: NON-DIFFERENTIAL USING WSP BUFFER

Scope

This procedure is used to lyse cells in samples intended for DNA analysis. It is suitable for sample types for which differential lysis is not necessary.

Procedure

1. Determine the number of samples to include in the extraction batch. The batch size shall not exceed the number of samples that can be extracted concurrently using the intended purification procedure.
2. Place sample into a microcentrifuge tube or tube/basket assembly.
3. Add the following reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
TNE	500
20% Sarkosyl	25
Proteinase K, 20 mg/ml	7.5
1 M DTT (optional)	15.6

   - For sexual assault samples, may add 15.6 µL of 1 M DTT.
4. Vortex briefly.
5. Incubate at 56 °C for a minimum 15 minutes not to exceed 24 hours.
6. Pulse spin.
7. Transfer cutting into a basket insert and place the basket back into the tube.
   - Certain samples may not require the use of a spin basket.
8. Centrifuge for 3 to 5 minutes at maximum speed.
9. Ensure no liquid remains in the basket, then remove and discard the basket and cutting.
10. Proceed to DNA purification by one of the following methods:
    - Purification: Organic extraction
    - Purification: EZ1 or EZ2 Connect Large Volume method

5. LYSIS: DIFFERENTIAL

Scope

This procedure allows for the selective lysis of spermatozoa from other cellular material for DNA analysis. It is suitable for samples suspected to contain semen.

Procedure

1. Determine the number of samples to include in the extraction batch. The batch size shall not exceed the number of samples that can be extracted concurrently using the intended purification procedure.
2. Place cutting into the microcentrifuge tube from a tube/basket assembly.
   - Do not use a Lyse&Spin assembly; spermatozoa may not flow through the basket during centrifugation.
3. If a PBS fraction is needed for serological testing, add 100–1,000 µl of PBS to each sample tube.
   - Differential lysis resumes at step 10.
4. Incubate at 37 °C for a minimum of 30 minutes.
5. Transfer cutting into the basket insert and place the basket back into the tube.
6. Centrifuge for 5 minutes.
7. Without disturbing the cell pellet, transfer most (all but ~50 µl) of the supernatant to a new, labeled tube.
   - Proceed with any serological tests using this PBS fraction or store frozen until use.
8. If needed, re-suspend the cell pellet for microscope slide preparation. Refer to the Sperm Search procedure.
9. Return the sample cutting to the tube with the cell pellet.
10. Add the following reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
TNE	400
Sterile dH_2O	50
20% Sarkosyl	25
Proteinase K, 20 mg/ml	2.5

11. Vortex briefly.
12. Incubate at 37 °C for 1 to 2 hours.
13. Transfer cutting into the basket insert and place the basket back into the tube.
14. Centrifuge for 5 to 7 minutes.
15. Remove and discard the basket insert and cutting.
16. Without disturbing the cell pellet, remove the supernatant fluid and place this first fraction into a new, labeled 2 ml tube.
    - Analysis of fraction 1 resumes at step 25.
17. Re-suspend the remaining sperm cell pellet in 500 µL sperm wash buffer.
18. Vortex briefly.
19. Centrifuge fraction 2 for 5 to 7 minutes.
20. Without disturbing the cell pellet, remove and discard the supernatant fluid.
21. Perform 2 additional washes. Remove and discard the supernatant after each wash.
    - A microscopic exam for spermatozoa may be done at this time. Refer to Sperm Search procedure.
    - If desired, PBS can be substituted for the last wash to aid in cellular staining.
    - 3 total washes are likely appropriate for the majority of samples.

- Additional washes may be beneficial if a large quantity of epithelial cells relative to sperm cells are observed during microscopic exam.
- Reducing the number of washes may be warranted (e.g., if few sperm are microscopically observed or quantification results indicate a low amount of male DNA).

22. Add the following reagents to each fraction 2 tube, including reagent blank(s):

Reagent	Volume (µL)
TNE	150
Sterile dH_2O	150
20% Sarkosyl	50
1 M DTT	15.6
Proteinase K, 20 mg/ml	5

23. Vortex briefly.
24. Incubate at 37 °C for a minimum 15 minutes, not to exceed 24 hours.
25. Proceed to DNA purification by one of the following methods:
    - Purification: Organic Extraction
    - Purification: EZ1 or EZ2 Connect Large Volume method

6. LYSIS: HAIR SAMPLES USING SPERM WASH BUFFER

Scope

This procedure is used to lyse cells in hair samples intended for DNA analysis. It is suitable for samples where an apparent root end has been collected.

Procedure

1. Cut off up to 10 mm of the root end and place in a microcentrifuge tube.
2. If a substrate control is needed, cut off the adjacent 5 to 10 mm of hair shaft and place it in a separate microcentrifuge tube.
   - Cellular material may be present on the surface of the hair.
3. Add the following reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
Sperm Wash Buffer	500
1 M DTT	20
Proteinase K, 20mg/ml	7.5

4. Vortex thoroughly.
   - Ensure each hair is fully submerged.
5. Incubate at 56 °C for a minimum 6 hours not to exceed 24 hours.
6. Pulse spin.
7. Proceed to DNA purification by one of the following methods:
   - Purification: Organic Extraction
   - Purification: EZ1 or EZ2 Connect Large Volume method

7. RECOVERING SLIDE-MOUNTED HAIRS OR SEMEN SMEARS

A scribe can be used to score the coverslip around the hair root portion to be removed, and either process below can be used to remove the scored section of coverslip:

1. The coverslip may be removed by carefully pipetting a suitable solvent (i.e., xylene or toluene*) around the edges of the coverslip. If the coverslip does not loosen and come off, the entire slide can be placed in a petri dish and covered with solvent for one or more hours until the coverslip has loosened. Beware of multiple hairs on one slide, however.
2. The coverslip may also be removed by freezing the slide in a -20°C freezer for at least 20 minutes, then prying the coverslip off with a scalpel.

Pick out the hair (or hair root or scrape the semen smear) and place in a 1.5 ml microcentrifuge tube. Wash in 500µl xylene or toluene to remove excess Permount. Follow this with a wash in 500µl ethanol followed by a final rinse in sterile dH_2O.

Proceed with the appropriate extraction procedure.

*NOTE: Solvents such as xylene and toluene are carcinogenic. Wear gloves and work in a fume hood. Take working with low level DNA precautions.

8. LYSIS: NON-DIFFERENTIAL USING G2 BUFFER

Scope

This procedure is used to lyse cells in samples intended for DNA analysis. It is suitable for sample types for which differential lysis is not necessary.

Procedure

Non-Hair Samples

1. Determine the number of samples to include in the extraction batch. The batch size shall not exceed the number of samples that can be extracted concurrently using the intended purification procedure.
2. Place sample into a microcentrifuge tube or tube/basket assembly.
3. Add the following reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
G2 buffer (neat or diluted)	190–590
Proteinase K, 20 mg/ml	10
1 M DTT (optional)	15.6

   - G2 buffer may be diluted 1:1 in sdH_2O if desired.
   - Sufficient volume of G2 buffer should be added to cover the sample.
   - For sexual assault samples, may add 15.6 µL of 1 M DTT.
   - Ensure the combined reagent volume does not exceed 600 µL.
   - The same amount of G2 buffer should be used for all samples in an extraction batch.
4. Vortex briefly.
5. Incubate at 56 ºC for a minimum of 15 minutes not to exceed 24 hours.
6. Pulse spin.
7. Transfer the cutting into a basket insert and then place the basket insert back into the tube.
   - If using the LySep, Lyse&Spin, or similar basket, the pulse spin and substrate transfer steps are not necessary.
   - Certain samples may not require the use of a spin basket.
   - Substrate removal is not required if the EZ1 or EZ2 Connect Tip Dance method will be used for purification.
8. Spin at maximum speed in a centrifuge for 5 minutes.
9. Ensure no liquid remains in the basket, then remove and discard the basket and cutting.
10. Proceed to DNA purification by one of the following methods based on lysate volume and presence of substrate:
    - Purification: EZ1 or EZ2 Connect Trace method
    - Purification: EZ1 or EZ2 Connect Tip Dance method
    - Purification: EZ1 or EZ2 Connect Large Volume method

9. LYSIS: HAIR SAMPLES USING G2 BUFFER

Scope

This procedure is used to lyse cells in hair samples intended for DNA analysis. It is suitable for samples where an apparent root end has been collected.

Procedure

1. Determine the number of samples to include in the extraction batch. The batch size shall not exceed the number of samples that can be extracted concurrently using the intended purification procedure.
1. Cut off up to 10 mm of the root end and place the hair in a microcentrifuge tube.
2. Add the following reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
G2 buffer, neat	180
Proteinase K, 20 mg/ml	10
1 M DTT	10

3. Vortex thoroughly.
    - Ensure each hair is fully submerged.
4. Incubate at 56 ºC for at least 6 hours.
5. Pulse spin.
6. Add the following additional reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
Proteinase K, 20 mg/ml	10
1 M DTT	10

7. Vortex thoroughly.
8. Incubate at 56 ºC for at least 2 hours.
    - The hair will be completely dissolved or broken into many small pieces.
    - Total lysis time in steps 4 and 8 should not exceed 24 hours.
9. Pulse spin.
10. Proceed to DNA purification by one of the following methods:
    - Purification: EZ1 or EZ2 Connect Trace method
    - Purification: EZ1 or EZ2 Connect Tip Dance method

10. PURIFICATION: ORGANIC EXTRACTION

Scope

This procedure is used to isolate DNA following cell lysis.

Procedure

1. Wear appropriate protective gear. This procedure shall be performed in an appropriate fume hood.
2. Add 500 µl phenol/chloroform/isoamyl alcohol to the digested sample.
3. Shake until a milky emulsion forms.
   - Use of a vortexer is optional.
4. Spin at maximum speed in a microcentrifuge for 3 to 5 minutes.
   - After centrifugation the DNA will be in the upper aqueous phase.
5. Transfer the upper aqueous layer into a new microcentrifuge tube.
   - Take care not to disturb the interface during the transfer.
   - Alternatively, the aqueous layer may be transferred directly into a concentration unit.
6. Repeat steps 2–5 as necessary until the interface is clean.
7. Purify and concentrate the sample(s) using the Microcon Concentration of DNA Extracts method.

11. QIAGEN BIOROBOT EZ1 AND EZ2 CONNECT WORKSTATIONS

The Qiagen BioRobot® EZ1, EZ1 Advanced, and EZ2 Connect workstations use technology based on DNA binding to magnetic beads followed by washing steps and elution for DNA extraction from forensic casework samples. This process takes the place of the organic phenol/chloroform/isoamyl alcohol extraction and subsequent purification (e.g. Microcon®) steps in forensic DNA analysis.

The sample undergoes lysis/digestion or pre-treatment to prepare it for DNA extraction on the Qiagen BioRobot® EZ1 or EZ2 Connect.

The DNA Investigator Card for the EZ1 workstation and EZ2 Connect software have 3 validated DNA purification procedures to select from depending on the desired application: the Trace Protocol, the Large Volume Protocol, and the Tip Dance Protocol. These three protocols may be used in conjunction with the 20, 25, 30, 40, 50, 100, and 200 µL elution volumes, as applicable based on the instrument and sample type.

The DNA purification protocols are designed for isolation of total DNA from forensic samples. The Trace Protocol allows for automated processing of starting volumes up to 200µl while the Large Volume Protocol accommodates starting volumes up to 600µl. The Trace Protocol can also be used to clean up organically extracted genomic DNA preparations that may have co-purified PCR inhibitors present. The Tip Dance Protocol can process solid materials (non-fluffy matrix samples such as hair roots and reference blood cards where the danger of the barrier pipette tip clogging is minimal) directly in the sample tube.

Certain versions of the Qiagen EZ2 Connect software include a programmed cooling protocol that launches at the conclusion of a DNA purification procedure. This cooling protocol is not required and may be aborted if desired.

Extraction Batch

A batch should generally not exceed 28 samples. When planning an extraction batch, ensure there are sufficient reagent blank samples included to adequately cover equal or better sensitivity for the amplifications and analysis to be performed on the extracted DNA. A batch may be processed using two BioRobot EZ1 or EZ2 Connect workstations running at the same time.

12. PURIFICATION: EZ1 TRACE METHOD

Scope

This procedure is used to isolate DNA in samples following cell lysis using the EZ1 instrument. It can also be used to clean up organically extracted samples that may have co-purified PCR inhibitors present. This procedure is suitable for starting volumes up to 200 µL.

Procedure

1. Label the appropriate number of tubes for elution.
2. If necessary, transfer the samples to compatible tubes.
3. For organic extract clean-up, add enough diluted G2 buffer to reach a starting volume of 195 µL
   - Dilute G2 buffer 1:1 with sdH_2O.
4. Add 1 µL carrier RNA to each sample tube.
   - Carrier RNA is not required for known reference samples.
5. Select “Trace Protocol” from the Protocols menu.
6. Follow the screen prompts to select the appropriate eluate and elution volume, and to set up the worktable.
   - Routinely elute samples in TE.
   - If combining several samples, elution in H_2O may be considered.
   - Extracts in H_2O should be used the same day or stored frozen.
7. Press “Start”.
8. Remove the elution tubes once the protocol has finished and as needed, inspect eluate for the presence of magnetic particles.
   - If magnetic particles are observed, apply the tube to a magnetic separator and transfer the extract to a new labeled tube.
9. Remove and discard the disposable labware.
   - Do not dispose of Investigator cartridges in any bleach or container which may contain bleach; cyanide gas may result due to the chaotropic salts.
10. Perform the Qiagen Biorobot EZ1 Workstation – Maintenance procedure following the run.
11. Conduct a 20-minute (minimum recommended time) UV decontamination if desired.
12. Proceed to DNA quantitation.
    - Store the extracts refrigerated or frozen. For long-term storage, freeze samples.
    - Avoid repeated freeze-thaw cycles.

13. PURIFICATION: EZ1 LARGE VOLUME METHOD

Scope

This procedure is used to isolate DNA in samples following cell lysis using the EZ1 instrument. It is suitable for starting volumes up to 600 µL.

Procedure

1. Label the appropriate number of elution tubes.
2. If necessary, transfer the samples to compatible tubes.
3. Add the following reagents to each sample tube, including reagent blank(s):

Reagent	Volume (µL)
MTL Buffer	400
Carrier RNA	1

   - Carrier RNA is not required for known reference samples.
4. Select “Large Volume Protocol” from the Protocols menu.
5. Follow the screen prompts to select the appropriate eluate and elution volume, and to set up the worktable.
   - Routinely elute samples in TE.
   - If combining several samples, elution in H_2O may be considered.
   - Extracts in H_2O should be used the same day or stored frozen.
6. Press “Start”.
7. Remove the elution tubes once the protocol has finished and as needed, inspect eluate for the presence of magnetic particles.
   - If magnetic particles are observed, apply the tube to a magnetic separator and transfer the extract to a new labeled tube.
8. Remove and discard the disposable labware.
   - Do not dispose of Investigator cartridges in any bleach or container which may contain bleach; cyanide gas may result due to the chaotropic salts.
9. Perform the Qiagen Biorobot EZ1 Workstation – Maintenance procedure following the run.
10. Conduct a 20-minute (minimum recommended time) UV decontamination if desired.
11. Proceed to DNA quantitation.
    - Store the extracts refrigerated or frozen. For long-term storage, freeze samples.
    - Avoid repeated freeze-thaw cycles.

14. PURIFICATION: EZ1 TIP DANCE METHOD

Scope

This procedure is used to isolate DNA in samples following cell lysis using the EZ1 instrument. It is suitable for starting volumes up to 200 µL with the solid material (hairs, reference blood card, etc.) still present in the sample tube.

Procedure

1. Label the appropriate number of elution tubes.
2. If necessary, transfer the samples to compatible tubes.
3. Add 1 µl carrier RNA to each sample.
    - Carrier RNA is not required for known reference samples.
4. Select “Trace TD Protocol” from the Protocols menu.
5. Follow the screen prompts to select the appropriate eluate and elution volume, and to set up the worktable.
    - Routinely elute samples in TE.
    - If combining several samples, elution in H_2O may be considered.
    - Extracts in H_2O should be used the same day or stored frozen.
6. Press “Start”.
7. Remove the elution tubes once the protocol has finished and as needed, inspect eluate for the presence of magnetic particles.
    - If magnetic particles are observed, apply the tube to a magnetic separator and transfer the extract to a new labeled tube.
8. Remove and discard the disposable labware.
    - Do not dispose of Investigator cartridges in any bleach or container which may contain bleach; cyanide gas may result due to the chaotropic salts.
9. Perform the Qiagen Biorobot EZ1 Workstation – Maintenance procedure following the run.
10. Conduct a 20-minute (minimum recommended time) UV decontamination if desired.
11. Proceed to DNA quantitation.
    - Store the extracts refrigerated or frozen. For long-term storage, freeze samples.
    - Avoid repeated freeze-thaw cycles.

15. PURIFICATION: EZ2 CONNECT TRACE METHOD

Scope

This procedure is used to isolate DNA in samples following cell lysis using the EZ2 Connect instrument. It can also be used to clean up organically extracted samples that may have co-purified PCR inhibitors present. This procedure is suitable for starting volumes up to 200 µL.

Procedure

1. Label the appropriate number of tubes for elution.
2. If necessary, transfer the samples to compatible tubes.
3. For organic extract clean-up, add enough diluted G2 buffer to reach a starting volume of 195 µL
   - Dilute G2 buffer 1:1 with sdH_2O.
4. Add 1 µL carrier RNA to each sample tube.
   - Carrier RNA is not required for known reference samples.
5. Log into the EZ2 Connect software
6. Select “DNA” under Applications
   - Alternatively, select a shortcut with the appropriate settings instead of manually selecting the settings in steps 6–12.
7. Select “DNA Investigator Kit”
8. Select “Trace” from the Protocols menu
9. Follow the screen prompts to select the appropriate eluate, rack type, and elution volume, and to set up the worktable.
   - Routinely elute samples in TE.
   - If combining several samples, elution in H_2O may be considered.
   - Extracts in H_2O should be used the same day or stored frozen.
10. Select at least one sample location when prompted.
11. Select “Generate missing samples IDs”.
12. Select “Skip load check”.
    - Optionally, create a shortcut with the settings selected in steps 6–12. Name the shortcut to include the applicable settings, e.g. “Trace – 50 µL – TE”.
13. Remove the elution tubes once the protocol has finished and as needed, inspect eluate for the presence of magnetic particles.
    - If magnetic particles are observed, apply the tube to a magnetic separator and transfer the extract to a new labeled tube.
14. Remove and discard the disposable labware.
    - Do not dispose of Investigator cartridges in any bleach or container which may contain bleach; cyanide gas may result due to the chaotropic salts.
15. Perform the Maintenance procedure following the run.
16. Conduct a UV decontamination if desired.
17. Proceed to DNA quantitation.
    - Store the extracts refrigerated or frozen. For long-term storage, freeze samples.
    - Avoid repeated freeze-thaw cycles.

Note: In the event of a run failure, consult the semi-automated recovery procedure from the Qiagen EZ2 Connect Recovery Procedure Instruction Manual for sample recovery steps. This requires careful note of affected sample location, where sample was dispensed, and the protocol step indicated in the Protocol Run Aborted dialog box on the instrument.

16. PURIFICATION: EZ2 CONNECT LARGE VOLUME METHOD

Scope

This procedure is used to isolate DNA in samples following cell lysis using the EZ2 Connect instrument. It is suitable for starting volumes up to 600 µL.

Procedure

1. Label the appropriate number of elution tubes.
2. If necessary, transfer the samples to compatible tubes.
3. Add the following reagents to each sample tube, including reagent blank(s)
    - Carrier RNA is not required for known reference samples.

Reagent	Volume (µL)
MTL Buffer	400
Carrier RNA	1

4. Log into the EZ2 Connect software.
5. Select “DNA” under Applications.
    - Alternatively, select a shortcut with the appropriate settings instead of manually selecting the settings in steps 5–11.
6. Select “DNA Investigator Kit”
7. Select “Large Volume” from the Protocols menu.
8. Follow the screen prompts to select the appropriate eluate and elution volume, and to set up the worktable.
    - Routinely elute samples in TE.
    - If combining several samples, elution in H_2O may be considered.
    - Extracts in H_2O should be used the same day or stored frozen.
9. Select at least one sample location when prompted.
10. Select “Generate missing samples IDs”.
11. Select “Skip load check”.
    - Optionally, create a shortcut with the settings selected in steps 5–11. Name the shortcut to include the applicable settings, e.g. “Large volume – 50 µL – TE”.
12. Remove the elution tubes once the protocol has finished and as needed, inspect eluate for the presence of magnetic particles.
    - If magnetic particles are observed, apply the tube to a magnetic separator and transfer the extract to a new labeled tube.
13. Remove and discard the disposable labware.
    - Do not dispose of Investigator cartridges in any bleach or container which may contain bleach; cyanide gas may result due to the chaotropic salts.
14. Perform the Maintenance procedure following the run.
15. Conduct a UV decontamination if desired.
16. Proceed to DNA quantitation.
    - Store the extracts refrigerated or frozen. For long-term storage, freeze samples.
    - Avoid repeated freeze-thaw cycles.

Note: In the event of a run failure, consult the semi-automated recovery procedure from the Qiagen EZ2 Connect Recovery Procedure Instruction Manual for sample recovery steps. This requires careful note of affected sample location, where sample was dispensed, and the protocol step indicated in the Protocol Run Aborted dialog box on the instrument.

17. PURIFICATION: EZ2 CONNECT TIP DANCE METHOD

Scope

This procedure is used to isolate DNA in samples following cell lysis using the EZ2 Connect instrument. It is suitable for starting volumes up to 200 µl with the solid material (hairs, reference blood card, etc.) still present in the sample tube.

Procedure

1. Label the appropriate number of elution tubes.
2. If necessary, transfer the samples to compatible tubes.
3. Add 1 µL carrier RNA to each sample.
    - Carrier RNA is not required for known reference samples.
4. Log into the EZ2 Connect software.
5. Select “DNA” under Applications.
    - Alternatively, select a shortcut with the appropriate settings instead of manually selecting the settings in steps 5–11.
6. Select “DNA Investigator Kit”.
7. Select “Tip Dance” from the Protocols menu.
8. Follow the screen prompts to select the appropriate eluate and elution volume, and to set up the worktable.
    - Routinely elute samples in TE.
    - If combining several samples, elution in H_2O may be considered.
    - Extracts in H_2O should be used the same day or stored frozen.
9. Select at least one sample location when prompted.
10. Select “Generate missing samples IDs”.
11. Select “Skip load check”.
    - Optionally, create a shortcut with the settings selected in steps 5–11. Name the shortcut to include the applicable settings, e.g. “Trace – 50 µL – TE”.
12. Remove the elution tubes once the protocol has finished and as needed, inspect eluate for the presence of magnetic particles.
    - If magnetic particles are observed, apply the tube to a magnetic separator and transfer the extract to a new labeled tube.
13. Remove and discard the disposable labware.
    - Do not dispose of the Investigator cartridges in any bleach or container which may contain bleach; cyanide gas may result due to the chaotropic salts.
14. Perform the Maintenance procedure following the run.
15. Conduct a UV decontamination if desired.
16. Proceed to DNA quantitation.
    - Store the extracts refrigerated or frozen. For long-term storage, freeze samples.
    - Avoid repeated freeze-thaw cycles.

Note: In the event of a run failure, consult the semi-automated recovery procedure from the Qiagen EZ2 Connect Recovery Procedure Instruction Manual for sample recovery steps. This requires careful note of affected sample location, where sample was dispensed, and the protocol step indicated in the Protocol Run Aborted dialog box on the instrument.

18. MICROCON CONCENTRATION OF DNA EXTRACTS

Scope

This procedure is used to purify phenol chloroform extracts. It may also be used to concentrate or combine TE-eluted DNA extracts.

Procedure

1. Insert a Microcon filter device into a provided tube.
2. Add 30–100 µL TE to the filter device, if desired.
3. Pipette DNA extract into the filter device.
   - If purifying a phenol chloroform extract, avoid pipetting organic solvent into the filter device.
   - Ensure the total volume in the filter device does not exceed 500 µL.
4. Cap the Microcon and centrifuge at ~500 to 5,000×g for at least 10–15 minutes.
   - Centrifuge settings can be adjusted according to centrifuge type and sample viscosity.
5. Remove the filter device from the assembly and discard the filtrate fluid from the tube. Return the filter device to the tube.
6. Add 200–500 µL TE to the filter device. Recap and spin the assembly in the centrifuge for ~10 minutes at ~500 to 5,000×g.
   - Additional washes may be necessary to remove inhibitors and should be done on samples of extremely limited quantity.
   - If the Microcon is being used to concentrate or reduce sample volume, a wash step is not necessary.
7. Add the desired final volume of TE (usually between 25 and 200 µL) to the filter device, as needed.
8. Remove the filter device from the tube and carefully invert it onto a new labeled retentate tube. Discard the filtrate tube.
9. Centrifuge the assembly at 1,000×g for 5 minutes.
10. Discard the filter device. Cap the retentate tube.
11. Proceed to DNA quantitation.

Store the extracts refrigerated or frozen. For long-term storage, freeze samples.

   - Avoid repeated freeze-thaw cycles.

19. VACUUM CENTRIFUGE CONCENTRATION OF DNA EXTRACTS

Scope

This procedure can be used to concentrate a sample to a desired volume or combine multiple DNA extracts in a single desired volume.

Procedure

1. Transfer any samples for combination into a single tube.
    - Samples eluted in TE shall not exceed a 15x concentration after combination and concentration.
    - As TE concentration increases, potential for inhibition during amplification increases; therefore, careful consideration should be given for low level or degraded samples.
    - Refer to the Microcon Concentration of DNA Extracts procedure when >15x TE concentration would result from combination of samples.
    - Do not exceed a 10x concentration of DNAstable when combining samples that were previously dried.
2. Add 50 µl of TE to any water-eluted extracts.
3. Ensure the correct program is selected for operation (e.g. V-AQ).
    - It is recommended that prior to first use of the day, the Vacufuge is run for 15 minutes to warm and prevent condensation.
4. Wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
5. Load opened sample tubes into the rotor symmetrically for balance.
    - Leave open spaces between the tubes if possible.
6. Close the lid and press Start.
7. Allow the samples to dry.
    - Evaporation time varies and is dependent on factors including ambient temperature, humidity, and starting volume of the sample.
8. Samples may be dried to a specific volume or to complete dryness.
    - Concentration to a specific volume
        i. Periodically check the sample to ensure the desired volume.
        ii. If over-drying has occurred, TE or sterile dH_2O can be added to the sample to bring the volume back to the desired amount.
9. Press Stop to end the run. The lid will unlock once the rotor stops.
10. Remove the samples.
11. Wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
12. Add at least 10 µl sterile dH_2O to any completely dried samples to achieve the desired volume.
13. Incubate rehydrated samples at room temperature for a minimum of 15 minutes.

20. VACUUM CENTRIFUGE PRESERVATION OF DNA EXTRACTS

Scope

This procedure applies to preparing samples for transfer from the laboratory and is to be used in conjunction with the DNA Extract and Work Product Transfer/Return procedure.

Procedure

1. Record the approximate volume of DNA extract remaining prior to drying in the case file.
    - This step is not required for remaining cellular work product not subjected to DNA extraction (e.g. cell pellets/substrates).
2. Add 50 µL of TE to any water-eluted extracts.
    - Vortex briefly.
3. Ensure the correct program is selected for operation (e.g. V-AQ).
    - It is recommended that prior to first use of the day, the Vacufuge is run for 15 minutes to warm and prevent condensation.
4. Wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
5. Load opened sample tubes into the rotor symmetrically for balance.
    - Leave open spaces between the tubes if possible.
6. Close the lid and press Start.
7. Allow the samples to evaporate to dryness.
    - Evaporation time varies and is dependent on factors including ambient temperature, humidity, and starting volume of the sample.
8. Press Stop to end the run. The lid will unlock once the rotor stops.
9. Ensure samples are completely dry.
    - If liquid remains in a sample, return it to the Vacufuge and repeat the drying process to complete desiccation.
10. Remove the samples.
11. Wipe down the inside of the Vacufuge and the rotor using an appropriate detergent.
12. Place the preserved DNA extract/work product tubes and a desiccant packet into a heat-sealable foil pouch or plastic bag.
13. Heat seal the pouch. Samples are now ready for transfer/return.

21. RECOVERY OF DNA EXTRACTS

Scope

This procedure applies to the rehydration of previously preserved DNA extracts and associated reagent blank(s) for additional analysis. This procedure applies to extracts and blanks submitted to the laboratory as well as those retained within a laboratory.

Procedure

1. Add at least 10 µl sterile dH_2O to the dried sample to reach the desired volume.
   - If the sample was stored in water with DNAstable prior to drying, it can be rehydrated with TE buffer.
   - Consider the previously recorded remaining volume, whether re-quantitation is appropriate, the desired amplification target, and potential future testing when selecting the recovery volume.
2. Incubate rehydrated samples at room temperature for a minimum of 15 minutes.
3. Vortex briefly.
4. Samples are now ready for re-quantification or amplification, as appropriate.
   - Rehydrated samples should be stored in the refrigerator.
   - Avoid repeating the drying/rehydrating process more than 3 times.

22. QUANTIFICATION: STANDARDS PREPARATION POWERQUANT® SYSTEM

Scope

This procedure is used to manually prepare a dilution series from the male gDNA standard supplied with the PowerQuant® System. Once prepared, this dilution series is used to generate a 4-point standard curve for estimating DNA concentration of unknown samples.

Procedure

1. This procedure shall be performed in a biological safety hood or other designated PCR setup area.
2. Vortex the gDNA standard tube for 15 seconds and centrifuge briefly.
   - Ensure a new tube of gDNA standard is thawed in a refrigerator overnight before use. Once thawed, the gDNA standard shall be stored refrigerated.
3. Prepare the quantification standards using the gDNA standard and Dilution Buffer provided in the PowerQuant® System kit as noted in the table below.
   - Use a new pipette tip for each transfer in the series.
   - Vortex each dilution for 10 seconds then briefly centrifuge prior to each transfer in the series.
   - Store the prepared standard series refrigerated for up to one month.

Standard	Concentration (ng/µL)	Volume Used (µL)		Dilution Factor
		gDNA [source]	Dilution Buffer	
Std 1	50	50 [undiluted gDNA]	NA	NA
Std 2	2	4 [undiluted gDNA]	96	25x
Std 3	0.08	4 [Std 2]	96	25x
Std 4	0.0032	4 [Std 3]	96	25x
NTC	NA	NA	100	NA

Note: NTC has been included in the standard dilution series; however, it may be excluded from the set and amplification grade water provided with the PowerQuant® System may be loaded to the NTC wells.

23. QUANTIFICATION: SAMPLE SET-UP POWERQUANT® SYSTEM

Scope

This procedure is used to manually prepare Y-screening lysates, purified DNA extracts, and Y23-intended direct amplification lysates for quantification using the PowerQuant® System with a 20 µL reaction volume.

Procedure

1. This procedure shall be performed in a biological safety hood or other designated PCR setup area.
2. Centrifuge the Master Mix, Primer Pair Mix, and Amplification Grade Water briefly to bring liquid to the bottom of the tube.
   - Ensure new tubes of the Master Mix, Primer Pair Mix, and Amplification Grade Water are completely thawed prior to first use.
   - Once thawed, the reagents shall be stored refrigerated. For optimal performance, the reagents should be used within one week. For longer storage, the reagents may be re-frozen but more than two freeze-thaw cycles should be avoided.
3. Vortex the reagent tubes for 15 seconds.
   - Do not centrifuge the Master Mix or Primer Pair Mix after mixing. This may cause a concentration gradient.
4. Determine the total number of samples, controls, and standards to be quantified. Add additional reactions (typically 1–4) to this number for reaction mix preparation to compensate for loss during pipetting.
   - The gDNA dilution standards shall be run in duplicate.
5. Prepare the qPCR reaction mix using the determined number of reactions and the reagent volumes from the table below.

qPCR Reaction Mix Component	Volume Per Reaction (µL)
2X Master Mix	10
20X Primer Pair Mix	1
Amplification Grade Water	7
Total Per Reaction	18

6. Vortex the prepared reaction mix for 5–10 seconds.
   - Do not centrifuge after mixing.
7. Aliquot 18 µL of prepared reaction mix into each well of the optical reaction plate that will be used.
8. Add 2 µL of gDNA dilution standard, sample, or control to the appropriate wells in the plate.
9. Seal the plate with an optical adhesive cover.
   - Ensure that all wells are adequately sealed to prevent evaporation during thermal cycling.
   - Avoid touching the bottom of the optical reaction plate.
10. Proceed to Quantification: PowerQuant® System.
    - Protect the plate from extended light exposure prior to thermal cycling.
    - Artifacts may occur if the time between qPCR setup and the start of thermal cycling exceeds 2 hours.

24. QUANTIFICATION: POWERQUANT™ SYSTEM

Scope

This procedure is used to perform qPCR on a 7500 Real-Time PCR System using the PowerQuant® System.

Procedure

1. Briefly centrifuge the prepared optical reaction plate.
2. Place the reaction plate on a 7500 Real-Time PCR System.
   - Ensure plate orientation is correct with well A1 in the upper left corner.
3. Select “Template” from the 7500 software landing page and navigate to the appropriate template. Confirm the thermal cycling protocol detailed in the table below.
   - The template can also be accessed through the File menu by selecting “New Experiment” and “From Template…”.

Thermal Cycling Protocol		
Hot Start	98 °C	2 min
39 cycles	98 °C	15 sec
	62 °C	35 sec

4. Import the appropriate plate setup file and verify the correct data was imported.
   - Any unused wells on the reaction plate may be assigned targets (Auto, Degradation, IPC, and Y). This ensures all well data is captured if the plate is loaded incorrectly.
5. Ensure “PQ_CXR” is selected as the passive reference.
6. Save the file to an appropriate location.
7. Start the run.
8. Remove the reaction plate from the 7500 Real-Time PCR System after quantification is complete.
9. Proceed to QUANTIFICATION: POWERQUANT™ SYSTEM DATA ANALYSIS

25. QUANTIFICATION: POWERQUANT™ SYSTEM DATA ANALYSIS

Scope

This procedure is used to analyze and export data generated by the 7500 Real-Time PCR System and evaluate it using the PowerQuant® Analysis Tool. The PowerQuant® Analysis Tool is a macro-enabled Excel workbook that is suitable for use with Results data exported as an .xls file from the 7500 Software.

The exported Results data include the following which are calculated by the PowerQuant® Analysis Tool:

- Standard curve slope, R^2, intercept, and efficiency values
- Auto/Y and Auto/D ratios
- Sample IPC shift

Procedure

1. Open the quantification results file using the 7500 Software.
2. For any unused wells on the plate:
    a. Select Setup from the Experiment Menu to the left.
    b. Select Assign Targets and Samples tab in the Experiment window.
    c. A warning [custom dye "PQ_CXR" has been deleted] message will appear. Select OK.
    d. Deselect all targets (Autosomal, Degradation, IPC, and Y) for all unused wells on the plate.
3. Select all wells to be analyzed then click the Analyze button on the top right in the Analysis view.
4. Select Standard Curve from the Analysis Experiment Menu to evaluate the standard curves based on the optimal ranges below:

Slope (Autosomal, degradation and Y curves):	-3.6 to -3.1
R^2:	>0.990

5. Consider one of the following adjustments for slope and/or R^2 results outside expected ranges. The standard curve must contain at least one data point for each standard.
    - Omit one outlier point (the autosomal, degradation and Y targets) from one standard curve set.
    - Omit one entire standard curve set (e.g. STDs 1, 2, 3, and 4 or STDs 1A, 2A, 3A, and 4A).
    - Re-analyze the data to incorporate any standard curve omissions.
    - Any edits to the standard curve shall be documented in the casefile.
6. Verify the following in cases where the slope and/or R^2 value cannot be brought within the expected range:
    - Standard dilution series preparation and load
    - Entered standard dilution series concentrations
    - Correct passive reference selection
    - Correct 7500 software analysis settings
    - No signs of evaporation during qPCR

    If the slope and/or R^2 value cannot be brought within the expected range, the data may be useable for limited conclusions. Consider consultation with a technical lead.
7. Ensure that all wells with data are highlighted in the plate map and export as an .xls file to an appropriate location.
    - Ensure "Results" is the selected data type in the Export Properties tab of the Export Data dialog box.
    - Select "One File" for export.
8. Open the PowerQuant® Analysis Tool and ensure that macros are enabled.

9. Select the “Import Quant” button and navigate to the appropriate .xls results file exported from the 7500 Software.
10. Enter a default volume for remaining extract in the Worksheet Settings box, if desired. Alternatively, a volume may be entered for individual samples.
11. Show or hide sample rows as desired.
12. Evaluate quantification results as described in QUANTIFICATION: POWERQUANT™ SYSTEM DATA INTERPRETATION

> - Quantification values outside of acceptable ranges defined in this manual are flagged in the PowerQuant® Analysis Tool to indicate samples that may need additional attention. Flags are also displayed to indicate any omitted standards or NTCs showing possible presence of DNA.

26. QUANTIFICATION: POWERQUANT™ SYSTEM DATA INTERPRETATION

Scope

This procedure is used to evaluate PowerQuant® System results generated by the 7500 Real-Time PCR System. It is used for assessing the quantity and quality of total human and human male DNA present in purified DNA extracts. It is also used for assessing amounts of total human and human male DNA in Y-screening lysates processed with the Casework Direct kit and direct amplification lysates.

Procedure

1. Evaluate all no DNA template controls (e.g. reagent blank, NTC) for signal. Signal equivalent to >1.0pg per 2µL input volume may indicate the presence of DNA.
   - In samples without a relatively smooth baseline, the automatic baselining algorithm in the 7500 software may occasionally result in signal not reflective of true amplification. To evaluate amplification curve:

   a. In the 7500 software, set the Graph Type in the Amplification Plot to Linear.
   b. Ensure the Plot Type is set to ΔRn vs Cycle.
   c. Under Options, select the Autosomal Target and ensure the Threshold is set to 0.2 (Auto not checked), and that Auto Baseline is selected.
   d. Select Show: Threshold.
   e. Select Show: Baseline (start and end).
   f. Examine the amplification curve for the Baseline Start (designated by a green square) and Baseline End (designated by a red square). A low Baseline End value and/or a difference of only a few cycles between Baseline Start and End along with a linear amplification curve indicate a possible baseline issue.
   g. Document this assessment in case file notes for any affected samples.

   - Consult the DNA QA manual for troubleshooting steps when contamination is suspected.
2. Evaluate the total human autosomal DNA and human Y concentration results for DNA samples.
   - Samples shall be reviewed to ensure the results are reasonable in the context of the sample being tested (e.g. robust quantity obtained from an apparent blood stain).
   - Purified DNA extracts and direct amplification lysates should be re-quantified if no DNA is detected for both the autosomal and Y targets.
   - If needed, evaluate the baseline and amplification curve as described in step 1.
3. Assess the [Auto]/[Y] results for DNA samples, as applicable.
   - A higher [Auto]/[Y] indicates less male DNA relative to total human DNA.
   - The ratio of total human autosomal DNA concentration to Y DNA concentration is not intended to identify minor female contribution in a male sample.

[Auto]/[Y] Result	Interpretation
No value	Male DNA not detected
<2.0	Male DNA detected. Female DNA may/may not be present
>2.0	A mix of male and female DNA is indicated

4. Assess DNA samples for inhibition and degradation using the following guidelines.
   - No detectable amplification for the autosomal, Y, and degradation targets with an IPC shift <0.3, in combination with an IPC curve that crosses the amplification threshold indicates that insufficient DNA template was added to the reaction.

	IPC Shift <0.3	IPC Shift >0.3 or Undetermined

[Auto]/[Deg] <2.0	No inhibition detected DNA not likely degraded	Inhibitor likely present DNA not likely degraded
[Auto]/[Deg] >2.0	No inhibition detected DNA likely degraded	Inhibitor likely present DNA possibly degraded
Undetermined [Auto]/[Deg]	No inhibition detected Likely severe DNA degradation	Inhibitor likely present DNA may be degraded

- Consider re-quantification using diluted sample, or sample clean-up when an IPC result indicates significant inhibition.
- Consider increasing the template target amount for amplification when:
  - Using Fusion 6C on a sample with [Auto]/[D] > 5
  - Using Y23 on a sample with [Auto]/[D] > 10

5. Use the following guidelines for further assessment of the relative amount of male DNA in purified DNA extracts, as applicable.
   - Where limited male DNA is present, Y-STR analysis in addition to, or in lieu of, autosomal STR testing may be most appropriate.

[Auto]/[Y] Result	**Testing Approach**
≤10.0	Autosomal STR testing is appropriate
10 to 50 **substantial male DNA present**	Autosomal* STR and/or Y-STR testing is appropriate
10 to 50 **limited male DNA present**	Y-STR testing is appropriate
≥50	Y-STR testing is appropriate

*Assessing the number of male contributors at the Y-STR loci included in the PowerPlex® Fusion 6C System may help determine if Y-STR testing is appropriate for a particular sample.

6. Evaluate Y-screen lysate results and consider the following guidelines when determining case approach.
   - Where additional analysis is not routinely recommended based on [Y] and [Auto]/[Y] results, further testing may be warranted based on case and sample information.
   - Serology testing results in combination with quantification results, relevant scenario information, and evidence collection timelines should be used to help determine the appropriate extraction method (differential vs non-differential).

[Y]	[Auto]/[Y]	Testing Approach
Not detected	No value	Additional analysis not routinely recommended.
<0.001 ng/µL	Any	Additional analysis not routinely recommended. Samples below this threshold have demonstrated low typing success rates regardless of extraction method.
≥0.001 ng/µL	≤60	Further testing is warranted.
	>60 Sample would undergo differential extraction	Further testing is warranted.
	>60 Sample would undergo non-differential extraction	Additional analysis not routinely recommended. Samples below this threshold have demonstrated low typing success rates when non-differentially extracted.

7. Determine which extract(s), if any, to amplify based on results as outlined in steps 1–5 and in conjunction with case approach considerations.
    - The reasons for stopping analysis shall be documented in the casefile notes (e.g., similar probative value, auto/Y too high).
    - Autosomal amplification with Fusion 6C requires a minimum autosomal template target of 100 pg.
    - Consider additional casework approaches to increase the amount of target DNA when low DNA quantities are obtained.
8. For purified DNA extracts and direct amplification lysates, proceed to the appropriate amplification procedure.
    - Store the extracts and lysates refrigerated or frozen. For long-term storage, freeze samples. Avoid repeated freeze-thaw cycles.

27. AMPLIFICATION: SAMPLE SET-UP POWERPLEX® FUSION 6C

Scope

This procedure is used to prepare samples manually for amplification using the PowerPlex Fusion 6C System with a 25 µL reaction volume. It is suitable for purified DNA extracts for which the autosomal template target meets the minimum 100 pg and maximum 5.0 ng requirement. The standard template target is approximately 1 ng.

Procedure

1. Based on quantification results, prepare DNA extracts and reagent blanks to appropriate concentrations as needed.
   - DNA extracts may require concentration or dilution with TE/water.
   - Ensure reagent blanks are amplified on the same instrument model as the corresponding samples.
2. This procedure shall be performed in a biological safety hood or other designated PCR setup area.
3. Centrifuge the Master Mix and Primer Pair Mix briefly to bring liquid to the bottom of the tube.
   - Ensure new tubes of the Master Mix, Primer Pair Mix, and Amplification Grade Water are completely thawed prior to first use. Once thawed, the reagents shall be stored refrigerated.
4. Vortex the reagent tubes for 15 seconds.
   - Do not centrifuge the Master Mix or Primer Pair Mix after vortexing. This may cause a concentration gradient.
5. Determine the total number of samples and controls to be amplified. Add 1–3 additional reactions to this number for reaction mix preparation to compensate for loss during pipetting.
6. Prepare a PCR reaction mix using the determined number of reactions and the reagent volumes from the table below.
   - The inclusion of amp grade water to the reaction mix is dependent on whether template DNA volume will total 15 µl or 10 µl.

	15 µL Template DNA	10 µL Template DNA
PCR Reaction Mix Component	Volume Per Reaction (µL)	
5X Master Mix	5	5
5X Primer Pair Mix	5	5
Amp Grade Water	0	5
Total Per Reaction	10	15

7. Vortex the prepared reaction mix for 5–10 seconds.
8. Aliquot the appropriate total volume of prepared reaction mix (per the above table) into each amplification tube or well.
9. Add the necessary volume of sample or reagent blank to the appropriately labeled tube or well.
   - Steps 9 and 10 may be performed in either order.
10. Add amp grade water or TE to bring the total volume of the reaction to 25 µl (as needed).
11. Repeat steps 8 and 9 for each sample and reagent blank.
    - Questioned and reference samples may be amplified in the same batch provided that reference samples are not brought to the set-up area until all questioned samples have been added to their respective reaction tubes, and tube lids have been firmly sealed.
12. Add diluted 2800M DNA standard to the labeled positive control tube or well.
    - Dilute the Fusion 6C 2800M DNA standard to target 1.0 ng of template DNA.

- The final concentration or template target shall be documented on the amplification worksheet.

13. Add amp grade water or TE to bring the total volume of the positive control to 25 µl (as needed).
14. Add amp grade water or TE to the labeled negative control tube or well to bring the total reaction volume to 25 µl.

28. AMPLIFICATION: POWERPLEX® FUSION 6C

Scope

This procedure is used to perform PCR on a GeneAmp™ PCR System 9700 or ProFlex™ PCR System thermal cycler. It is suitable for purified extracts prepared using the PowerPlex® Fusion 6C system with a 25 µL reaction volume.

Procedure

1. As needed, briefly centrifuge amplification tubes or 96-well plate to bring contents to the bottom of tubes or wells.
2. Place amplification tubes or 96-well plate on a thermal cycler.
3. Select the appropriate program that reflects the 25 µL reaction volume and the thermal cycling protocol detailed in the table below.
    - Ensure that *Max Mode* is selected for ramp speed if using a GeneAmp™ PCR System 9700.
    - Ensure the *GeneAmp PCR System 9700 Simulation Mode* is selected if using a ProFlex™.

Thermal Cycling Protocol		
Hot Start	96 °C	1 min
29 Cycles	96 °C	5 sec
	60 °C	1 min
Incubation	60 °C	10 min
Hold	4 °C	Indefinite

4. Remove samples from the thermal cycler after amplification is complete.
5. Proceed to Amplification Product Preparation: Fusion 6C, or store samples in a refrigerator or freezer.
    - Long-term storage of amplicons at ≥4 °C may result in artifacts (e.g. n+1).

29. DIRECT AMPLIFICATION SET-UP: POWERPLEX® FUSION 6C

Scope

This procedure is used to lyse and prepare reference samples for amplification without the need for purification and quantitation. This procedure prepares samples for amplification using the PowerPlex Fusion 6C System with a 12.5 µL reaction volume. It is suitable for blood or buccal known reference samples that have been collected onto FTA paper, filter paper, fabric, or swabs.

Lysates generated from this procedure may be used with the Y23 Amplification method following quantitation.

Procedure

1. Place the reference sample in a microcentrifuge tube.
    - One quarter of one swab or at least 3x3 mm of a stained substrate are typical sample sizes.
2. Add sufficient SwabSolution™ to cover the sample (100–1,000 µL). A reagent blank shall be initiated for each batch.
    - Ensure reagent blank is amplified on the same instrument model as the corresponding samples.
3. Incubate at 70 °C for at least 30 minutes.
    - Lysates can be stored for up to 24 months at ~4 °C.
4. Vortex and briefly centrifuge the sample and reagent blank tubes.
5. The remaining procedure steps shall be performed in a biological hood or other designated PCR setup area.
6. Centrifuge the Fusion 6C Master Mix and Primer Pair Mix briefly to bring liquid to the bottom of the tube.
    - Ensure new tubes of the Master Mix, Primer Pair Mix, and Amplification Grade Water are completely thawed prior to first use. Once thawed, the reagents shall be stored refrigerated.
7. Vortex the reagent tubes for 15 seconds.
    - Do not centrifuge the Master Mix or Primer Pair Mix after vortexing. This may cause a concentration gradient.
8. Determine the total number of samples and controls to be amplified. Add 1–3 additional reactions to this number for reaction mix preparation to compensate for loss during pipetting.
9. Prepare a PCR reaction mix using the determined number of needed reactions and the reagent volumes from the table below.

PCR Reaction Mix Component	Volume Per Reaction (µL)
Amplification Grade Water	5.5
5X Master Mix	2.5
5X Primer Pair Mix	2.5
Total Per Reaction	10.5

10. Vortex the prepared reaction mix for 5–10 seconds.
11. Aliquot 10.5 µL of prepared reaction mix into each amplification tube.
12. Add 2 µL of sample or reagent blank to each appropriately labeled tube.
13. Add diluted 2800M DNA standard to the labeled positive control tube.
    - Dilute the Fusion 6C 2800M DNA standard to target 2.0–10 ng template DNA.

- The final concentration or template target shall be documented on the direct amplification worksheet.

14. Add 2 µL of amp grade water to the labeled negative control tube.
15. Pipette mix or briefly vortex all prepared tubes.
16. Proceed to the Direct Amplification: PowerPlex® Fusion 6C method.

30. DIRECT AMPLIFICATION: POWERPLEX® FUSION 6C

Scope

This procedure is used to perform PCR on a GeneAmp™ PCR System 9700 or ProFlex™ PCR System thermal cycler. It is suitable for direct amplification lysates prepared using the PowerPlex® Fusion 6C system with a 12.5 µL reaction volume.

Procedure

1. As needed, briefly centrifuge amplification tubes or 96-well plate to bring contents to the bottom of tubes or wells.
2. Place the amplification tubes or 96-well plate on a thermal cycler.
3. Select the appropriate program that reflects a 12 µL reaction volume and the thermal cycling protocol detailed in the table below.
    - Ensure that *Max Mode* is selected for ramp speed if using a GeneAmp™ PCR System 9700.
    - Ensure the *GeneAmp PCR System 9700 Simulation Mode* is selected if using a ProFlex™.

Thermal Cycling Protocol		
Hot Start	96 °C	1 min
26 Cycles	96 °C	5 sec
	60 °C	1 min
Incubation	60 °C	10 min
Hold	4 °C	Indefinite

4. Remove samples from the thermal cycler once amplification is complete.
5. Proceed with Amplification Product Preparation: Fusion 6C, or store samples in a refrigerator or freezer.
    - Long-term storage of amplicons at ≥4 °C may result in artifacts (e.g. increased n+1)

31. AMPLIFICATION PRODUCT PREPARATION: FUSION 6C

Formamide is a teratogen – wear gloves

1. Prepare a master mix of Hi-Di™ formamide and WEN ILS 500.
    a. (# of samples + 1) x 9.5 µL formamide
    b. (# of samples + 1) x 0.5–1.0 µL WEN ILS 500

    If an injection will include wells with no samples, add formamide (or formamide+WEN mixture) to the blank wells.

    Note: The volume of internal lane standard used in the loading master mix can be adjusted to change the intensity of the size standard peaks based on laboratory preferences. If increasingly greater volume of internal lane standard is necessary over a period of time, this may indicate a decrease in CE sensitivity and should be evaluated before performing additional casework analysis on the instrument.

2. Vortex and spin briefly. If making a stock tube of mixed formamide and size standard, label the tube with the lot number of WEN added along with the date and analyst's initials.

3. Dispense 10µl of formamide+WEN mix into the appropriate wells of the CE plate.

4. Add 1µl allelic ladder or amplification product to the appropriate wells, being careful to not introduce bubbles.

5. Cover the plate with a septa cover.

6. Spin briefly to remove any bubbles.

7. Denature samples at 95°C for 3 minutes.

8. Chill the samples for 3 minutes (use a freezer plate block, crushed ice, or ice-water bath).

9. Place plate in a plate base and cover with a plate retainer, being mindful of plate alignment.

An injection time of 15 seconds shall be used for forensic unknown samples that will be deconvoluted in STRmix™. For amplicons with excessive peak heights (e.g. causing -A shoulder artifacts or saturation):

- A smaller amplicon volume between 0.5µl and 1µl can be loaded.
- A maximum dilution of 1:10 in either formamide or TE buffer may be used.
- Reduced injection times may be used for samples that will not be analyzed in STRmix™ v2.8.

Use of any of these methods shall be documented in the case file.

32. AUTOMATED SAMPLE PROCESSING USING THE HAMILTON AUTOLYS

Introduction

The Hamilton Microlab AutoLys STAR workstation along with the Hamilton Microlab STAR Venus Software and AutoLys Software is used by the Washington State Patrol Crime Laboratory Division laboratories to set up Y-screening, quantification, and amplification reactions. The AutoLys will also perform lysis, extraction and purification of samples, followed by normalization or samples for amplification. The AutoLys workstation utilizes Hamilton AutoLys tubes and FlipTubes (A) for a fully automated, hands-free process. AutoLys tubes come in two options, filterless for differential lysis of samples potentially containing sperm cells, and standard configuration with filter for all other applications.

When using the AutoLys, please refer to the protocol in use for the proper AutoLys deck setup.

Report files are generated and saved on the AutoLys computer following each run on the robot. The report files will be routinely archived following the laboratory's data archival procedure (e.g. quarterly backup).

Y-Screen Setup

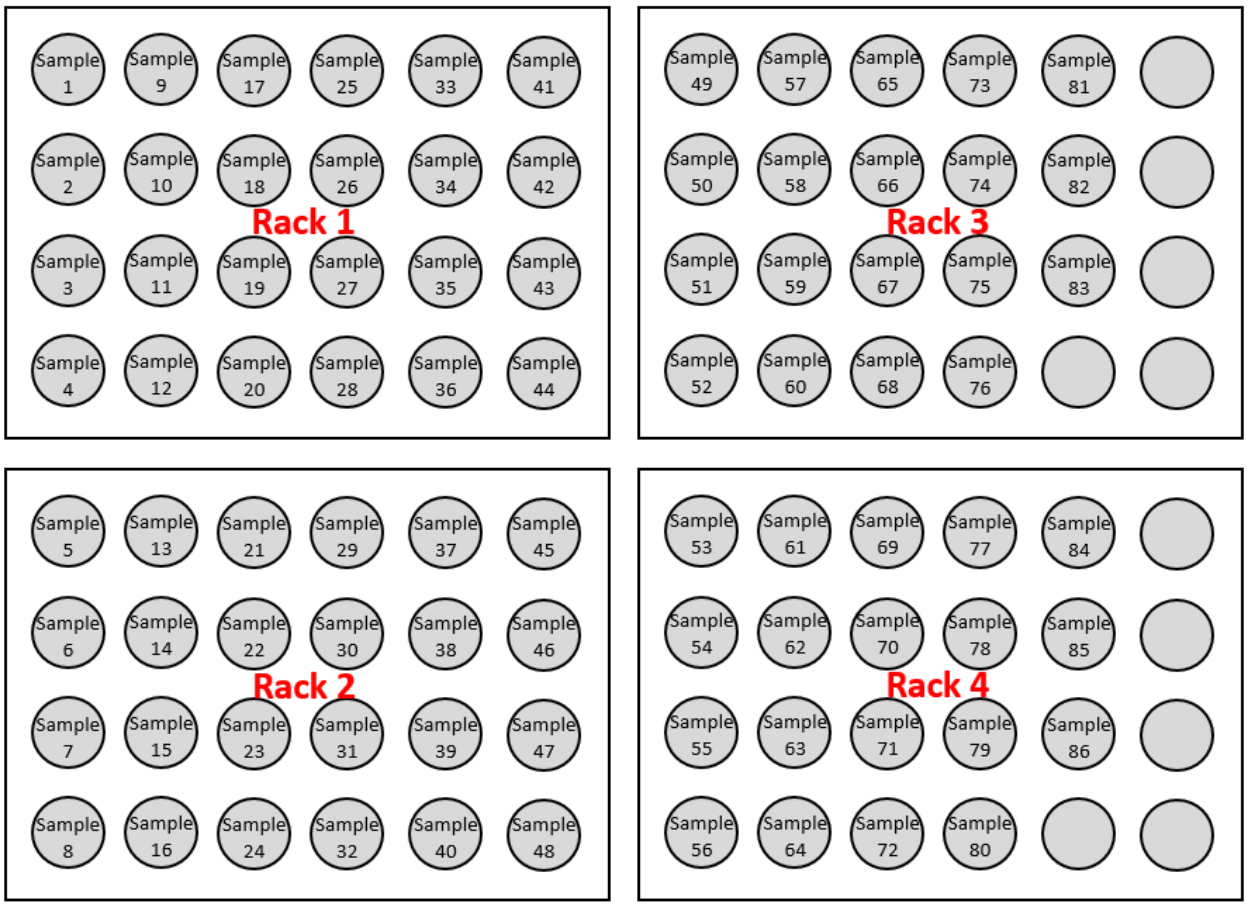
1. Prepare the samples for Y-screening by placing 1/8 to 1/4 of the swab material into a standard AutoLys tube (with filter). Ensure that the swab material is in the bottom of the AutoLys tube.

   Note: Do not place labels on the AutoLys tubes as these labels will cause the tubes to stick in the AutoLys heat blocks. Labeling of tubes should be done directly on the tube with ink or other suitable alternative.

2. Place the AutoLys tubes, including at least one reagent blank, into AutoLys sample racks and ensure the racks are balanced. The AutoLys Y-Screening program is capable of processing a maximum of 86 samples, the remaining 10 sample rack positions are reserved for quantification standards and controls in the downstream quantification plate set-up. AutoLys sample rack configuration is shown below in Figure 1. Samples are added from top to bottom starting on the left then moving right. If the last column of samples contains an even number fewer than 8 (i.e. 2,4, or 6) then the samples must be placed in the upper and lower racks to mirror each other so that the racks are balanced for centrifugation purposes. If the last column contains an odd number of samples, then an AutoLys tube with 400 µL water must be used as a balance. This balance AutoLys tube, if needed, does NOT count towards the total number of samples being tested when prompted to enter this value.

3. Place the sample racks in their designated deck location on the AutoLys. See Figure 2.

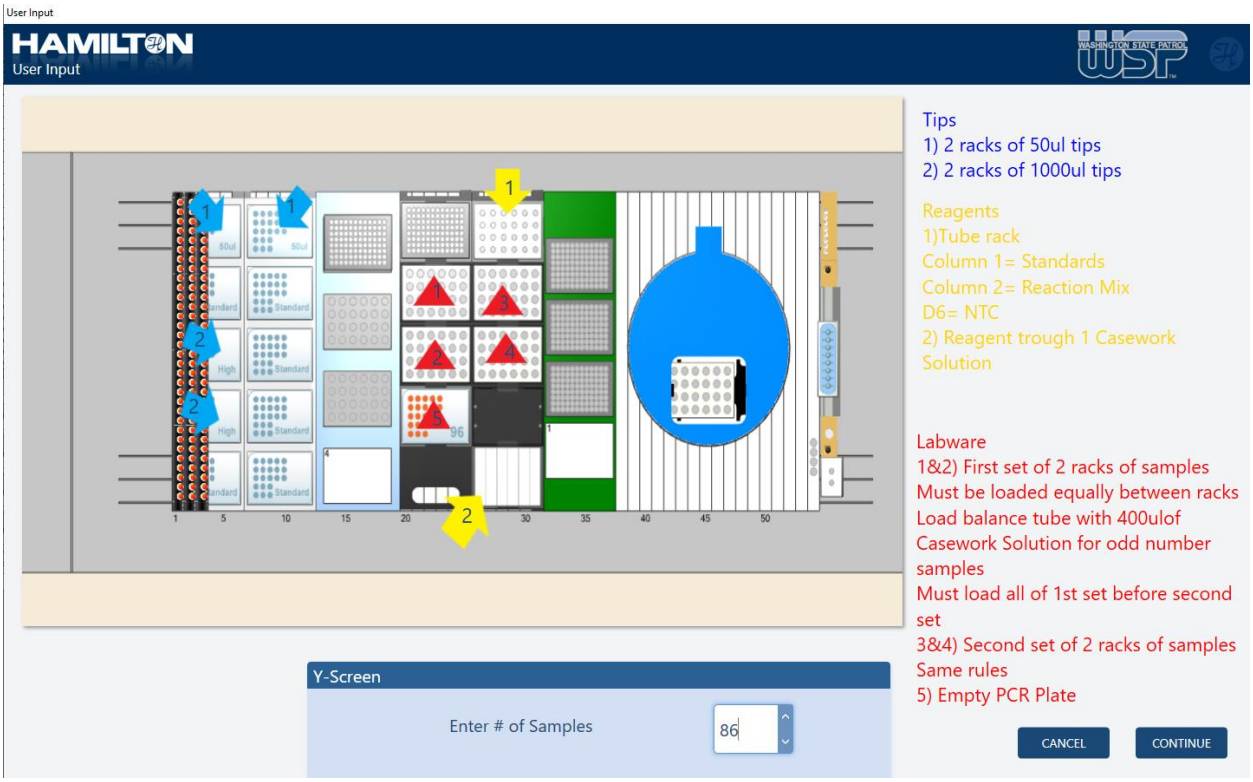
Figure 1. Sample placement example in AutoLys racks.



4. Prepare Casework Direct Solution for N+6 samples and quantification reaction mix for N+12 samples, to allow for the necessary dead volumes required by the robot. Refer to Y-SCREENING FOR SEXUAL ASSAULT EVIDENCE.

5. On the AutoLys computer select the Hamilton Method Manager icon. Select the Y-Screening method from the screen menu and follow the instrument prompt instructions, also described below.

Figure 2. AutoLys deck layout for automated Y-Screening method.

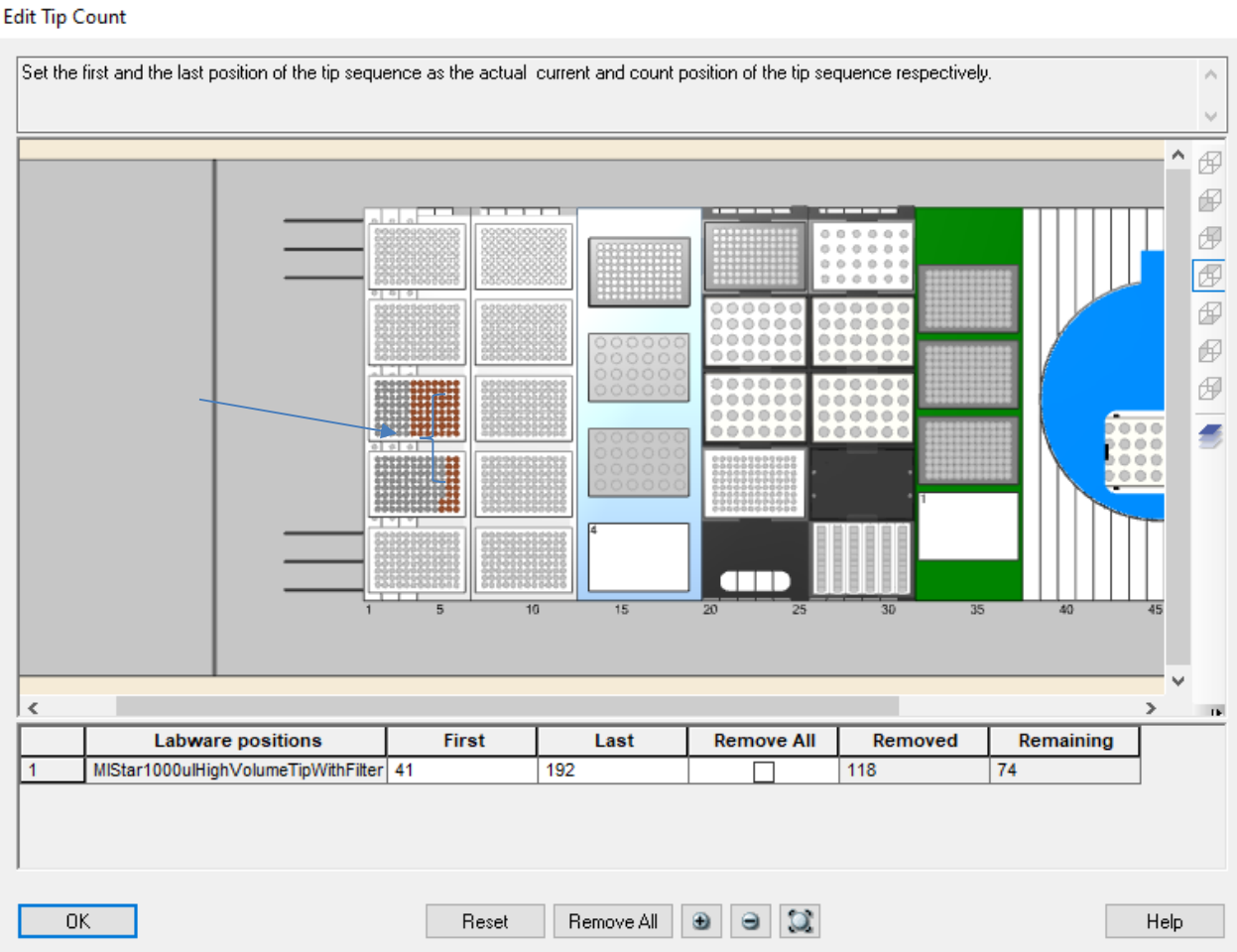


6. Transfer the Casework Direct Solution to the appropriate trough and place the trough in the dedicated location on the AutoLys, see Figure 2. Ensure that any bubbles are removed prior to starting the run.

7. Input the number of samples to be tested, up to a maximum of 86. (Standards, NTCs, and balance AutoLys tubes do NOT count towards the total sample count).

8. When prompted, verify sufficient number and correct location of 1000µL pipette tips between the software display and physical locations in positions 3 and 4 of Carrier 1, see Figure 3. Ensure the barcodes of the pipette trays are facing towards the laser scanner. (Minimum number of 1000µL tips needed = number of samples).

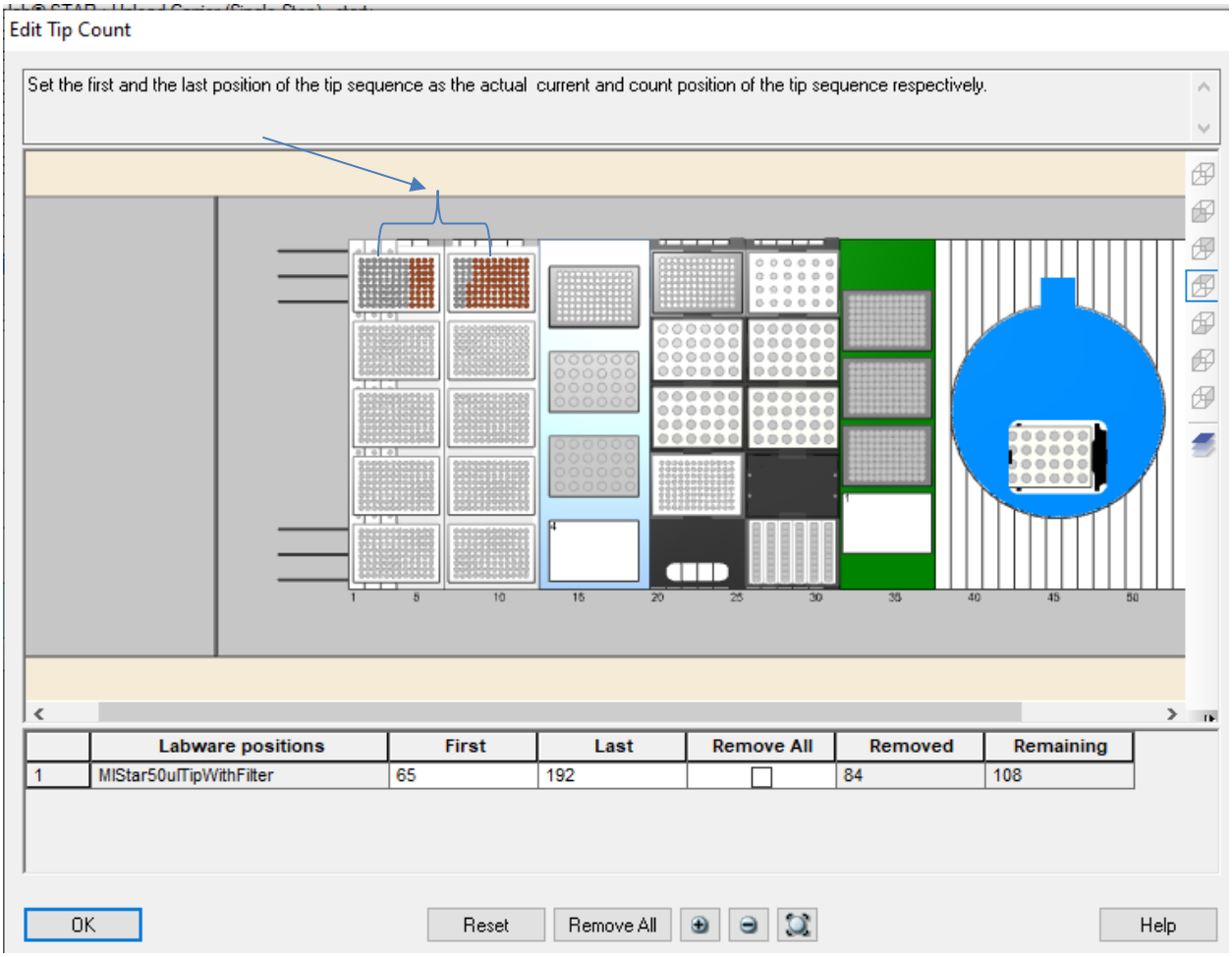
Figure 3. Display location for 1000µL pipette tips.



9. When prompted, verify sufficient number and correct location of 50µL pipette tips between the software display and physical location in position 1 of Carrier 1 and position 1 of Carrier 2, see Figure 4. Ensure the barcodes of the pipette trays are facing towards the laser scanner. (Minimum number of 50µL tips needed = number of samples + 17).

   Note: After the 50 µL tips are verified, the method starts processing samples. All sample tubes, tips, and Casework Direct reagents need to be in place prior to finishing this verification.

Figure 4. Display location for 50µL pipette tips.



10. When the incubation steps have completed, the AutoLys will pause and prompt the analyst to place fresh quantification reagents, NTCs, and the 96-well PCR plate on the deck, see Figure 5. Prepare quantification reaction mix for N+12 samples, to allow for the necessary dead volume required by the robot. Refer to DNA QUANTIFICATION: POWERQUANT™ SYSTEM REACTION PREPARATION.

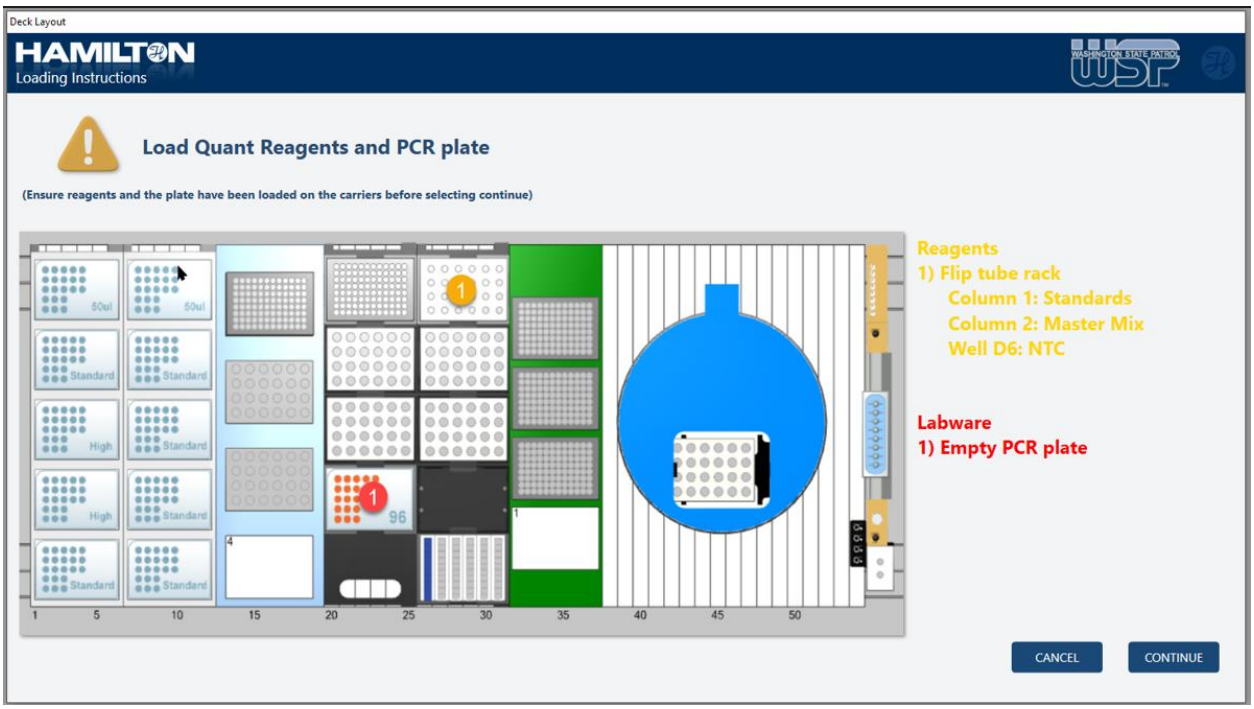
11. Transfer the quantification reaction mix in equal volumes between four FlipTubes (A) and place the tubes in their dedicated location on the AutoLys, see Figure 5. Ensure that any bubbles are removed prior to starting the run.

12. Prepare quantification standards and NTC in FlipTubes and place the tubes in their dedicated location on the AutoLys, see Figure 5. Ensure that any bubbles are removed prior to resuming the run.

13. Place a 96-well optical PCR plate in the dedicated location on the AutoLys, see Figure 5.

14. Ensure the quantification reagents, NTCs, and 96-well PCR plate are placed on the AutoLys, then click “Continue”.

Figure 5. Pause screen and display location for quantification reagents, NTCs, and 96-well PCR plate.



15. When the method has completed, seal the 96-well PCR plate with an optical adhesive cover, and briefly centrifuge the plate if any bubbles are present. The plate is now ready to proceed to DNA quantification.
16.

Note: The Y-screening program places the PowerQuant standards in wells A01-H01 and the NTCs in wells G12-H12. Samples are placed in wells A02-F12.

Quantification Using PowerQuant

1. Open the Hamilton Method Manager on the AutoLys computer and select the lysis and purification method > Start (green arrow)
2. Follow the prompts as described below
    a. Select to have the method begin and end with QPCR
    b. Enter the number of samples to be quantified, not including standards or NTCs, with a maximum of 86 samples

Figure 1. AutoLys lysis and purification sample screen.

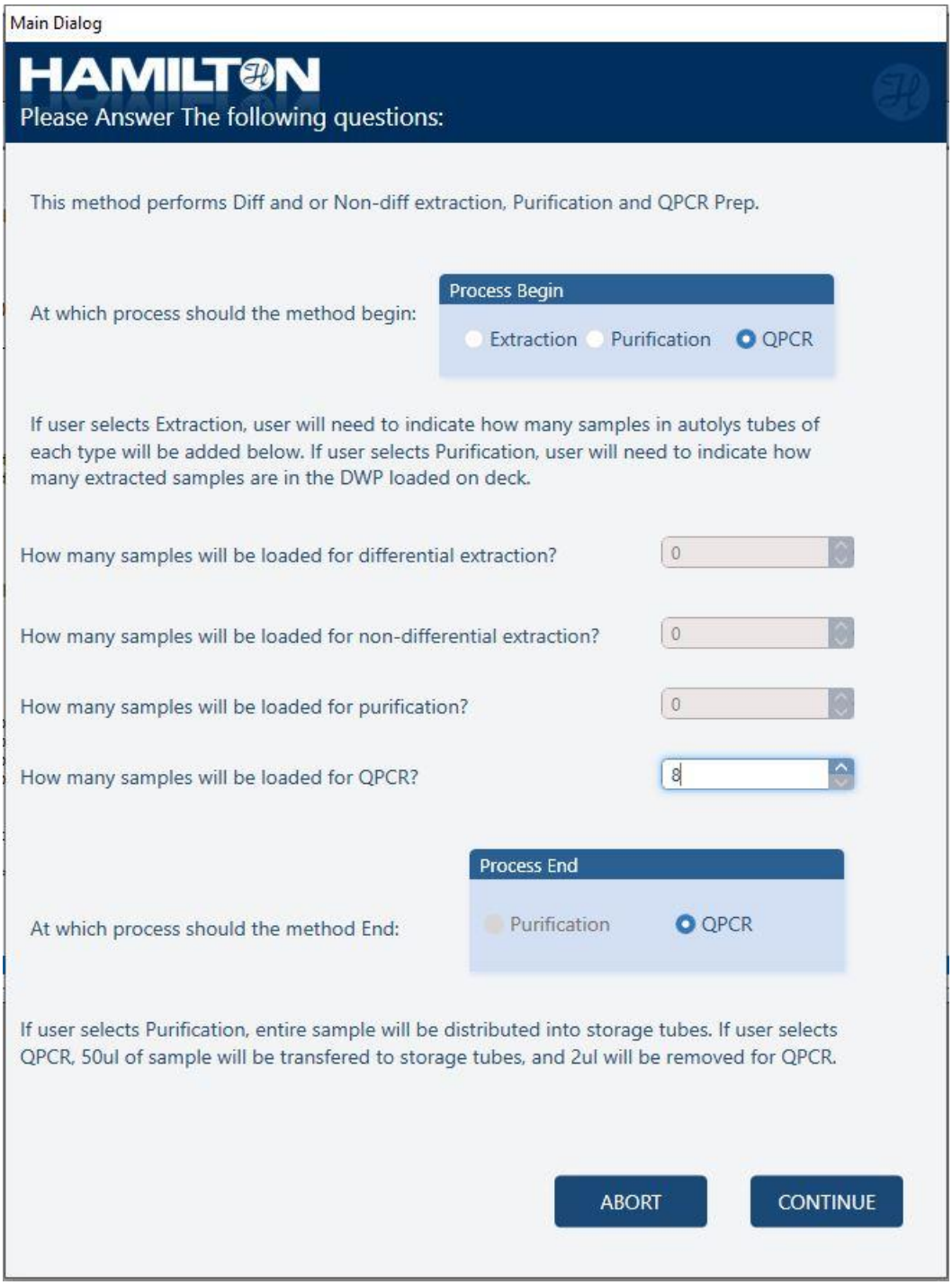
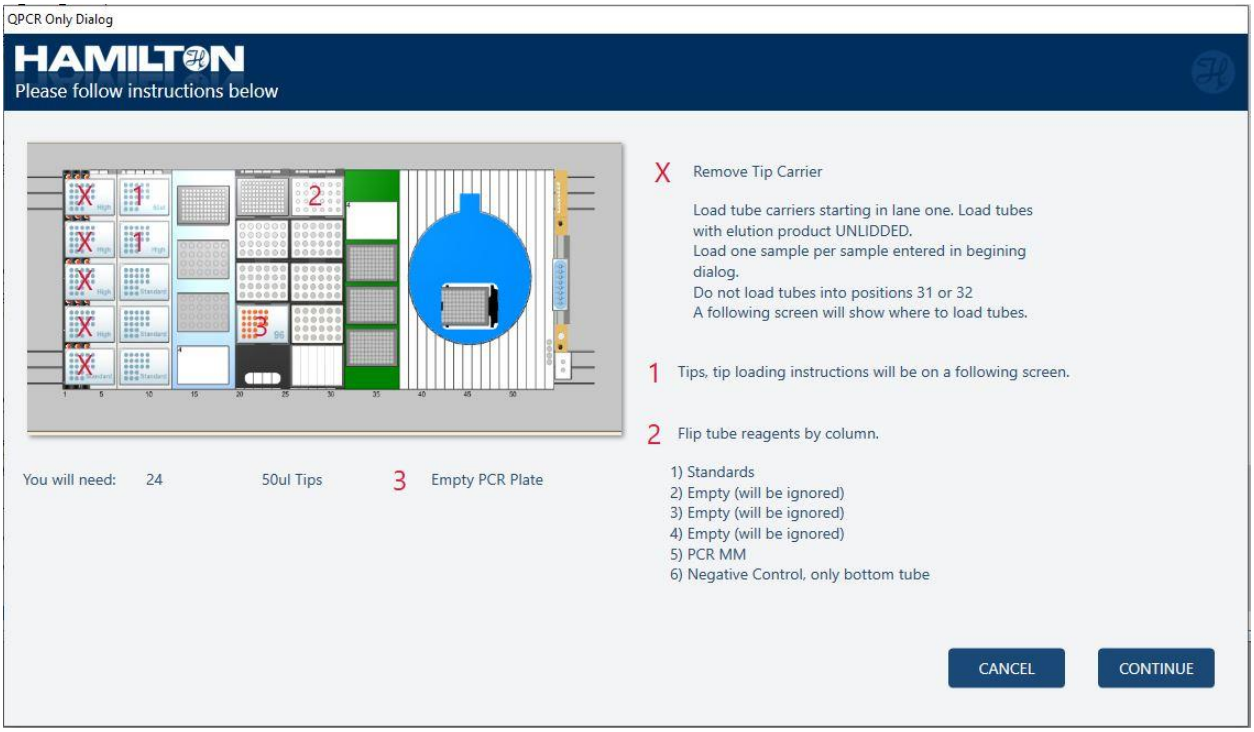


Figure 2. AutoLys deck layout for automated quantification set-up.



3. Prepare the quantification reaction mix (see DNA Quantification: PowerQuant System Reaction Preparation)

4. Aliquot equal volumes of the quantification reaction mix to four FlipTubes and place the tubes in their dedicated location on the AutoLys (see Figure 2). Remove any bubbles prior to starting the run.

5. Prepare quantification standards and NTC in FlipTubes and place the tubes in their dedicated location on the AutoLys (see Figure 2). Remove any bubbles prior to starting the run.

6. Place a 96-well optical PCR plate in the dedicated location on the AutoLys (see Figure 2).

7. Place the purified DNA extract samples in skirted sample elution tubes (with cap removed) into one or more AutoLys tube carriers. Load the carriers on the AutoLys in their dedicated location (see Figures 2 and 4).

8. Note the number of 50 µL tips required for the quantification process. This information is presented on the deck set-up screen.

9. When prompted, verify sufficient number and correct location of 50 µL pipette tips between the software display and physical location (see Figure 3). Ensure the barcodes of the pipette trays face toward the laser scanner.

Note: After the sample tube locations are verified the method starts processing samples. All sample tubes, reagents, tips, and deep well plates need to be in place prior to finishing this verification.Figure 3. Display location for 50 µL pipette tips.

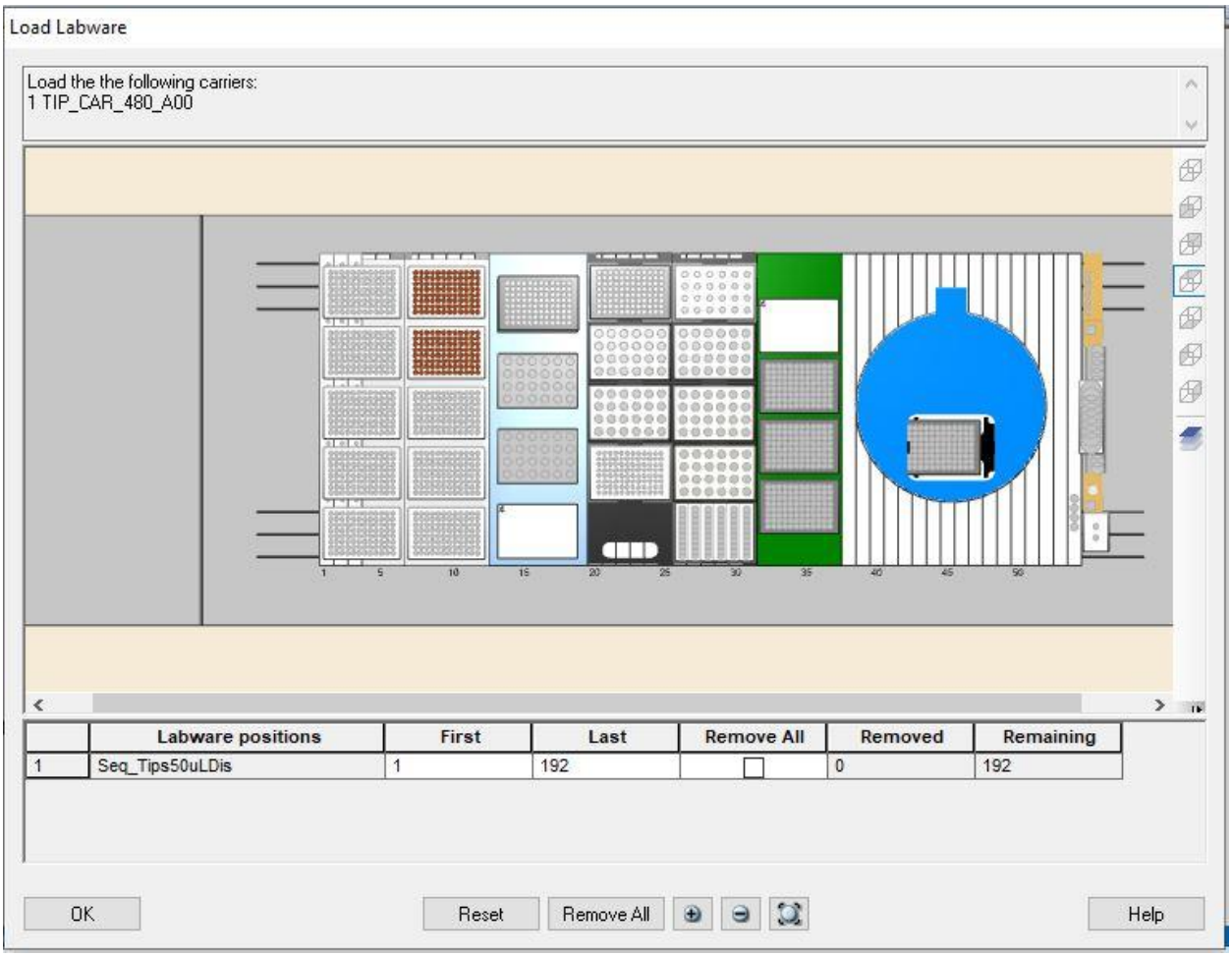
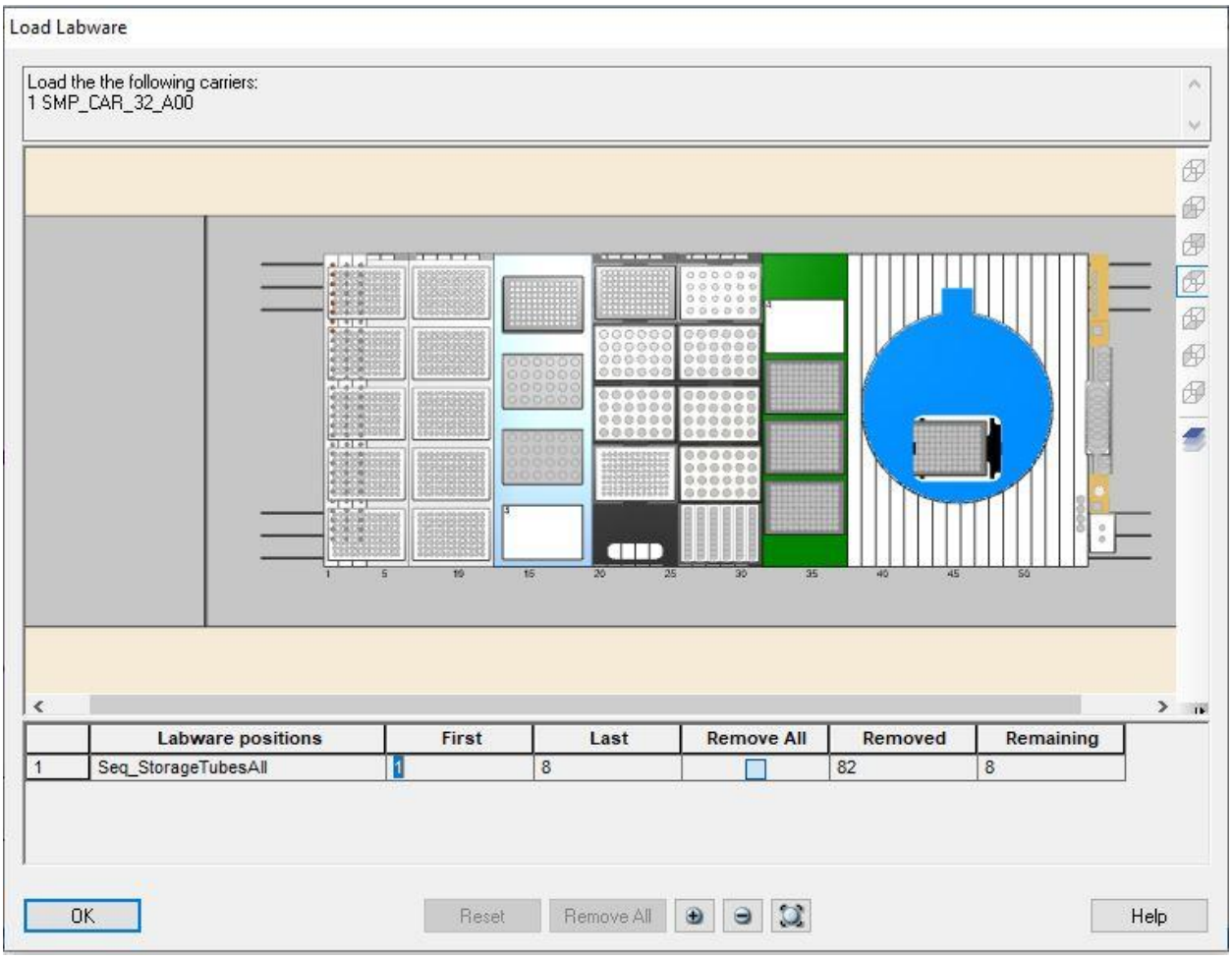


Figure 4. Display location for sample elution tubes.



10. When the method has completed, seal the 96-well PCR plate with an optical adhesive cover, and briefly centrifuge the plate if any bubbles are present. The plate is now ready to proceed to DNA quantification. The sample tubes should be closed and stored in appropriate storage conditions.

Note: The AutoLys quantification method places the PowerQuant standards in wells A01–H01 and the NTCs in wells G12–H12. Samples are placed in wells A02–F12.

Normalization and Amplification Using Fusion 6C

1. Complete the PowerQuant Autosomal Normalization form and export the .csv file. The reagent calculations include any necessary dead volumes required by the robot.

2. Open the Hamilton Method Manager on the AutoLys computer. Select the normalization and amplification method > Start (green arrow)

3. Follow the prompts as described below

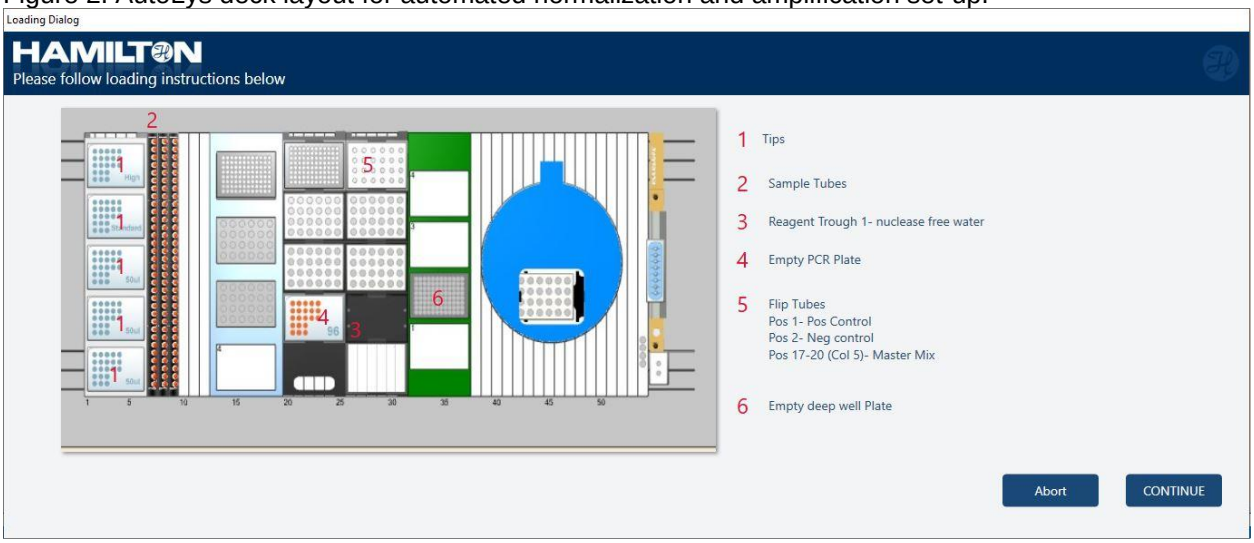
4. Import the .csv file from step 1, as prompted in Figure 1

Figure 1. Import .csv file.

5. Place the previously quantified sample tubes (with cap removed) into one or more AutoLys tube carriers in the order specified on the PowerQuant Autosomal Normalization form.

6. Load the carriers on the AutoLys in their dedicated location (see Figures 2 and 6).

Figure 2. AutoLys deck layout for automated normalization and amplification set-up.



7. Transfer amplification grade water to the appropriate trough and place the trough in the dedicated location on the AutoLys (see Figure 2).

8. Place a 96-well optical PCR plate in the dedicated location on the AutoLys, see Figure 2.

9. Prepare the amplification reaction mix (see Amplification of STR Loci: Fusion 6C)

10. Aliquot the amplification reaction mix in equal volumes to four FlipTubes and place the tubes in their dedicated location on the AutoLys (see Figure 2). Remove any bubbles prior to starting the run.
11. Prepare amplification positive and negative control samples in FlipTubes and place the tubes in their dedicated location on the AutoLys, see Figure 2. Remove any bubbles prior to starting the run.

12. If sample dilutions are needed, place a deep well plate in the dedicated location on the AutoLys (see Figure 2).

13. Note the number of 1000 µL, 300 µL, and 50 µL tips required for the normalization/amplification process. This information is presented on the deck set-up screen.

14. Follow the prompts to verify the location and quantity of pipette tips (see Figures 3–5). Ensure the barcodes of the pipette trays face toward the laser scanner.

Note: After the sample tube locations are verified the method starts processing samples. All sample tubes, reagents, tips, and deep well plates need to be in place prior to finishing this verification.

Figure 3. Display location for 1000 µL pipette tips.

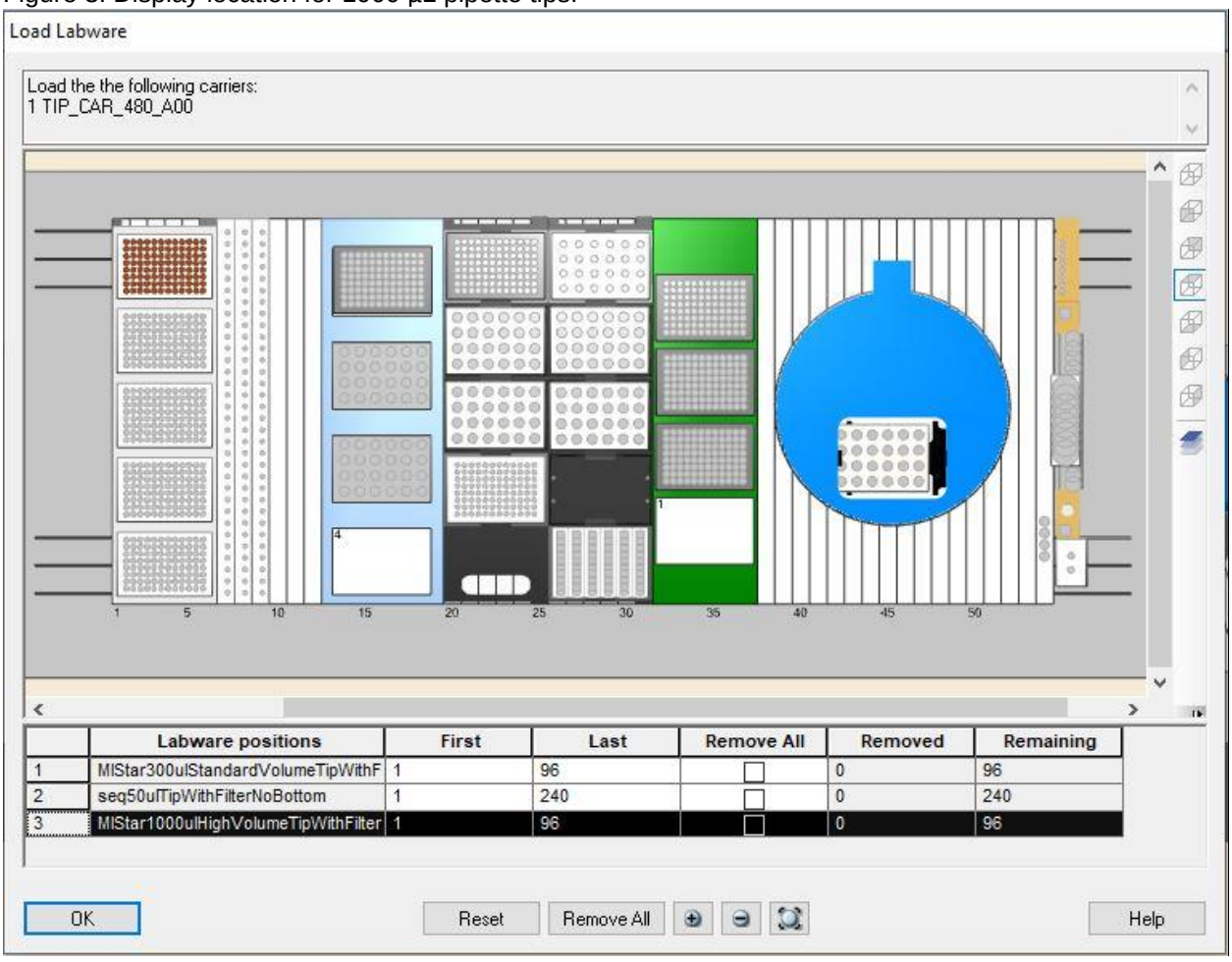


Figure 4. Display location for 300 µL pipette tips.

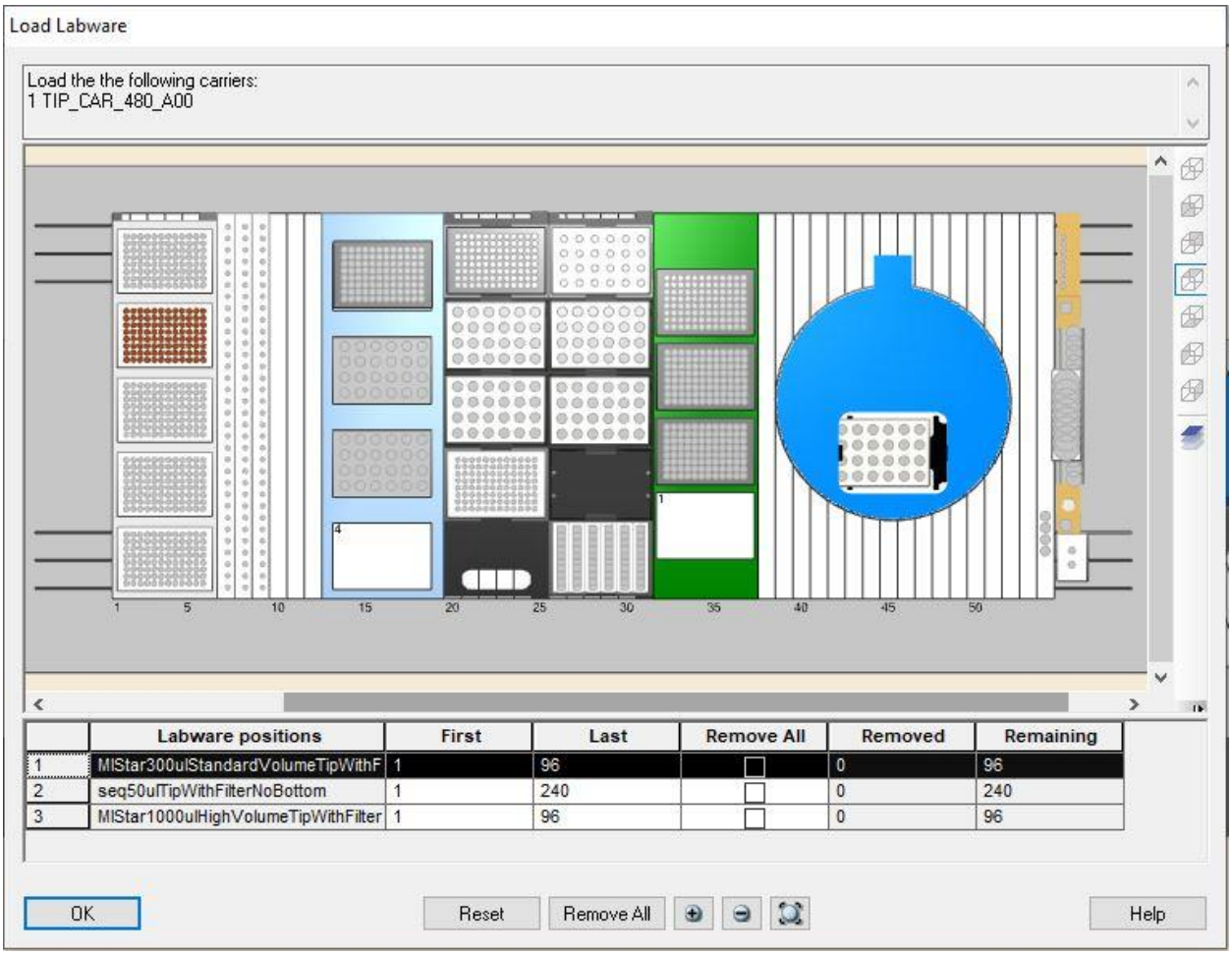


Figure 5. Display location for 50 µL pipette tips.

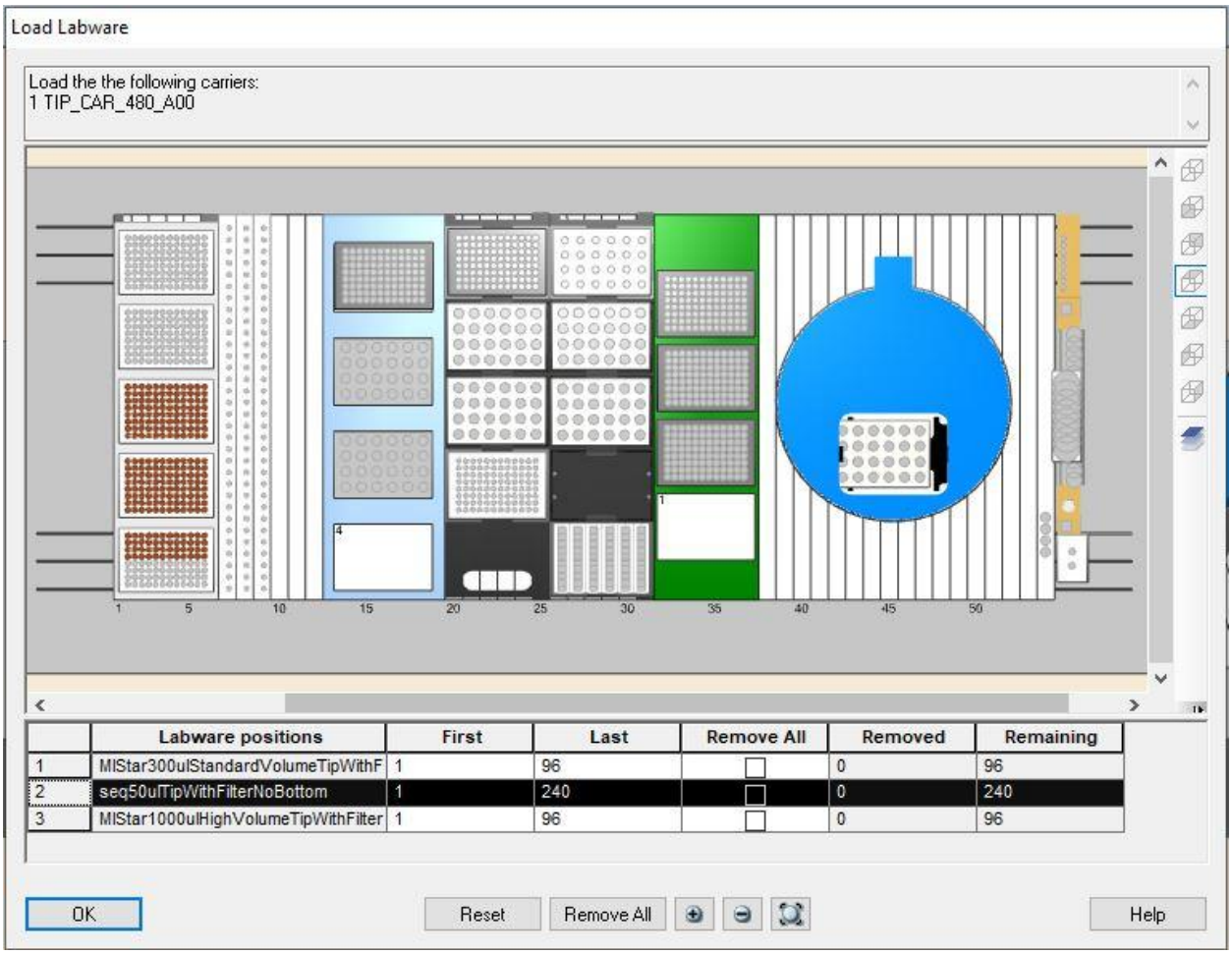
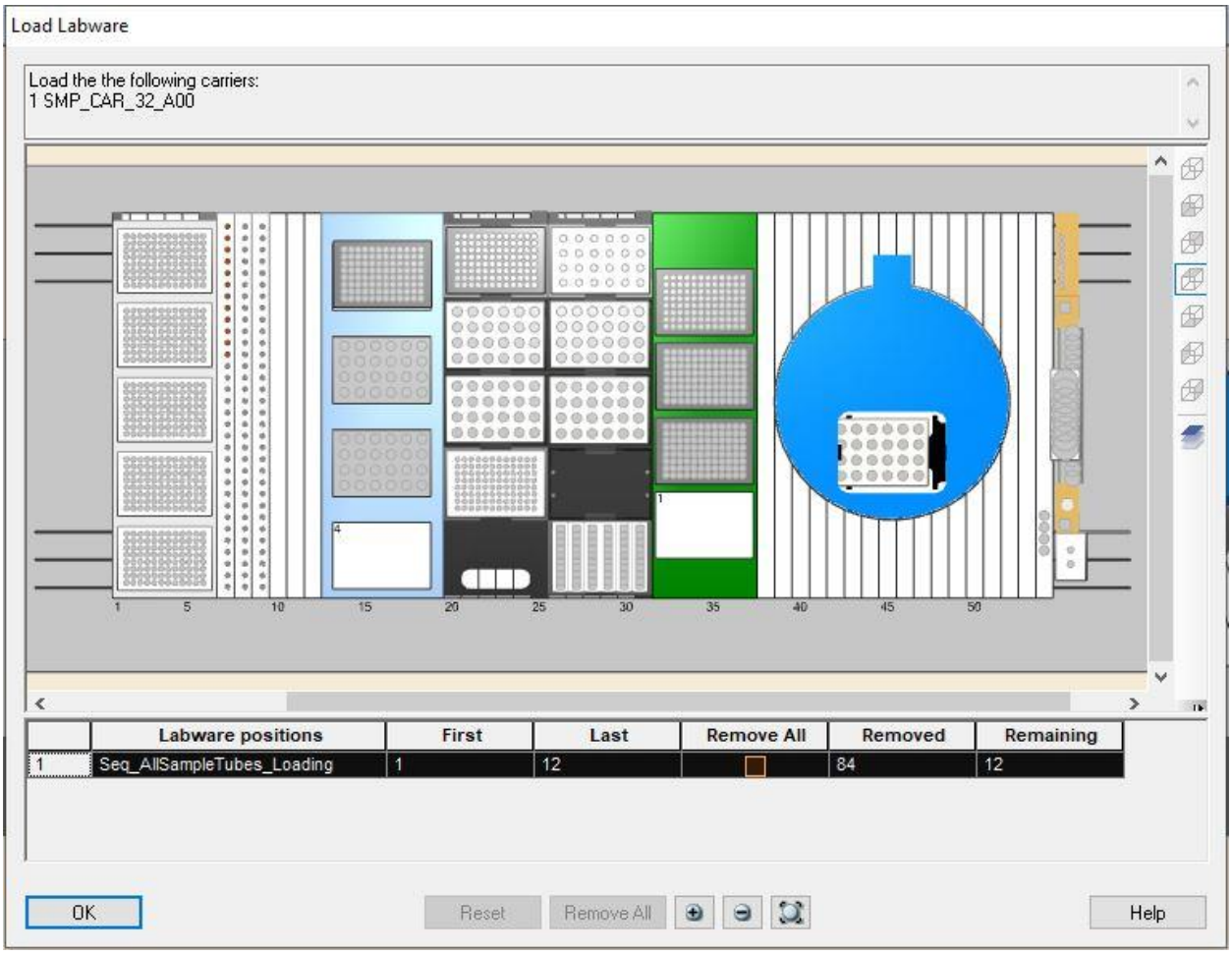


Figure 6. Display location for sample tubes.



15. When the method has completed, seal the 96-well PCR plate with a foil cover. Briefly centrifuge the plate if any bubbles are present. The plate is now ready to proceed to DNA amplification. The sample tubes should be closed and stored in appropriate storage conditions.

Note: The AutoLys normalization and amplification method places positive controls in wells B01 and D01, and negative controls in wells C01 and E01. Wells A01, A03, A05, A07, A09, and A11 are left empty as place holders for allelic ladder wells for CE loading.

33. HAMILTON MICROLAB STARLET

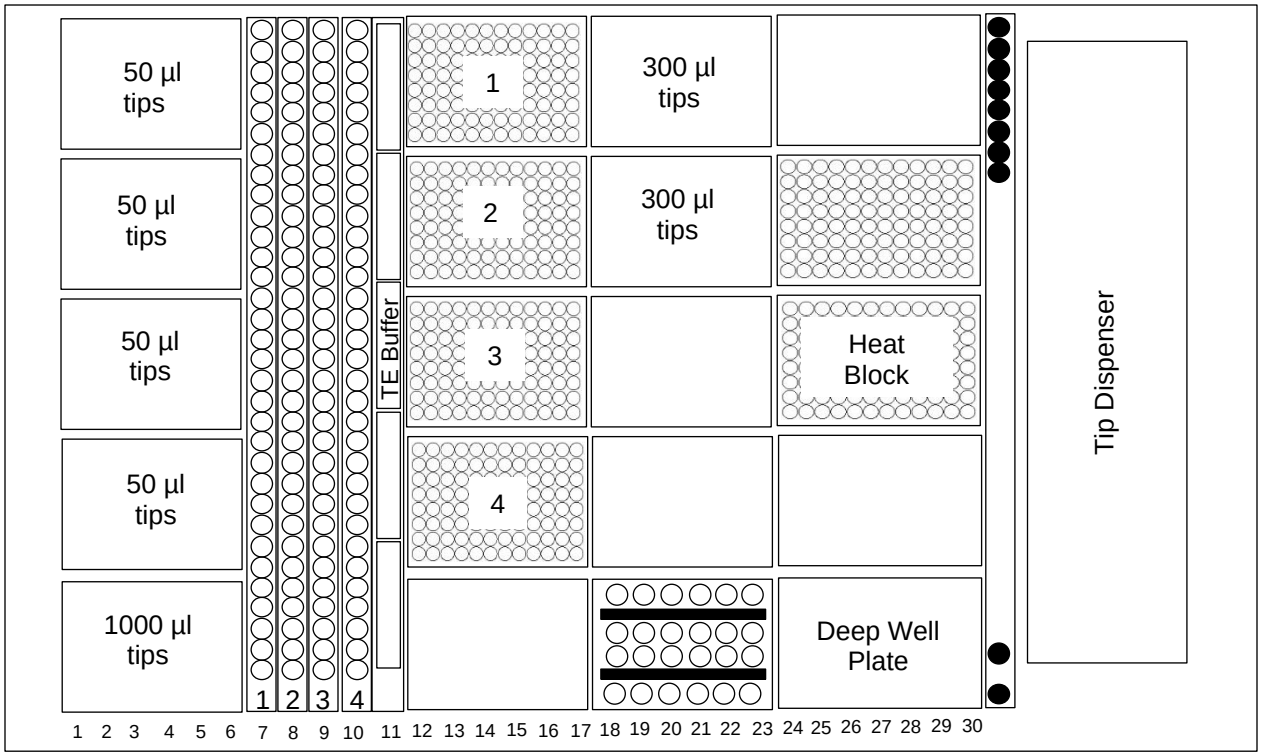
Scope

This procedure is used for sample processing on the Hamilton Microlab STARlet automated liquid handling platform. It is suitable for automated preparation of standard DNA dilution series, preparation of quantification reactions using the PowerQuant® System with a 20 µL reaction volume, normalization of purified DNA extract concentrations and preparation for amplification using PowerPlex Fusion 6C System with a 25 µL reaction volume. It is also suitable for preparation of direct amplification reactions using PowerPlex Fusion 6C System with a 12.5 µL reaction volume.

The STARlet uses Hamilton Run Protocol software to manage the robot and allow the development and execution of each protocol. The layout of the instrument surface contains 30 positions designated in numerical carrier positions from left to right (see Figure 1).

Figure 1: Carrier position system on the STARlet.
Positions for 96-well reaction plates labeled 1, 2, 3, and 4.

Back of Robot



Front of Robot

STARlet Protocols

Prior to running any protocols, check for and perform any necessary maintenance. Run the UV program, if equipped, when all protocols have been completed for the day.

Preparation of PowerQuant™ Standard Dilution Series

1. Vortex the gDNA standard tube for 15 seconds and centrifuge briefly to bring the liquid to the bottom of the tube.

- Ensure a new tube of gDNA standard is thawed overnight in the refrigerator prior to first use. Once thawed, the gDNA standard shall be stored refrigerated.

2. In the Hamilton Run Protocol software, select “Quant Standard Dilutions”.
3. Follow the directions given by the software.
4. The prepared standard dilution series may be stored refrigerated for up to one month.

Quantification Preparation for PowerQuant System

1. The Starlet Quant worksheet shall be used with this procedure.
2. Centrifuge the Master Mix, Primer Pair Mix, and Amplification Grade Water briefly to bring liquid to the bottom of the tube.
   - Ensure new tubes of the Master Mix, Primer Pair Mix, and Amplification Grade Water are completely thawed prior to first use.
   - Once thawed, the reagents shall be stored refrigerated. For optimal performance, the reagents should be used within one week. For longer storage, the reagents may be re-frozen but more than two freeze-thaw cycles should be avoided.
3. Vortex the reagent tubes for 15 seconds.
   - Do not centrifuge the Master Mix or Primer Pair Mix after mixing. This may cause a concentration gradient.
4. In the Hamilton Run Protocol software, select “Quant Plate Setup”.
5. Follow the directions given by the software. Placement of items on the STARlet work deck is depicted graphically and a description will appear when the computer cursor is hovered over each item.
   - Do not count quantification standards when entering the number of samples
   - Ensure there is enough volume of each reagent before moving forward.
6. Following completion of the protocol, seal the plate with an optical adhesive cover.
   - Ensure that all wells are adequately sealed to prevent evaporation during thermal cycling.
   - Avoid touching the bottom of the optical reaction plate.
7. If necessary, spin the plate to remove any bubbles.
8. Proceed to DNA QUANTIFICATION: POWERQUANT SYSTEM.
   - Protect the plate from extended light exposure prior to thermal cycling.
   - Artifacts may occur if time between qPCR setup and the start of thermal cycling exceeds 2 hours.

Normalization and PowerPlex® Fusion 6C STR Amplification Setup

1. In the Hamilton Run Protocol software, select “Norm Amp Plate Setup”.
2. Follow the directions given by the software:
   a. Use a dilution of the 2800M positive control (+C) DNA appropriate for a 15 µL load volume (2800M is provided at 10ng/µL):
   b. Load samples in the carrier and positions indicated by the STARlet Normalization worksheet. If samples are already loaded following a quantification assay setup, remove any samples that will not be processed.
   c. Import the sample information from the .csv file created using the STARlet Normalization worksheet in the PowerQuant Results workbook when prompted.
   d. If consumptive samples are being amplified on the Starlet, complete the Normalization worksheet to reflect the amplification parameters applicable to the sample. Do not place the sample in the Starlet sample carrier. The Starlet will distribute reaction mix into all wells associated with a sample to be amplified. Manually add the consumptive samples to the plate following completion of the protocol.
   e. Ensure there is enough volume of each reagent before moving forward.
3. Following completion of the protocol, seal all columns containing samples.
4. Proceed to DNA amplification.

PowerPlex® Fusion 6C Direct Amplification Setup

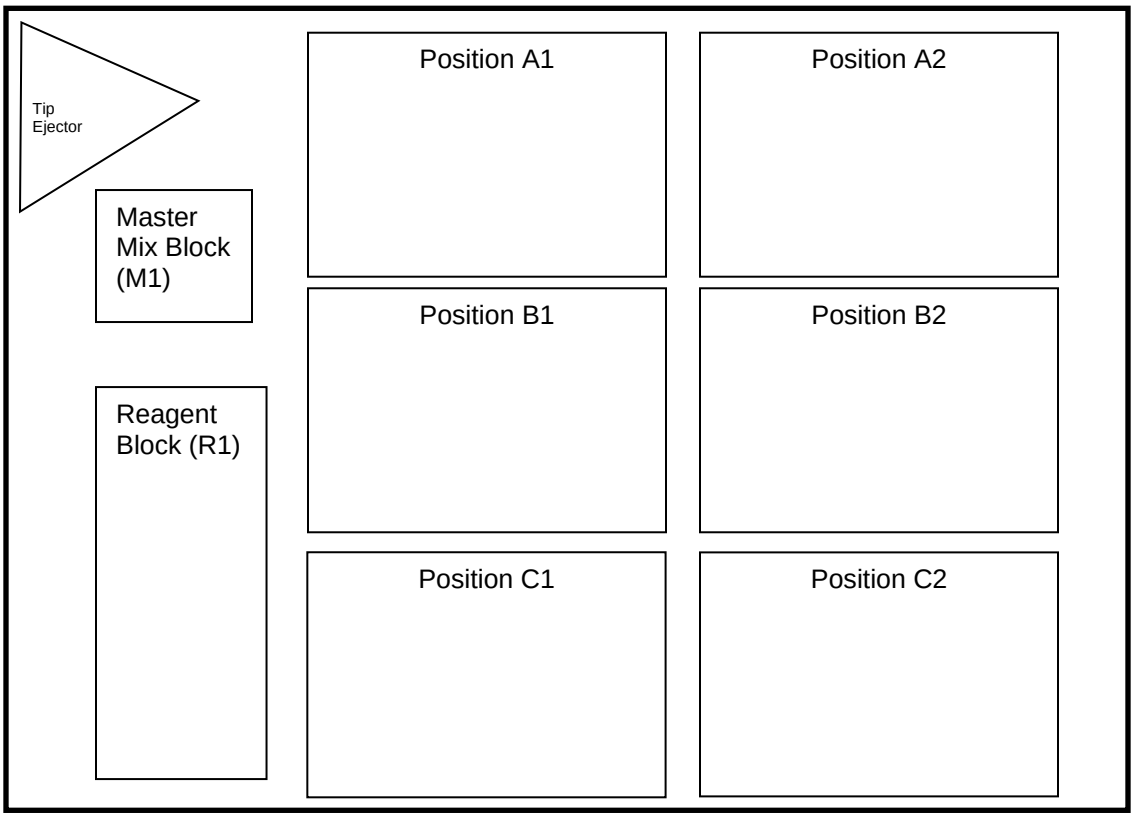
1. Prepare samples as outlined in the Direct Amplification of STR Loci: Fusion 6C, Sample Preparation section.
2. In the Hamilton Run Protocol software, select “Direct Reference Amp” (the sample substrate does not need to be removed from the tube prior to running this protocol).
3. Follow the directions given by the software:
    a. Ensure there is enough volume of each reagent before moving forward.
4. Following completion of the protocol, seal all columns containing samples.
5. Proceed to AMPLIFICATION: POWERPLEX FUSION 6C.

34. QIAGEN QIAGILITY INSTRUCTIONS

Introduction

The QIAGEN QIAgility along with the QIAgility Software is used by the Washington State Patrol Crime Laboratory Division laboratories to set up quantification reactions, normalize extracts and set-up amplifications, and set up CE plates for detection.

When using the QIAgility, please refer to the protocol in use for proper QIAgility deck setup.



Quantification Setup

PowerQuant™ Quantification Assay Setup

1. Enter sample names into the QIAgility template sheet of the “4-point PowerQuant™” workbook. This template mirrors the 4 x 8 well racks of the instrument deck. The sample names and load order will automatically be transferred to the sample bank sheet(s) matching the 96-well quantification plate setup and the Plate Template sheet when the “Populate Data” button on the Plate Document sheet is clicked. Save the sample bank sheet(s) and the Plate Document sheets as .txt files.

2. Mix the appropriate volumes of reagents in a 5 mL tube. Place this tube into position C on the Master Mix Block.

3. Place the appropriate DNA Standards and NTC in the proper positions on the QIAgility Deck.

4. Load samples in EZ1&2 screw cap tubes into the sample bank racks in the position designated by the QIAgility template sheet. If your samples are in different tubes, be mindful that the instrument is calibrated to the EZ1&2 screw cap tube, or transfer sample into an EZ1&2 screw cap elution tube.

5. Place a 96-well optical reaction plate in the appropriate location of the QIAgility Deck.

6. Open the appropriate protocol from the QProtocols folder.

7. In the Software, select/highlight the rack where your samples are located. Click on the “Import” button. Click on the box to the right of the Import File drop-down menu and navigate to the QIAgility.txt file generated above (step 1). Repeat this step as needed if additional sample blocks are in use.

8. Generate a sample bank, making sure the following values are correct:

   “From Row:” = 1 (indicates the beginning of the samples to import by skipping header)
   “To Well:” = A1
   “Limit Sample Count = 64”
   “Sample Name = Column 1”
   Uncheck the “Load Conc. From Column” box.
   Under “Sample Bank Options, select “Add Filtered Rows to Sample Bank” and select “QF Samples” under Existing Bank.
   Click the “Import” button, then “Finish.”
   In the sample bank window the number following bank “Bank QF Samples” should now correspond to the number of samples in the sample racks.

   Note: If a third sample bank is used and this sample is being utilized please make sure the following values are correct:

   “From Row:” = 1 (indicates the beginning of the samples to import by skipping header)
   “To Well:” = A1
   “Limit Sample Count = 32”
   “Sample Name = Column 1”
   Uncheck the “Load Conc. From Column” box.
   Under “Sample Bank Options, select “Add Filtered Rows to Sample Bank” and select “QF Samples-2” under Existing Bank.
   Click the “Import” button, then “Finish.”
   In the sample bank window the number following bank “Bank QF Samples-2” should now correspond to the number of samples in the sample racks.

   Note: If samples appear to have imported incorrectly, the sample bank can be edited by selecting the sample bank from the list in the top right window, clicking on “Edit Bank”, then under Sample Selection of the Update Sample Bank window, click on “Bank Wells” to select all the samples and “Delete Selection.” This should be followed by hitting the “Clear” button above the samples list. Then, re-import your samples following the directions in steps7-8.

9. On the QIAgility deck map within the software, double-check reaction steps; depending on the protocol in use this can be done by selecting the destination plate. The “Pipette 2µl of QF samples…” reaction listed should be active (formerly grayed out) after populating the QF Samples sample bank. Delete any pipetting function not in use (grayed out).

10. A pre-run report may be used to confirm that tips, tubes, plates and liquids have been set up correctly. It can be accessed by clicking the icon on the toolbar or the Pre-run Report button at the bottom of the checklist window after clicking the green arrow.

11. Click the green arrow at the top to start. When prompted to Save, create a folder with the batch date. The default name for the file can be accepted. Save the file to the newly created folder.

12. Check the boxes in the checklist menu as the actions are completed.

Normalization and Amplification Setup

PowerQuant™ – QIAgility Data Converter

Helpful instructions are also on the "Instructions" sheet of the "WSP QIAgility" workbook.

1. Using the 7500 Software, select all used wells and click "Export". Export Properties should be:
    a. Select data to export: only "Results" should be checked
    b. Select one file or separate files: pick "One File"
    c. Enter the export file properties: Export File Name: "_data.xls"
    d. Choose an appropriate location

2. Open the "WSP QIAgility" workbook

3. On the "Samples" sheet enter the sample names in the order they will be loaded on the QIAgility. Positive and negative amplification controls do not need to be listed. The sample names must be unique and appear exactly as they were on the quantification. The maximum number of samples that can be entered is 62.

4. Enter the "Amp Batch Name" on the "Samples" sheet

5. Enter the "Plate Name" on the "CE Plate" sheet

6. Click the "Choose Folder for Run Files" button on the Samples sheet to save the generated files appropriately

7. Click on the "Import 7500 Results" button on the "Wash. State Patrol" tab in the menu bar

8. The target values on the "Samples" sheet automatically populate to "1" but can be manually updated as appropriate. An amp target that is not possible will automatically be highlighted.

    Note: A sample with a quantification value >50 ng/µL will need manually diluted prior to using the QIAgility for normalization. The corresponding quantification value will need updated manually before proceeding to the next step. Likewise, the quantification value for a concentrated sample will need updated manually before proceeding to the next step. Caution should be taken to ensure the volume required by the QIAgility (up to 15 µL) does not exceed the volume in a sample extract.

9. Click the "Create Files" button on the "Wash. State Patrol" tab in the menu bar which will automatically populate the "CE Plate" sheet of the workbook and save the file needed for the QIAgility (amp batch name "_6C_QGility.csv") and 3500 CE setup file (CE plate name "_3500File.txt")

QIAgility Normalization and Amplification Setup

1. Mix the appropriate volumes of amplification reagents in a 5 mL tube. Place this tube into position C on the Master Mix Block.

2. Place the appropriate diluents in 5 mL tubes, dilution plate (if needed), and positive and negative amplification controls in the proper positions on the QIAgility Deck.

3. Skipping wells A1 and A2, load samples in EZ1&2 screw cap tubes into the Sample banks racks in the order designated by the “WSP_QIAgility” Samples sheet. If your samples are in different tubes, be mindful that the instrument is calibrated to the EZ1&2 screw cap tube, or transfer sample into an EZ1&2 screw cap elution tube.

4. Place an appropriate amplification plate (or tubes with detached caps) in the appropriate location of the QIAgility Deck.

5. Open the appropriate QProtocols

6. In the Software, select/highlight the rack where your samples are located. Click on the “Import” button. Click on the box to the right of the Import File drop-down menu and navigate to the **amp batch name “_6C_QGility.csv” file** generated above (step 9).

7. Generate the sample banks, making sure the following values are correct:

   “From Row:” = 2
   “To Well:” = A3
   “Limit Sample Count to:” = 64
   “Sample name from column:” = 1
   “Load Conc. from column:” = 2
   Under “Sample Bank Options,” select “Add Filtered Rows to Sample Bank” and “Banks specified in column:” = 3
   Click the “Import” button, then “Finish.”

8. On the QIAgility deck map within the software, double-check reaction steps; depending on the protocol in use this can be done by selecting the destination plate. Delete any pipetting function not in use (grayed out).

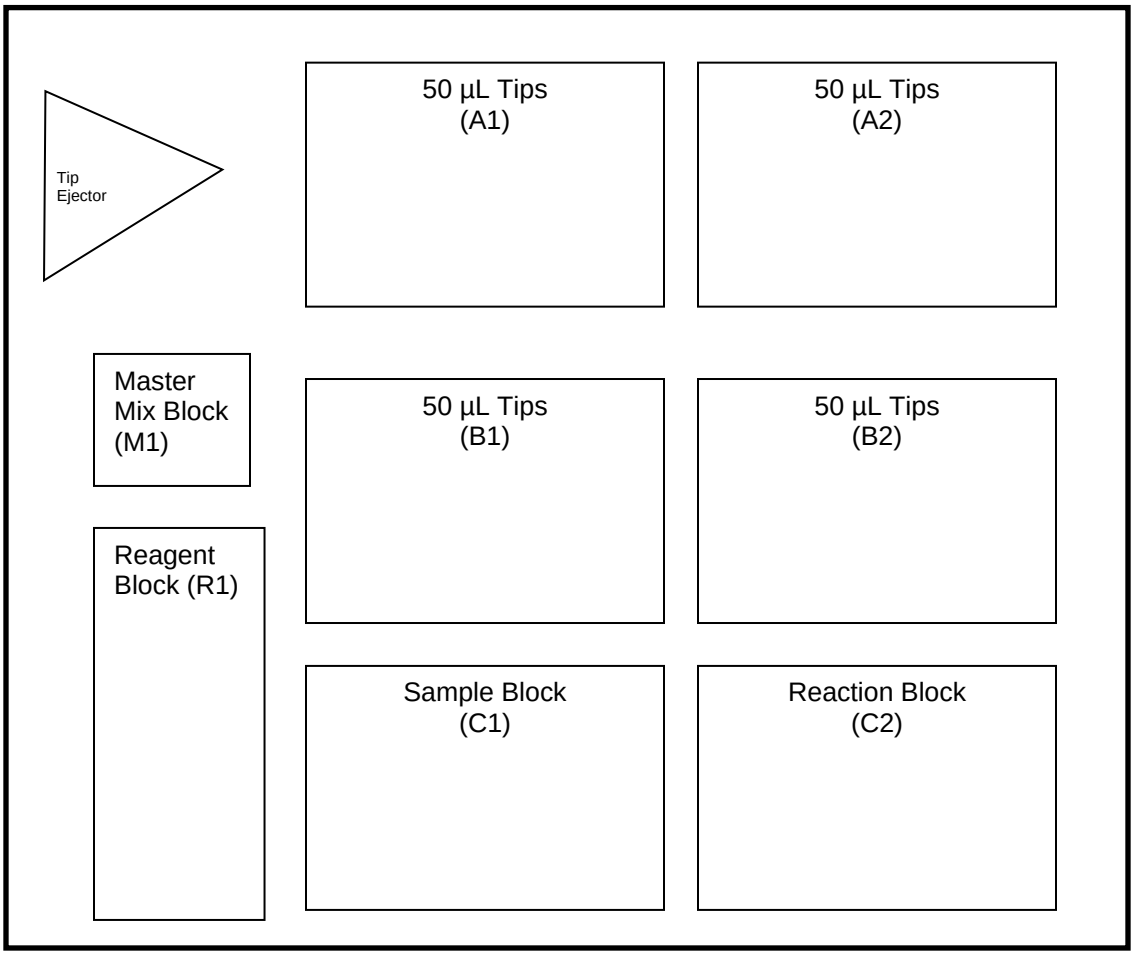
9. A pre-run report may be used to confirm that tips, tubes, plates and liquids have been set up correctly. It can be accessed by clicking the icon on the toolbar or the Pre-run Report button at the bottom of the checklist window after clicking the green arrow.

10. Click the green arrow at the top to start. When prompted to Save, create a folder with the batch date. The default name for the file can be accepted. Save the file to the newly created folder.

11. Check the boxes in the checklist menu as the actions are completed.

12. When the run is complete, cap and store the remaining sample extracts as appropriate (the dilution plate may be discarded). Remove the amplification plate/tubes and cover the reaction wells and proceed with the amplification process.

CE Plate Setup



Procedure

1. Turn on the QIAgility and associated computer. Ensure that the lid of the QIAgility is closed and start the QIAgility software.
2. Open the appropriate plate loading protocol.
3. ~~A~~djust the number and location of samples in the sample bank to reflect the number and location of samples in the 96 well amplification plate. Select the Sample Block (robot position C1) then select “Bank Samples” and edit the sample bank by adding or removing samples as necessary.
4. Ensure that the CE does not inject from an empty well by adjusting the number of additional formamide/ILS wells in the second to the last step of the reaction list.
5. Place supplies in the appropriate locations as indicated by the protocol or as follows:
    a. Place an uncapped 1.5 mL tube of prepared Formamide/ILS (refer to the “Amplification Product Preparation” section of this manual) into the appropriate position of the Master Mix Block. ~~.~~Ensure that there is an appropriate volume of this reagent for the number of samples to be added to the plate.
    b. Place an uncapped tube of the appropriate ladder in the appropriate position of the Reagent Block. Ensure that there is an appropriate volume of ladder present in the tube.

   c. Remove the caps from the 96 well amplification plate/tube containing amplified samples and place the plate/tubes into the Sample Block (robot position C1) with well position A1 in the upper left corner.
   d. Place a new 96 well CE plate into the Reaction Block (robot position C2) with well position A1 in the upper left corner.
   e. Ensure that there are an appropriate number of 50µL tips present in the robot.
6. Click on the run icon to start the protocol. Save the file
7. A pre-run “Checklist” dialog box will appear. Check the boxes next to the messages that appear in the checklist. All boxes must be checked before the “OK” button will be active allowing the run to proceed. If desired, the “Pre-Run Report” can be viewed by clicking the appropriate button. The number of tips and amounts of other consumables required are listed in the Pre-Run Report.
8. At the completion of the run, cover the CE plate with septa. Refer to the “Amplification Product Preparation” section of this manual to finish preparing the loaded plate.

35. SAMPLE SWITCH DETECTION

Scope

This procedure shall be used when two or more reference samples with the same suspected or observed Amelogenin genotype are processed for the same case (using STR or Y-STR testing), and where there is an inclusionary result for at least one of the references.

Procedure

At least one of the following three sample switch detection procedures shall be used in applicable cases. If a combination of more than one approach is employed, a confirmation sample or sampling (similar to SCS) need only occur from the last witnessed or separately analyzed processing step. This procedure must be completed prior to release of the report.

STR Confirmatory Sample (SCS)

1. Collect a second sample from each reference with an inclusionary result.
    - When two reference samples are processed concurrently, a second sample may be collected from either reference during the initial sampling. This approach fulfills the SCS requirement for both references.
2. Process this second sampling for confirmation of the original result.
3. All documentation associated with confirmation testing shall be retained in the casefile.

Witnessing

1. A DNA staff member shall observe the processing and track sample name(s) and case number(s) for the pertinent sample(s) during the following steps:
    - Sampling, including verification that sample name matches the tube label.
    - Manual steps and transfers during extraction, amplification set-up, and CE set-up.
    - Witnessing is not necessary during CE set-up if a multi-channel pipette is used to transfer from strip tubes or an amplification plate.
2. Witnessing shall be indicated by the initials of the witness on each applicable (i.e. witnessed step) page of the casefile.

Separate Analysis

1. Process each applicable reference separately.
    - Separate analysis entails different start times and separate batches (to include separate reagent blanks) for sampling and extraction, as well as separate amplification and CE load times.

36. EVALUATING DNA PROFILE DATA

Guidelines are provided for evaluating DNA typing profiles obtained by STR analysis using the Promega PowerPlex® Fusion 6C and Promega PowerPlex® Y23 System PCR amplification kits, an AB Genetic Analyzer, and GeneMapper® *ID-X*.

GeneMapper® ID-X: Creating a Project

1. In the new Project window, select the **Add Samples to Project** icon (or select Add Samples to Project from the Edit menu). Under the Files tab, select the run folder(s) containing the sample files, then click **Add to List** followed by **Add**.

2. In the Project window, select a desired view from the Table Setting drop-down list.

3. Select the **Samples** tab. Assign as appropriate the following parameters: sample type, analysis method, panel, size standard, and custom control. For sample type, reagent blanks and negative amplification controls are designated “Negative Control”, positive amplification controls and custom controls are designated “Positive Control”, ladders are designated “Allelic Ladders”, and all other samples are designated “Sample”. The positive control, custom control or an allelic ladder assigned as a sample can be used as a genotyping control to serve as a QC function as needed.

4. An allelic ladder must be present in each run folder in the project.

5. Once all parameters have been assigned, click on the analyze button (green arrow). Complete the fields in the Save Project dialog box. Click OK to save the project.

   NOTE: Depending on the Project Options that are set for analysis, the software may not complete the analysis of the project if one or more analysis requirements are not met. This is indicated by a yellow triangle flag (Analysis Requirements Not Met (ARNM)) in the samples. The offending sample(s) are filtered into the Analysis Requirements Summary (ARS) dialog box. Place the cursor over the yellow ARNM flag(s) to display the reason (tooltip) and make the appropriate edit(s) to the sample(s). Repeat step 5.

6. In the navigation pane, expand the run folder. Select the **Raw Data** tab in the content pane then scroll through each of the listed samples in the expanded run folder. Verify the presence of primer peaks in all samples. The raw data may also help evaluate any anomalies, start and stop points for analysis, and the causes of poor size-calling.

GeneMapper® ID-X: Quality Assessment

The GMID-X software program analyzes the CE data and allows the analyst to evaluate allelic ladder, control, and sample quality; investigate sample-level process quality values (PQVs) and marker-level PQVs; review sample plots and edit peak labels; and adjust plot displays to determine the source of artifacts.

The PQV results of the quality assessment are displayed as color-coded flags
- Pass (green square)
- Check (yellow triangle)
- Low Quality (red octagon)

Evaluate the quality flags. It is sometimes necessary to confirm a called artifact, label an artifact, evaluate flagged imbalanced peak heights, review/verify a potentially mixed-source sample, and investigate other low quality flags. Once the data has been evaluated, the sample data can be accepted with the CGQ overridden or the sample data can be rejected and deleted from the project.

Peak Edits

Allows individual peaks to be examined and edited. Left-click to select the peak to be examined and then right-click to open a drop-down menu containing peak edit options.

- **Delete Label(s)** – allows the user to delete the label for a particular peak. This peak edit will only be used for preparing CODIS export files or when re-interpreting a peak.
- **Add Allele Label** – allows the user to add a custom allele label. The custom allele will be added to the marker and will be entered as an allele in the Genotypes table.
- **Add Artifact Label** – allows the user to add a custom or predefined artifact label to a peak.
- **Rename Artifact Label** – allows the user to customize an artifact label and add it to a predefined list. The custom artifact label is not added to the Genotypes table. This peak edit will only be used for original observations. It will not be used when re-interpreting a peak.
- **Peak Raw Data** – allows the user to view the peak morphology in the raw data plot in the Project window.

NOTE: The software automatically labels spikes. Any peak labeled as an artifact by the software or the user is not listed in the Genotypes table.

Original observations regarding peak labels will be recorded using the **Rename Artifact Label** and **Add Allele Label** options. Subsequent changes to peak labels shall be recorded by deleting then adding a new label to the peak and entering a reason for the change when prompted by the software.

Overriding the GQ and CGQ PQV

Once the edit(s) are complete for the marker(s), right-click the GQ flag in the highlighted marker row of the Genotypes table and select Yes in the dialog box to override the genotype quality for the marker.

1. The GQ PQV turns to a passing flag (green square).
2. All other PQVs for the marker turn gray and maintain their original shape to indicate the marker was overridden.
3. Once all the PQVs for a sample have been addressed, a dialog box will appear stating that the Genotype Quality (GQ) flag is green for all markers in the sample and ask if the user wants to override the Composite Genotype Quality (CGQ) for the sample. Select **Yes** to override the CGQ.

Overriding the CGQ PQV Only

Alternatively, once the sample has been evaluated for peak height imbalances and all artifact peaks have been re-labeled, right click the yellow triangle or red octagon shape under the CGQ heading. Click Yes in the dialog box to override the Composite Genotype Quality flag for the profile.

Mark Sample for Deletion

Allows the user to mark a sample for deletion and continue to evaluate data. To delete a sample from the project, select the **Mark Sample for Deletion** box at the top right of any dye pane for the sample. Click the **Bring Marked Samples to Top** icon in the Samples plot toolbar. Prior to closing the plot window, verify that the sample(s) marked for deletion is to be deleted. The sample(s) will be deleted from the project when the Samples plot is closed.

GeneMapper® ID-X: Data Evaluation

All samples regardless of quality assessment flag are examined. Controls and ladders displaying a green passing quality flag may not need to be visually inspected. If a control displays a yellow or red quality flag, it shall be visually examined.

If a load volume and/or injection time other than the validated defaults were employed for the analyzed sample(s), including quality control samples, then those parameters must be documented in the case file.

WEN Internal Size Standard – Fusion 6C

If the sizing quality (SQ) flag is red, evaluate the WEN ILS-500 data to confirm peaks are properly labeled. If there is mislabeling of peaks due to a fast or slow run, create a new “fast” or “slow” version of the analysis method that begins evaluating data sooner or later, as appropriate. If the data indicates a faulty injection, re-inject the sample.

The allelic ladder has PCR products up to approximately 485 bp so the 475 and 500 bp peaks of WEN should be present. If they are not, confirm that there are at least 2 WEN peaks larger than the largest allele in the profile. Allowances will be made for runs that do not include the 500 bp peak if the sample does not have peaks greater than 475 bp. Allowances will also be made for the allelic ladder run. If there are peaks above 475 bp in a sample amplified with the Fusion 6C kit, and no 500 bp WEN peak is present, then the sample must be re-injected. An appropriate allelic ladder with the WEN 500 bp peak must also be used. Ensure that WEN peaks are above the detection threshold (have not dropped out). If needed, the orange detection threshold in the analysis method may be lowered to the applicable analytical threshold to detect additional peaks. Document any modifications to the size standard or analysis method in the case file.

Raw or Unanalyzed Data

The raw or unanalyzed data for each sample should be examined. Typically, the primer peak should rise and fall abruptly and the baseline should be flat and smooth. If the primer peak trails excessively or the baseline is excessively elevated and the genotype data quality is adversely affected, the sample should be re-injected.

Excessive or Saturated Peak Heights

Excessive peak heights at Amelogenin and up to two additional loci are acceptable, provided that any resulting artifacts do not affect interpretation of the profile. Deconvolution results shall be evaluated carefully to ensure they have not been adversely affected by the saturated data. Attempts shall be made to resolve excessive peak heights that adversely affect interpretation or that are present at more than the acceptable number of loci (refer to the “Amplification Product Preparation”). Documentation of approaches used to mitigate excessive peak heights shall be recorded in the case file.

Allelic Ladder

An allelic ladder will be included with every set of samples run on the CE unit. Ensure that the allelic ladder displays a green passing flag. If a run folder contains no passing ladders, the samples cannot be used for interpretation and must be re-injected or re-loaded with the same or new ladder samples.

Quality Control Samples

Verification that samples have been extracted and amplified correctly is through assessment of control samples. Passing data does not need to be manually reviewed; non-passing data shall be manually reviewed.

All no template DNA samples (negative control samples and reagent blanks) shall be analyzed with peak detection set at analytical threshold to assist in the detection of contamination.

Positive control samples and custom control samples shall be analyzed with peak detection set at stochastic threshold.

- Reagent Blanks: No PCR product should be detected in the reagent blank samples. If reproducible peaks are present, the contamination flow chart in the DNA Quality Assurance manual should be consulted. The reagent blank shall be amplified utilizing the same primers, amplification instrument model and concentration conditions as required by the sample(s) containing the least amount of DNA and shall be typed utilizing the same analysis instrument model, injection conditions and most sensitive volume conditions of the extraction set.
- Confirmatory Samples: When used, the STR confirmatory sample (SCS: DNA from a case reference sample previously run) shall yield the correct allele designations and be free of contamination.
- Positive Amplification Control (+C): Confirm that the alleles in the positive amplification control (+C) are correct and no contamination is present. If the positive control has peaks below stochastic threshold but a second positive control sample (SCS or verified known sample) has normal heights, inaccurate pipetting during +C preparation is indicated, and the sample set can be re-amplified or interpretation of the results can be made with caution based on a case-by-case assessment. If the positive control displays peaks below stochastic threshold over multiple amplifications, this may indicate a decrease in CE sensitivity and should be evaluated before performing additional casework analysis on the instrument.
- Negative Amplification Control (-C): No PCR product should be detected in the negative amplification control (TE buffer/ -C). If the -C has reproducible peaks, the contamination flow chart in the DNA Quality Assurance manual should be consulted and the corresponding samples may need to be re-amplified (if possible). The -C must be loaded and injected at least as long as the corresponding +C.

STR DNA Profile Data

All non-artifact peaks that are sharp, distinct and greater than or equal to analytical threshold will be considered alleles, documented, and considered during profile determination.

The determination of a profile or individual genotype is aided using thresholds established via validation.

- **Analytical Threshold: 70 RFU (120 RFU for direct amplification samples)**

  The analytical threshold (or detection threshold) is the level at which a peak can be reliably differentiated from background fluorescence as either an allele or artifact as determined via validation.

- **Stochastic Threshold: 600 RFU (500 RFU for direct amplification samples)**

  The stochastic threshold is the level at which a sister peak to an allele that meets or exceeds this threshold should be detected above the analytical threshold. Alleles detected between 70 RFU and 599 RFU could have an undetected heterozygous sister allele due to stochastic effects of the PCR amplification process. Allelic dropout, the presence of more than one contributor, and elevated artifacts or background signal shall be considered in the evaluation of the profile data. The stochastic threshold aids in the determination of genotypes within a profile.

Reference samples amplified using standard procedures should be analyzed with peak detection set at the stochastic threshold (600 RFU for standard amplification or 500 RFU for direct amplification). Peak detection set at the corresponding analytical threshold may be used for analysis of reference samples, however all loci with single alleles less than the stochastic threshold must be considered partial. In such situations it is recommended that the sample be reworked to get a complete reference profile.

PCR and Genetic Analyzer Artifacts

If an artifact can be confidently characterized as one of the following anomalies or the peak morphology is not consistent with an allele, the sample does not need to be re-injected. Any uncertainty about an anomalous peak requires at least reinjection to ensure quality and accuracy of allele calls.

Artifacts which may interfere with possible alleles shall be considered and documented.

An artifact (spike, shoulder, pull-up or other anomaly) is acceptable provided:

1. It meets the criteria outlined below and is appropriately edited by the analyst in the software.
2. The peer reviewer of the data agrees that the artifact has been appropriately characterized by the analyst, documented, and is not due to contamination or another problem.

- **Stutter**
  Stutter is a phenomenon that occurs during PCR due to strand slippage. As a characteristic small peak, one repeat unit less in size than the allele, it is a known biological amplification phenomenon. Established stutter percentages aid in discriminating between stutter and potential extra alleles or some other anomaly (e.g. weak heterozygous or tri-allelic peaks, contamination).

  Stutter filters set in GeneMapper® *ID-X* using the values below may be used to filter out potential stutter peaks. In a mixed source sample, stutter peaks may need to be considered. Refer to GeneMapper® Analysis Methods in this manual for the application of stutter filters in sample analysis.

  Stutter threshold values (% of alleles) are as follows:

Fusion 6C*			
Locus	Back stutter (%)	Half back stutter (%)	Forward stutter (%)
Amelogenin	--	--	--
D3S1358	13.5	--	1.7
D1S1656	14.3	3.6	2.3
D2S441	9	--	1.8
D10S1248	13	--	1.3
D13S317	10.3	--	2.2
Penta E	7.2	--	1.9
D16S539	12	--	3
D18S51	14.6	--	2.8
D2S1338	13.6	--	2.2
CSF1PO	11.1	--	3.7
Penta D	4.5	--	3.7
TH01	4.8	--	1.5
vWA	14.4	--	2.7
D21S11	12.7	--	2.8
D7S820	9.7	2 *	1.8
D5S818	11	--	2.3
TPOX	5.4	--	1.1
D8S1179	11.8	--	3.4
D12S391	17.4	--	2.7
D19S433	12.1	1.4	2.6
SE33	16.1	6.6	3.3
D22S1045	16.8	--	9
DYS391	9.4	--	2

FGA	12.4	1.2	2.8
DYS576	12.5	--	3.4
DYS570	13	--	2.4

Values from Promega's developmental validation, confirmed through internal validation
* Values from internal validation studies

Peaks in both the minus and plus stutter positions with ratios greater than the above values may indicate the presence of a mixture. Consider heterozygote allele pairing expectations, the number of contributors based on the other loci, and the estimated proportions when evaluating profiles to determine if the elevated or observed stutter could be an allele.

Peaks in the n-8 stutter positions were observed during validation of the Fusion 6C amplification kit; however, they were less common. Refer to internal validation studies for n-8 stutter instances. When determining whether a peak in the n-8 position is stutter or an allele, consider heterozygote allele pairing expectations, the number of contributors based on the other loci, and the estimated proportions.

- **DNA-Dependent Artifacts in Fusion 6C**

Locus	**Artifact Size**
Amelogenin	n-1
D1S1656	n-1, n-2
D13S317	n-2
D18S51	n-2
vWA	n-2, elevated baseline in the locus
D7S820	n-2
D5S818	n-2
D19S433	n-2
SE33	n-2
DYS391	n-1
FGA	n-1, n-2

- **Spikes**
  Spikes have a general appearance of being needle-like and are randomly seen in samples. Spikes typically disappear with re-injection. Commonly, spikes are observed in most or all of the dye colors, and generally at the same base pair location. Occasionally, single color spikes may also be observed. The heights of spikes (in RFU) usually vary between dye colors.

  GeneMapper® *ID-X* will automatically flag spikes within the analyzed range. Evaluate the flag and confirm the spike. Spikes may sometimes be flagged as something other than spike. Pull-up can sometimes be labeled as a spike also. Evaluate the artifact and follow the peak edit steps to appropriately flag the artifact.

- **Formamide/Dye Blobs**
  Raised baseline areas may represent formamide/dye blobs which usually occur at the same base pair location in multiple samples of the run.

  GeneMapper® *ID-X* may label a formamide/dye blob that falls within the analyzed range as an allele or with a quality flag. Evaluate the artifact and follow the peak edit steps to appropriately flag the artifact. If a dye blob falls outside of the marker range but not between markers, GeneMapper® *ID-X* will not always flag the artifact. In this instance, the artifact can be ignored.

- **Pull-Up**

Pull-up artifacts are peaks that show up at the same base pair size or close (i.e. about a base equivalent on either side of the allele) but in another color. Pull-up usually occurs under peaks with excessive signal or strong alleles. It may also occur between peaks of another color.

GeneMapper® *ID-X* may label a pull-up that falls within the analyzed range as an allele or with a quality flag. Evaluate the artifact and follow the peak edit steps to appropriately flag the artifact. If a pull-up falls outside of the marker range but not between markers, GeneMapper® *ID-X* will not always flag the artifact. In this instance, the artifact can be ignored.

- **Shoulders**
  Shoulders (-A or +A) flank the sides (one or both) of an allele. GeneMapper® *ID-X* will not always flag shoulder peaks. If shoulders are present greater than the stochastic threshold and are given an allele designation by GeneMapper® *ID-X*, the sample should be considered for a re-run at a shorter injection time (reference samples only), run following re-extension, or re-amplified using less template DNA. If shoulders greater than the stochastic threshold are present in known reference DNA samples and positive controls, then a re-run or re-amp is not necessary if interpretation of the DNA profile is unhindered. If shoulders are at no more than one locus in single source evidence samples then a re-run or re-amp is not necessary if interpretation of the allele(s) at that locus is unhindered and any resulting artifact(s) (pull up, etc.) does not affect interpretation of other loci. The artifact shall be evaluated and appropriately addressed in the software.

Variant Alleles

Tri-allelic patterns and allelic peaks that do not fit into the current range defined by the GeneMapper ID-X bins for that locus are considered variant alleles. When a potential variant allele is observed:

1. Assess sample migration and sizing quality.
   - If sample migration or sizing quality is poor, the sample shall be reinjected.
2. Check the NIST STRBase website to see if the variant has been previously reported using Fusion 6C and a CE platform.
3. If previously reported on STRBase, a variant may be accepted without further analysis. The relevant STRBase reference shall be included in the case record.
4. Consider reinjection if a variant allele has not been reported on STRbase. A variant allele not reported in STRbase may be accepted without reinjection provided that sample sizing and migration have been carefully assessed.
   - The same variant allele observed in multiple unknown samples does not require confirmation.
   - Abnormal sex chromosome variants (e.g. XXY) or variants present in an uninterpretable profile do not require confirmation.

Unresolved Peaks

Unresolved allelic and stutter peaks can result in unintuitive STRmix results. If a one base pair difference between two peaks is not resolved (e.g. a minor 20 peak unresolved from a major 19.3 allele):

1. Consider reinjection, as in some cases resolution may occur.
2. Assess sample migration and sizing quality.
   - If sample migration or sizing is poor, the sample shall be reinjected.
3. The Polynomial Degree and Peak Window size of the appropriate analysis method may be adjusted to help peak resolution.
   - This analysis parameter modification shall be documented in the case file.
   - The modified analysis method shall be exported and maintained in the electronic case record.

4. The size and height information for the unresolved peak may be manually added to the STRmix™ input file in cases where the apex of the peak is discernible.
    - A technical lead shall be consulted for this option.
5. The affected locus may be omitted from deconvolution if the above methods are unsuccessful and unintuitive STRmix™ results are obtained.

37. INTERPRETATION OF STR PROFILES

The interpretation of STR profiles takes into account multiple factors. To reduce the risk of potential bias in interpretation, analysts perform the interpretation of unknown samples to determine number of contributors, contributor genotypes, and which loci are suitable to be used for statistical calculations prior to making any comparisons to reference sample data except a conditioned or assumed profile.

Three zones for expected heterozygous peak height ratios (PHR) were established for Fusion 6C. Where at least one allele

- is above 4000 RFU, the PHR is expected to be at or above 60%.
- is between 1300 RFU and 3999 RFU, the PHR is expected to be at or above 40%.
- is between 600 RFU and 1299 RFU, the PHR is expected to be at or above 25%.

Profiles (whether single or multiple contributor) with alleles within and near the stochastic range will be interpreted with caution. The possibility of allelic dropout (not observing a heterozygous sister peak) increases in this range.

The following interpretations can be made to single and multiple contributor samples:

- **Match (Inclusion)**
  Any situation where there are no forensically significant (unexplained genetic) differences between the allele calls obtained from the reference sample and the questioned sample. Statistical analysis must be performed in support of any inclusion that is determined to be relevant in the context of a case. Statistical analysis is not required for the support of any inclusion to either non-probative samples (e.g. fraction 1 from victim's vaginal swab), elimination samples (e.g. consensual partner, husband or boyfriend) or assumed contributors (e.g. owner of the item). Even though statistics are not required as part of the conclusion for the above sample types, a description of the item (and therefore its relevance) must be included in the report.

- **Non-Match (Exclusion)**
  If at least one allele does not match for any locus between the reference sample and the question sample, and in the absence of any scientific explanation of the non-match, the donor of the reference sample may be excluded as a possible contributor of/to the questioned sample.

- **Not Interpretable, Inconclusive or Limited Interpretation**
  Some DNA typing profiles are too low level or are too complex for a variety of reasons (number of contributors, degradation, possibility of dropout, etc.).

Mixtures with an Expected DNA Profile

In mixtures where a contributor is known or expected to be present (i.e. intimate samples), assuming the presence of the known contributor's alleles may allow for determination of the remaining profile.

The following will be considered for the presence of a known contributor(s):

- Assumption of ownership for a known contributor can be made for
  - intimate items (e.g. underwear, body swabs, etc.)
  - other personal items (e.g. clothing, bedding, etc.) as long as there is a reasonable expectation of ownership documented in case file (phone notes, etc.). This assumption will be clearly stated in the report.
- Additional known contributor (e.g. consensual partner) when appropriate. This assumption will be clearly stated in the report.
- A single contributor profile obtained in one fraction of a differential extraction may be applied to the other fraction where carryover/premature lysis is suspected.

Determining Number of Contributors (NOC)

STRmix™ requires an input of the estimated number of DNA contributors in a sample on which to condition its analysis. Assumptions as to the number of contributors shall be based on the most reasonable interpretation of the data.

An estimate of the NOC is based on the locus that exhibits the greatest number of allelic peaks. Additional information such as expected peak height ratios at other loci may assist the analyst in determining the minimum NOC.

Data may be analyzed using GMID-X analysis methods with or without stutter filters enabled. However, analysis of complex mixtures or mixtures with greater than two contributors shall use a stutter filter-enabled analysis method to assist with NOC determination and shall be retained in the final GMID-X project.

The following steps will be used by the analyst when estimating the NOC and may also aid in assessing intuitiveness of STRmix results:

1. The profile should be reviewed as a whole, assessing the level of degradation, presence of low-level peaks, noisy or clean baseline, and general quality of the profile.
2. Likely stutter peaks (both forward and back) should be considered with reference to the internally validated per allele stutter ratio expectations.
3. The locus with the highest number of unambiguous allele peaks should be identified. The number of alleles present at this locus (or the number of alleles plus one if an odd number of alleles are present) divided by 2 shall be used to determine the initial postulated NOC.
4. Peak height imbalances shall be reviewed at the most informative locus/loci (greatest number of alleles). Consideration of any imbalances, along with the possibility of allele sharing or ‘stacking’, may indicate the likely presence of an additional contributor above that indicated by allele count alone. An attempt may be made to visually “pair” alleles and assign them as contributors by taking into account expected peak height/stutter ratios.
5. Possible contributor proportions across the entire profile should be considered; if one or more contributors at an informative locus was either at a trace level or was a clear major, this pattern should be checked to ensure it is represented at other loci.
    a. For evaluative purposes only, a lower contributor allele estimate can be manually calculated across multiple loci and may be used to estimate the expected peak height for that contributor at a particular locus.
        i. For each locus where a lower contributor is observed, divide the RFU value of the single observed lower contributor allele (or ½ the height if suspected to be a homozygote) by the sum of all allele heights observed at that locus. Multiply this value by 100%.
        ii. Determine the average across all loci used.
6. The general pattern of contributors (number and proportion) should be applied to all loci in the profile to determine if the NOC assigned to the profile appears reliable or if the addition or subtraction of one contributor may be more appropriate. Genetic variants such as trisomy should also be considered at loci where the general pattern does not hold across the profile.

Typically, the presence of one or two alleles above the analytical threshold should not be the sole reason for inferring an additional contributor. However, peaks observed below the analytical threshold, not in known artifact positions, and/or poor STRmix diagnostics may support the inference of a higher NOC.

The risk of false inclusions increases when additional contributors (more than the true NOC) are assumed to be present in the mixture, while the risk of false exclusions increases when fewer contributors are assumed. Therefore, it is usually more appropriate to bias estimates of NOC towards fewer contributors. Note that incorrect assignment of NOC typically only affects the likelihood ratio and genotype assignments for low level contributors.

If the NOC is ambiguous, consider re-amplification at a higher target. If all available biological methods for addressing the ambiguity have been attempted, use of varNOC may be appropriate. See the STRmix™ 2.8 procedure for additional guidance.

If the profile is determined to be from 5 or more contributors, the profile shall be reported as uninterpretable.

In rare cases where only a few alleles are present above analytical threshold, NOC assignment for STRmix may not be feasible. Consult with an FS4 or FS5. The profile may be reported as uninterpretable with Technical Leader approval.

Profiles obtained from differential extractions may be manually assessed to determine if one fraction is non-probative (e.g. fraction 1 from victim's vaginal swabs matches victim or is primarily from victim with carryover from fraction 2). Non-probative profiles and non-probative fractions of profiles do not need to be analyzed using STRmix, but shall be documented on sample or mixture analysis forms, as appropriate, if not analyzed using STRmix™.

An analyst may perform manual comparisons for simple exclusions to robust single source profiles or 2-person mixtures. Alternatively, STRmix™ may be used for all comparisons.

38. STRMIX™ V2.8

Scope

These procedures detail the use of STRmix™ software v2.8 for interpretation of PowerPlex® Fusion 6C DNA profiles. STRmix™ is used to aid the analyst in mixture analysis and likelihood ratio calculations for two-, three-, and four-person DNA profiles. STRmix™ is also used to assess (as needed) and calculate likelihood ratios for single-source profiles.

STRmix 2.8 may be used to calculate likelihood ratios for deconvolutions generated using STRmix 2.5.11 without the need to repeat the deconvolution in version 2.8.

Introduction

STRmix™ software applies a fully continuous probabilistic genotyping approach to interpretation of forensic DNA profiles. It uses biological modeling, statistical theory, computer algorithms, and probability distributions to suggest statistically weighted genotype possibilities and calculate likelihood ratios (LRs). One key component of this process involves the Markov chain Monte Carlo (MCMC) class of algorithms for sampling from a probability distribution.

Procedures

Preparing Data for STRmix™ Analysis

Prior to running a deconvolution in STRmix™ the DNA profile must be evaluated and edited appropriately, and the number of contributors (NOC) shall be assessed. Refer to the Interpretation of STR Profiles section for details on profile evaluation prior to STRmix™ analysis.

- **Evidentiary Profiles**
  1. Rename the .hid files for evidence profiles to be interpreted in STRmix to include “STRmix” at the end of the filename.
  2. All potentially probative evidence profiles shall be analyzed in GMID-X using the appropriate analysis method to ensure the below-listed stutter is retained for STRmix™ input. All other stutters shall be given an appropriate artifact label.
     a. If multiple matching complete single-source profiles are obtained in the same casefile, only one shall be analyzed using STRmix™. This deconvolution shall be used for reporting on all corresponding profiles.

	Stutter Modeled by STRmix™ v2.8				
Locus	Back	Forward	Double Back	Half Back	6bp Back
D3S1358	•	•	•		
D1S1656	•	•	•	•	
D2S441	•	•	•		
D10S1248	•	•	•		
D13S317	•	•	•		
Penta E	•	•	•		
D16S539	•	•	•		
D18S51	•	•	•	•	
D2S1338	•	•	•		
CSF1P0	•	•	•		

Penta D	•	•			
TH01	•	•	•		
vWA	•	•	•		
D21S11	•	•	•		
D7S820	•	•	•	•	
D5S818	•	•	•		
TPOX	•	•	•		
D8S1179	•	•	•		
D12S391	•	•	•		
D19S433	•	•	•		
SE33	•	•	•	•	•
D22S1045	•	•	•		
FGA	•	•	•		

3. Ensure all artifacts other than appropriate stutter are labeled.
4. Ensure any OL designations have been labeled as the appropriate artifact or with their actual allelic size designation; i.e. 30.1 (not >30).
5. Ensure there are no unresolved peaks or that unresolved peaks have been appropriately considered.
6. Export the data from the Genotypes tab using the “Export to STRmix 2.8” Table Setting.

- **Reference Profiles**
  1. All reference profiles shall be analyzed in GMID-X using the appropriate analysis method so that stutter is filtered out of the profile. No stutter peaks should be included in the STRmix™ input file.
  2. Ensure all artifacts have been labeled; i.e. pull-up.
  3. Ensure any OL designations have been labeled as the appropriate artifact or with their actual allelic size designation; i.e. 30.1 (not >30).
  4. Export the data from the Genotypes tab using the “Export to STRmix 2.8” Table Setting.

- **Manual Creation of Reference Input Files**
  Reference profiles generated using a legacy amplification kit should be re-amplified using Fusion 6C if possible. Otherwise, these profiles as well as reference profiles generated by an external laboratory, and discrete profiles from mixtures to be used for conditioning or comparison will be formatted for use in STRmix™.

  1. Open form 7238 – STRmix Ref Input. If needed, select from the bottom tabs to choose from the Fusion 6C or Identifiler Plus amplification kits.
  2. Fill the sample name in all rows of the Sample Name column.
  3. Input the profile into the Allele columns. Homozygotes need only be entered once into the Allele 1 column.
  4. Any loci with partial genotypes shall be left blank in the input file or ignored during the deconvolution. STRmix will read any locus with one allele as a homozygous locus.
  5. Save the entry as a Text (Tab delimited) file.

- **STRmix™ Analysis Initial STRmix Deconvolution**
  1. Select the Interpretation module from the main menu.
  2. In the Interpretation window, designate the following:
     a. Case Number
     b. Sample ID
     c. Case Notes (optional)

   d. Number of Contributors (utilizing a range of contributors is discussed in the varNOC section)
3. Analysis of more complex mixtures may slow down your computer performance. As needed, the Low Memory Mode may be used, and can be selected in the Run Settings.
4. Verify the selected Profiling Kit is: WSP_combined_Fusion6C_3500.
5. If a locus needs to be omitted for analysis, select the corresponding box in the IGNORE column under the LOCI heading of the Kit Settings.
   a. If a tri-allelic genotype is observed that locus shall be ignored for STRmix™ analysis.
6. Add the DNA profile data to the Evidence Profile Data box.
7. If performing analysis of a single source profile where comparison to a reference is desired, add the pertinent profile to the Reference Profile Data box. Do not add reference profiles intended for comparison to mixed profiles.
8. If an individual can be reasonably assumed, add the pertinent profile to the Reference Profile Data box.
   a. Check the Contributor to: HD box to reflect the conditioning.
   b. If there is uncertainty regarding whether a reasonably assumable individual is included as a contributor to an evidentiary profile, an unconditioned deconvolution can be performed. The assumable individual should then be compared to that deconvolution. See the LR From Previous section for additional details.
      i. If the resulting LR supports exclusion or is uninformative, the original deconvolution shall be reported. This comparison result shall also be reported.
      ii. If the resulting LR supports inclusion, then a conditioned deconvolution should be run and can be reported. This deconvolution shall be carefully examined for unintuitive genotype combinations, especially as inclusionary LR approaches 1.0. Both deconvolutions shall be saved in the case record. If the conditioned deconvolution is not reported, the inclusionary result shall be reported.
9. Once analysis has been initiated, a pop-up dialog box may appear that warns of missing expected stutter. If this occurs, review the profile and input data to confirm the specified stutter peak was not inadvertently removed during profile evaluation, and that peak resolution has occurred.
10. If an analysis does not complete (i.e. Out of Memory error) or is on pace to take too long, the analysis shall be rerun in low memory mode (see Step 3 above) and/or using a computer with additional memory.
    a. If the analysis is still unable to run to completion it will be reported as uninterpretable. See the Profile Exceeds Computational Power conclusion statement.

- **Batch Sample Analysis**
  The Batch Mode function can be used to perform deconvolutions for multiple samples consecutively. Directions for use can be found in the *STRmix v2.8 Operation Manual*.

- **varNOC**
  VarNOC may be used to deconvolute mixed DNA profiles for which a single NOC estimate cannot be determined with confidence, and it is not appropriate to use the lowest NOC estimate. VarNOC allows for a mixed DNA profile to be interpreted using a range of values for the NOC. VarNOC produces a weighting under each model of NOC that can be incorporated into an LR calculation. This provides a way to directly compare two deconvolutions for the same mixture with different NOC values.

  The varNOC function is intended for use only on rare occasions. Prior to its use the analyst should attempt all available troubleshooting methods to reduce ambiguity in the NOC; i.e. reamplification and/or resampling.

Use of VarNOC requires documented consultation with a technical lead or supervisor and requires Technical Leader notification.

varNOC Deconvolution

1. Select the Interpretation module from the main menu.
2. In the Interpretation window, designate the following:
    a. Case Number
    b. Sample ID
    c. Case Notes (optional)
3. Check the Contributor Range box.
4. Enter the determined minimum and maximum NOC. This range of contributors shall not exceed ±1.
5. Select the FBI_extended_Cauc Population for Range.
6. Verify the selected Profiling Kit is: WSP_combined_Fusion6C_3500.
7. If needed, under the LOCI header of the Kit Settings, select any loci that need to be ignored during the deconvolution.
8. Add your DNA profile data to the Evidence Profile Data box.
9. If conditioning on a reference profile, add the pertinent profile to the Reference Profile Data box.
    a. Check the Contributor to: HD box to reflect the conditioning.

    Note: The varNOC function encompasses two separate deconvolutions and an additional MC process; the run time is thus expectedly longer than that of most single deconvolutions.

Evaluation of STRmix Results

The interpretation report shall be reviewed by assessing the analysis diagnostics to determine how successfully STRmix was able to model the observed DNA profile. Diagnostics that are not intuitive or shifted from expectations may indicate that:

- The DNA profile needs further scrutiny (i.e. appropriate designation of artifacts, incomplete peak resolution, incorrect NOC)
- The deconvolution has not reached the appropriate sample space and additional iterations may be needed (failure of the MCMC chains to converge)
- The DNA profile is of poor quality

A single skewed diagnostic may not indicate an unsuccessful deconvolution. Intuitive mixture proportion estimates and genotype pairings and weights are the strongest indicator that STRmix was able to successfully model the inputted data.

Although diagnostic information helps the analyst assess the deconvolution results and guide re-assessment of data, secondary diagnostics cannot be directly compared between deconvolutions with different NOC values to troubleshoot uncertainty in NOC determination.

- **Reviewing Primary Diagnostics**
    - The proposed genotype pairings and weights at each locus shall be assessed for intuitiveness based on the observed DNA profile.
    - Mixture proportions and template amounts for each contributor should correspond to the observed DNA profile.
        a. The template amounts (RFU) for each contributor should roughly reflect the heterozygous alleles at the smaller loci.
        b. An apparent, trace level contributor with a single major contributor could cause STRmix™ to produce unintuitive contributor proportion estimates and genotype weights.

i. Re-running the deconvolution using a higher number of iterations may resolve the issue. If not, the "User Informed Mx Priors" feature may be used (see Mx Priors section below).

- The degradation values and plot should align with the observed DNA profile.
  a. For highly degraded samples, the default threshold for maximum degradation (deg max) may result in skewed diagnostics, a locus efficiencies plot that displays a steep "ski slope" pattern, contributor degradation values approaching 0.01, and/or unintuitive genotype weights.
  b. In these cases, consideration can be given to increasing the degradation max to 0.10. This can be done in the Kit Settings prior to beginning a deconvolution and shall be documented in the case file.

- **Reviewing Secondary Diagnostics**
  The following diagnostics may also be used to determine if the deconvolution has successfully converged on the best MCMC sampling space.

  - The Gelman-Rubin Convergence Diagnostic (GR)
    - The GR value indicates how well the MCMC chains have converged on the final profile deconvolution. Ideally, each of the 8 chains will be sampling the same space after burn-in.
    - A GR greater than 1.2 can indicate that the chains did not converge during analysis.
    - When the GR is greater than 1.2, STRmix will automatically run an extra 10,000 post burn-in accepts. This may improve the deconvolution and decrease the GR.
    - An elevated GR value that is less than 1.5 may not be cause for concern if primary diagnostics are intuitive, especially in the case of a more complex mixture (e.g. 3- or 4-person).
    - GR values greater than 1.5 likely warrant additional scrutiny.
      - The analysis can be re-run to see if a new seed resolves the issue.
      - The analysis can be re-run using the same seed with a 10x increase in burn-in accepts.
      - The seed can be specified under run settings by un-checking the Random box and entering the desired seed.
      - If poor diagnostics persist, consult with a technical lead, supervisor, or the DNA Technical Leader.
  - Log(likelihood)
    - This value should be greater than zero.
    - The larger this value, the better STRmix™ has been able to describe the observed data. A low or negative value suggests STRmix has not been able to describe the observed data given the information provided.
      - In these instances, further scrutiny of the profile and deconvolution may be appropriate. Reasons for a low or negative value include:
        - The profile is very low level and there is very little data making up the likelihood value.
        - The assigned NOC is wrong resulting in forced stochastic events in the STRmix™ run (e.g. large heterozygote peak imbalances or variation in mixture proportions across the profile).
        - Data has been removed that was real, particularly stutter peaks, and must now be described in STRmix™ by dropout.
        - Artifact peaks have been included in the input file and must now be accounted for in STRmix™ as drop-in.
  - Allele and stutter variance constants
    - These values indicate how well the data has been explained using the STRmix biological models.
    - If a variance constant (c^2 for allele variance and k^2 for stutter variance) has increased markedly from its respective mode, this may indicate that the data is not

being explained well by the STRmix biological models. This issue may result when a DNA profile's quality is sub-optimal or when the number of assigned contributors is incorrect.
    - Observed in conjunction with a low/negative log(likelihood) diagnostic, a large allele or stutter variance constant can indicate the deconvolution requires further scrutiny.
    - If the sample is simply low level, a low log(likelihood) and variance constants close to the mode will likely result.
    - If non-artifacts have been omitted, artifacts have been left in, or the profile was otherwise misinterpreted, a low log(likelihood) and high allele and/or stutter variances could result.

- **Discrete Contributors**
    - A single, unambiguous genotype at all examined loci in the "Component ≥99%" column of the STRmix Interpretation Report is considered a fully deduced, or discrete contributor.
    - Discrete contributors may be identified as an individual (e.g. Individual A).
    - Only discrete contributors may be compared to other profiles in the case, manually or via calculation of a likelihood ratio as appropriate.
    - If the same discrete contributor is observed in multiple samples, this "match" may be confirmed manually.
    - All comparisons to discrete contributors shall be reported; however, a statistic shall not be reported. The "LR From Previous" report shall be retained in the case record. Verbal qualifiers shall not be reported for these comparisons.

- **Mx Priors**
  Mx Priors may also aid in the interpretation of mixtures where STRmix has difficulty determining contributor proportions. Using Mx Priors for anything other than a 2-person mixture with a minor contributor less than 10% (see Step 1 below) requires approval by the DNA Technical Leader.

  Configuring Mx Priors
    - To estimate the minor contributor proportion:
        a. Divide the combined height of the unambiguous minor/trace contributor alleles (e.g. not in stutter position) by the total RFU at that locus.
        b. The total RFU at each locus is the sum of all the alleles present at that locus.
        c. Repeat for each locus where there are unambiguous minor/trace alleles and average the results. This is the estimated minor contributor proportion.
        d. Calculate the estimated major contributor proportion by subtracting the value obtained in step c from 1.0.
    - In the STRmix Run Settings, check the Use Mx Priors box
    - Select Contributor 1 from the top drop-down box.
    - Slide the "Mean" scale to the value that represents the calculated major contributor's estimated proportion.
    - Slide the "Variance" scale to 9.7656E-4.
    - Select Contributor 2 from the top drop-down box.
    - Slide the "Mean" bar to the value that represents the calculated minor contributor's estimated proportion.
    - Slide the "Variance" scale to 9.7656E-4.
    - Proceed with the remaining steps to perform a STRmix analysis.
    - If this does not result in an intuitive deconvolution, consult with a supervisor, technical lead, or the DNA Technical Leader.

<u>Troubleshooting unintuitive deconvolutions</u>

Troubleshooting approaches for cases where the deconvolution does not seem to have modeled the data well can include:

- Check for errors in the input data. Some common ones are:

  - A true allele was removed
  - An artifact was inadvertently included
  - Poor peak resolution resulted in the omission of a stutter or allelic peak that STRmix™ is expecting to be present
- Saturated peaks may lead to unintuitive deconvolution results.
- Unintuitive genotype pairings or weights at vWA, especially in combination with elevated G-R or allele variance constants, can be the result of a known issue with the stutter associated with modeling of allele 14. The true stutter may be higher that what is modeled by STRmix, resulting in the 13 peak incorrectly assigned as largely allelic. If investigation determines this to be the cause of unintuitive results, the vWA locus shall be omitted from the deconvolution.
- Re-run the STRmix™ deconvolution with an increased number of burn-in and/or post burn-in accepts.
  - Complex mixtures may benefit from 10x extra burn-in accepts to provide additional time for the MCMC analysis to reach equilibrium.
  - Post burn-in accepts may be increased by 10x or 100x (500,000 or 5,000,000 per chain).
- Re-run the STRmix™ deconvolution using “User Informed Mx Priors” or increasing the deg max setting (see above).
- Perform additional work on the sample to try to biologically improve data quality or reduce ambiguity (e.g. re-amplify or re-extract).

If STRmix™ is run with increased number of iterations, the deconvolution and likelihood ratios from the higher iteration run shall be used for interpretation. Results from other deconvolutions for the same sample need only be retained in the electronic case record.

If a STRmix analysis is inadvertently run using incorrect parameters, and the error is discovered after the technical review process has begun, the erroneous result shall be retained in the electronic case record.

Rejection of an electronically retained analysis (e.g. not suitable due to input errors) shall be documented in accordance with the QOM.

Calculation of a Likelihood Ratio

STRmix™ software shall be used for calculation of likelihood ratios. The allele frequencies used for statistical calculations in STRmix are derived from Moretti et al. (2016) and are provided with the software. No minimum allele frequency is used in statistical calculations. Instead, STRmix™ applies a “1/k+1” prior distribution for allele frequencies at each locus to calculate a posterior mean frequency for each allele.

Likelihood ratios are calculated using LR from Previous, which may be accessed directly or via Batch Mode or Investigation Batch.

1. Select LR From Previous from the Investigation module.
2. Browse to the deconvolution of interest or drag-and-drop a deconvolution folder into the dialog box.
3. Edit the Sample ID or Case Notes as desired.
4. Low memory mode can be selected under the Run Settings
5. Add the reference profile(s) for comparison to the Reference Profile Data box.
   a. Multiple reference samples may be selected to accommodate various LR propositions, however generally each reference will be compared individually.
   b. If more than one reference profile yields an inclusionary LR to the same evidentiary profile, it is recommended that a “combined LR” be calculated to determine if all such references can be included together (e.g. H_p: Reference 1 + Reference 2 and H_d: 2 unknowns).

c. If only 2 reference samples are included and this "combined LR" does not support their combined inclusion a comment shall be added to the report to reflect that, while both individuals are included, they cannot both be in the mixture.
d. If >2 two reference samples are included and this "combined LR" does not support their combined inclusion consult with a supervisor, technical lead, or the DNA Technical Leader to determine the appropriate additional LR propositions to compare.
e. The "combined LR" report need only be maintained in the electronic data and the statistic will not be reported.

6. Loci can be omitted from the comparison by selecting Kit Settings and then the LOCI heading. Check the appropriate locus boxes under the Ignore column.
    a. Partial and tri-allelic loci, when present in a reference profile, shall be ignored for the comparison.
7. For a varNOC deconvolution, select a Contributor Range Priors Method (in the LR Settings). Choosing to calculate either a stratified or maximum likelihood estimate LR will depend on the scenario.
        a. The Stratified LR assigns a weighted average LR across the numbers of contributors.
            - This is appropriate for cases when there is an inability to reasonably assign a single NOC to the profile, particularly when low level DNA is present, and no assumptions can be made for potential contributors.
        b. The Maximum Likelihood Estimate LR (MLE) assigns an LR using the contributors with the highest probability under Hp and Hd. This can result in a different NOC in the numerator and denominator of the LR.
            - This may be appropriate in cases where additional contextual information such as scenario, sample collection and known contributors is available, or where one NOC within the range gives a clear advantage to one hypothesis over the other
8. The lowest 99% 1-Sided Lower HPD interval LR shall be reported to support the inclusion or exclusion of a reference profile.
    a. For varNOC analysis, these values are located in the "Summary of LR (Variable Number of Contributors)" section of the LR report.

Evaluation of the Likelihood Ratio

The LR shall be assessed to determine if the person of interest is included, excluded, or can be neither included nor excluded. These values are based on the 2018 SWGDAM recommendations on Genotyping Results Reported as Likelihood Ratios and LR estimates for profiles at the limits of interpretation that were observed in validation.

Likelihood Ratio	**Reporting Convention**
LR ≥ 2.0	LR truncated to 2 significant figures, reported in words, e.g. 5.9 million (10^6)
1 < LR < 2.0	Uninformative
LR = 0	Exclusion (see below)
0.5 < LR < 1	Uninformative
0.01 < LR ≤ 0.5	$\frac{1}{LR}$ rounded up to 2 significant figures, reported in words. Reported as support for H_d.
LR ≤ 0.01	Exclusion

If the 99% 1-Sided Lower HPD Interval LR = 0, there are two possible explanations. Both should be evaluated before reporting the result.

- If the majority of loci yield an LR >1.0, but a few result in an LR of zero. Those loci should be evaluated for input errors, incorrect number of contributors assumed, or if an intuitively valid genotype combination was not considered by STRmix™. Rectifying errors, changing the number of contributors, or increasing the number of iterations may resolve these loci. Justification for these actions shall be documented in the case file. If resolution is not achieved by the above options consult a Supervisor, Technical Lead, or the DNA Technical Leader.
- If most loci yield a LR <1.0 and the final LR is zero, this will be reported as an exclusion.
- Occasionally, the 99% 1-Sided Lower HPD Interval will be significantly lower than the sub-source LR. This may happen when most loci result in a LR >1.0, but a small number support an exclusion and rare alleles are in the profile. If this is observed, the input profile should be checked for errors. If none are found, changing the number of contributors or increasing the number of iterations should be considered. If none of these resolve the discrepancy, the lowest sub-source LR may be reported after consultation with a Supervisor, Technical Lead, or the DNA Technical Leader.

Database Searching

The database searching feature allows for the search of an evidentiary profile against a database of DNA profiles. Each of the individuals in the database are considered a potential contributor under the two hypotheses:

H_P: Database individual and N-1 known contributors
H_D: N unknown contributors

To improve run time, the LRs from this feature do not account for theta (FST). This feature is used with the WSP elimination DNA database and LR calculations are done using the NIST Combined population. An LR threshold of 5,000 is routinely used to reduce risk of adventitious hits.

Database searches are required for all unidentified deconvolution components that are suitable for comparisons.

- **Performing A Database Search**
  - Navigate to Database Search from the Investigation module
  - Drag-and-drop a previous deconvolution folder into the Previous Interpretation box.
  - As needed, verify the settings.

- **Evaluation of Database Search Results**
  - Review the search report for any matches.
  - If a match is returned:
    a. Navigate to the “WSP STRmix Elimination Database” file and obtain the matching profile.
    b. Create a manual STRmix input file for the elimination profile.
    c. Alternatively, the “WSP STRmix Elimination Database” file can be added to the LR From Previous Reference Profile Data box, and the profile of interest selected.
    d. Calculate the LR From Previous and evaluate the results.
    e. If the LR is >5,000, contact the CODIS unit to determine the source of the elimination profile. This may also be done for LRs <5,000.
    f. Based on this source information and the LR from Previous results, determine if a quality variance report is appropriate.
       i. Reporting a quality variance is not limited to only LR from Previous results >5,000.
       ii. If a quality variance report is needed, the database search report shall be retained in the case file.
  - All STRmix database search output files shall be retained in the case record.

39. STRLITE FOR STRMIX 2.8

STRlite is an Excel workbook that aids in the analysis and documentation of mixed DNA profiles. STRlite provides worksheets to document DNA profiles, summarizes STRmix™ deconvolution diagnostics, and utilizes STRmix™ deconvolution results to aid in the determination of CODIS profiles.

STRlite is compatible for use with STRmix™ v2.8.

Using STRlite

Analyst information and the date may be entered on the STRlite Navigation tab of the Excel Ribbon.

The following functions can be accessed via the "Import" button on the STRlite Navigation tab or the menus on the Master tab:

Pre-STRmix

1. Import the GeneMapper genotype table by selecting "Import Genotypes".
2. Select the "Import Genotype Table" button and navigate to the same .txt input file used for STRmix™ analysis.
    a. Profiles within an exported genotype table can be added here as a sample (unknown) or to the references (known) worksheet, as appropriate.
3. From the *Pre-STRmix Summary* worksheet, the genotype table for any reference samples used for conditioning can be imported using the "Import Reference for Conditioning" button.
    a. This addition can be removed by selecting "Hide".
4. Optionally, the analyst may utilize the number of contributors and likelihood ratio propositions section of this worksheet for documentation purposes. The number of contributors and number of proposition sets may be changed using the appropriate buttons. The sample names or unknowns may be entered manually for each proposition.

Deconvolution (Decon)

1. Import a single or multiple STRmix deconvolutions using the respective option and navigating to the STRmix™ decon folder(s) of interest.
2. STRlite will check for an associated database search for each imported decon. Address any pop-up dialog windows regarding this as needed.
3. Use the "Send to CODIS" buttons to export profiles to the *CODIS* worksheet as appropriate. Up to two profiles from the same deconvolution can be sent to one *CODIS* worksheet.

Likelihood Ratios

1. Import an LR by selecting "Import LR".
2. Navigate to the desired STRmix LR folder and click "Add LR".
    a. Multiple LRs can be imported for inclusion on the same *STR Likelihood Ratio Summary* worksheet.
    b. STRlite will include LRs from different decons on the same *STR Likelihood Ratio Summary* worksheet when imported together.
    c. Once all the desired LR folders have been added, select "Import LRs".
3. The "Add LR" and "Remove LR" buttons on the *STR Likelihood Ratio Summary* worksheet may also be used to manually display the desired number of likelihood ratio rows.
    a. For each LR use the "Import LR" button to import the STRmix LR folder.
4. If more than eight spaces are needed for LRs, the full Likelihood Ratio worksheet can be saved for the case file (e.g. printed to PDF), then the "Clear All LRs" button can be used to allow for the importing of additional LRs following the above steps.

CODIS

1. The CODIS worksheet can be used to determine a profile's MME or MRE, as needed. Edit the CODIS sample and recheck the MME or MRE, if necessary. Loci that have been manually edited will be highlighted in yellow.
2. For MRE calculation, remove the 'M' (from the column to the right of the allele calls) at the applicable loci to designate high stringency. An 'M' designates moderate stringency.
3. Profiles for CODIS entry can be exported directly from the CODIS worksheet.
    a. Select STRlite Settings from the STRlite Navigation Tab of the Excel ribbon to specify user-specific CODIS information.
        i. Cells highlighted in green can be modified for customization.
        ii. Enter your CODIS user ID (also your GeneMapper user name) into cell G13.
        iii. Enter your lab CODIS ORI into cell G14.
    b. Enter an Item Description onto the CODIS worksheet and ensure the sample Category is correct.
    c. Indicate whether the profile matches a known profile.
    d. Click Export CMF. Note, Y loci are not included in the exported file.
    e. Proceed to the *Importing Data to CODIS – STR Data Entry* procedure for importing the CMF file to the CODIS software.

References (Standards)

1. Import the GeneMapper genotype table by selecting "Import Standards".
2. Select the "Import Genotype Table" button and navigate to the same .txt input file used for STRmix™ analysis.
3. Add the appropriate samples via the "Add to References" button.

CODIS Match Estimation

This worksheet may be used to calculate the MME and MRE for a DNA profile without needing to import a deconvolution. Profiles are entered as on the *CODIS* worksheet.

After retaining the desired worksheets for the case file, the STRlite workbook may be discarded.

40. LEGACY DATA

Occasionally, a DNA typing result that was developed using a protocol that is no longer in use may need to be further evaluated for comparison to newly submitted samples or to assess a candidate match. For Profiler Plus® and COfiler®, the analyst will refer to Revision 12 of the WSP Casework STR Analysis Procedures to find information and interpretation guidelines for these kits. For autosomal data generated using AmpFℓSTR Identifiler® Plus amplification kit, the analyst will refer to Revision 28 of the WSP Casework STR Analysis Procedures to find analysis settings and interpretation guidelines. For autosomal data generated using Promega PowerPlex® Fusion 6C and interpreted using the semi-quantitative binary approach, the analyst will refer to Revision 36 for interpretation guidelines. For Y-STR data generated using AmpFℓSTR Yfiler®, the analyst will refer to Revision 34 to find analysis settings and interpretation guidelines. The SOP revision number will be recorded in the case notes.

For autosomal data generated on an Applied Biosystems 3130 Genetic Analyzer, the analyst will refer to Revision 23 of the WSP Casework STR Analysis Procedures to find analysis settings and interpretation guidelines. For Y-STR data generated on an Applied Biosystems 3130 Genetic Analyzer, the analyst will refer to Revision 22 of the WSP Casework STR Analysis Procedures to find analysis setting and interpretation guidelines. Since the 3130 and 3500 are considered the same platform, a requalification is not necessary.

Other tasks allowed without documented requalification include:

1) Evaluating a candidate match within CODIS from a high stringency search that just involves a comparison between two single source CODIS entries that match at high stringency.
2) Comparing a casework (forensic) DNA profile interpretation that previously had the genotypes of the possible contributors documented in the mixture interpretation worksheets compared to the results of moderate stringency search candidate matches.

Tasks that require documented requalification include:

1) Moderate stringency matches between a candidate and target DNA profile where: (1) one or both of the DNA profile(s) originate from legacy data; and (2) the match involves comparisons of the original image(s) or electropherogram(s) to assess the match; are considered reinterpretation.
2) Assessing/evaluating allele calls, genotype calls (to include potential allelic drop-out), a change in the assumptions used, or removing alleles (or entire loci) from statistical estimates from legacy amplification test kit data, are all considered reinterpretation.

An analyst remains qualified for reinterpretation of data for 2 years after the last proficiency test of a legacy kit (or the last requalification, see below). If two or more years have passed the analyst must requalify following the below process prior to performing reinterpretation of legacy data.

To requalify an analyst who has been previously qualified in the legacy kit(s) complete the following training plan:

1) Review the pertinent validation data (internal lab and system write-ups)
2) Review the standard operating procedures appropriate for the typing kit as designated above. Archived procedures may be found on SharePoint.
3) Document completion of the training plan for the legacy amplification kit(s) using the Work Authorization form. Include the relevant legacy kit and platform model in the authorization details. Route the form for approval as specified for authorizations in the CLD Quality Operations Manual.

To qualify an analyst in a legacy amplification kit(s) that is proficiency tested in the current amplification kit(s) but was never previously qualified in the legacy amplification kit(s) the following procedure is followed.

1) Prepare a training plan for DNA Technical Leader approval which includes the review of the validation data and standard operating procedures of the legacy kit(s), training by a previously qualified analyst, and an interpretation competency test.

2) Complete the training plan.
3) Document completion of the training plan for the legacy amplification kit(s) using the Work Authorization form. Include the relevant legacy kit and platform model in the authorization details. Route the form for approval as specified for authorizations in the CLD Quality Operations Manual.

Technical reviewers of reinterpretations of the legacy data are held to the same training requirements as the analyst reinterpreting the data.

41. CODIS & CODIS MATCH PREDICTION

Once peer reviewed, eligible profiles that meet the guidelines developed for CODIS use will be uploaded and searched unless determined to be unsuitable for upload. All partial and mixture profiles for CODIS use will be assessed for compliance with moderate match estimation (MME) threshold requirements to determine the appropriate specimen category using the CODIS software or STRlite CODIS worksheet. Profiles that do not meet the required MME for NDIS will be assessed for compliance with the match rarity estimate (MRE) threshold requirements for the NDIS forensic targeted specimen category using the CODIS software. A local CODIS administrator shall approve entry of a sample in the NDIS forensic targeted specimen category.

Forensic unknown profiles from homicides, sexual assault/rape, kidnapping, and terrorism with a Source ID of No can be further assessed for the virtual DNA Index of Special Concern (DISC) and DISC-enabled. This virtual index requires additional case metadata to be entered into CODIS by the local CODIS administrator.

If an evidence profile is not uploaded to CODIS, the reason shall be documented in the case file. The MME, MRE, and core loci on the STRlite CODIS worksheet may serve as this documentation. If an eligible profile meets MME/MRE requirements but is deemed unsuitable for upload based on other available profiles in the case (e.g. duplicate profiles), this shall be documented in the case file.

Mixed profiles may be entered in their entirety or individual components may be deduced for CODIS entry. The Match Estimation Tool in the CODIS software or STRlite CODIS worksheet may be utilized to assess the discriminating power of mixture components to reduce the number of adventitious matches. Any CODIS match to a complex mixed profile shall be evaluated based on the peak height ratios, contributor ratios, and assumptions initially used to evaluate the profile. If it is not possible to make exclusions to the profile, then the profile is considered not interpretable and is not CODIS eligible.

See the Washington State Patrol Crime Laboratory Division Convicted Offender/CODIS Program Standard Operating Procedures for additional CODIS procedures.

Deducing Profiles for CODIS Entry

STRmix™ may be used to assist in the deduction of a profile for CODIS entry using the following rules. These are applied per contributor and locus, and the proposed genotypes for each locus are considered in order of descending weight (as determined by STRmix). If using STRlite to document a deconvolution, these rules are automatically applied when utilizing the “CODIS Contributor Summary” on the Deconvolution worksheet.

1. The cumulative weight of genotypes must reach at least 99%. STRlite stops adding possible genotypes when 99% cumulative weight for a locus is reached, however additional genotype possibilities listed on the deconvolution report may be included in the CODIS profile if their inclusion does not cause the profile to become ineligible for CODIS.
    - In rare cases, it may be warranted to slightly reduce the required cumulative genotype weight from 99% at one or two loci to allow for searching of additional indices in CODIS. This shall only occur after careful consideration of the associated risk for missing potential matches.
2. The list of genotypes must include no more than 4 different alleles.
3. An allele present in every genotype set reaching the 99% weight is designated as an obligate allele. Obligate allele designations may be omitted from the CODIS profile if omission does not cause the profile to become ineligible for CODIS.
4. If the list of genotypes for a locus includes a genotype with dropout, there must be an obligate allele in the list, and only the obligate allele may be entered into CODIS.

Note: it may be possible to combine genotype combinations from two probative contributors to enter the combined profile as a Forensic Mixture. This is more likely to occur when the contributor proportions approach 1:1.

When a mixed profile has been interpreted using varNOC, any deduced contributor component from either associated deconvolution may be entered into CODIS if deemed suitable after MME/MRE assessment. Any potential candidate matches to a single-source profile (e.g. convicted offender) shall be dispositioned by performing an LR From Previous calculation in STRmix.

For CODIS searching purposes only, it may be appropriate to repeat a deconvolution of a profile and condition on a probative known individual or an unknown individual identified elsewhere in the case to deduce a profile of another unknown individual for CODIS entry.

1. Known individual - situations where the original deconvolution and subsequent comparison of the known reference sample has determined that the known individual is a possible contributor to another mixed profile in the case (LR supports inclusion). It would then be possible to repeat the deconvolution of the profile, conditioning on the known individual, in an attempt to deduce another contributor for CODIS entry.
2. Unknown individual – situations where an unknown individual (e.g. Individual A) is identified within a case and appears to also be a contributor to a mixed profile. The deconvolution of the mixed profile may be repeated, conditioning on Individual A, to attempt to deduce a profile for another unknown individual (e.g. Individual B) or component for CODIS entry. Individual A must be a discrete contributor with a genotype weight of ≥99.00% at all loci.

The following rules are applied to deduce Y-STR alleles for CODIS entry in a multiple-male contributor mixture:

- A major allele at a Y-STR locus in F6C shall be:
  - Greater than or equal to 600 RFU
  - At least two times greater (RFU) than the lesser Y-STR allele
- If only one Y-STR allele is present and that allele is greater than or equal to 70 RFU, it can be attributed to the major.
- The Y-STR markers will be interpreted per locus (no assumption of major can be applied across the Y-STR markers).
- If the F6C major profile is female and the minor component is a two male contributor mixture, the above criteria can be applied to assess if there is a sufficient major to minor proportion difference between the two male contributors.

Disposition of SDIS Matches

If a profile is entered into an SDIS-only category and a potential candidate match to a convicted offender is returned, the following process shall be followed prior to requesting match confirmation from the CODIS lab. This process is not required for outsourced cases.

1. Create a reference input file using the convicted offender genotype on the Match Detail Report (see “Manual creation of files for STRmix™ input”).
2. Use the “LR from Previous” function to compare the convicted offender profile to the original mixture deconvolution.
3. If the convicted offender profile is not typed in Fusion 6C, the candidate match should be manually assessed for consistency in genotype weights regardless of the STRmix™ result. Consideration should be given to requesting the offender sample be re-typed in Fusion 6C.
   a. If the offender sample is re-typed in Fusion 6C, the updated profile should be used to create a STRmix reference input file and compared to the original mixture deconvolution as described in steps 1 and 2 above.

4. The results shall be technically reviewed (to ensure input data and LR are correct) and the date and initials of the technical reviewer shall be documented on the LR from Previous Report.
    a. If the lowest LR generated is an inclusion proceed with requesting match confirmation as appropriate.
    b. If the lowest LR generated is an “exclusion” or “supports exclusion”, the candidate match will be dispositioned as a “No Match” and will not be sent for match confirmation or reported.
5. Documentation retained in the case record shall include:
    a. If the LR result is an inclusion, the Match Detail Report and LR from Previous Report shall be retained in the casefile. The STRlite LR worksheet may be included in the casefile in lieu of the LR from Previous Report. The associated STRmix files shall be retained in the case record.
    b. If the LR result is an “exclusion” or “supports exclusion”, neither the Match Detail Report nor the LR from Previous Report shall be retained.

42. LABORATORY REPORTS

General report requirements may be found in the *CLD Quality Operations Manual* and the *DNA Analysis Quality Assurance Manual*. This section specifies the language used to report results of DNA analysis. Analysts should consult a technical lead forensic scientist or supervisor for reporting in situations not addressed by this policy.

Itemization of evidence items/samples

Evidence items and test samples are itemized in LIMS as needed to facilitate reporting. Item descriptions will not imply the body fluid source of DNA or activities that may have led to the deposition of DNA. Terms such as "touch," "wearer," and "blood/semen/saliva stain" will not be used to describe items or samples.

The following descriptors may be used in referring to items and samples. This list is not exhaustive and is intended to provide examples of acceptable descriptors. Combinations of the below descriptors and others not provided as examples may be used in accordance with this policy.

- Physical characteristics, e.g. "Red-brown stain collected from item 1"
- Test results, e.g. "AP-positive stain collected from item 1"
- Collection method, e.g. "M-Vac sample from item 1"
- Location, e.g. "Sample from trigger of item 1"

Quantification conclusion statements

Reports shall include a quantification conclusion statement for each sample that was Y-screened. A quantification conclusion shall also be included for each sample that is analyzed through quantification and not tested further.

Y-screening result	**Post-extraction DNA quantification result**	**Quantification conclusion statement**
No human and/or male DNA detected	Not extracted	Preliminary screening indicated no [male] DNA [on *item/sample description*].
	No male DNA detected	No male DNA was detected [on *item/sample description*].
Male DNA below extraction threshold (<0.001 ng/µL or Auto:Y >60)[5]	Not extracted	Preliminary screening indicated a low amount of male DNA [compared to the total amount of human DNA] [on *item/sample description*]. Due to the limited quantity of male DNA, this sample was not

[5] The applicable threshold depends on the intended extraction method. Refer to QUANTIFICATION: POWERQUANT™ SYSTEM DATA INTERPRETATION.

		tested further at this time.
	Male quantity sufficient for STR analysis	Male DNA was detected [on *item/sample description*].
	Male quantity insufficient for further testing	Male DNA insufficient for further testing was detected [on *item/sample description*].
	Male quantity unsuitable for STR analysis	Male DNA was detected [on *item/sample description*] at a level unsuitable for standard STR testing. [This/these sample[s] may be suitable for Y-STR testing].
	No male DNA detected	No male DNA was detected [on *item/sample description*].
Male DNA meets extraction threshold (≥0.001 ng/µL or Auto:Y ≤60)[5]	Not extracted	Preliminary screening indicated the presence of male DNA [on *item/sample description*].
	Male quantity sufficient for STR analysis	Male DNA was detected [on *item/sample description*].
	Male quantity insufficient for further testing	Male DNA insufficient for further testing was detected [on *item/sample description*].
	Male quantity unsuitable for STR analysis	Male DNA was detected [on *item/sample description*] at a level unsuitable for standard STR testing. [This/these sample[s] may be suitable for Y-STR testing].
	No male DNA detected	Preliminary screening indicated the presence of male DNA [on *item/sample description*]; however, upon additional

		analysis, no male DNA was detected.
Not tested	DNA quantity sufficient for STR analysis, no further testing	[Human] DNA [including male DNA] was detected [on *item/sample description*].
	Insufficient human or male quantity for further testing	[Human/Male] DNA insufficient for further testing was detected [on *item/sample description*].
	Human and/or male DNA insufficient for STR testing, sufficient for Y-STR testing	[Human and male/Male] DNA was detected [on *item/sample description*] at a level unsuitable for standard STR testing. [This/these sample[s] may be suitable for Y-STR testing.]
	Human DNA detected, but no male DNA detected	[Human] DNA was detected [on *item/sample description*]; however, no male DNA was detected.
	No human DNA detected	No human DNA was detected [on *item/sample description*].

DNA profile conclusion statements

Reports shall include a conclusion statement for the final interpretation of each amplified DNA sample. Conclusions shall address all of the component contributors for differentially extracted samples. This may be accomplished by reporting a single holistic conclusion for both fractions, or by reporting separate conclusions for each fraction.

Conclusion statements shall consist of the following components in order:

1. Profile summary statement
2. Discrete individual statement(s), if designating an unknown individual
3. Comparison result for each comparison made to the profile, consisting of:
    a. Likelihood ratio or non-probative association statement, when applicable
    b. Verbal scale statement (not applicable to non-probative associations unless LR is reported)
4. Compound LR exclusion statement, when applicable
5. CODIS information for each evidence profile[6], consisting of:
    a. Statement of upload, no upload, or one-time search.

[6] A CODIS statement is not required for profiles from non-probative fractions where CODIS information has been reported for the other associated fraction profile.

b. CODIS search result

Typical statements used for each component of the conclusion are provided below. Brackets "[ ]" are used to indicate text that is not applicable to all samples or that must be customized based on the sample or results. Italic text within brackets is a placeholder and intended to be replaced with sample- or result-specific information. Analysts may modify the statements for grammatical correctness as needed.

Profile summary statements

Profile result	Statement
Interpretable evidence profile obtained[7]	The DNA profile obtained [from *item description/number*/fraction *number*] was interpreted as originating from [*number of contributors*] individual[s]. [*Assumed individual(s)*] [is an/are] assumed contributor[s] to the profile.
No profile obtained	No DNA profile was obtained [from *item description/number*].
Uninterpretable profile, originates from 5 or more individuals	The DNA profile obtained [from *item description/number*] was interpreted as originating from at least five individuals and is not suitable for comparisons.
Uninterpretable profile, exceeds computational power	The DNA profile obtained [from *item description/number*] was interpreted as originating from [*number of contributors*] individual[s]. This profile is unsuitable for further interpretation due to computational limitations.
Uninterpretable profile, other reasons requiring approval by DNA technical leader	The DNA profile obtained [from *item description/number*] is not suitable for interpretation.
Interpretable reference profile obtained	A [male/female] DNA profile was obtained [from *item description/number*].

Discrete contributor statements

Discrete contributor	Statement
Discrete contributor initially designated (choose from options)	1. An unknown individual, designated Individual [*Letter*], was deduced. 2. This individual was designated Individual [*Letter*].
Discrete contributor compared (choose from options)	1. Individual [*Letter*] is included as a possible contributor to this profile. 2. Individual [*Letter*] is excluded as a contributor to this profile. 3. The comparison of Individual [*Letter*] to this profile was uninformative due to equal support for inclusion and exclusion as a possible contributor to this profile.

Multiple discrete contributor statements may be combined into one sentence, e.g. "Two unknown individuals, designated Individuals A and B, were deduced."

[7] For single source profiles, male/female designation may be included in the profile summary statement, e.g. "The DNA profile was interpreted as originating from one male individual." For mixed profiles, the presence of a male contributor within the mixture may be included in the profile summary statement, e.g. "The DNA profile obtained was interpreted as originating from two individuals, including at least one male contributor".

Likelihood ratio statements

LR result	Statement
Inclusion LR ≥ 2.0	The DNA profile is [*LR*] times more likely if [*assumed individual* and] [*compared individual*] [and *number of unknown contributors* unrelated individuals] [is/are] the contributor[s], rather than if [*assumed individual* and] [*number of unknown contributors*] unrelated individual[s] selected at random from the U.S. population [is/are] the contributor[s].
Uninformative 1.0≤LR<2.0	The DNA profile is equally likely if [*assumed individual* and] [*compared individual*] [and *number of unknown contributors* unrelated individuals] [is/are] the contributor[s], rather than if [*assumed individual* and] [*number of unknown contributors*] unrelated individual[s] selected at random from the U.S. population [is/are] the contributor[s].
Support for exclusion 0.01<LR<0.5	The DNA profile is [*1/LR*] times more likely if [*assumed individual* and] [*number of unknown contributors*] unrelated individual[s] selected at random from the U.S. population [is/are] the contributor[s], rather than if [*assumed individual* and] [*compared individual*] [and *number of unknown contributors* unrelated individuals] [is/are] the contributor[s].
Exclusion LR=0, LR≤0.01	[*No likelihood ratio reported*]
Non-probative inclusions/associations	[*No likelihood ratio reported, e.g. "This profile matches [donor of vaginal swabs]" or "This DNA profile is consistent with [donor of vaginal swabs] and contributor(s) to fraction 2."*]

[*Number of contributors*] is the total number of contributors to a mixture. This may be a single number for a standard deconvolution or a range of numbers (e.g. "3 or 4") for a varNOC deconvolution.

[*Number of unknown contributors*] is the number of contributors not accounted for as an assumed or compared individual in an LR proposition. This may be a single number or range, as for *number of contributors*.

For number of contributors to varNOC deconvolutions utilizing MLE priors, reference the number of contributors listed in the "Chosen Number of Contributors Under H_P/H_D Proposition" section of the LR report.

Verbal scale statements

LR result	Statement
LR reported	This analysis provides [*verbal qualifier*] support for the proposition that [*compared individual*] [is/is not] [a/the] contributor to this profile as compared to the proposition that [*compared individual*] [is not/is] [a/the] contributor.
Exclusion, LR=0, LR≤0.01	[*Compared individual(s)*] [is/are] excluded as [a/the] contributor[s] to this profile.
Uninformative	This analysis is uninformative because it equally supports the propositions that [*compared individual*] is a contributor and is not a contributor.

Compound LR exclusion statement

Condition	Statement
Two compared individuals are included individually but not together	However, [*compared individual 1*] and [*compared individual 2*] cannot both be included in the mixture together.

CODIS upload information

See Reports Involving CODIS for CODIS upload and CODIS search result statements.

Remarks statements

The following remarks shall be included in the remarks section of the report when the corresponding conditions are met unless noted as optional.

Condition	Statement
All analytical reports for which DNA evidence was submitted	All evidence was received sealed unless otherwise noted.
DNA work product generated	DNA work product was packaged in item [*item number*] for return to the submitting agency.
Submitted item consumed	This evidence item was consumed during analysis.
DNA extract consumed	This DNA extract was consumed during analysis.
SAK contains untested items	The sexual assault evidence collection kit also contained the following untested items: [*list items*].
Additional DNA evidence submitted, but not examined	Additional evidence was not analyzed.
All analytical reports for which DNA evidence was submitted	All items of evidence will be returned to the submitting agency. Any items pending additional testing will be routed to the appropriate laboratory prior to return.
Unresolved minor differences in spelling of names in case documentation	Differences in the names of individuals were observed on the submitted case documentation and/or evidence. Alternative names observed were [*list names*].
Microscope slide prepared but not examined	A microscope slide was prepared for the [*sample*] sample(s); however the microscopic evaluation for the presence of sperm cells was not performed. This analysis may be performed if requested.
Further testing pending consumption authorization (optional)	Further testing of this DNA extract was halted pending consumption authorization.
Y-STR testing may be appropriate or recommended (optional)	Samples in this case may be suitable for Y-STR testing. Contact the Washington State Patrol Crime Laboratory for more information.
CODIS upload	Routine searches of the CODIS database are performed. Should a potentially probative match occur, a subsequent report will be issued.
Contamination attributable to an elimination profile detected in sample or control, re-work not possible	A quality assurance elimination profile was identified in a [sample from/control sample associated with] [*item descriptions/numbers*]. This was considered in the interpretation of the sample[s].

<table>
<tr><td>Contamination not attributable to an elimination profile detected in a negative control, re-work not possible</td><td>DNA was detected in a negative control sample associated with [item description/numbers]. This was considered in the interpretation of the sample[s].</td></tr>
<tr><td>CODIS profile eligibility requires request for elimination samples – Consensual partner</td><td>Please submit reference samples from any individuals with whom [the victim or listed individual] may have had recent consensual sexual contact (within 7 days of evidence collection), as available.</td></tr>
<tr><td>Reference sample(s) needed for comparison (optional)</td><td>Please submit reference samples for comparison and/or elimination purposes as available.</td></tr>
<tr><td>Probative reference comparisons performed, regardless of LR</td><td>Statements of support for inclusionary or exclusionary propositions are based on the likelihood ratio statistic and the following ranges recommended by the Scientific Working Group on DNA Analysis Methods:

<table>
<tr><th>Verbal Qualifier</th><th>Likelihood Ratio (LR)</th></tr>
<tr><td>Very strong support for inclusion</td><td>≥1,000,000</td></tr>
<tr><td>Strong support for inclusion</td><td>10,000–999,999</td></tr>
<tr><td>Moderate support for inclusion</td><td>100–9,999</td></tr>
<tr><td>Limited support for inclusion</td><td>2.0–99</td></tr>
<tr><td>Uninformative</td><td>≥1.0 and <2.0</td></tr>
<tr><td>Limited support for exclusion (1/LR)</td><td>2.0–99</td></tr>
<tr><td>Exclusion – LR not reported (1/LR)</td><td>≥100</td></tr>
</table></td></tr>
<tr><td>Probative reference comparisons performed, regardless of LR</td><td>Likelihood ratios compare the probabilities of observing a DNA profile under two proposed explanations of the evidence. These propositions are based on relevant information available to the analyst at the time of examination. If new information is received that would alter these assumptions or alternate propositions are required, a new interpretation may be necessary.</td></tr>
<tr><td>DNA profile(s) obtained</td><td>DNA interpretations cannot determine how or when the DNA was deposited, nor can it identify an individual as the source of the DNA deposited.</td></tr>
<tr><td>Male and/or female designations are included in a conclusion</td><td>Male and female designations are based on the respective detection or absence of the Y-chromosome. Genetic testing results may or may not correspond with an individual's assigned sex at birth and/or gender.</td></tr>
<tr><td>All analytical reports</td><td>This report does not contain all the documentation associated with the work performed. Independent analysis and interpretation of the data requires a review of the full case record.</td></tr>
<tr><td>All analytical reports</td><td>The results and conclusions presented in this report include the interpretations of the forensic scientist whose signature appears on the report.</td></tr>
<tr><td>Report encompasses work under multiple requests</td><td>This report includes analysis performed for request(s) [additional request number(s)].</td></tr>
<tr><td>Report includes reference to work from a previous request (optional)</td><td>For previous analysis, see Washington State Patrol Crime Laboratory Report for case [applicable case number and request number(s)]</td></tr>
</table>

Methods statements

The following statements shall be included in the methods section of the report when the corresponding conditions are met.

Condition	Statement
Extracted samples	Sample(s) were extracted for DNA content.
Quantified samples	Sample(s) were quantified for human and male DNA content.
Amplified samples	Short tandem repeat (STR) DNA profiles were developed using the PowerPlex Fusion 6C amplification kit and a 3500 Genetic Analyzer.
STRmix used for profile interpretation/statistics	STR DNA profile interpretation and statistical calculations are performed using STRmix software, version 2.8.0.

Glossary definitions

The following statements shall be included in the glossary section of the report when the corresponding conditions are met.

Condition	Statement
Differential extraction performed	Items separated into fraction 1 and fraction 2 were processed using a differential extraction method that aims to separate non-sperm cell DNA (fraction 1) from sperm cell DNA (fraction 2), if present. The use of this method and subsequent detection or absence of male DNA alone does not imply the presence or absence of semen.

43. REPORTS INVOLVING CODIS

This section specifies the language used to report CODIS uploads and search results See Laboratory Reports for DNA analysis reporting requirements. Analysts should consult a technical lead forensic scientist or supervisor for reporting in situations not addressed by this policy. Analysts may modify the statements for grammatical correctness as needed.

Reports shall clearly convey information on CODIS upload/search, ineligibility, and search results. A report shall be generated in the following situations:

- The first time an evidence profile is a) entered in the database or b) deemed ineligible or unsuitable for upload.
- Any time a DNA profile is searched as a one-time event.
- Any time a positive association is made with an entered DNA profile
    - Conviction matches where a) a reference sample was previously typed or b) there was a previous offender hit do not require reporting.

Prior to reporting conviction matches, resolution of the case shall be verified either via communication with the LEA or in a local court system, or via criminal history records.

Positive associations involving a solved/unsolved case(s) within the same agency may be reported in one report. Both case files will retain the necessary CODIS paperwork. The analyst not generating the report will indicate in their documentation which laboratory request contains this reported information.

Likelihood ratios calculated to determine CODIS match dispositions shall not be reported.

CODIS Conclusion Statements

Result	Conclusion Statement
Interpretable profile or component(s) of interpretable mixture not uploaded (choose from options)	1. This DNA profile is not [eligible/suitable] for entry into the Combined DNA Index System (CODIS). 2. No [deduced/additional/remaining] components of this DNA profile are [eligible/suitable] for entry into the Combined DNA Index System (CODIS). 3. No DNA profile was entered into the Combined DNA Index System (CODIS).
No hit upon initial upload	[[An] eligible component[s] of] this DNA profile [obtained from *item description/number*] was entered into the Combined DNA Index System (CODIS) and searched. No [probative] matches were found.
No hit upon 1x suspect search	A one-time search of this DNA profile was performed against the state level of the Combined DNA Index System (CODIS) and no [probative] matches were found.

Hit to offender sample upon initial upload	[[An] eligible component[s] of] this DNA profile [obtained from [*item description/number*] was entered into the Combined DNA Index System (CODIS) and searched. A [match/possible association] was declared with the following individual: Name: [*Offender Name]* [DOB: *Offender date of birth*] [SID/DOC Number/FBI Number: *Identifying number*] Please submit a known reference sample from this individual to the Washington State Patrol [*location*] Crime Laboratory for confirmation. [No [matches/associations] occurred to the remaining component[s].]
UHR hit to offender sample upon initial upload	This DNA profile [obtained from [*item description/number*] was entered into the Combined DNA Index System (CODIS) and searched. A [match/possible association] was declared with the following individual: Name: [*Offender Name]* [DOB: *Offender date of birth*] [SID/DOC Number/FBI Number: *Identifying number*] Please provide this investigative information to the appropriate legal authority (e.g. medical examiner, coroner, or state anthropologist) to assist with the identification of the remains. If an identification is made, please provide that information (e.g. a death certificate or other notification) to the Washington State Patrol Crime Laboratory.
Case to case hit upon initial upload NOTE: Inclusion of the agency contact information may not be necessary if both DNA profiles originated from cases within the same agency	[[An] eligible component[s] of] this DNA profile [obtained from *item description/number*] was entered into the Combined DNA Index System (CODIS) and searched. A [match/possible association] was declared with a DNA profile obtained from the following case: Item: [*item description/number*] Agency: [*Agency Name*] Agency case: [a*gency case number*] Agency representative: [a*gency representative name]* Contact information: [*phone number and/or email address]* For additional information please contact the agency representative listed above. [No [matches/associations] occurred to the remaining component[s].]

UHR hit to forensic/missing persons profile upon initial upload NOTE 1: Inclusion of the agency contact information may not be necessary if both DNA profiles originated from cases within the same agency. NOTE 2: The legal authority statement should be omitted for associations that do not identify an individual (e.g. a hit to a forensic mixture or profile with a Source ID of "No").	This DNA profile [obtained from *item description/number*] was entered into the Combined DNA Index System (CODIS) and searched. A [match/possible association] was declared with a DNA profile obtained from the following case: Item: [*item description/number*] Agency: [*Agency Name*] Agency case: [a*gency case number*] Agency representative: [a*gency representative name]* Contact information: [*phone number and/or email address]* For additional information please contact the agency representative listed above. Please provide this investigative information to the appropriate legal authority (e.g. medical examiner, coroner, or state anthropologist) to assist with the identification of the remains. If an identification is made, please provide that information (e.g. a death certificate or other notification) to the Washington State Patrol Crime Laboratory.
Hit upon one-time suspect search NOTE: Inclusion of the agency contact information may not be necessary if both DNA profiles originated from cases within the same agency	A one-time search of this DNA profile was performed against the state level of the Combined DNA Index System (CODIS) and a match was declared with a DNA profile obtained from the following case: Item: [*item description/number*] Agency: [*Agency Name*] Agency case: [a*gency case number*] Agency representative: [a*gency representative name]* Contact information: [*phone number and/or email address]* For additional information please contact the agency representative listed above.
Conviction match upon initial upload - Only where there is no previously typed reference or previous offender hit	[An eligible component[s] of] this DNA profile [obtained from *item description/number*] was entered into the Combined DNA Index System (CODIS) and searched. A possible association occurred to the following individual: Name: [*Offender name*] [DOB: *Offender date of birth*] [SID/DOC Number/FBI Number: *Identifying number*] Based on information received, this individual has already been associated with this case. If confirmation is required, please submit a known reference sample from [*offender's name*] to the Washington State Patrol [*location*] Crime Laboratory.
Ownership review case hit	It is requested that a known reference sample from [*offender name*] be submitted to [*vendor laboratory name*]. Please contact the Washington State Patrol Crime Laboratory at dnaoutsourcing@wsp.wa.gov to arrange for this submission.

Match to a sample from the elimination database	This DNA profile [obtained from *item description/number*/of Individual *Letter*] was searched against the state level of the Combined DNA Index System (CODIS) and a [match/possible association] to a profile in the elimination database was declared. The elimination database contains DNA profiles from WSP Crime Laboratory Division staff, law enforcement representatives, and other known individuals. The DNA profile [obtained from *item description/number*] was removed from CODIS.
CODIS Match Request for hit to offender sample	As a result of ongoing, automated searching of the Combined DNA Index System (CODIS), a [match/possible association] occurred between the DNA profile previously obtained from [*item description/number*] and the following individual: Name: [*Offender Name]* [DOB: *Offender date of birth*] [SID/DOC Number/FBI Number: *Identifying number*] Please submit a known reference sample from this individual to the Washington State Patrol [*location*] Crime Laboratory for confirmation. For previous analysis [of [*item description/number]]*, see Washington State Patrol Crime Laboratory Report for case [*case number and request number(s)].*
CODIS Match Request for UHR hit to offender sample	As a result of ongoing, automated searching of the Combined DNA Index System (CODIS), a [match/possible association] occurred between the DNA profile previously obtained from [*item description/number*] and the following individual: Name: [*Offender Name]* [DOB: *Offender date of birth*] [SID/DOC Number/FBI Number: *Identifying number*] Please provide this investigative information to the appropriate legal authority (e.g. medical examiner, coroner, or state anthropologist) to assist with the identification of the remains. If an identification is made, please provide that information (e.g. a death certificate or other notification) to the Washington State Patrol Crime Laboratory.

CODIS Match Request for case-to-case hit NOTE: Inclusion of the agency contact information may not be necessary if both DNA profiles originated from cases within the same agency	As a result of ongoing, automated searching of the Combined DNA Index System (CODIS), a [match/possible association] occurred between the DNA profile previously obtained from [*item description/number*] and the following: Item: [*item description/number*] Agency: [*Agency Name*] Agency case: [a*gency case number*] Agency representative: [a*gency representative name]* Contact information: [*phone number and/or email address]* For additional information please contact the agency representative listed above. For previous analysis [of [*item description/number]]*, see Washington State Patrol Crime Laboratory Report for case [*case number and request number(s)].*
CODIS Match Request for UHR hit to forensic/missing persons profile NOTE 1: Inclusion of the agency contact information may not be necessary if both DNA profiles originated from cases within the same agency. NOTE 2: The legal authority statement should be omitted for associations that do not identify an individual (e.g. a hit to a forensic mixture or profile with a Source ID of "No").	As a result of ongoing, automated searching of the Combined DNA Index System (CODIS), a [match/possible association] occurred between the DNA profile previously obtained from [*item description/number*] and the following: Item: [*item description/number*] Agency: [*Agency Name*] Agency case: [a*gency case number*] Agency representative: [a*gency representative name]* Contact information: [*phone number and/or email address]* For additional information please contact the agency representative listed above. Please provide this investigative information to the appropriate legal authority (e.g. medical examiner, coroner, or state anthropologist) to assist with the identification of the remains. If an identification is made, please provide that information (e.g. a death certificate or other notification) to the Washington State Patrol Crime Laboratory.

CODIS Match Request for ownership review case	As a result of ongoing, automated searching of the Combined DNA Index System (CODIS), a [match/possible association] occurred between the DNA profile previously obtained from [*item description/number*] and the following: Name: [*Offender Name]* [DOB: *Offender date of birth*] [SID/DOC Number/FBI Number: *Identifying number*] It is requested that a known reference sample from [*offender name*] be submitted to [*vendor laboratory name*]. Please contact the Washington State Patrol Crime Laboratory at dnaoutsourcing@wsp.wa.gov to arrange for this submission. For previous analysis [of [*item description/number]]*, see Washington State Patrol Crime Laboratory Report for case [*case number and request number(s)].*

44. KINSHIP ANALYSIS

In cases involving questioned parentage or identity, there may be a need to provide kinship calculations to evaluate support for a biological parentage, full sibling, half sibling, aunt/uncle-niece/nephew, or grandparent-grandchild relationship based on the DNA profiles obtained. For scenarios not covered, contact the DNA Technical Lead for assistance.

In the cases of alleged parentage, the data is initially assessed for an inheritance pattern. When an exclusion cannot be made, the kinship index (KI) in the form of a likelihood ratio (LR) shall be calculated using CODIS Popstats and reported with the appropriate verbal qualifier as described below.

Though linkage between vWA and D12S391 can affect the KI in some situations, in simple cases where each tested individual has no more than one tested offspring, linkage is not expected to have an effect. In these cases, both vWA and D12S391 shall be included for the parentage calculations in Popstats.

Inclusionary LRs shall be reported by truncating the smallest LR from the three required populations to the second significant digit. When reported, exclusionary LRs shall be reported by taking the lowest LR from the three required populations, then rounding $\frac{1}{LR}$ up to the second significant digit.

The allele frequencies used for statistical calculations in Popstats are derived from Moretti et al. (2016) and are provided with the software. A 5/2N minimum allele frequency is applied within Popstats.

One-parent kinship index

This analysis is applicable for cases where one known parent is tested: Paternity determination with a known mother, child, and alleged biological father, or maternity determination with a known father, child, and alleged biological mother.

1. Compare the known parent and child DNA profiles at each locus to deduce the alleles contributed by the unknown parent:
    a. Where the known parent and child share just one allele, the other allele found in the child is from the unknown parent.
    b. Where the known parent and child are homozygous, this allele is also from the unknown parent.
    c. Where the known parent and child are heterozygous and share both alleles, either allele could be from the unknown parent.

2. Compare the alleged parent's DNA profile to the deduced haplotype of the unknown parent.
    a. If the DNA profiles match at all loci, the alleged parent cannot be excluded as a biological parent of the child, and an LR shall be calculated.
    b. If the alleged parent profile and deduced parental haplotype share one allele in common at all except one or two loci, the alleged parent cannot be excluded as the biological parent of the child. An LR incorporating the probability of mutations at any non-matching loci shall be calculated.
        - The locus LR accounting for the possibility of mutation is calculated as $\frac{\mu}{\bar{A}}$, where μ is the locus-specific mutation rate and $\bar{A}$ is the mean power of exclusion.
    c. If the alleged parent profile and deduced parental haplotype do not have one allele in common at three or more loci, the alleged parent is excluded as the biological parent.

3. Calculate the Single Parentage PI Using Popstats
    a. Select Parentage from the Popstats Calculations menu.
    b. Select the Trio subtype and enter all alleles for each locus to be used in the calculation.

- If a mutation is observed between the known parent and child, but no mismatch is observed at that locus in the alleged parent, this locus shall be omitted from the calculation. This is necessary to accommodate limitations within Popstats.
- If a mutation is observed between the known parent and child and a mismatch is also observed at that locus in the alleged parent, then the Zero parent kinship index shall be calculated for the child and alleged parent.
- If a mutation is observed between only the child and alleged parent, then proceed with the calculation.

c. Select Calculate.
d. Use the default mutation rate and mean power of exclusion where applicable.
e. Retain the Popstats Single Parentage Statistics report for the case file.

4. Conclusion Statements
   a. Inclusion
   [Tested child]'s DNA profile is [*Total Parentage Index*] times more likely if [*Known Parent*] and [*Alleged Parent*] are the biological parents, rather than if [*Known Parent*] and an unrelated individual selected at random from the U.S. population are the biological parents. This analysis provides [*Verbal Scale*] support for the proposition that [*Alleged Parent*] is the biological [father/mother] of [*Tested Child*] as compared to the proposition that [*Alleged Parent*] is not the biological [father/mother].
   b. Exclusion
   [*Alleged Parent]* is excluded as the biological father/mother of [*Tested Child*].

Zero-parent kinship index

This analysis is applicable for cases where no known parents are tested, i.e. only the child's and an alleged parent's reference samples are available.

1. Compare the alleged parent's DNA profile to the child's DNA profile.
   a. If the DNA profiles share at least one allele at all loci, the alleged parent cannot be excluded as a biological parent of the child and an LR shall be calculated.
   b. If the alleged parent and child profiles share at least one allele in common at all except one or two loci, the alleged parent cannot be excluded as the biological parent of the child. An LR incorporating the probability of mutations at any non-matching loci shall be calculated.
      - The locus LR accounting for the possibility of mutation is calculated as $\frac{\mu}{\bar{A}}$, where μ is the locus-specific mutation rate and $\bar{A}$ is the mean power of exclusion.
   c. If the alleged parent and child profiles do not have at least one allele in common at three or more loci, the alleged parent is excluded as the biological parent.

2. Calculate the Zero-Parent PI Using Popstats
   a. Select Kinship from the Popstats Calculations menu.
   b. Ensure only the PO (Parent-Offspring) kinship relationship is selected for the calculation.
   c. Enter all alleles for each locus to be used in the calculation.
   d. Select Calculate.
   e. Use the default mutation rate and mean power of exclusion where applicable.
   f. Retain the Popstats Single Parentage Statistics report for the case file.

3. Conclusion Statements
   a. Inclusion
   It is [*Single Parentage Index*] times more likely to observe the DNA profile obtained from [*Tested Child*] if [*Alleged Parent*] is the biological [father/mother] of [*Tested Child*], rather than if an unrelated individual selected at random from the U.S. population is the biological [father/mother]. This analysis provides [verbal scale] support for the

proposition that [*Alleged Parent*] is the biological [father/mother] of [*Tested Child*] as compared to the proposition that [*Alleged Parent*] is not the biological [father/mother].

b. Exclusion
[*Alleged Parent]* is excluded as the biological father/mother of [*Tested Child*].

Reverse parentage kinship index

This analysis is applicable for cases where neither tested parent can be assumed as a known parent, such as parentage determination for a child and two alleged parents and is often used for missing person identification.

1. Compare the alleged parents' DNA profiles to the child's DNA profile.
    a. If the child's DNA profile can be explained by sharing at least one allele with each parent at all loci, the alleged parents cannot be excluded as the biological parents of the child and an LR shall be calculated.
    b. If the child's DNA profile can be explained by sharing at least one allele with each parent at all except one or two loci, the alleged parents cannot be excluded as the biological parents of the child and mutations must be considered at those loci when calculating the LR.
    c. If the child's DNA profile does not share at least one allele with each parent at three or more loci, the alleged parents are excluded as the biological parents.

2. Calculate the Reverse PI Using Popstats
    a. Select Parentage from the Popstats Calculations menu.
    b. Select the Reverse subtype and enter all alleles for each locus to be used in the calculation.
        - Popstats cannot accommodate mutations within reverse parentage calculations. If a mismatch is observed between an alleged parent and the child, then the Zero Parentage KI shall be calculated for the child and each alleged parent.
    c. Select Calculate.
    d. Retain the report for the case file.

3. Conclusion Statements
    a. Inclusion
    It is [*Reverse Parentage Index*] times more likely to observe the DNA profile obtained from [*Tested Child*] if [*Alleged Parent 1*] and [*Alleged Parent 2*] are the biological parents of [*Tested Child*], rather than if two unrelated individuals selected at random from the U.S. population are the biological parents. This analysis provides [verbal scale] support for the proposition that [*Alleged Parent 1*] and [*Alleged Parent 2*] are the biological parents of [*Tested Child*] as compared to the proposition that [*Alleged Parent 1*] and [*Alleged Parent 2*] are not the biological parents.
    b. Exclusion
    [*Alleged Parent 1*] and [*Alleged Parent 2*] are excluded as the biological parents of [*Tested Child*].

Two-person kinship index for full sibling and second-degree relationships

This analysis applies to relationship determination with a test individual and an alleged full sibling, half sibling, aunt/uncle/niece/nephew, or grandparent/grandchild.

Full siblings and second-degree relatives are not required to share any genetic material due to inheritance, so initial comparison between the two DNA profiles need not be performed. The kinship index in the form of an LR will be calculated using Popstats and reported along with the appropriate verbal qualifier as described below.

1. Calculate the Kinship Index (KI) for full siblings, half siblings, aunt/uncle/niece/nephew or grandparent/grandchild relationships using FBI Popstats.
    a. Select Kinship from the Popstats Calculations menu.
    b. Select the pertinent relationship with unrelated parents.
    c. Enter all alleles for each locus to be used in the calculation.
    d. Omit vWA from the calculation due to possible linkage disequilibrium with D12S391.
    e. Select Calculate.
    f. Retain the “Kinship Statistics Summary” for the case file. Alternatively, if individual locus LRs are needed, the “Kinship Locus Report” may be retained.

2. Conclusion Statements
    a. Inclusion
    The DNA result is [*Likelihood Ratio*] times more likely if [*Reference 1*] is the [*Tested Relationship*] of [*Reference 2*], rather than if an unrelated individual selected at random from the U.S. population is the [*Tested Relationship*]. This analysis provides [*verbal scale*] support for the proposition that [*Reference 1*] and [*Reference 2*] are [*Tested Relationship*] as compared to the proposition that [*Reference 1*] and [*Reference 2*] are not [*Tested Relationship*].

    b. Exclusion (LR=0, LR<0.01)
    [Reference 1] is excluded as a/the [Tested Relationship] of [Reference 2].

    c. Support for Exclusion (0.01<LR<0.5)
    The DNA result is [1/*Likelihood Ratio*] times more likely if [*Reference 2*] is the [*Tested Relationship*] of an unrelated individual selected at random from the U.S. population, rather than if [*Reference 1]* is the [*Tested Relationship*]. This analysis provides [*verbal scale*] support for the proposition that [*Reference 1*] and [*Reference 2*] are not [*Tested Relationship*] as compared to the proposition that that [*Reference 1*] and [*Reference 2*] are [*Tested Relationship*].

    d. Uninformative (1.0≤LR<2.0)
    The DNA result is equally likely if [*Reference 1*] is the [*Tested Relationship*] of [*Reference 2*], rather than if an unrelated individual selected at random from the U.S. population is the [*Tested Relationship*]. This analysis is uninformative because it equally supports the propositions that [*Reference 1] and* [*Reference 2*] are and are not [*Tested Relationship*].

Methods statements

The following statements shall be included in the methods section of the report when the corresponding conditions are met. Refer to 42 for required statements not specific to kinship testing.

Condition	Statement
Kinship calculations performed	Statistical calculations for kinship analysis were computed by Popstats.

Remarks statements

The following remarks shall be included in reports that include kinship analysis when the corresponding conditions are met. Refer to Laboratory Reports for required remarks not specific to kinship testing.

Condition	Statement

<table>
<tr><td>Kinship calculations performed, regardless of LR</td><td>Statements of support for inclusionary or exclusionary propositions are based on the likelihood ratio statistic and the following ranges recommended by the Scientific Working Group on DNA Analysis Methods:

<table><tr><th>Verbal Qualifier</th><th>Likelihood Ratio (LR)</th></tr><tr><td>Very strong support for inclusion</td><td>≥1,000,000</td></tr><tr><td>Strong support for inclusion</td><td>10,000–999,999</td></tr><tr><td>Moderate support for inclusion</td><td>100–9,999</td></tr><tr><td>Limited support for inclusion</td><td>2.0–99</td></tr><tr><td>Uninformative</td><td>≥1.0 and <2.0</td></tr><tr><td>Limited support for exclusion (1/LR)</td><td>2.0–99</td></tr><tr><td>Exclusion – LR not reported (1/LR)</td><td>≥100</td></tr></table></td></tr>
<tr><td>Possible mutation observed</td><td>The calculated parentage statistic accounted for possible mutation between the alleged parent and child.</td></tr>
</table>

45. CODIS EXPORT

To successfully execute this procedure, the analysis of data and creation of the CODIS export file must be done using a GMIDx login that is the same as the user's CODIS login name.

Establish Source and Destination Labs for CODIS Export Files

1.) In GMIDx, go to Tools > CODIS Export Manager.
2.) Under Source Lab IDs, enter the source lab ORI and select 'add'.
3.) Under Destination Lab IDs, enter the destination lab ORI and select 'add'.
    a. Note: the source lab and destination lab ORI will be the same and will be the ORI of the lab that generated the profile being imported into the CODIS software.
    b. You can now delete the "srclab" source lab and "destlab" destination lab IDs in CODIS Export Manager.
4.) Click "OK".
    a. The added lab IDs will stay with GMIDx from this point forward.

Preparation for creating a CODIS export file

1.) Prior to attempting to create a CODIS export file, complete any needed GMIDx artifact edits to the profile(s) of interest.
2.) It is recommended that you now save a "copy" of your GMIDx project, adding the designation "CODIS" to the name. The "CODIS" designated project will be a temporary project used only for exporting profiles to CODIS and will not be retained.
3.) Make any additional allele edits to the profile(s) of interest working from your "CODIS" designated project. This may include deleting peaks not intended for CODIS export or adding allele labels to stutter peaks that should be exported.
4.) From the GMIDx Samples tab, select the "CODIS Export" table setting (if you do not have the CODIS Export table setting option, you can import it from the GMIDx Database Defaults Table Settings folder using the GMIDx Manager window).
5.) For the profile(s) of interest, choose the correct Specimen Category from the drop-down (e.g. "Forensic, Unknown", "Forensic Mixture", "Suspect"). The specimen category can be updated if needed once the profile is imported into CODIS.
6.) Leave the Specimen Category as "No Export" for any samples not to be included in the CODIS export file. If exporting profiles for STR Data Entry, multiple profiles can be exported in one CODIS export file. If exporting profiles for Popstats Calculations, each profile needs to be exported into a separate CODIS export file.
7.) Optional: type the sample name, as it is desired to appear in CODIS, into each sample's corresponding UD1 column.

NOTE: No brackets or parentheses may be present on peaks to be exported. GMIDx will not allow the OL designation to be exported, so if a true allele, OL's must be given an allele designation prior to creating the CODIS export file.

Creating a CODIS Export File

1.) Following completion of the "Preparation for creating a CODIS export file" steps above**,** from the GMIDx Samples tab, choose File > Export Table for CODIS.
2.) Select export file type CMF 3.2 (.xml).
3.) Select the appropriate source and destination CODIS laboratory IDs, if not already defaulting correctly.
4.) Enter the desired file name.
5.) Navigate to the desired export file location. e.g. Computer > Local Disk (USB Thumb Drive).
6.) Click "Export".

NOTE: Once a CMF file has been exported from GMIDx, it cannot be edited – CODIS will not accept the file if it has been altered after creation.

Importing Data to CODIS – STR Data Entry

1.) In the CODIS Software, open Specimen Manager from the Workbench Explorer.
2.) At the top of the window, click on the Specimen Manager drop-down and select "Import Specimens".
    a. Navigate to and select the desired file to be imported from your portable media storage device.
    b. Click "Open".
    c. Ensure the Import Type is "Data Import" and the Assign to User field is associated with the analyst that produced the DNA profile.
    d. Click "OK".
        i. A message that the file imported successfully should appear, click "OK". The profile(s) have now been imported into the CODIS Software, but have not yet been entered as specimens in Specimen Manager.
3.) Open Message Center.
    a. Double-click "Import STR Files" in the navigation pane.
        i. The imported file should be present in bold – select by double-clicking on the file. If the imported file is not present in bold, click 'Refresh' in the Import STR Files drop-down at the top of the window.
        ii. In this step, the CODIS software is validating the import file for suitability. If the validation passes, the profile(s) contained in the import file will now be entered as specimens in Specimen Manager.
    b. Double-click "Import Reports" in the navigation pane.
        i. The imported file report should be present in bold – select by double-clicking on the file. If the imported file report is not present in bold, click 'Refresh' in the Import STR Files drop-down at the top of the window.
        ii. Two reports will now open, the LDIS Import Reconciliation Summary and Details Reports. Review both reports for accuracy and for the presence of any error codes. If any error codes are present, see your local CODIS Administrator. (Error codes will show up on the Details Report to the left of each profile's sample name).
        iii. Close the Reconciliation Reports after completion of review.
4.) Open STR Data Entry.
    a. Query for the imported specimen(s).
    b. Select the appropriate sample and click "OK".
    c. Update the Specimen ID, Specimen Category, Source ID, and Partial Profile Indicators as necessary.
    d. Save the LDIS Specimen Detail Report for the case file.

Importing Data to CODIS – Popstats

1.) In the CODIS Software, open Popstats.
2.) Choose the desired Popstats Calculations option (e.g. Forensic Single Source, Forensic Mixture, Match Estimation).
3.) In the Target Profile window, click "Import".
4.) Navigate to and select the desired file to be imported from your portable media storage device.
5.) Click "Open".
6.) Continue with calculations according to current casework practices.
    1. For calculations that require more than one input (e.g. Forensic Mixture: Mixture, H1, H2), GMIDx can be used to create a tailored CODIS export file for each input with allele labels added to account for stutter or peaks deleted as needed.

46. CASE DOCUMENTATION

Scope

This policy describes requirements for documenting evidence examinations/laboratory analyses and the format and storage of case documentation.

Generating case documentation

Laboratory reports are generated in LIMS. Other case documentation may be handwritten or generated digitally.

Handwritten and other physical case documentation will be converted to digital format by scanning to a PDF file in a format representing the document (i.e. black and white, or color) at a resolution of 300 dpi or greater. Physical documentation is considered a draft copy and will be destroyed after administrative review of the case file.

Case files generated for RFLEs received on or after January 1, 2025, will consist of PDF documents. The draft case file will be compiled prior to technical review. The draft case file should be printed to PDF to "flatten" it prior to submitting it for technical review. Throughout the technical and administrative review processes, the draft case file is edited/annotated as needed using comment features in Acrobat. All edits/annotations will be identified with the date of the change and initials of the individual making the change. The final case file and analytical data will be moved to the completed case folder upon completion of administrative review.

Case record storage locations

The case record consists of all administrative documentation and examination documentation that apply to a case. Examples of administrative documentation commonly associated with a DNA case include:

- RFLE/SAK RFLE
- Police report
- Authorization to consume evidence/extracts
- DNA Supplemental Information form
- Emails, phone conversation notes, and other documentation of communications

Technical records are all records related to the case except administrative documentation, including:

- Examination documentation
- Analytical data
- Laboratory reports

Case records are retained in the following locations for long-term storage:

1. Administrative documentation other than the RFLE/SAK RFLE is retained in the case file, the completed case folder, uploaded as an attachment to the case in LIMS, or documented in the Case Info – Synopsis field in LIMS.
    - Refer to the QOM for RFLE/SAK RFLE retention requirements.
2. Examination documentation is retained in the case file.
3. The case file and analytical data are retained in a completed case folder at the following network location: \\FileShare\WSP\FLSB\CLD\Functional Areas\DNA\[Year] Completed Cases\[Case Number]\.
    a. The completed case folder name will follow the format "LYY-NNNNNN-RRRR", where LYY-NNNNNN is the nine-digit case number and RRRR is the four-digit request number corresponding to the request number used for the laboratory report, as assigned in LIMS.

   b. Access to this storage location will be limited to read and write permissions to avoid inadvertent data loss. Designated individuals will be granted modify permissions to facilitate deleting and organizing files as needed or by request.
4. Draft laboratory reports and technical reviewer observations are retained in a “Review” folder within the completed case folder.
5. Laboratory reports are retained in LIMS.

Case file

The case file contains the primary information needed to understand the evidence examined, analysis performed, and the results and interpretations of testing.

Case file format and administrative requirements

Case files will adhere to the following requirements:
1. Case files will be named as follows, where LYY-NNNNNN-RRRR represents the case and request numbers as assigned in LIMS: “LYY-NNNNNN-RRRR-Case File”
   a. If a case file contains work performed for multiple requests, the case file name will use the request number corresponding to the laboratory report.
2. Each page of the case file will be marked with:
   a. Date the documentation was generated/analytical process was performed
   b. Initials of the case analyst
   c. Page number
3. The first page will be marked with the total number of pages in the case file
4. All annotations in the final case file must be visible on the case file pages and not retained solely as metadata (e.g. Acrobat sticky notes)

Case file content

Case files will contain the following documentation when applicable:
1. Evidence examination notes, sketches, and photographs
2. DNA analysis worksheets for the following processes:
   a. Extraction
   b. Quantitation
   c. Amplification
   d. CE detection, sample location, and analysis review
   e. Evidence and reference sample analysis (e.g., mixture or single source profile worksheet, STRmix Evidence Input File table from the deconvolution report, STRlite Pre-STRmix worksheet, STRlite Standards worksheet, or allele table)
3. STRmix documentation for the final interpretation of each sample:
   a. The STRlite Deconvolution worksheet
      - Alternatively, the STRmix Deconvolution Report, up to and including the evidence input file may be used to document a deconvolution. Pages that contain only “Component Interpretation” of unknown, CODIS ineligible contributors may be omitted from the case file.
   b. The STRmix database search report for database searches that resulted in any matches
   c. The STRlite Likelihood Ratios worksheet documenting at minimum, all comparisons for which a numerical LR is reported.
      - Alternatively, the LR From Previous Report, up to and including the LR for all racial groups may be used to document LRs.
4. Statistical reports for Y-STR comparisons and kinship testing
5. CODIS-related data sheets and match documentation generated prior to the completion of administrative review

The case file may also include administrative documentation and other documentation relevant to the examination/analysis performed generated prior to completion of administrative review. Such documentation produced after administrative review shall be stored in LIMS or the completed case folder as described above.

Analytical data and other digital records

The following items will be retained in the completed case folder for each case when applicable:

1. Digital photographs
2. Quantification data (*.eds* files)
3. CE run folders containing CE data files for all samples and controls (*.hid* files*)*
4. GeneMapper ID-X projects containing all final analyzed files for all samples (*.ser* files)
5. GeneMapper size standard, and analysis methods files (*.xml* files)
6. STRmix deconvolution and database search folders containing the files generated by STRmix
7. Draft laboratory reports
    a. The date the draft was saved to the Review folder will be added to the filename
    b. Draft laboratory reports will be retained in a “Review” folder within the completed case folder
8. Technical reviewer observations
    a. Technical reviewer observations will be retained in a “Review” folder within the completed case folder
        - See Technical Review for details regarding generation and retention of technical reviewer observations.
9. Other documentation relevant to the examination/analysis may be retained in the completed case folder.

Analytical data and other digital records that are retained but not used for interpretation or reporting purposes should be placed in a “Not used” or similarly named folder in the completed case folder.

The analytical data and other digital records will be moved to the completed cases folder upon completion of administrative review.

47. TECHNICAL REVIEW

Scope

This policy describes requirements for performing and documenting technical review specific to the DNA functional area. For general technical review requirements, see the CLD *Quality Operations Manual*.

Policy

Technical reviews will be performed for each testing request. All administrative and examination documentation related to the request, including each of the electronic data files used to support a conclusion, will be evaluated to ensure that the analysis and interpretations were performed in accordance with CLD policies and procedures.

Procedure

1. The technical reviewer will review the case record as described in the section below.
2. As needed, the technical reviewer will use annotations in Adobe Acrobat to leave comments on draft laboratory reports and case file documentation within the respective documents.
   - Technical reviewers shall record their original observations contemporaneously. A technical reviewer observation is considered original when it differs from the analyst's observation.
   - The identity of the reviewer and date of observations will be recorded by Acrobat.
3. The analyst and technical reviewer will resolve any differences between the analyst's and technical reviewer's original observations and any other requests for changes to the case file.
   a. Changes made to technical records will be noted using annotations in Acrobat, along with the identity of the individual making the change and the date of the change. If the change constitutes a rejected observation, data, or calculation, a reason will also be recorded.
   b. When a technical reviewer's original observation is rejected, the reason for the rejection, the identity of the person rejecting, and the date will be recorded as an annotation in Acrobat (e.g., by replying to the technical reviewer's observation as a comment).
4. The technical reviewer will ensure all comments in the draft report and case file have been resolved, then remove any comments that are not original observations.
5. The technical reviewer will use the "Create Comment Summary" function in Acrobat to export the technical reviewer's original observations.
   a. The "Document and comments with connector lines on single pages" layout will be used.
   b. Only pages containing comments will be exported (the option to include pages containing no comments will be unchecked).
   c. The file will be saved in the *review* folder with the following naming convention, where LYY-NNNNNN-RRRR represents the case and request numbers as assigned in LIMS: LYY-NNNNNN-RRRR-Technical Review
6. The technical reviewer will remove all comments from the case file and save the document.

Review of DNA Profile Data

Edits made by the analyst during evaluation of the data can be examined by the reviewer using the tools and features within the GeneMapper® *ID-X* software.

<u>View Samples with Manual Edits and Overrides</u>

1. Open the GeneMapper® *ID-X* project.
2. In the Project window, select **View CGQ Overrides** from the Table Setting drop-down list. Sample(s) displayed in this list were manually accepted by the analyst with or without edits.

3. In the Project window, select View Edited Samples from the Table Setting drop-down list. Sample(s) with at least one allele or artifact label edit are displayed.
4. Verify the peak edits.

View Edits in the Label Edit Viewer

The label edit viewer contains a list of edits made to the allele and artifact labels displayed in the sample electropherogram plots of the Samples plot. View the label edit viewer from the project window or the samples plot.

- The label edit viewer may not show all edits made to a sample when multiple sample files have the same filename. To avoid this, the duplicate STRmix analysis sample files are renamed with "STRmix" at the end of the filename. All allele edits may also be viewed using the audit history viewer if needed, regardless of duplicate filenames or sample names.

1. In the Project window, make sure **View Edited Samples** is selected from the Table Setting drop-down list.
2. Select the edited sample in the filtered Samples table and select to **Display Plots**.
3. In the Samples plot, select the View Label Edits setting from the Plot Setting drop-down list to display the plots above the list of edits in the Label Edit Viewer table.
4. Verify the peak edits are appropriate and that a reason is entered for any re-interpreted peaks. Select a row in the Label Edit Viewer table and the corresponding edited peak will be highlighted.
    - Re-interpretation occurs when a peak label is changed after the analyst's original observation.

View Edits from the Project Window

1. In the Project window, make sure **View Edited Samples** is selected from the Table Setting drop-down list.
2. Select the edited sample in the filtered Samples table and select to open the **Label Edit Viewer** in a separate window.
3. Verify the peak edits.

View Allele Edits and Comments in the Genotypes Table and Genotypes Plot

Genotypes Table

1. In the Project window, select the **Project** node in the navigation pane then select the **Genotypes** tab.
2. Make sure the **View Edited Samples** setting is selected from the Table Setting drop-down list.
3. Review the entries in the following table columns:
    a. **Allele Edit (AE) Reason for Change**: Displays the last reason for change entered for an edit that yields an allele label.
    b. **Marker Edit Comment (MEC)**: Displays the reason for change entered for an edit that yields an artifact label or when alleles are deleted.
    c. **Marker Edit (ME) flag**: Displays a green check mark (true) is allele or artifact labels are edited within a marker size range.
4. Verify the peak edits.

Genotypes Plot

1. In the Project window, Shift-click to select all rows in the Genotypes table then select to **Display Plots**.
2. In the Genotypes plot, select the **Traditional Genotype Plot** setting from the Plot setting drop-down list. This plot setting displays one marker per pane for each of the markers selected in the Genotypes table.
3. Select **File** and **Close Plot Window** to return to the Project window.

4. Verify the peak edits.

Review of Analytical Data and Digital Case Records

1. Ensure run folders contain only sample files pertaining to the case in review.
2. Ensure all of the amplified samples are represented on the GMID Sample Location sheet.
3. Ensure all of the listed samples on the GMID Sample Location sheet are referenced to their appropriate run folder and are present within the GeneMapper project.
4. Ensure that the appropriate analysis method files used for analysis are present
5. Examine each sample file saved in the GeneMapper® project as appropriate (i.e. raw data, size standard examination, injection duration).
6. Ensure the appropriate sample analysis worksheets (i.e. single source or mixture sheets) are present and have been filled out.
7. An independent analysis of the evidence sample(s) shall be performed. The reviewer should agree with the statements about the number of possible contributors, interpretations, and conclusions of the analyst.
8. When applicable, statistics reports for comparisons are present.
9. When applicable, CODIS search and match documentation is present.
10. The allele table(s) in the case file (if present) will be compared to the GeneMapper results to ensure consistency.
11. Ensure any verbal reports conform to the policy and procedures outlined in the CLD *Quality Operations Manual*. If permission is obtained to issue a verbal DNA interpretation report, the report and associated documentation must be technically reviewed prior to release. The written version of the verbal report will be maintained in the case file and used to check for consistency when the final written report is reviewed. A technical review is not necessary for verbal communications to the investigator of non-comparative results (such as serology test results). This communication shall be documented in the case file notes.

Review of STRmix Interpretations

1. Input information documented on the deconvolution report(s), likelihood ratio reports, and STRlite reports shall be checked against GeneMapper ID-X.
2. Check that the sample alleles, deconvolution, likelihood ratios, CODIS profiles, and reference samples are documented on the appropriate worksheets or reports and are included in the case file.
3. Evaluate the deconvolution diagnostics for alignment with intuitive expectations.
4. If a CODIS profile was determined, check that the entered genotypes make intuitive sense, are supported by the STRmix and GeneMapper ID-X data, and comply with the Deducing Profiles for CODIS Entry procedures specified in this manual.
5. Ensure all necessary files are included in the electronic data.

Review of Case Record

Verify the following during technical review:

1. Consumption authorization is documented if required.
2. Item testing from notes matches the report.
3. Correct conclusions have been documented in the case file and match the report
4. Any photographs taken during testing are saved in the electronic data.
5. All reagent lot numbers are current and correct.
6. Sample names and reagent blank dates are consistent through the file.
7. Work product disposition is noted and extraction consumption as noted is verified.
8. All quantification files are included in the electronic data. Standard curve slopes and R^2 are within expected range, or if otherwise has been noted.

9. Verify values and calculations on amplification sheets. Verify that reagent blanks were amplified at the appropriate volumes.
10. All CE runs are reflected on the CE worksheet.
11. Variant alleles have been confirmed when required.
12. The correct statistics have been performed and are included in the report.
13. The LDIS form is complete and approval for upload/one-time search has been documented (when applicable).
14. CODIS upload/one-time search was performed, is reflected in the documentation (with LDIS administrator's initials), and is reflected in the report (when applicable).
15. The reference sample switch detection protocol has been completed when required.

48. OUTSOURCED OWNERSHIP REVIEW

The ownership review of outsourced samples being submitted for CODIS entry will encompass standard WSP case file considerations and will be focused on the sample(s) under consideration for CODIS eligibility. In addition, all of the criteria for data evaluation outlined in the technical specifications section of the Service Agreement that are put in place for the specific vendor will be evaluated. A vendor specific ownership review form will be filled out by the reviewing scientist and retained in the case file. The electronic data of a DNA profile deemed eligible for CODIS upload shall be re-analyzed (by using the vendor created GMID-X project). Vendor specific Service Agreements are found in the Outsourcing Documents folder on SharePoint. The DNA F6C Case Ownership Review Checklist is found in the Forms and Templates folder on SharePoint.

Ownership Review of Casework for contracted samples

A vendor laboratory case file will contain a scanned copy of the entire case file and report, and a folder(s) containing the electrophoresis data. The outsource coordinator (or designee) will normally download, save and provide case availability for reviewers from an accessible location. Vendor laboratory case batches may share extraction and amplification controls. Controls may be reviewed once and then accepted for the entire batch. Ownership review of CODIS eligible samples will include evaluation of the criteria below, which are specific to the amplification kit used to generate the data and to the Scope of Work (Exhibit A) section of the Biological Screening & DNA Testing Services Agreements with the appropriate vendor laboratory SOPs.

The DNA F6C Case Ownership Review Checklist will be completed by the ownership reviewer, signed, and retained in each file. CDs for electronic data are not required as these electronic files are separately backed up through an offsite server. Vendor laboratory cases that are ineligible for CODIS entry or serology/Y-screening negative do not require ownership review. Ineligible cases will only undergo ownership review if the CODIS upload status later changes to CODIS eligible. For ownership review of Identifiler® Plus vendor laboratory outsourced data, the analyst will refer to legacy protocols in Revision 30 of the WSP Casework STR Analysis Procedures to find information and interpretation guidelines.

<u>Fusion 6C</u>

Examine the case file documentation (.pdf files) to verify that all appropriate records and the run folders for the review to be completed are present. The ownership review addresses the following points:

1. Verify the case information has been entered correctly in LIMS.
2. Verify that the correct sample results for each CODIS sample are stated in the report.
3. Verify the CODIS sample packaging, description, and sampling is documented in the laboratory notes. Verify the CODIS sample can be tracked through all processing stages (accessioning, itemization, serology, Y-screening, sampling, extraction, quantification, normalization, concentration, amplification, capillary electrophoresis) of the laboratory notes.
4. Verify sample remains for reanalysis. If not, verify that authorization to consume is present in case file.

   Data review note: analysis methods, panels, bins, and stutter files have been created for Sorenson Forensics cases and provided for Bode Technology Group cases for use in GMID-X. These must be imported for use prior to analysis of any Sorenson Forensics or Bode Technology Group data.

5. Import the vendor laboratory .ser file(s) into GMID-X. Projects contain all question and reference samples for the case and allelic ladders used. The yellow status arrow may

appear in GMID-X when the project is viewed for select vendor laboratories. Change the "Panel" to the appropriate vendor laboratory specific panel as needed. Do not choose "analyze" in GMID-X, as this will erase any vendor laboratory edits for the project. Confirm that the allelic ladders passed.

6. View the CODIS sample and any additional profiles used in the deduction of the CODIS sample profile (e.g. fraction 1 profile or reference profile used to deduce profile from fraction 2). Verify that the analyst agrees with all allele calls and that the allele calls match those reported in the vendor laboratory allele table(s) and any probabilistic genotyping documentation.
7. Use the electropherograms in the Sorenson Forensics provided .pdf file to verify the reagent blank and positive and negative amplification controls associated with the CODIS sample all passed in the .ser. If these controls are not present in the .ser, electropherograms provided in the .pdf files may be used.

   If shared controls in a batch are verified by a single analyst, these shall be documented in a control log located on SharePoint. The control log documentation for these controls may be reviewed/verified by other ownership reviewers in lieu of independent control verification.

8. Verify no contamination was detected in the samples or controls. If there was a contamination or processing error in the case, it should be addressed and a log/report describing the incident included in the case file documentation.
9. Verify that any technical and/or administrative review forms are present, complete, and signed.
10. When verifying the allele calls of the CODIS sample(s):
    a. For single source profiles, as designated by the vendor laboratory, the allele table or (Sorenson Forensics only) profile provided on electropherogram printouts can be used.
    b. For Sorenson Forensics mixture profiles with a clear CODIS eligible major profile (defined as meeting the Sorenson Forensics major/minor disparity percentages at all loci AND no ambiguity at any locus) or a clear deduction profile with no locus ambiguity, the appropriate Sorenson Forensics allele table or deduced profile provided on printout of the electropherogram can be used.
    c. For all other Sorenson Forensics mixture profiles, the appropriate Mixture Analysis form shall be used.
    d. For mixture profiles using probabilistic genotyping interpretation:
        i. Check allele calls (stutter calls optional) to verify the correct sample was imported (Evidence Input Files section).
        ii. Verify correct sample imported for any references used (Reference Files section).
        iii. Verify intuitiveness of the deduced profile under Component Interpretation and/or Component Interpretation Summary.
11. If additional alleles are deemed CODIS eligible, this will be documented in the case file and technically reviewed by a second qualified analyst prior to CODIS upload. Y-STR loci and other CODIS related edits may be added to existing allele tables or probabilistic genotyping profiles.
12. Verify that the CODIS sample profile meets CODIS eligibility requirements and select the appropriate specimen category and upload level following WSP protocols for sample entry.
13. The DNA F6C Case Ownership Review Checklist will be used to document the ownership review of the CODIS sample(s) and related data, the CODIS sample entry,

and the final case technical and administrative review steps.

14. LIMS activities are used for tracking purposes. The administrative reviewer will verify LIMS activities. One relevant activity is to be entered for every profile uploaded and/or for every hit/case association obtained.
    a. Request(s) assigned to a vendor laboratory:
        i. Data Received, Negative, Ineligible (entered by outsource coordinator or designee)
        ii. CODIS Upload (entered by ownership reviewer)
    b. Request(s) assigned to ownership review:
        i. CODIS Hit, CODIS Conviction Match, CODIS Case to Case hit (entered by ownership reviewer)

NOTE: CODIS upload and CODIS Hit activities shall be removed for any association determined to be to a consenting partner

49. Y-STR CASEWORK

Quality Assurance & Control

The following quality procedures are to be followed in addition to the quality assurance practices outlined in the DNA Analysis Quality Assurance Manual and the DNA Casework STR Analysis Procedures for autosomal STR typing.

Controls and Standards

Reagent Blank

A reagent blank that was extracted with the original sample set must also be amplified with the Y23 kit. The reagent blank will be amplified using the same concentration and instrument conditions (load volume and injection times) as required by the sample in the extraction set containing the least amount of DNA. (See the Controls and Standards Section of the DNA Casework STR Analysis Procedures manual for exceptions regarding the use of a reagent blank.)

Amplification Controls

Each Y23 amplification set will include two amplification controls: the male Control 2800M and a negative control containing only amplification grade water.

Allelic Ladder

The Y23 allelic ladder will be included with every set of Y23 Y-STR samples run on the CE unit. This allelic ladder contains the most common alleles for each locus amplified in the kit.

Sample Switch Detection Procedure

At least one, or a combination of more than one, sample switch detection protocols must be used if there is a need to process two or more male reference samples from the same case. Please refer to the Quality Assurance/Quality Control, Controls and Standards section of this manual for additional information.

50. Y-STR AMPLIFICATION

The desired amplification target for male template DNA for the Y23 kit is approximately 0.25 – 0.5ng using Y quantification data. Lower targets of male template DNA may be amplified, with no lower limit. Amplifying quantities greater than 1 ng should be performed with caution as they may result in saturated Y-STR profiles.

Y23 Kit

1. Preparation of DNA Samples

    a. Using the quantification results, calculate the volume needed for each test sample and reagent blank to provide the desired amount of DNA for amplification.
        - Ensure reagent blanks are amplified on the same instrument model as the corresponding samples.

    b. If required, a reduction in the volume and/or combination of DNA extracts can be performed before amplification. (See the Vacufuge™ Procedure – Concentration, Preservation, And Recovery of DNA Extracts and/or Microcon® Concentration of DNA protocols of the DNA Casework STR Analysis Procedures manual for further information.)
    c. An amplification grade water/DNA mixture of the appropriate DNA concentration may be prepared at this step if desired. Alternatively, the water and DNA can be added to the amplification tubes separately. The total volume of water and DNA sample added to the PCR reaction is 17.5 µL.

    Note: If the DNA template has a TE buffer concentration greater than 15X, the volume of sample added should not exceed 5 µL.

2. Amplification Set-Up

    a. Prepare the components from the amplification kit by vortexing for 15 seconds before each use. (The following shall be performed in a dedicated PCR amplification hood.)
    b. Preparation of PCR Amplification Mix:

Component	Vol. per Reaction (µL)
Y23 5X Master Mix	5.0
Y23 10X Primer Pair Mix	2.5

    Note: Additional sample reactions can be added into the calculation. The extra volume compensates for loss that may occur during pipette transfers.

    c. Aliquot 7.5 µL of master mix per tube.
    d. Add 17.5 µL of the water (or amplification grade water)/DNA template samples to their appropriate tubes.
    e. Add the Amplification Controls:
    f. For the positive control, briefly vortex the tube of 2800M Control DNA (10ng/µl), and then dilute an aliquot to the desired DNA concentration. Add 0.5 ng of diluted 2800M Control DNA to the appropriate tube.
    g. For the negative amplification control, pipet amplification grade water instead of template DNA into the appropriate tube.

3. Load the samples onto the thermal cycler and start the appropriate amplification protocol. Ensure that Max Mode is selected for ramp speed and 25 µL reaction volume is selected. Ensure that the “GeneAmp PCR System 9700” Simulation Mode is selected if using the ProFlex.

4. Y23 thermal cycler parameters:
   96 °C for 2 minutes, then:
   94 °C for 10 seconds
   61 °C for 1 minute
   72 °C for 30 seconds
   for 30 cycles, then:
   60 °C for 20 minutes
   4 °C soak

51. Y-STR AMPLIFICATION PRODUCT PREPARATION

Procedure

1. Prepare a formamide/ILS mix for the appropriate number of wells as follows:
   [(0.5 µL WEN ILS 500 Y23) x (# samples)] + [(9.5 µL formamide) x (# samples)]
   Note: WEN ILS 500 Y23 is not the same ILS used in the PowerPlex Fusion 6C Product Preparation
2. Vortex for 10–15 seconds
3. Pipet 10 µL of formamide/ILS into each well
4. Add 1 µL of amplified sample (or Y23 Allelic Ladder Mix) to each well. Cover wells with septa.
5. Centrifuge the plate briefly to remove air bubbles.
6. Denature samples at 95 °C for 3 minutes, then immediately snap cool for 3 minutes.

Note: It is recommended that only 3–4 injections of samples amplified with Y23 be run on the 3500 instrument in any given run, as some sample/ILS sloping may begin to occur the longer the CE plate is left on the instrument.

Optional: A stabilizer can be added to the formamide/ILS mix if run are anticipated to be over 3–4 injections. The preparation for the reaction mix with stabilizer is as follows:

1. Prepare a formamide/ILS/stabilizer mix for the appropriate number of wells as follows:
   [(0.5 µL WEN ILS 500 Y23) x (# of samples)] + [(9.5 µL of formamide) x (# of samples)] + [(0.5 µL Y23 Stabilizer) x (# of samples)]
2. Vortex for 10–15 seconds to mix.
3. Pipet 10.5 µL of formamide/ILS/Stabilizer into each well.
4. Add 1 uL of amplified sample (or Y23 Allelic Ladder Mix) to each well. Cover wells with septa.
5. Centrifuge the plate briefly to remove air bubbles.
6. Denature the samples at 95 °C for 3 minutes, then immediately snap cool for 3 minutes.

In addition to using injection times less than the default injection times for amplicons with excessive peak heights (e.g. causing –A shoulder artifacts or saturation), a smaller amplicon volume as described above can be loaded, or a dilution in either formamide or TE up to 1:10 can be used. If any of these methods or combinations thereof is used for the analyzed sample, it shall be documented in the case file.

<u>Y-STR Panels and Bins</u>

GeneMapper® ID-X requires chemistry-specific panels and bins. For Y23, these have been acquired from Promega and imported into GMID-X. In the Panel Manager, check the “Y Marker” boxes for all loci except for DYS385.

52. Y-STR GUIDELINES FOR EVALUATING GENEMAPPER® DATA

Follow the guidelines and workflow provided for the analysis of autosomal STR data with the addition of the following information for interpreting Y-STRs.

Evaluation of Quality Control Samples

Amplification controls and the allelic ladder are reviewed under the Allelic Ladder Quality and Control Quality sections of the Analysis Summary tab. Verify that the allelic ladders and controls have passed the quality system criteria. Passing data does not need to be manually reviewed; the non-passing data shall be manually reviewed. Analysis of negative controls utilizes the GMID-X software as an expert assistant and ensures that any evidence of contamination that may be present within a negative control will be flagged by the software for the analyst to review.

Evaluation of Y-STR Profiles

Designation of True Alleles

All non-artifact peaks that are sharp, distinct and greater than or equal to analytical threshold will be considered alleles, documented, and considered during profile determination. Peaks greater than or equal to the analytical threshold for Y23 are alleles that can be considered for inclusionary purposes and statistical assessment. (See Y-STR Mixtures for further interpretation requirements for the reporting of major and minor components.)

The determination of a profile or individual genotype is aided by the use of thresholds established via validation.

Y23

- **Analytical Threshold: 100 RFU**
  The analytical threshold is the level at which a peak can be reliably differentiated from background fluorescence as either an allele or artifact as determined via validation.

The appropriate analysis method shall be applied to the analysis of samples.

- Evidence samples shall be analyzed with peak detection set at 100 RFU.
- Reference samples should be analyzed with peak detection set at 200 RFU.
  - Reference samples may be analyzed with peak detection set at 100 RFU to address lower peak heights. Consider rework if a partial profile is obtained.

Determination of a Single Source Y-STR Profile

A Y23 DNA typing result can be considered to be from a single contributor if there is no more than 1 allele (single copy) that is greater than or equal to the analytical threshold at each locus. The exception is the duplicated locus DYS385 (multi-copy) which will often exhibit the presence of two balanced alleles (>50% for peaks >600RFU) for a single individual. If the Y23 DNA typing result is determined to be from a single contributor, alleles that meet or exceed the analytical threshold can be reported in the Y23 profile and be used for inclusionary and exclusionary purposes. If two alleles are observed above the analytical threshold at DYS385, they may be used for inclusionary and exclusionary purposes. If only one allele is observed at DYS385, it must be equal to, or greater than, 600 RFU to be used for inclusionary purposes. If a single allele is observed below 600 RFU, drop-out of a second allele is possible so the single visible allele should only be used for exclusionary purposes.

Note: DYS389I/II is also duplicated locus (multi-copy) but looks like a single copy result as it produces a single peak for DY389I and a single peak for DYS389II with the Y23 kit.

Interpreting Mixed Y-STR Profiles

The Y23 DNA typing result is considered to be from a mixed source if two or more alleles are present at two or more single copy loci. More than 2 alleles present at DYS385 would indicate a mixture. The analyst should also consider the presence of multiple stutter peaks that are greater in percentage than what has been established in the validation. It should also be taken into consideration that loci other than DYS385 can experience duplications that will produce more than 1 allele.

Mixtures with three or more contributors are generally un-interpretable; however, a major component can sometimes be present. If a major component is suspected, interpretation of the sample can be done. Consultation with and approval by the DNA Technical Leader, Supervisor, or Technical Lead following agreement between analyst and reviewer shall be documented in the case file. Evaluation of these profiles must account for potential allele stacking.

Considerations for the Occurrence of Duplications

Typically a single copy Y-STR locus will exhibit a single PCR product. There are instances of duplications of Y-STR loci where more than one peak will appear (or more than two peaks will occur for the multi-copy DYS385). The possibility of duplications may present potential complications for determining if a Y-STR profile is a single source contributor or a mixed sample. The following are some guidelines to aid in this evaluation:

- The greater the number of loci with multiple alleles, the more likely it is a mixture.
- If there is more than one locus with multiple alleles, the further apart these loci are physically located on the Y chromosome, the more likely it is a mixture.
- Duplications will typically have a single repeat difference. Greater than a single repeat difference may indicate a mixture.

The alleles at duplicated and multi-copy loci, such as DYS385, should be treated as genotypes and documented in the allele table as such. (Example: 11, 14)

Variant Alleles

If a variant allele or multi-allele (i.e. duplication) pattern occurs, the sample will be re-injected to confirm the result. Alternatively, other matching samples from the same contributor can also qualify as confirmation. The analyst will also check the NIST STRbase web site (https://strbase.nist.gov/), to see if the variant has been previously reported. If the variant has been previously reported, a copy of this information shall be saved in the case record. Variant alleles confirmed in the WSP system and not previously seen should be reported to the NIST STRbase web site using the appropriate online procedures. A Y-STR variant is calculated in the same way as an autosomal variant, keeping in mind that not all Y-STR loci are tetranucleotide repeats. Y-STR duplications and deletions are not considered variant alleles but should still be reported to STRbase if not previously reported.

A variant allele present in an uninterpretable profile or at RFU levels consistent with the uninterpretable portion of a mixture do not need to be confirmed.

Anticipated Artifacts

In addition to the normal artifacts (spikes, dye blobs, pull-up, and –A shoulders) that occur in autosomal STR's, the following should be taken into consideration:

Stutter

In addition to the normal one repeat smaller stutter usually seen, the occurrence of the following can occur:

- The presence of plus stutter is more prevalent than in autosomal STR's and occasional N+4 stutter can occur.
- For Y23 increased forward stutter (i.e., n+4 and n+8) can be observed at the DYS389I and DYS389II loci when using the cell-line derived 2800M Control DNA. This forward stutter is inherent to this cell line DNA and is not seen in amplification of DNA from human blood, body fluid, or other human-derived samples.

Y23 - The following stutter threshold percentages established during validation will be used:

Locus	**N-4**	**N-8**	**N+4**
DYS576	16%	2%	4%
DYS389 I	8%		
DYS448	(N-6) 4%		
DYS389 II	16%	2%	
DYS19	(N-2) 10%	(N-4) 11%	(N+2) 4%
DYS391	10%		
DYS481	(N-3) 28%	(N-6) 6%	(N+3) 5%
DYS549	12%	1%	2%
DYS533	12%		3%
DYS438	(N-5) 5%		
DYS437	7%		
DYS570	15%	1%	3%
DYS635	12%		4%
DYS390	12%		
DYS439	11%		4%
DYS392	(N-3) 15%		(N+3) 10%
DYS643	(N-5) 5%		
DYS393	15%	2%	3%
DYS458	17%	2%	2%
DYS385 a/b	16%	3%	4%
DYS456	15%	1%	6%
Y-GATA-H4	11%		3%

N-2 and N+2 stutter is set at 1.25 to 2.75 bp range
N-3 and N+3 stutter is set at 2.25 to 3.75 bp range
N-4 and N+4 stutter is set at 3.25 to 4.75 bp range
N-5 stutter is set at 4.25 to 5.75 bp range
N-6 stutter is set at 5.25 to 6.75 bp range
N-8 stutter is set at 7.25 to 8.5 bp range

Effects from Female DNA

Non-specific amplification of female DNA in samples with high levels of female DNA may occasionally be seen. These amplification products will normally not affect the interpretation of the Y-STR profile. The

known female reference associated with a case sample can be amplified to aid in the interpretation of non-specific amplification products if needed.

Validation studies show that there is a correlation between the amount of female DNA in a sample and the performance of a Y-STR amplification. An increase in the amount of female DNA in a sample will generally result in a decrease of peak heights in the Y-STR profile. Samples with extreme levels of female DNA could result in complete inhibition of the male DNA. This should be taken into consideration when making casework decisions regarding choice of sample and combining of samples. The case sample extract can be diluted to aid in decreasing the amount of inhibiting female DNA if necessary.

Documentation of Artifacts

Artifacts meeting or exceeding analytical threshold will be documented for all questioned samples, reagent blanks, and the negative amplification control. For all known samples and the positive male amplification control, artifacts meeting or exceeding the reference analysis method threshold (Y23) will be documented.

Interpretations

The following interpretations may be made to Y-STR profiles:

Inclusion

- The Y-STR profile of the known reference matches the single-source, major component, or eligible minor component Y-STR profile of the questioned sample.
- There are no forensically significant differences between the allele calls obtained from the reference sample and the questioned sample.
- If a full profile is not generated for the questioned sample, inclusions and a statistic can still be made to the reportable loci. This can be done if the statistics for the typing results providing the most genetic information are reported and a resulting statistic rarer than one in one can be provided.

Exclusion

- If the Y-STR profile of the known reference does not match the single-source, major component, or eligible minor component Y-STR profile of the questioned sample at all loci (barring a scientific explanation), then the donor of the reference sample is excluded as the source of the questioned sample.
- If the haplotype of the known reference is not present at any of the pertinent loci of an eligible indistinguishable mixture, then the donor of the reference sample can be excluded as a contributor to the profile.

Inconclusive

- When incomplete results are obtained, inclusions and exclusions may still be possible, but caution should be used.
- Peaks below the analytical threshold (Y23) shall not be used for inclusions, but can be carefully considered for exclusions.
- In some cases, no conclusions can be made due to the complexity or the limited genetic information of the Y-STR profile.

53. Y-STR MIXTURE DECONVOLUTION

Mixtures with Major Components

When a major component can be distinguished from the mixture (generally a two person mixture), inclusions and exclusions can be made between known reference samples and the major contributor profile. A population database search to generate statistics can then be performed on the major profile. A major contributor will be deduced locus by locus. At each locus, if the allele from the tallest peak is greater than or equal to 800 RFU for Y23 and the shorter allele peak height is less than 50% of the taller allele peak height, then the tallest peak may represent the major contributor. A locus may only be included in the statistics when these conditions are met.

Caution should be used when interpreting DYS385 where each contributor may have up to 2 alleles. At this locus, alleles may only be attributed to the major contributor under the following circumstances:

- If 4 alleles are present, the two larger alleles must both meet the above guidelines in relation to the two smaller alleles.
- If only 3 alleles are present, one larger than the other two, the larger allele must be at least 1600 RFU for Y23 and the combined height of the two minor alleles must be no more than 50% of the height of the larger allele.
- If only 3 alleles are present, two larger than the other one, both the larger alleles must be at least 800 RFU for Y23 and the height of the smaller allele must be no more than 50% of the height of each of the larger alleles.
- If only 2 alleles are present, it is often not possible to determine the major contributor's profile due to significant peak height imbalance being observed in low level, single-source samples. The greatest imbalance observed during validation was the height of one allele being 32% (Y23) of the height of the second allele from the same contributor. Therefore, if the height of the smaller allele is no more than 30% of the height of the larger allele, and the larger allele is greater than or equal to 800 RFU for Y23, the larger allele may be attributed to the major contributor. Additionally, if both peaks are greater than or equal to 800 RFU for Y23 and are within 50% or greater balance of each other, then both peaks can be attributed to the major contributor.
- If only 1 allele is present and it is greater than or equal to 800 RFU for Y23, this allele can be attributed to the major contributor.

Mixtures with a Minor Component

There will be no inclusions made or statistics generated for the minor component of a Y-STR genotype originating from more than two individuals.

When a minor component profile can be distinguished from a two person mixture, inclusions and exclusions can be made between known reference samples and the minor contributor profile. A population database search to generate statistics can then be performed on the deduced minor component. A minor contributor will be deduced locus by locus. Alleles may only be attributed to the minor contributor under the following circumstances:

1) A reportable major contributor has been declared at the locus
2) The minor allele at the locus is greater than or equal to the analytical threshold
3) If only one allele is present at a locus, the following steps should be taken to attempt to deduce the minor contributor's genotype:
    a. The average percent contribution of the deduced contributor should first be calculated. This can be done by finding the loci in which the deduced contributor does not share with the assumable profile (i.e. a unique allele) and dividing the allele peak height by the total RFU at the locus. The calculated values from each locus should then be averaged.

b. The average percent contribution value in step1 should be multiplied by 30% to account for the maximum peak height variation observed in validation between a mixture of contributors at equal proportions. This value is the lowest expected deduced allele height percentage – referred to as the deduced allele estimate (DAE) percentage.
c. The DAE percentage will be used at the loci where only one allele is present. The total RFU observed at the locus will be multiplied by the DAE percentage to obtain the DAE peak height value. If the value is larger than the analytical threshold, the analyst can be confident that the deduced contributor's allele has not dropped out.
d. The final step is to evaluate any stutter peaks present and determine whether or not the stutter peaks may possibly be masking a contributor.
    i. If the peak in stutter position does not exceed the stutter filter and is less than the DAE, it can be assumed that the stutter is not elevated and that both contributors share the same allele.
    ii. If the peak in stutter position does not exceed the stutter filter and is greater than the DAE, the minor contributor's allele may be masked; in this case the locus should be considered inconclusive for the minor contributor.
e. Extreme caution should be used when interpreting mixtures with a total male DNA input of 0.06ng or less. As the amount of DNA input decreases, stochastic and amplification artifacts increase. At these levels, highly elevated stutter peaks may be observed that appeared to be part of the deduced profile, but actually are not.

4) A minor contributor can be deduced at DYS385 using the following guidelines:
    a. The deduced minor alleles all meet the analytical threshold. The calculated DAE, if used, should be halved.
    b. All of the interpretation guidelines previously outlined for deduction of a major component from DYS385 are also met.

Mixtures with a Known Contributor

In instances where a contributor is known or expected to be present (such as intimate samples, consensual partners, etc.), subtracting out the known contributor's alleles can allow for determination of the remaining deduced probative profile. The remaining profile may be used for a population database search to generate statistics. At those loci where only one allele has been identified, it can be assumed that both the known and deduced contributors have donated the same allele if it can be shown that the average percent contribution of the deduced contributor (as determined from all loci where the known and deduced contributors have separate alleles), would result in an allele that is greater than or equal to the analytical (Y23) threshold at that locus. If the deduced contributor could be masked in stutter, the procedure for consideration of stutter outlined under "Mixtures with a Minor Component" should be followed.

Indistinguishable Mixtures

No inclusions will be made to mixtures where a major, minor, or deduced component cannot be distinguished. This is due to the inability to provide an evidentiary statistical weight to indistinguishable Y-STR mixtures. These situations will be reported out as "inconclusive". Exclusionary comparisons made to indistinguishable mixtures may be done with caution. Considerations for exclusionary comparisons should include number of contributors, RFU levels, total number of loci with data above reporting threshold, the presence of inhibition, degradation and the possibility of drop-out.

54. Y-STR LABORATORY REPORTS

Y-STR laboratory reports will contain comparable information as outlined for autosomal STR DNA reports in the Laboratory Reports section of this manual. The specific guidelines for conclusions and report writing for Y-STR analysis are listed below.

Conclusion statements shall contain the following components in order:

1. A profile summary statement
2. A deduced contributor statement, if designated
3. Comparison result for each comparison made to the profile, consisting of:
    a. The direct result: inclusion or exclusion
    b. The calculated Y-HRD profile frequency estimate, when applicable

Profile summary statements

Profile Result	Statement
Interpretable profile obtained	The [partial] Y-STR DNA profile obtained [from *item description/number*] was interpreted as originating from [at least] [*number of contributors*] male individual[s].
Profile suitable for exclusions but not inclusions	The [partial] Y-STR DNA profile obtained [from *item description/number*] was interpreted as originating from [*number of contributors*] male individuals. Due to its complexity, this profile is suitable only for exclusions. No inclusions may be made to this profile.
Uninterpretable profile due to complexity; i.e. number of contributors, mixture proportions	The [partial] Y-STR DNA profile obtained [from *item description/number*] was interpreted as originating from [at least] [*number of contributors*] male individuals. Due to its complexity, this profile is not suitable for comparisons.
Uninterpretable profile due to limited genetic information	The [partial] Y-STR DNA profile obtained [from *item description/number*] is of limited genetic information to which no comparisons can be made.
No profile	No Y-STR DNA profile was obtained [from *item description/number*]
Interpretable reference profile obtained	A [male] Y-STR DNA profile was obtained [from *item description/number]*.

Deduced contributor statements

Condition	Statement
Major and minor profiles deduced	A [partial] major male contributor was deduced [and designated Individual *Letter*].

	A [partial] minor male contributor was deduced [and designated Individual *Letter*].
Major deduced, minor not suitable for comparisons due to limited genetic information	A [partial] major male contributor was deduced [and designated Individual *Letter*]. Due to limited genetic information, the remaining minor component is not suitable for comparisons.
Major deduced, minor not suitable for comparisons due to the mixture being at least 3 male contributors.	A [partial] major male contributor was deduced [and designated Individual *Letter*]. Due to complexity, the remaining minor component is not suitable for comparisons.
Foreign deduction using an expected Y-STR profile	Assuming [*assumed individual*], an additional male contributor was deduced [and designated Individual *Letter*].

Comparison and statistical support statements

Condition	Statement
Exclusion	[*Compared individual*] is excluded as the contributor of the [major/minor] male DNA in this sample.
Inclusion	This [major/minor] Y-STR profile matches the Y-STR profile of [*compared individual*]. Therefore, neither [*compared individual*] nor his paternal male relatives can be excluded from this profile. This [major/minor] Y-STR profile has [not] been observed [*number of observations*] times in the U.S. national database of the Y Chromosome Haplotype Reference Database, and is not expected to occur more frequently than [*haplotype frequency*] male individuals in the U.S. population.
Inconclusive; Cannot be excluded from an exclusions-only profile	Comparison to the profile for [*compared individual*] is inconclusive. No inclusionary or exclusionary statement can be made regarding [*compared individual*] as a contributor to this Y-STR DNA profile.

Remarks statements

The following remarks shall be included in the report when the corresponding conditions are met unless noted as optional. Refer to Laboratory Reports for required remarks not specific to Y-STR testing.

Condition	Statement
YHRD statistics differ between comparisons (optional)	Differences between reported statistics for DNA profiles may occur based on the number of loci used to determine the association.

Other required content

The following statements shall be included when the corresponding conditions are met.

Condition	Statement
Amplified samples	Y chromosome short tandem repeat (Y-STR) DNA profiles were developed using the PowerPlex Y23 amplification kit and a 3500 Genetic Analyzer.
Statistics calculated using YHRD	Statistics for Y-STR results were calculated using the Y Chromosome Haplotype Reference Database, release [*R#*].

55. Y-STR STATISTICAL CALCULATIONS

Scope

This procedure is used to calculate haplotype frequencies and match probabilities for Y-STR profiles when a comparison results in an inclusion.

Introduction

The publicly available YHRD database is used to search for matching haplotypes and to calculate haplotype frequencies and match probabilities.

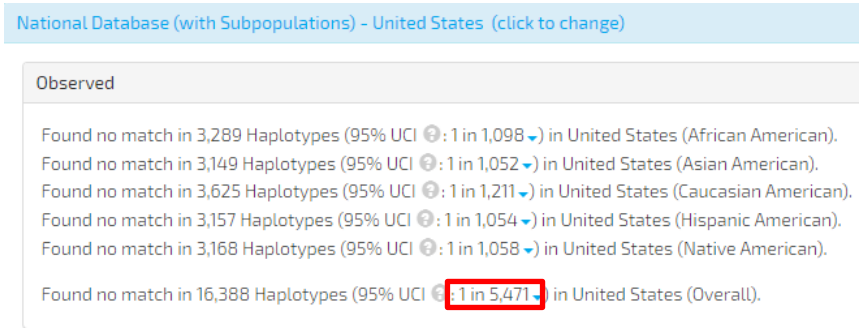
Haplotype frequencies are calculated from the number of matches found and the database size. The frequency is then corrected to account for database size and sampling variation by calculating the Clopper and Pearson 95% upper confidence interval (UCI) of the haplotype frequency.

Haplotype match probabilities are calculated by applying a correction for population substructure to the 95% UCI of the haplotype frequency: $\Pr(A|A) = \theta + (1-\theta) \times p_A$, where θ is the population substructure correction factor and p_A is the 95% UCI of the haplotype frequency.

Database searches are performed using subsets of the database, termed datasets. Datasets contain only those haplotypes that have results at all loci defined for that dataset. Therefore, datasets contain varying numbers of haplotypes and it is possible to obtain a less discriminating haplotype frequency from a more discriminating typing kit due to a small dataset size. In order to report the most discriminating statistic, database searches are repeated using the next dataset with fewer loci. This is referred to as a reduced loci search. When performing a reduced loci search, YHRD filters matches based on the additional loci in the haplotype.

Procedure for calculating Y-STR haplotype frequency

1. Open https://yhrd.org and navigate to the database search function
2. For manual haplotype entry:
    a. Select the option to manually enter a haplotype
    b. On the haplotype entry page select the PowerPlex Y23 kit, enter the haplotype, and select Search
3. For haplotype upload, select the option to upload a haplotype and select the file to upload.
4. On the Report page, confirm the entered haplotype is correct, Y23 is selected as the dataset, and PowerPlex Y23 is selected as the kit
5. Click Add feature to this Report and select National Database (with Subpopulations, 2014 SWGDAM-compliant)
6. Remove the Worldwide database results from the report
7. Save a copy of the report for the case file.
8. Select the Y17 dataset
9. Click Add feature to this Report and select National Database (with Subpopulations, 2014 SWGDAM-compliant)
10. Remove the Worldwide database results from the report
11. Save a copy of the report for the case file.
12. Compare the United States (Overall) 95% UCI haplotype frequencies obtained from the Y23 and Y17 datasets. The more discriminating (rarer) 95% UCI haplotype frequency truncated to two significant figures shall be used for reporting the strength of the inclusion (unless reporting a match probability).



13. The report containing the reported statistic shall be included in the case file. The other search report shall be saved in the case electronic data.

56. DNA EXTRACT AND WORK PRODUCT TRANSFER/RETURN

This procedure is used in conjunction with the VACUUM CENTRIFUGE PRESERVATION OF DNA EXTRACTS procedure within this manual.

DNA extract(s) or work product(s) shall be preserved prior to transfer to another laboratory for additional analysis.

All DNA work product produced during sample analysis, including remaining DNA extracts from evidence (excluding reference samples), associated reagent blank(s), microscope slides, and sample cuttings or cellular material not subjected to DNA extraction, shall be preserved and returned to the submitting law enforcement agency as a new item. Amplicons, direct amplification lysates, and Y-screen samples are not considered work product and may be discarded once testing in the case has concluded.

The preserved/dried DNA extracts, cell pellets, and sample cuttings shall be sealed in a plastic or foil bag for transfer/return. The new item containing the preserved DNA extracts packet and other work product(s) will be created in LIMS and transferred/returned.

If there is only one case associated with a reagent blank(s), the reagent blank will be included with the DNA extract(s) to be transferred/returned. Each preserved DNA extract and associated reagent blank can be in a separate plastic or foil bag or they all may be placed in one bag. If there is more than one case associated with a reagent blank, the reagent blank will be preserved and retained at the lab at room temperature. Reagent blanks from extractions where there is no remaining DNA may be discarded.

The case notes and report will specify the new item(s) that contains the returned DNA extracts and/or other work product. Additionally, where possible, evidence packaging will be labeled (i.e. stickers or other method) to indicate that DNA work product is enclosed.

57. QUALITY ASSURANCE/QUALITY CONTROL

DNA Isolation Areas

1. All solutions used in DNA isolation are prepared according to the protocols in the Reagent Preparation section of this manual.

2. All glassware to be used in preparation of critical reagents will be autoclaved. If a solution itself is to be autoclaved, the glassware used in its preparation need not be autoclaved. Pipette tips and microcentrifuge tubes that are sterilized (as in the manufacturing process) need not be autoclaved.

3. Gloves, masks and lab coats will be worn where appropriate.

4. Instruments used to cut or handle stains (scissors, forceps, scalpels, Harris punch) should be cleaned before each use. Cut each sample on a clean sheet of paper.

5. Use plugged pipette tips when pipetting any solutions.

6. Care should be exercised when inserting pipettors into reagent containers. Reagents should be poured from the reagent bottle into a smaller container from which pipetting should be done. Avoid touching the container with the barrel of the pipettor.

7. The work area should be cleaned after each use with 10% bleach followed by a rinse with water or wiped down with another appropriate cleaner.

8. The interiors of microcentrifuges should be cleaned periodically.

9. DNA from reference standards should be isolated at a different time or a different place than DNA from evidence samples.

PCR Set-Up Area

1. Gloves will be worn when working in the PCR set-up hood.

2. Set up the PCRs according to protocol. Use only the pipettes dedicated to the PCR set-up area. Use sterile, plugged pipette tips. Dispose of pipette tips in waste container in hood.

3. Reference standards and evidence samples may be amplified together provided the following precaution is taken: Add DNA isolated from evidence samples to PCR tubes before reference standard DNA is brought to the set-up area. Make sure each reaction tube is sealed after the addition of DNA.

4. The hood will be cleaned on a regular basis with a 10% bleach solution (or other appropriate cleaner) and then rinsed with water or wiped down with another appropriate cleaner. Pipette barrels will also be wiped regularly with a 10% bleach solution (or other appropriate cleaner). Additionally, the UV light in the hood (if so equipped) can be left on a few hours after each use of the hood and pipettes.

Amplification and Product Analysis Area

1. All solutions used in the amplification and product analysis area are prepared according to the reagent manual protocol.

2. Gloves will be worn where appropriate when working in the amplification room.

3. Only dedicated lab coats will be worn when working in this area.

4. Handling of PCR products will be done with dedicated pipettors and plugged pipette tips.

5. Benchtop protective paper will be changed on a regular basis.

6. Any PCR product or other items contaminated with PCR product will be discarded in the appropriate container in the PCR amplification/analysis work area.

7. PCR product may only be stored in a dedicated refrigerator or freezer.

Controls and Standards

Reagent Blank

A reagent blank(s) will be prepared each time a set of DNA samples is extracted. There must be sufficient reagent blank(s) present to accommodate all amplification volumes and kits run with the extraction set; therefore, it may be appropriate to have multiple reagent blanks. The reagent blank will be prepared like the other samples being extracted, except no source of DNA will be added to this sample. Reagent blanks shall be extracted concurrently with the corresponding evidence samples. For differential extractions, reagent blanks corresponding to fractions 1 and 2 will be generated. Any generated reagent blank shall be amplified using the same primers, instrument model and concentration conditions as required by the sample(s) containing the least amount of DNA. If only one kit is amplified, then only reagent blanks for that kit need be run. If multiple kits are amplified, then the appropriate reagent blanks for each kit must be run. The amplified reagent blank shall be typed using the same instrument model, injection conditions and most sensitive volume conditions of the extraction set.

When combining samples (e.g. cuttings from an M-Vac filter separated into multiple tubes, or several swabs from a single item split between tubes) there must be an equal volume of reagent blanks combined as well. For instance, if three sample extracts are combined, three reagent blanks with a combined volume equal to the combined sample extract volume must be combined as well.

If multiple reagent blanks are used within an extraction set, each reagent blank must be quantified and at least one must be amplified if any of the specimens associated with the extraction set will be amplified. If multiple reagent blanks are used and quantified within an extraction set, at a minimum, the reagent blank that demonstrates the greatest signal (if any) must be amplified and characterized.

If a reagent blank associated with an extraction set or sample being amplified is depleted, an analyst shall not continue on to a different amplification test kit without a reagent blank. The volume of sample amplified cannot exceed that of its reagent blank. Follow the analysis completed retention policy for the samples associated with the depleted reagent blank.

For samples extracted prior to July 1, 2009, every attempt shall be made to follow the above guidelines regarding the amplification of a reagent blank with its corresponding extracted DNA sample. However, this may not always be possible. A sample extracted prior to July 1, 2009, may be amplified with a different amplification test kit even if the associated reagent blank has been previously depleted. If any volume of reagent blank remains, it shall be amplified with the new kit. In those cases where the reagent blank has been partially or fully consumed by past analysis with similar technologies (i.e., PCR), the analyst should make every attempt to ensure that there were no past issues with the reagent blank (contamination or otherwise). This information shall be documented in the case file.

Positive and Negative Controls

At the amplification step, two extra tubes containing the amplification master mix will be prepared. To one tube, the appropriate volume of positive control DNA (9947A or 2800M) will be added, and to the other an equal volume of TE or amplification grade water will be added.

A positive and negative control will be included for each kit run in every set of amplifications conducted.

Internal Size Standards

An appropriate internal size standard will be included with every sample prepared to be run on the CE unit. The data collected from this size standard will be used to verify the quality of a particular injection and also be used to estimate the size of any DNA fragments present in the run. It is essential to have this data in order to process samples in the GeneMapper® *ID-X* program.

Allelic Ladder

An appropriate ladder will be included with every set of samples run on the CE unit.

Substrate Sample

Substrate samples can, on occasion, provide useful information in an analysis. However, the relevance of the data generated by a substrate sample may not be intuitively obvious. Any data generated from a substrate sample should be evaluated very carefully as to its significance. The fact that DNA consistent with another individual is recovered in a "substrate sample" is not a clear indicator of the involvement of that individual in the particular incident under investigation. A substrate sample may be included in the samples run at the discretion of the analyst. However, if one is run, the analyst should be very clear as to the purpose of this sample, how it bears on the analysis and investigation, and what the significance of the results are. This shall be clearly documented in the analyst's notes.

58. REAGENT QUALITY CONTROL

Quality Control Testing

All reagents used in DNA analysis are evaluated routinely. The reagent blanks and positive and negative controls used during testing continually verify the suitability of the reagents.

In addition, the following reagents need to be evaluated prior to use in casework:

- dH_2O used for extraction or amplification
- Proteinase K
- Phosphate buffered saline (PBS)
- Dithiothreitol (DTT)
- Phenol/Chloroform/Isoamyl alcohol
- 20% Sarkosyl
- TE
- Tris/EDTA/NaCl (TNE)
- Sperm wash buffer (SWB)
- QIAGEN Buffer MTL
- QIAGEN EZ1&2® DNA Investigator kit
- Promega Casework Direct Kit
- Promega SwabSolution™ Kit
- Promega PowerQuant™ System
- Promega PowerPlex® Fusion 6C
- Promega PowerPlex® Y23

See individual laboratory protocols for evaluation of all other reagents used in STR testing.

A known DNA sample shall be used to perform quality control testing (QC) of a new lot of reagent, kit or kit component before it can be used for casework. The known DNA sample shall be analyzed using the current laboratory protocols.

For all reagents listed above with the exception of quantification and Y-screening kits, 1) the correct genotype must be obtained from the known DNA sample, 2) all data analysis parameters as required in the current laboratory protocols must be met, and 3) good inter-locus and intra-locus peak height balances in the DNA profile must be observed in order for the QC to pass.

The QC of amplification kits shall include amplification of a known DNA sample at both a standard template target and a reduced template target of 0.30 ng for Fusion 6C or 0.15 ng (male DNA) for Y23.

The QC of quantification and Y-screening kits requires that the quantification standard curve meets all data analysis parameters as required in the current laboratory protocols. This QC does not require amplification of the known DNA sample.

If the sample does not type correctly or the analysis parameters are not met, the reagent, kit or kit component shall not be used for casework until the problem is resolved and/or new reagents or kit materials are obtained. Scientifically valid exceptions pertaining to meeting the analysis parameters can be made if approved by the DNA unit supervisor in consultation with the DNA Technical Leader. The successful completion of the QC shall be documented on the reagent, kit or kit component and on the appropriate QC form (maintained electronically).

Quality control testing will be verified by a qualified DNA Forensic Scientist to ensure a quality DNA profile (or analysis parameters) was obtained, any negative controls are free of contamination, all data is maintained electronically in the appropriate location, and the corresponding QC form is filled out

completely and accurately. The verifier's initials will then be added to the QC form, which will then be saved as a read only .pdf file and stored with the QC data for that reagent.

59. REAGENT LOT NUMBERS AND EXPIRATION DATES

To facilitate traceability and quality control of reagents, the WSP DNA laboratories shall use the following conventions.

- Critical reagents (internally prepared and commercially prepared) shall have an expiration date. If a commercially prepared reagent does not have a manufacturer-provided expiration date, an expiration date shall be assigned and will be three years from the received date. Critical reagents shall be checked prior to use in casework.
- When a reagent (critical or not) has an expiration date, the expiration date shall serve as the lot number for the reagent (i.e., mmddyy).
- For those reagents that do not have an expiration date, a lot number shall be assigned. The lot number can be a numbering system consisting of the last two digits of the year received followed by sequential numbering (i.e., yy-1, yy-2, yy-3, etc.) or using a lab-assigned expiration date to ensure turnover of the reagent.

The following table lists expiration dates for the reagents used in the DNA section. Reagents are stored at room temperature unless otherwise noted.

Reagent	**Expiration**
Dithiothreitol (1 M DTT)	3 years (stored frozen)
Phenol Chloroform Isoamyl Alcohol	3 years, ~4°C
Promega Casework Direct Kit	Mfr provided, -20ºC upon receipt, ~4ºC after initial use
Phosphate Buffered Saline (PBS)	Mfr provided or 3 years
Promega PowerPlex ® Y23	Mfr provided, -20ºC upon receipt, ~4ºC after initial use
Promega PowerPlex ® Y23 Stabilizer	2 years or Mfr provided, 2°C - 10°C
Promega PowerPlex® Matrix Standard	Mfr provided, ~4ºC
Promega PowerPlex® Fusion 6C amplification kit	Mfr provided, -20ºC upon receipt, ~4ºC after initial use for up to 6 months.
Promega SwabSolution™ Kit	Mfr provided, upon arrival thaw completely, ~4°C after initial use
Promega PowerQuant™ Calibration Kit	Mfr provided, -30°C to -10°C; short term storage (less than 1 week) at ~4°C; minimize number of freeze-thaw cycles
Promega PowerQuant™ System	Mfr provided, -30°C to -10°C; after first thaw, store at ~4°C; for optimal performance, the reagents should be used within one week. For longer storage, the reagents may be re-frozen but more than two freeze-thaw cycles should be avoided.
Promega PowerQuant™ Male gDNA Standard (provided with PowerQuant™ System kit)	Mfr provided, -30°C to -10°C upon receipt, ~4°C after initial use; do not refreeze
Proteinase K (from EZ1&2 DNA Investigator Kit)	Mfr provided
EZ1&2® DNA Investigator kit w/ carrier RNA and G2 Buffer	Mfr provided, cRNA frozen after reconstitution
Buffer MTL	3 years
20% Sarkosyl	1 year
Sperm Wash Buffer (SWB)	1 year
Sterile Deionized Water (sdH_2O)	3 years
Sterile Distilled Water	3 years
TE Buffer	2 years
Tris/EDTA/NaCl (TNE)	1 year

60. REAGENT PREPARATION

Use reagent grade chemicals unless otherwise noted. Premade, reagent grade chemicals can be substituted for many of the recipes below. Prepare all solutions using deionized water (dH_2O). Wear gloves and follow safety recommendations provided by the manufacturer for handling chemicals. Comply with any and all laws, regulations, or orders with respect to the disposal of any hazardous or toxic chemical, material, substance or waste. Store all reagents at room temperature unless otherwise noted. Reagents are prepared as follows:

Solution	Volume	Method of Preparation	Comments
1 M Tris-HCl, pH 8.0	1 L	1. 98 g Tris-HCl 2. 46 g Tris base 3. 800 ml of dH_2O 4. Check pH [should be 8.0 (+/- 0.2)]. 5. Adjust volume to 1 liter with dH_2O. 6. Autoclave.	*Stock solution*
0.5 M EDTA Disodium ethylene diamine tetraacetic acid dihydrate ($Na_2EDTA{\cdot}2H_2O$)	1 L	1. 186.1g $Na_2EDTA{\cdot}2H_2O$ 2. 800ml of dH_2O 3. Stir with magnetic stirrer 4. pH to 8.0 (+/- 0.2) by adding ~20g of NaOH pellets* 5. Check pH (add 5N or 10N NaOH if needed) 6. Adjust volume to 1 liter with dH_2O 7. Autoclave or filter through a sterile 0.2 μm filter. *The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.	*Stock solution* Warning: EDTA is an irritant. Wear lab coat, gloves, mask, and protective eyewear. Warning: NaOH is corrosive and toxic. Wear lab coat, gloves, and protective eyewear. Use caution when handling. NaOH, when combined with water, results in an exothermic reaction. Dissolve the NaOH pellets in the water gradually. Allow the NaOH to dissolve completely and cool between each addition.
20% (w/v) SDS Sodium dodecyl sulfate	1 L	1. 200 g electrophoresis-grade (ultra pure) SDS 2. 800 ml dH_2O 3. Adjust volume to 1 liter with dH_2O. OR 4. SDS may be purchased pre-made from SERI.	*Stock solution* Warning: Wear protective mask when weighing SDS. Warming (e.g. in a 37° C water bath) may be necessary to dissolve solids completely.

TNE (TRIS/EDTA/NaCL) 10 mM Tris-HCl (pH 8.0) 100 mM NaCl 1mM EDTA (pH 8.0)	100 ml	1. Add 1 ml of 1 M Tris-HCl pH 8.0 (*stock solution) to approximately 75 ml of dH_2O. 2. To this solution add 0.584 g NaCl and 200 µl 0.5M EDTA (*stock solution). 3. Stir until dissolved. 4. Adjust the pH to 8.0 (if necessary) with 0.4 N NaOH. 5. Adjust volume to 100 ml with dH_2O. 6. Autoclave.	***Extraction reagent*** Expiration = 1 year
20% Sarkosyl	100 ml	1. Add 20 g N-lauroylsarcosine to dH_2O. 2. Stir until dissolved. 3. Adjust volume of 100 ml with dH_2O 4. Filter through a sterile 0.45 µM filter.	***Extraction reagent*** Expiration = 1 year
1.0 M Dithiothreitol (DTT)	10 ml	1. Add 1.542 g of DTT to 10 ml dH_2O. 2. Aliquot into 1 ml portions. 3. Store frozen.	***Extraction reagent*** Expiration = 3 years
Sperm Wash Buffer (SWB) 10 mM Tris-HCl, pH 8.0 10 mM EDTA 50 mM NaCl 2% SDS	500 ml	1. 5 ml 1 M Tris-HCl, pH 8.0 (stock solution) 2. 10 ml 0.5 EDTA (stock solution) 3. 1.46 g NaCl 4. 50 ml 20% SDS (stock solution) 5. 435 ml of dH_2O 6. Check pH - adjust to 8.0 with HCl if needed 7. Autoclave	***Extraction reagent*** Expiration = 1 year
Reconstituted carrier RNA (1 µg/µL)		1. Add 310 µL of autoclaved dH_2O to carrier RNA 2. Vortex to ensure carrier RNA is thoroughly dissolved 3. Aliquot into small portions for use Store frozen	***Extraction reagent*** Lyophilized carrier RNA is provided as a component of the QIAGEN DNA Investigator Kit
TE Buffer 10 mM Tris-HCl, pH 8.0 0.1 mM EDTA	1 L	1. 10 ml of 1 M Tris-HCl, pH 8.0 (stock solution) 2. 0.2 ml of 0.5 M EDTA (stock solution) 3. 990 ml dH_2O 4. Check pH - adjust to 8.0 with HCl if needed 5. Autoclave	***Extraction reagent*** Expiration = 2 years
Deionized Formamide Alternatively, the formamide can be aliquoted directly		1. 10 g of AG 501-X8 resin 2. 100 g of formamide 3. Stir with magnetic stir bar gently for at least 30 minutes 4. Check pH using pH paper. • If the pH is not greater than 7.0, decant the formamide • Add an additional 10 g of resin. • Stir for another 30 minutes.	Warning: Formamide should neither be inhaled nor contact the skin. Wear gloves and work in a fume hood. DO NOT remove the beads by filtration. The filter paper will dissolve in the formamide and replace

from the bottle and frozen.		5. When the pH is greater than 7.0, aliquot into 1.0 ml portions. 6. Store frozen. Throw away any tubes that do not freeze at –20º C.	any impurities which had been previously removed. Either decant it into a clean container, or let the beads settle out and aliquot directly from the container with the beads. The beads are coarse and will not pass through the opening of a 1 ml pipette tip.

61. CALIBRATION OF INSTRUMENTS

Checking the calibration of equipment and instruments demonstrates they are working in the proper fashion and will produce the expected results. For each refrigerator/freezer/oven/water bath, variance shall be established as per the Equipment Calibration and Maintenance section of the DNA Analysis Quality Assurance manual. If the instrument is functioning outside of its expected range, use of the instrument in forensic testing should be stopped immediately and measures taken to return the instrument to function within its accepted range. The following equipment/instruments may affect testing in DNA, and written documentation of their calibrations and checks (below) shall be maintained.

General

1. pH meters will be calibrated according to the manufacturers' instructions before they are used. The calibration including buffers used and, if recommended by the manufacturer, the slope shall be recorded.

2. Balances will be checked at least yearly by an external provider using NIST Traceable weights.

3. The operating temperature of refrigerators, freezers, and any other storage unit or area used to store reagents or evidence that would degrade if not maintained at or below a given temperature will be monitored and recorded each working day using the TempGenius system. If manual recording is necessary, a thermometer will be employed, and the temperature recorded each working day. Semiannually, the TempGenius sensors (or thermometers) used to record these temperatures will be verified against a NIST traceable thermometer. The sensor (or thermometer) can remain in service if it is within ± 4˚ C of the NIST traceable thermometer. Alternatively, a thermometer can be used that is NIST traceable and certified for a specific time period as long as it is replaced or re-certified before that period has expired.

4. The operating temperature of ovens, water baths, or other apparatus that is used to maintain a constant temperature of reagents or a reaction shall be monitored and recorded each working day using the TempGenius system. If the oven, water bath, or other apparatus is intermittently used for reactions, the operating temperature shall be monitored and recorded prior to its use. If manual recording is necessary, a thermometer will be employed, and the temperature recorded each working day or prior to use. Semiannually, the TempGenius sensors (or thermometers) used to record these temperatures will be verified against a NIST traceable thermometer. The sensors (or thermometers) can remain in service if they are within ± 4˚ C of the NIST traceable thermometer unless otherwise stated in the specific application protocol. Alternatively, a thermometer can be used that is NIST traceable and certified for a specific time period as long as it is replaced or re-certified before that period has expired.

    a. The operating temperatures of apparatus used solely for non-critical processes (e.g. denaturing/snap-cooling of amplification product) do not require daily recording of temperatures. The sensors (or thermometers) of these apparatus shall be verified against a NIST traceable thermometer at least annually.

5. On a bi-annual basis (2x per year), the thermal cyclers shall be calibrated using the appropriate calibrating probe and the directions provided by the manufacturer for the temperature verification test and temperature non-uniformity test. For the ProFlex thermal cyclers, the Heated Cover test and the Self Verification Test shall also be performed.

    a. If a thermal cycler fails calibration, consider confirming the result with a second (different) calibration probe to rule out a probe issue. If calibration issues persist, contact the appropriate manufacturer.

In addition, the wells and heated cover will be cleaned bi-annually according to manufacturer specifications. If a contamination event occurs related to the thermal cycler, it shall be cleaned according to manufacturer specifications.

When a new thermal cycler is installed, a performance verification plan addressing reliability will be drafted and submitted to the DNA Technical Leader for approval.

The results of the performance verification tests will be summarized and retained in the instrument log book or in the appropriate electronic folder on an agency approved server.

6. Certified traceable thermometers are critical equipment and may be used for the duration of their certification.

7. Thermal cycler temperature verification systems shall be calibrated annually by an appropriately qualified external vendor. The annual calibration serves as a performance check.

8. Pipettes shall have their calibration checked at least once a year by a qualified external agency. The annual calibration serves as a performance check.

The following instruments shall be performance checked prior to implementation for casework purposes and following routine maintenance or repair by qualified technicians.

- Qiagen EZ1 BioRobot
- Qiagen EZ2 Connect
- ABI Prism 7500 Real Time PCR instrument
- ABI GeneAmp PCR System 9700 thermal cycler
- Applied Biosystems ProFlex PCR System
- Hamilton Microlab AutoLys STAR Workstation
- Hamilton Microlab STARlet
- Qiagen QIAgility BioRobot
- Genetic analyzers

Records of the above checks and calibrations will either be maintained within the DNA sections or have a reference to where they are retained within the laboratory.

62. TEMPGENIUS – SETUP AND MAINTENANCE

Installation and Setup

The TempGenius system wirelessly records the temperature for refrigerators, freezers, water baths, and ovens in the laboratory. TempGenius provides constant monitoring of these units and if the temperature of a unit falls outside of a given range, will alert the user and record the event.

General Installation and Operation

Refer to the TempGenius "Installation Guide and Operating Manual".

Setting Up Alert Parameters

Alert parameters for each sensor must be set-up separately. In order for an event to trigger an alert, a temperature outside the designated range must be maintained for the designated time "span". For each sensor, parameters for three separate sets of alert criteria can be designated. Access the alert parameters for a sensor via Dashboard → Set-up button → Sensor Set-up tab.

Alert Schedules

Alerts can be delivered via phone/voicemail, fax, text, or email. A single user can be notified multiple ways if desired, though the schedule for each delivery method must be set-up separately. Any assigned user(s) will receive alerts for all sensors during their alert schedule. To set up an alert schedule for a user, navigate to the Alert Set-up tab via Dashboard → Set-up button → Alert Set-Up tab.

Monitoring and Alert Resolution

TempGenius monitors temperatures and automatically sends alerts to the assigned user(s) and the TempGenius Dashboard.

Alert Resolution

When an alert is generated, a resolution (or "Corrective Action" as termed by TempGenius) shall be recorded. Alert resolutions are handled via the Corrective Actions tab in the Dashboard. Enter the resolution description and initials of the user in the highlighted fields, and click "Save".

Maintenance

Semiannually

A NIST probe reading for each monitored unit will be recorded semiannually to ensure the accuracy of the temperature readings recorded by TempGenius. If the TempGenius reading varies from the NIST reading outside of the allowable range, another reading will be taken at a different time. If the TempGenius reading persists outside of range, the manufacturer will be contacted.

Annually

The manufacturer defines the annual calibration as the adjustment (or replacement) of a sensor due to temperature variability against a NIST probe. The average offset for a sensor will be determined based on the total readings for the year. If the annual average offset of a sensor is in excess of 4°C, an adjustment is mandatory and the manufacturer shall be contacted. A laboratory may choose to make an adjustment if the offset between a sensor and NIST probe is +/- 1°C.

If any adjustment is made to the sensor, a reading with a NIST probe will be taken the following work day to verify the adjustment. All adjustments will be documented on the TempGenius maintenance sheet.

Every Two Years or As Needed

Replace the batteries in the sensors by unscrewing the transmitter cover. Use lithium batteries to ensure performance at cold temperatures.

63. QIAGEN BIOROBOT EZ1 AND EZ2 CONNECT – MAINTENANCE

After Each Run

1. Clean the worktable and cartridge rack with dH_2O followed by ethanol.
2. Close the workstation door and follow the prompts to clean the piercing unit.
3. Open the workstation door and wipe the piercing unit using a tissue moistened with dH_2O followed by a tissue moistened with ethanol.
4. Clean the metal plate(s) underneath the worktable with dH_2O followed by ethanol.
5. Close the workstation door.

Monthly (or more often as needed)

Grease the O-rings:

1. EZ1: Remove the worktable and cartridge rack and push back the tray holder.
   EZ2: Follow the weekly maintenance prompts
2. Add a small amount of silicon grease to each pipettor head O-ring using any method that minimizes the amount of grease added.
3. Wipe off any excess grease.

NOTE: One method that can be used is to add a small amount of silicon grease to the inside top edge of an unused filter-tip. Place this tip onto the pipettor head, and rotate the tip over the O-rings.

Annually

On a yearly basis, the instruments shall be serviced by a Qiagen technician.

Performance Check

A performance check shall be conducted prior to use on casework for each new EZ1 and EZ2 Connect instrument. This performance verification shall address reliability and test for contamination and shall be documented.

Following yearly maintenance or repair that directly affects results of analysis, the instrument shall be performance checked by extracting/purifying a known sample and a reagent blank followed by quantification. The extracted samples shall yield sufficient DNA to target a robust amplification and the blank shall be free of DNA (i.e., Undet.); however, amplification is not required if these two conditions are met. If amplified, the known sample shall genotype correctly and the blank shall be free of DNA.

For repairs that do not directly affect analysis results, such as door magnet or UV bulb replacement, the performance check need only demonstrate that the repair or service was successful.

The results of all performance verifications of the EZ1 and EZ2 instruments shall be summarized and maintained.

64. AB7500 REAL TIME PCR SYSTEMS INSTRUMENT – SETUP

PowerQuant™ System

A plate template file (.edt file) can be used to store the dye information, target names and tasks; well locations and DNA concentrations for the DNA standards; run method, and analysis settings.

In all templates created for casework analysis, ensure that the DNA quantification standards are run in duplicate.

1. Turn on the computer associated with the 7500 instrument. Turn on the 7500 instrument then launch the AB 7500 Software v.2.3.

2. On the *Home* screen, select the *Advanced Setup* icon (or select "Advanced Setup" from the *New Experiment* button).

3. In the *Experiment Properties* form, specify the following parameters
   - Enter an appropriate name for the template
   - Select the following: "7500 (96 Wells)" for instrument, "Quantitation – Standard Curve" for experiment, "TaqMan® Reagents" for reagents to use, and "Standard (~2 hours to complete a run)" for ramp speed.

4. Select "Plate Setup" from the *Setup* menu in the left panel. Select the *Define Targets and Samples* tab. Click three times on "Add New Target", and enter the following information:

Target Name	Reporter	Quencher
Autosomal	PQ_FAM	NFQ-MGB
Y	PQ_CFG540	NFQ-MGB
Degradation	PQ_Q670	NFQ-MGB
IPC	PQ_TMR	NFQ-MGB

5. Select the *Assign Targets and Samples* tab and highlight the wells in use on the *View Plate Layout* tab by dragging the pointer over the plate wells. Assign all four targets to the selected wells by selecting the boxes under "Assign".

6. Highlight the wells containing the DNA standards on the *View Plate Layout* tab and select "S" as the Task for the autosomal, Y and degradation targets. The Task for the IPC should be "U".

7. Enter the concentration for each DNA standard in the Quantity field without the unit of measure (e.g. enter 50 for 50ng/µl).

8. Highlight the wells containing the NTC samples on the *View Plate Layout* tab and select "U" as the task for all targets.

Notes:
- Multiple wells with DNA standards at the same DNA concentration can be highlighted at the same time so that values need to be entered only once.
- The *Define and Setup Standards* tool will not work for assigning a standard curve to multiple targets in the same sample.

9. Select "PQ_CXR" from the *Select the dye to use as the passive reference* drop-down menu

10. From the *Analysis* menu at the top of the screen, select "Analysis Settings".

11. On the *CT Settings* tab in the *Select a Target* panel, select the Autosomal target. In the *CT Settings for Autosomal* section, uncheck the *Use Default Settings* box and uncheck the *Automatic Threshold* box. Enter 0.2 for the threshold. Leave the *Automatic Baseline* box checked. Repeat this for each target with the following threshold values:

    Autosomal: 0.2
    Degradation: 0.2
    IPC: 0.03
    Y: 0.2

12. Select “Run Method” under “Setup” in the left panel and modify the default run method as follows:
    - Enter “20” for “Reaction Volume Per Well”
    - Select the first Holding Stage. Right click using the mouse and choose “Delete Selected” to delete this stage.
    - Change the single remaining Holding Stage to 2 minutes at 98°C.
    - Enter “39” for “Number of Cycles” under “Cycling Stage”
    - Change Step 1 to 98°C for 15 seconds and Step 2 to 62°C for 35 seconds
    - Ensure that the *Data Collection On* icon is active for Step 2 of the Cycling Stage

    Note: The *Data Collection On* icon is shown in the legend at the bottom of the screen.

13. Save the template to an appropriate location using the “Save as Template” option from the file menu. The instrument setup and thermal cycling program can be saved as a template for future use.

65. AB 7500 REAL TIME PCR SYSTEM INSTRUMENT – MAINTENANCE

The AB 7500 Real Time PCR System is a PCR instrument used for the quantification assay. The instrument has the following maintenance schedule.

On at least a yearly basis or if the instrument is moved to a new laboratory location, the following checks shall be performed: 1) the Regions of Interest (ROI) calibration, and 2) the Dye Calibration. These yearly checks may be performed by ABI service personnel as part of the regular instrument maintenance schedule.

After approximately 2000 hours of use, the bulb shall be replaced. The following checks shall be performed each time the bulb is replaced: 1) calibrate the ROI, 2) run a background calibration, 3) perform an Optical calibration and 4) run the Dye calibration.

Monthly

Background Run/Check Sample Block For Well Contamination

1. Reboot the instrument
2. Turn on the 7500 instrument and launch the 7500 Software v2.3.
3. Create a new background plate by selecting the “Instrument” tab > “Instrument Maintenance Manager” > “Background” tab > **Start Calibration**
4. Complete the calibration as instructed by the wizard (to include background plate loading)
5. Place a 96-well background tray (provided in the Spectral Dye Kit) into the instrument. Shake the plate and centrifuge as necessary to remove bubbles.
6. The background run may take 10 minutes. Do not set up any new plate documents, open existing files, or perform other processes on the system during the background run to avoid compromising the background data file.
7. When the run is complete, the background data will display, indicating the calibration status (passed/failed).
8. The plotted lines should form a tight band with fluorescent signals for filters A, B, C and D below 72,000 fluorescent standard units (FSU) and filter E below 90,000 FSU. Any outlier wells indicate potential well contamination and shall be cleaned using a swab with distilled/deionized water and, if needed, with a 10% bleach solution, followed by ethanol. The background run must be rerun to confirm whether the contaminant has been successfully removed.

Document the performance, result, and any actions taken for the background run. A service call may be necessary if the instrument cannot produce a passing background run.

Monitoring the Lamp

1. Turn on the 7500 instrument and launch the 7500 Software v2.3.
2. Click the “Instrument” tab > “Instrument Maintenance Manager” > Lamp Status/Replacement.
3. Check that usage is less than 2000 hours.

Biannual

Optical Calibration

1. Turn on the 7500 instrument and allow to warm up for at least 10 minutes before starting the calibration.
2. Launch the 7500 Software v2.3.
3. Create a new optical plate by selecting the “Instrument” tab > “Instrument Maintenance Manager” > “Optical” tab > Start Calibration

4. Complete the calibration as instructed by the wizard (to include optical plate loading)
5. Remove a ROI calibration plate from a spectral calibration kit (stored in freezer). Allow the plate to come to room temperature. Maintain the plate in the packaging until ready to use. Vortex the plate and centrifuge to ensure that no bubbles are in any of the wells.
6. The optical calibration may take 10 minutes. Do not set up any new plate documents, open existing files, or perform other processes on the system during the optical run to avoid compromising the optical data file.
7. When the run is complete, the optical data will display, indicating the calibration status (passed/failed).

Annual

Dye Calibration

Before using the PowerQuant™ system, the instrument must be calibrated for FAM, CAL Fluor® Gold 540 (CFG540), TMR, Quasar® 670 (Q670), and CXR dyes. Use the PowerQuant™ System Calibration kit which includes each of the dyes at a 100X concentration along with a calibration buffer for use as a diluent to create a spectral dye calibration plate for each dye.

Prior to performing the PowerQuant™ dye calibration, perform the ROI calibration, background calibration and optical calibration as per this manual.

Dye calibration shall be performed before initial use of the PowerQuant™ System and following bulb replacement.

Creating a PowerQuant™ Calibration Plate

1. Thaw the five PowerQuant™ calibration standards (FAM, CFG540, TMR, Q670 and CXR) and the calibration buffer.

2. Vortex the concentrated calibration standards and calibration buffer for 10 seconds to mix. DO NOT centrifuge the Calibration Standards after mixing.

3. Dilute each PQ calibration standard 100-fold in PQ calibration buffer in a separate tube as detailed in the table below.

Reagent	*FAM*	*CFG540*	*TMR*	*Q670*	*CXR*
Cal Std FAM	22µl	--	--	--	--
Cal Std CFG540	--	22µl	--	--	--
Cal Std TMR	--	--	22µl	--	--
Cal Std Q670	--	--	--	22µl	--
Cal Std CXR	--	--	--	--	22µl
Calibration Buffer	2178µl	2178µl	2178µl	2178µl	2178µl
Total Volume	2200µl	2200µl	2200µl	2200µl	2200µl

4. Vortex the diluted calibration standards for 10 seconds to mix. DO NOT centrifuge after mixing.

5. Set aside five 96-well optical plates, each marked with a respective calibration standard. For each diluted calibration standard, dispense 20 µl to all 96 wells of a 96-well optical plate reserved for the appropriate dye-specific plate.

6. Seal each plate with an optical adhesive film. Protect plates from light.

7. Centrifuge plates briefly.

8. Prepared calibration plates may be used immediately and stored protected from light at -20 °C for up to 4 months for future use.

Performing a Dye Calibration

Ensure a background calibration has been done prior to dye calibration.

The protocol followed can be found in the PowerQuant™ Technical Manual and is the Calibration protocol for the AB 7500 Software, version 2.0.6 and 2.3.

1. Turn on the computer associated with the 7500 instrument. Turn on the 7500 instrument, and then launch the AB 7500 Software v.2.3

2. From the *Instrument* menu at the top of the screen, select “Instrument Maintenance Manager”. Select the *Dye* section from the left panel. Select “Custom Dye Calibration”, and choose “Start Calibration.”

3. In the *Dye Calibration* window that appears, select *New Dye* button. In the *Dye Library* window that appears, select the *New* button.

4. Enter PQ_FAM as the new dye name, select the *Reporter* radio button under “Type” and then select “OK”. Repeat this process to define the remaining dyes: PQ_CFG540, PQ_TMR, PQ_Q670, and PQ_CXR.

5. In the *Custom Dye Calibration* Setup window, select the dye to be calibrated from the *Dye* Name drop-down list.

NOTE: If using a previously prepared and stored calibration plate, vortex and centrifuge to ensure there are no bubbles.

6. Place the dye plate to be calibrated into the instrument. Select “Next”, then “Start Run”.

7. Once the calibration run is complete, select “Next” in the bottom right corner of the window. The software will display the spectra and indicate whether the spectra are acceptable under “Status”.

8. Select “Finish”, and repeat steps 5-7 for each plate to calibrate the other dyes.

Note: FAM should give highest signal in Filter 1, CFG540 should give highest signal in Filter 2, TMR should give highest signal in Filter 3, Q670 should give highest signal in Filter 5, and CXR should give highest signal in Filter 4.

9. Document the successful performance of the calibration. If the custom dye calibration fails, prepare new standard calibration plate(s) and recalibrate.

NOTE: After calibration, store the calibration plate for up to 4 months at -20°C, protected from light. Reuse the plate when reanalysis is necessary.

Performance Check

For each new AB 7500 Real Time PCR quantitation instrument, a performance check shall be conducted prior to use on casework. This performance verification shall address sensitivity and shall be documented and retained in the instrument logbook.

Following routine yearly maintenance, a performance check will be performed, documented, and retained and consist of the following:

1. Regions of Interest (ROI) and Dye calibration tests (may be run by service technician as part of maintenance) and
2. A quantification plate containing previously quantified samples to address reproducibility.

If an instrument is moved to a new laboratory location, the ROI and Dye calibration tests shall be performed.

66. HAMILTON AUTOLYS – MAINTENANCE

Select the Microlab STAR Maintenance & Verification icon on the AutoLys computer to perform the daily and weekly maintenance. Some daily maintenance tasks are included in the weekly maintenance and do not need to be repeated when weekly maintenance is performed. Maintenance reports are generated and saved on the AutoLys computer and will be routinely archived following the laboratory's data archival procedure (e.g. quarterly backup).

Daily

1. Clean the deck and carriers with detergent solution.
2. Empty the tip waste and liquid waste, as needed.
3. Clean the tip eject sleeve with detergent solution.
4. Ensure the tightness check of the 1000µl pipetting channels passes.
5. Verify the cLLD functions properly (1000µl pipetting channels).
6. UV irradiate the tube inserts and tube carriers between runs using a crosslinker. Irradiation may be performed using a UV light in a hood for a minimum of 15 minutes when a crosslinker is not available.

Weekly

1. Clean the deck and carriers with detergent solution.
2. Check the condition of all carriers and that they appear in proper working order.
3. Clean the tip eject sleeve with detergent solution.
4. Check the laser scanner window and gently wipe down with 70% ethanol and a lint-free cloth, as needed.

Monthly

1. Empty the liquid waste container. Clean the liquid waste container and its tubing with detergent solution, followed by three water rinses.
2. Soak the tube inserts in detergent solution, rinse with water, and allow to dry.

Semiannually

Preventative maintenance will be performed twice a year by a Hamilton trained field service engineer.

As needed

If the AutoLys channels encounter problems with picking up the AutoLys tubes, the calibration file may be adjusted to realign the channels. The calibration file should also be evaluated following semiannual maintenance.

1. Navigate to the calibration file ShiftValuesText.xls within the Program Files folder on the AutoLys computer.
2. Save a copy of the original file by adding the date to the file name prior to making adjustments.
3. Adjust the X, Y, and/or Z values, as needed.
4. Run the MedChannelShiftCal protocol to evaluate the adjustments.
5. Run the centrifuge method to ensure the trays transfer correctly and complete a performance check of each AutoLys protocol that utilizes the AutoLys channels and tubes.

Performance check

A performance check of each new Hamilton AutoLys that encompasses all the protocols intended for use on that instrument shall be conducted prior to use on casework. This performance verification shall address reliability, test for contamination, and be documented.

Following semiannual maintenance, repair that directly affects results of analysis, or adjustments to the AutoLys channels calibration file, a performance check will be performed and documented prior to the instrument being returned to use on casework.

The performance check shall consist of a set of known samples run through all protocols intended for use on that instrument (e.g. Y-screening, DNA lysis/purification, etc.) with the expected results obtained. A performance check will also be performed and documented on an individual protocol if a significant protocol revision occurs. This performance check will be completed before the revised protocol can be used on casework and shall consist of a set of known samples run through the revised protocol with expected results obtained. The former protocol will be removed from service and archived following the laboratory's data archival procedure (e.g. quarterly backup).

For repairs that do not directly affect analysis results, such as door strut or UV bulb replacement, the performance check need only demonstrate that the repair was successful.

67. HAMILTON MICROLAB STARLET – MAINTENANCE

Selecting the MicroLab Maintenance and Verification Run icon will allow the user to view the current maintenance procedures that are due and all maintenance procedures available to the laboratory staff.

An electronic log of the maintenance performed is maintained on the computer associated with the robot. The electronic log will be archived at an appropriate interval.

Day-of-Use Maintenance

Daily and weekly maintenance shall be performed prior to the first use of each day or week, respectively. Daily and weekly maintenance is not required on days or weeks in which the STARlet is not used. Weekly maintenance includes the daily maintenance tasks and fulfills the daily maintenance requirement for the day on which it is performed.

1. Open the maintenance software.
2. Select “Daily” or “Weekly” maintenance as appropriate.
3. Select “Process” on the top bar
4. Select “Run”
5. Follow the directions given by the software.

After use of the robot, clean all surfaces with 70-100% ethanol and run the UV program, if equipped.

Monthly

If the instrument is not equipped with a UV lamp, place tube holders and reagent block into a hood with a UV light and run for a minimum of 15 minutes.

Semiannual Maintenance

Preventative maintenance shall be performed semiannually by a Hamilton Service Representative.

Performance Check

A performance verification of each new Hamilton Microlab STARlet shall be performed prior to use on casework. Additional performance verification shall be performed after semiannual preventative maintenance or repair that directly affects results of analysis. For repairs that do not directly affect analysis results, such as door strut or UV bulb replacement, the performance check need only demonstrate that the repair was successful. Refer to Equipment Performance Verification procedures in the CLD Quality Operations manual.

Revisions to the STARlet’s programming are a modification of the method and may require performance verification and/or validation. Refer to Method Validation procedures in the CLD Quality Operations and DNA Quality Assurance manuals. After a revision to the programming, the “Log Files” folder on the STARlet computer will be archived to a server storage location.

68. QIAGEN QIAGILITY – MAINTENANCE

Daily (following use)

1. Remove all samples, plastic-ware and consumables from the instrument deck.
2. Empty discarded tips from the tip disposal box if needed.
3. Wipe the surfaces of the instrument deck, sample racks and plate trays with a towel moistened with a detergent solution. Alternately, the racks and trays can be rinsed with water and dried.
4. A UV decontamination step may be selected following the cleaning procedure.

Yearly

On a yearly basis, the QIAgility BioRobot instruments will be serviced by a QIAGEN technician.

Performance Check

A performance check of each new QIAgility BioRobot shall be conducted prior to use on casework. This performance verification shall address reliability and test for contamination and shall be documented.

Following yearly maintenance or repair that directly affects results of analysis, , or a significant protocol revision a performance check will be performed and documented. The performance check shall consist of a set of known samples run through the appropriate protocols (quant set up or the CE plate set up for the instruments in the post PCR rooms) with the expected results obtained. Following a protocol revision, the former protocol will be removed and archived following the laboratory's data archival procedure (e.g. quarterly backup). For repairs that do not directly affect analysis results, such as door strut or UV bulb replacement, the performance check need only demonstrate that the repair was successful.

If the QIAgility pipettor head is replaced during the yearly maintenance or for a repair, the p-axis values may need to be verified against the Volume Calibration certificate for the new pipettor head. This can be done by selecting Option 1 of the Volume Calibration Wizard under Options/Robot Setup/Calibrate Volumes and checking that the P-axis values listed match those on the certificate. It may be necessary re-calibrate the plate positions and heights.

Calibrations

Calibrations are required following yearly maintenance or if changing the plastic-ware used. The software will indicate calibration is needed by displaying a red exclamation point in a box in the top right corner of the plate windows.

<u>Calibrating plate position</u>

Note: Tip Rack Holder calibrations must be performed before any other plate type can be calibrated.

Caution: The Y-arm will move during position calibration when the hood is raised. Do not click on any software buttons while parts of your body are within the instrument worktable.

1. Open the instrument hood and place the plates to be calibrated on the instrument worktable.
2. Ensure that tips are available in at least one Tip Rack Holder.
3. Open the plate position calibration screen by selecting "Options/Robot Setup/Calibrate plate positions".
4. Select a plate from the "Plate Selection" list. Note: Tip Rack Holders are listed as 96-well plates.
5. Use the "Lower Tip" and "Raise Tip" buttons in the "Tip Control" panel to move the pipetting head so that it is just above the plate surface.

6. Adjust the “X-position” and “Y-position” in the “Plate Origin” panel so that the pipetting head is directly above the center of the well indicated in red.
7. Click “Save.”

Calibrating Plate Heights

1. Ensure that tips are available, that the Tip Rack Holder is calibrated, and that the correct tips are set as available.
2. Place empty tubes in the first 4 wells of each plate.
3. Select “Options/Robot Setup/Calibrate plate height”. The “Configure Plate Heights” dialog box will appear.
4. Check the boxes next to the plates to be calibrated in the “Plates to Calibrate” list.
5. Define the number of wells from which to take an average. It is recommended that 4 wells are used to calculate the average.
6. Click on the “Autodetect (checked items)” button to initiate calibration and “Yes” to the “Ready to auto-detect well heights” in the pop-up window.
7. Click “Ok” to the auto-detecting heights for the 96 well plate...pop-up window.
8. If “Require confirmation from user?” was selected, the following message Bottom of well detected at xxx, based off the average of 4 samples” will appear. Click on the “Yes” button to accept the calibration, if the averages for a plate are within 10 units of each other. If the averages are not within 10 units of each other, click on the “No” button to reject the current calibration and then repeat steps 4–8.

69. AB 3500 GENETIC ANALYZER – SETUP AND MAINTENANCE

Setup

Spatial Calibration

The spatial calibration maps the positional relationship between the capillaries and the CCD camera. This calibration allows the instrument software to determine what fluorescent emissions are coming from which capillary. A spatial calibration must be done every time a capillary array is removed and replaced, the detector door is opened, the detection cell is moved, or the instrument is moved.

1. In the Maintenance navigation pane, select the spatial calibration.

2. Select **No Fill** or **Fill** to fill the array with polymer.

3. Start calibration.

4. Evaluate the spatial calibration. Ensure that you see
    a. One sharp peak for each capillary (small shoulders are acceptable).
    b. One marker (+) at the apex of every peak. No off-apex markers.
    c. An even peak profile (all peaks about the same height).

5. Accept the results if the above criteria are met. Refer to the 3500 User Guide if the spatial calibration fails.

Spectral Calibration

The spectral calibration is applied during sample detection to calculate the spectral overlap and separate the raw fluorescent signals into individual color signals. Perform a spectral calibration after major maintenance on the system, such as changing the laser, calibrating or replacing the CCD camera, changing the polymer or capillary array, or if the instrument is moved to a new location. It may be necessary to repeat the spectral calibration if pull-up/pull-down increases or approximately every 6 months.

Fusion 6C

At first use, thaw the 6C Matrix Mix and Matrix Dilution Buffer completely. After first use, store the reagents between 2-10ºC.

1. Pre-heat the oven to 60ºC (at least 30 minutes prior to the first injection).
2. Vortex the 6C Matrix Mix for 10-15 seconds prior to use. Add 10µl of the 6C Matrix Mix to one tube of Matrix Dilution Buffer. Vortex for 10-15 seconds. The diluted 6C Matrix Mix can be stored for up to 1 week between 2-10ºC.
3. Add 10µl of the diluted 6C Matrix Mix prepared in step 2 to 500µl of Hi-Di™ formamide. Vortex for 10-15 seconds.
4. Add 15µl of the 6C Matrix Mix with formamide prepared in step 3 to wells A1 through H1 of the 96-well plate. Briefly centrifuge the plate to remove bubbles. DO NOT HEAT DENATURE.
5. Place the assembled plate on the instrument. Select the Spectral Calibration in the Maintenance navigation pane. Select “Matrix” for the chemistry standard and “Promega J6” for the Dye set. Ensure that the box is checked to allow borrowing.
6. Start the calibration.
7. Evaluate the spectral calibration data and accept or reject. Passing and failing capillaries are shown in green and red respectively. Borrowed capillaries are shown in yellow with an arrow indicating the capillary borrowed from.

NOTE: Refer to the PowerPlex® 6C Matrix Standard Technical Manual for instructions regarding creating a new Dye Set.

Y23

At first use, thaw the 5C Matrix Mix and Matrix Dilution Buffer completely. After first use, store the reagents between 2-10ºC.

1. Pre-heat the oven to 60ºC (at least 30 minutes prior to the first injection).
2. Vortex the 5C Matrix Mix for 10-15 seconds prior to use. Add 10µl of the 5C Matrix Mix to one tube of Matrix Dilution Buffer. Vortex for 10-15 seconds. The diluted 5C Matrix Mix can be stored for up to 1 week between 2-10ºC.
3. Add 10µl of the diluted 5C Matrix Mix prepared in step 2 to 500µl of Hi-Di™ formamide. Vortex for 10-15 seconds.
4. Add 15µl of the 5C Matrix Mix with formamide prepared in step 3 to wells A1 through H1 of the 96-well plate. Briefly centrifuge the plate to remove bubbles. DO NOT HEAT DENATURE.
5. Place the assembled plate on the instrument. Select the Spectral Calibration in the Maintenance navigation pane. Select “Matrix” for the chemistry standard and “Promega G5” for the Dye set. Ensure that the box is checked to allow borrowing.
6. Start the calibration.
7. Evaluate the spectral calibration data and accept or reject. Passing and failing capillaries are shown in green and red respectively. Borrowed capillaries are shown in yellow with an arrow indicating the capillary borrowed from.

NOTE: Refer to the PowerPlex® 5C Matrix Standard Technical Manual for instructions regarding creating a new Dye Set.

Maintenance

The Data Collection software installed on the computers associated with the genetic analyzers employs wizards to assist the user in maintenance procedures. Access the appropriate wizard and follow the prompts to complete the required general maintenance tasks.

Before each run

Check for bubbles in the pump block and all lines; remove using software wizards if necessary. Replace polymer, anode/cathode buffers if necessary.

Monthly

- Perform a water wash to flush the pump chamber and channels. This shall be done via the software wizard. The wizard requires Conditioning Reagent.
- Replace the polymer.
- Flush the water trap (pump trap).
- Replace the cathode buffer septa.
- Restart the instrument and computer.

Quarterly

- Backup run data and monitor the instrument database.

Annually

Preventative maintenance will be performed by a qualified technician. If an instrument is permanently taken out of service it does not require the annual maintenance to be performed in the year it is removed.

As needed

- If polymer has been stagnant in the chamber/channels/array for an extended period between water washes and increased artifacts are observed, the array can be filled with fresh polymer (via the Fill Array Wizard) or a water wash can be performed.
- Replace the capillary array whenever poor precision or allele calling is noted, or when resolution or signal intensity decreases. It is acceptable to use a capillary array past the manufacturer's expiration date.
- Perform a spatial calibration whenever the capillary array has been removed/replaced, the detection cell door has been opened, the detection cell of the cap array is moved, or the instrument was moved.
- Perform a spectral calibration whenever a new capillary array is installed, a new dye set is used, after any optical alignment, or if pull-up/pull-down increases.
- Restart the instrument and computer.

Performance Check

A performance check shall be done prior to use for casework purposes for each CE unit that is moved, or when an AB technician provides routine maintenance or repair that directly affects results of analysis.

The performance check will consist of successfully running a +C amplification positive or other known sample, an amplification negative, and a ladder on the instrument.

For repairs that do not directly affect analysis results, such as door magnet replacement, the performance check need only demonstrate that the repair or service was successful.

A new CE instrument also requires a 1 base pair precision check (± 0.5) with a minimum of data from 6 ladders run per capillary.

Verify that the sample genotyped correctly and meets all data analysis parameters as required in the current laboratory protocols. Documentation of the performance check will be recorded in the instrument logbook.

A spatial calibration is required if repair/maintenance performed involved removing or replacing the instrument capillary (also if relocating the instrument). A spectral calibration is required if repair/maintenance involved the optics of the instrument (laser or CCD).

70. GENEMAPPER® ID-X SETUP

Size Standard

The size standard for use with PowerPlex® Fusion 6C and PowerPlex® Y23 is WEN_ILS_500_CS.

Fusion 6C Panels and Bins

GMID-X requires chemistry-specific panels and bins. For Fusion 6C and Y23, these have been acquired from Promega and imported into GMID-X. Custom panels and bins specific for STRmix analysis have been created from those acquired panels and bins from Promega. For Fusion 6C, in the Panel Manager, check the Y Marker boxes for DYS391, DYS576, and DYS570 to indicate they are Y markers. For Y23, check the Y Marker boxes for all markers except DYS385.

Analysis Methods

The Analysis Method defines peak detection, sizing, genotyping, and quality assessment parameters applied during the analysis of sample data. Instructions for creating Analysis Methods are below.

Creating or Changing an Analysis Method – Fusion 6C

1. In GeneMapper® *ID-X*, select GeneMapper® *ID-X* Manager.

2. Select the **Analysis Methods** tab, then click **New**.

3. Select the **General** tab.
    a. Enter a name for the method. Instructions provided below for methods named
        i. **Fusion6C_70rfu_1.2** (for unknown/question samples, optional for single source profiles or simple mixtures that are analyzed using the 70_STRmix_F6C_061022 method) and negative controls)
        ii. **600_F6C_072723** (for known/reference samples, positive controls, and ladders).
        iii. **70_STRmix_F6C_061022** (for ladders and unknown/question samples for STRMix)
        iv. **600_STRmix_F6C_061022** (for ladders for STRmix, optional)
        v. **120_F6C_DA_072723** (for direct amplification reference samples (optional) and negative controls)
        vi. **500_F6C_DA_072723** (for direct amplification reference samples (optional), positive controls, and ladders (alternatively, the associated direct amplification ladder may be analyzed using 600_F6C_072723))

4. From the drop-down list for Security Group, select **GeneMapper ID-X Security Group**.

Note: Analysis of negative controls using the Fusion6C_70rfu_1.2 or 120_F6C_DA_072723 method utilizes the GMID-X software as an expert assistant and ensures that any evidence of contamination that may be present within a negative control will be flagged by the software for the analyst to review.

5. Select the **Allele** tab.

    a. For **Fusion6C_70rfu_1.2, 600_F6C_072723, 120_F6C_DA_072723,** and **500_F6C_DA_072723** methods
        i. Select “PowerPlex_Fusion_6C_Bins_IDX-v1.2” from the Bin set drop-down menu.
        ii. Click on the box to select **Use marker specific stutter ratio if available**.
        iii. All other boxes, including the Amelogenin Cutoff box should have “0.0” entered.

   b. For **70_STRmix_F6C_061022** and **600_STRmix_F6C_061022** methods
      i. Select "Fusion 6C Bins-STRmix-061022" from the bin set drop-down menu.
      ii. Click on the box to select **Use marker specific stutter ratio if available**. A custom stutter file has been created to filter stutter that is not modeled by STRmix v2.8 (n-2 at D19S433 and FGA).
      iii. All other boxes, including the Amelogenin Cutoff box should have "0.0" entered.

6. Select the **Peak Detector** tab.
   a. In the **Ranges** box, select **Full Range** from the drop-down menu. Select **Partial Sizes** from the drop-down menu; designate the start point at 60, stop point at 500.
   b. In the **Smoothing and Baselining** box, select **Light** for Smoothing and type in "51" for the Baseline Window.
   c. Select **Local Southern Method** in the **Size Calling Method**.
   d. In the **Peak Detection** box, set the **Min. Peak Half Width** at 2 pts, the **Polynomial Degree** at 3, and the **Peak Window Size** at 15 pts; Set the **Slope Threshold Peak Start** and **End** both at 0.0.
      i. For **Fusion6C_70rfu_1.2** and **70_STRmix_F6C_061022** methods, set the peak amplitude thresholds at "70" for B, G, Y, R, and P dye filters and "600" for the O dye filter.
      ii. For **600_F6C_072723 and 600_STRmix_F6C_061022** methods: set the peak amplitude thresholds at "600" for all dye filters.
      iii. For 120_F6C_DA_072723method, set the peak amplitude thresholds at "120" for all dye filters.
      iv. For 500_F6C_DA_072723method, set the peak amplitude thresholds at "500" for B, G, Y, R, and P dye filters and "120" for the O dye filter.

7. Select the Peak Quality tab. In the Min/Max Peak Height (LPH/MPH) box, enter "600.0" for Fusion6C_70rfu method and enter "500.0" for 120_F6C_DA_072723 and 500_F6C_DA_072723 methods for both Homozygous min peak height and Heterozygous min peak height. Enter "1500.0" for Homozygous min peak height and "600.0" for Heterozygous min peak height for 600_F6C_072723 method. Enter "40000" for Max Peak Height (MPH). Enter "0.5" for Min peak height ratio in the Peak Height Ratio (PHR) box for Fusion6C_70rfu and 600_F6C_072723 methods and enter "0.6" for 120_F6C_DA_072723 and 500_F6C_DA_072723 methods, "1.5" for Max peak width (basepairs) in the Broad Peak (BD) box, "2" for Max expected alleles in the Allele Number (AN) box, and "0.2" for the Cut-off Value in the Allelic Ladder Spike box.

8. Do not alter the default settings in the **SQ and GQ Settings** tab.

9. After completing these settings, click Save to save the changes and close the Analysis Method Editor.

The analysis method(s) can be exported to other copies of GeneMapper® *ID-X*.

<u>Creating or Changing an Analysis Method – Y23</u>

1. In GeneMapper® *ID-X*, select GeneMapper® *ID-X* Manager.
2. Select the **Analysis Methods** tab, then click **New**.
3. **General** tab.
   a. Enter a name for the method. Instructions provided below for methods named or similarly named: "Y23_100rfu_1.2" (used for unknown/questioned, negative control/reagent blank) and "Y23_200rfu_1.2" (used for known/reference, positive male control, and ladder).
   b. From the drop-down list for Security Group, select **GeneMapper ID-X Security Group**.
   c. Instrument type and a description may be entered.
4. **Allele** tab.
   a. Select "PowerPlexY23_Bins_IDX_v1.2" from the Bin Set drop-down menu.

b. **Select "Use marker-specific stutter ratio and distance if available"**.
c. Enter the following:

Global Minus Stutter Distance	Tri	Tetra	Penta	Hexa
From	2.25	3.25	4.25	5.25
To	3.75	4.75	5.75	6.75

d. All other boxes, including Amelogenin Cutoff should have "0.0" entered.

5. **Peak Detector** tab.
   a. In the **Ranges** box, for Analysis select **Full Range** from the drop-down menu. For Sizing select **Partial Sizes** from the drop-down menu; set the start size at 60 and the stop size at 500.
   b. In the **Smoothing and Baselining** box, select **Light** for Smoothing and type in "51" for the Baseline Window.
   c. For **Size Calling Method** select **Local Southern Method**.
   d. In the **Peak Detection** box, set the **Min. Peak Half Width** at 2 pts, the **Polynomial Degree** at 3, and the **Peak Window Size** at 15 pts; Set the **Slope Threshold Peak Start** and **End** both at 0.0.
      i. For **Y23_100rfu_1.2**: set the peak amplitude thresholds for B, G, Y, R, and P at "100", also set O at "100".
      ii. For **Y23_200rfu_1.2**: set the peak amplitude thresholds for B, G, Y, R, and P at "200", set O at "100".
   e. Ensure the Use Normalization box is not checked.

NOTE: If a one base difference between two alleles cannot be resolved (i.e. minor peak 1 base from major peak), then the Polynomial Degree and Peak Window Size can be adjusted to help resolve that specific sample. This change of analysis parameters shall be documented in the case file and the modified analysis method shall be exported and maintained in the electronic case record.

6. Select the **Peak Quality** tab. In the **Min/Max Peak Height (LPH/MPH)** box, enter "100.0" for both **Homozygous min peak height** and **Heterozygous min peak height**. Enter "40000" for **Max Peak Height (MPH)**. Enter "0.5" for Min peak height ratio in the Peak Height Ratio (PHR) box, "1.5" for Max peak width (basepairs) in the **Broad Peak (BD)** box. Under the **Allele Number (AN)** section, keep "2" for autosomal markers and set "1" for Y markers. Set "0.2" for the Cut-off Value in the **Allelic Ladder Spike** box and enable Spike Detection for both Allelic Ladders and Samples.

NOTE: Locus DYS385 is listed as an autosomal locus in the panels to differentiate that it can have up to two possible alleles in a single source Y sample. All other loci will be flagged as a mixture if more than one allele is present.

7. Do not alter the default settings in the SQ and GQ Settings tab.
8. After completing these settings, click Save to save the changes and close the Analysis Method Editor.

GeneMapper® ID-X Audit Manager Settings

1. Select Audit Manager from the Admin menu.
2. Select Setting and enter the same Login Name and Password used to log into GeneMapper *ID-X*.
3. From the Audit Map Objects list select Allele.
4. Set the State for *modified* to Silent. Enter a reason for the change in each ensuing dialog box.
5. Set the State for *deleted* and *created* to On. Enter a reason for the change in each ensuing dialog box.
6. Restart the software to allow these changes to take effect.

Genemapper® ID-X Custom Control Import

As a quality control feature, GeneMapper® *ID-X* has the capacity to house up to 300 total analyzed lab reference samples, custom controls, and QC sample profiles in two separate databases (each with a 150 profile capacity).

IMPORTANT! Before adding custom control samples, review the samples manually and edit allele labels as needed to ensure that the profile is accurate. Profiles that include OL labels are not imported into the Profile Manager. Profiles that include numeric allele labels on peaks that are not true DNA peaks will affect concordance results.

Importing the Reference Project

1. Open GeneMapper® *ID-X*. Select the GeneMapper® *ID-X* **Manager** icon from the Project window toolbar (or from the Tools menu, select GeneMapper® *ID-X* Manager).

2. Select the **Projects** tab, then click **Import**.

3. Navigate to and select the appropriate project containing the profiles to be imported. Click **Import**. Ensure the appropriate Security Group is selected and click **OK**. Click **Done** to close the GeneMapper® *ID-X* Manager.

Importing the Reference Profile(s)

1. In the Project window, click the **Open Project** icon. Select the imported project, then click **OK**.

2. From the Table Setting drop-down list, select **Import Reference Profiles**. The Sample table view changes to display only those columns required to add reference profiles to the GeneMapper® *ID-X* database.

3. In the Profile ID column of the Samples tab, click each cell and enter an appropriate Profile ID name (profiles are stored in the database under Profile ID not Sample Name).

4. Select the appropriate rows, then select **Tools**, **Add Profile**. Select the appropriate profile type: **Custom Control** or **Lab Reference**.

5. Click Close in the Add Profile Results dialog box to save the profile to the GeneMapper® *ID-X* database.

Viewing Profiles in the Profile Manager

1. In the Project window, select **Tools**, **Profile Manager**.
2. View the list of profiles in the Profile Manager window. Click '+' beside the Profile ID to view the genotypes stored in the GeneMapper® *ID-X* database.
3. Click **Close** to close the Profile Manager window and return to the Project window.

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72. STR FORMS

The worksheets approved for use in STR casework can be found on the FLSB SharePoint site.

73. APPENDIX A: KINSHIP STATISTICAL REFERENCE

Table 1 – One-Parent Parentage Index Formulas

Mother	Child	Alleged Father	Prob(E | M,C,F)	Prob(E | M,C)	LR
AA	AA	AA	$P_A^2 \times P_A^2$	$P_A^2 \times P_A^2 \times P_A$	$1/P_A$
AA	AA	AB	$P_A^2 \times 2\,P_AP_B \times ½$	$P_A^2 \times 2\,P_AP_B \times P_A$	$1/(2P_A)$
AA	AB	BB	$P_A^2 \times P_B^2$	$P_A^2 \times P_B^2 \times P_B$	$1/P_B$
AA	AB	AB	$P_A^2 \times 2\,P_AP_B \times ½$	$P_A^2 \times 2\,P_AP_B \times P_B$	$1/(2P_B)$
AA	AB	BC	$P_A^2 \times 2\,P_BP_C \times ½$	$P_A^2 \times 2\,P_BP_C \times P_B$	$1/(2P_B)$
AB	AB	BB	$2\,P_AP_B \times P_B^2 \times ½$	$2\,P_AP_B \times P_B^2 \times ½ \times (P_A + P_B)$	$1/(P_A + P_B)$
AB	AB	AB	$2\,P_AP_B \times 2\,P_AP_B \times (¼ + ¼)$	$2P_AP_B \times 2P_AP_B \times ½ \times (P_A+P_B)$	$1/(P_A + P_B)$
AB	AB	AC	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times ½$	$2P_AP_B \times 2P_AP_C \times ½ \times (P_A+P_B)$	$1/(2P_A + 2P_B)$
AB	AA	AA	$2\,P_AP_B \times P_A^2 \times ½$	$2P_AP_B \times P_A^2 \times ½ \times P_A$	$1/P_A$
AB	AA	AB	$2\,P_AP_B \times 2\,P_AP_B \times ½ \times ½$	$2\,P_AP_B \times 2\,P_AP_B \times ½ \times P_A$	$1/(2P_A)$
AB	AA	AC	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times ½$	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times P_A$	$1/(2P_A)$
AB	AC	CC	$2\,P_AP_B \times P_C^2 \times ½$	$2\,P_AP_B \times P_C^2 \times ½ \times P_C$	$1/P_C$
AB	AC	BC	$2\,P_AP_B \times 2\,P_BP_C \times ½ \times ½$	$2\,P_AP_B \times 2\,P_BP_C \times ½ \times P_C$	$1/(2P_C)$
AB	AC	AC	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times ½$	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times P_C$	$1/(2P_C)$
AB	AC	CD	$2\,P_AP_B \times 2\,P_CP_D \times ½ \times ½$	$2\,P_AP_B \times 2\,P_CP_D \times ½ \times P_C$	$1/(2P_C)$

Where KP is the known parent, C is the child, and AP is the alleged parent:

$P(C|KP, AP)$ is the probability of observing a child with the tested genotype if the known parent and alleged parent are the biological parents.

$P(C|KP, U)$ is the probability of observing a child with the tested genotype if the known parent and an unknown, randomly selected individual are the biological parents.

The parentage index (PI) is the likelihood ratio calculated by dividing the two probabilities:

$$\frac{P(C|KP, AP)}{P(C|KP, U)}$$

Table 2 – Reverse Parentage Index Formulas

Mother	Q	Father	Prob(E | M, F,Q)	Prob(E | M, F, U)	LR
AA	AA	AA	$P_A^2 \times P_A^2$	$P_A^2 \times P_A^2 \times P_A^2$	$1/P_A^2$
AA	AA	AB	$P_A^2 \times 2\,P_AP_B \times ½$	$P_A^2 \times 2\,P_AP_B \times P_A^2$	$1/(2P_A^2)$
AA	AB	BB	$P_A^2 \times P_B^2$	$P_A^2 \times P_B^2 \times 2P_AP_B$	$1/(2P_AP_B)$
AA	AB	AB	$P_A^2 \times 2\,P_AP_B \times ½$	$P_A^2 \times 2\,P_AP_B \times 2P_AP_B$	$1/(4P_AP_B)$
AA	AB	BC	$P_A^2 \times 2\,P_BP_C \times ½$	$P_A^2 \times 2\,P_BP_C \times 2P_AP_B$	$1/(4P_AP_B)$
AB	AB	BB	$2\,P_AP_B \times P_B^2 \times ½$	$2\,P_AP_B \times P_B^2 \times 2P_AP_B$	$1/(4P_AP_B)$
AB	AB	AB	$2\,P_AP_B \times 2\,P_AP_B \times (¼ + ¼)$	$2P_AP_B \times 2P_AP_B \times 2P_AP_B$	$1/(4P_AP_B)$
AB	AB	AC	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times ½$	$2P_AP_B \times 2P_AP_C \times 2P_AP_B$	$1/(8P_AP_B)$
AB	AA	AA	$2\,P_AP_B \times P_A^2 \times ½$	$2P_AP_B \times P_A^2 \times P_A^2$	$1/(2P_A^2)$
AB	AA	AB	$2\,P_AP_B \times 2\,P_AP_B \times ½ \times ½$	$2\,P_AP_B \times 2\,P_AP_B \times P_A^2$	$1/(4P_A^2)$
AB	AA	AC	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times ½$	$2\,P_AP_B \times 2\,P_AP_C \times P_A^2$	$1/(4P_A^2)$
AB	AC	CC	$2\,P_AP_B \times P_C^2 \times ½$	$2\,P_AP_B \times P_C^2 \times 2P_AP_C$	$1/(4P_AP_C)$
AB	AC	BC	$2\,P_AP_B \times 2\,P_BP_C \times ½ \times ½$	$2\,P_AP_B \times 2\,P_BP_C \times 2P_AP_C$	$1/(8P_AP_C)$
AB	AC	AC	$2\,P_AP_B \times 2\,P_AP_C \times ½ \times ½$	$2\,P_AP_B \times 2\,P_AP_C \times 2P_AP_C$	$1/(8P_AP_C)$
AB	AC	CD	$2\,P_AP_B \times 2\,P_CP_D \times ½ \times ½$	$2\,P_AP_B \times 2\,P_CP_D \times 2P_AP_C$	$1/(8P_AP_C)$

Where AP_1 and AP_2 are the alleged parents and C is the child:

$P(C|AP_1, AP_2)$ is the probability of observing a child with the tested genotype if the alleged parents are the biological parents.

$P(C|U_1, U_2)$ is the probability of observing a child with the tested genotype if two unknown randomly selected people are the biological parents.

The Reverse Parentage Index is the likelihood ratio calculated by dividing the two probabilities:

$$\frac{P(C|AP_1, AP_2)}{P(C|U_1, U_2)}$$

Table 3 – Zero-Parent Parentage Index Formulas

Child	Alleged Parent	LR
AA	AA	$1/P_A$
AA	AB	$1/(2P_A)$
AB	AA	$1/(2P_A)$
AB	AB	$(P_A + P_B) / (4P_AP_B)$
AB	AC	$1/(4P_A)$

Where C is the child and AP is the alleged parent:

$P(C|AP, U)$ is the probability of observing a child with the tested genotype if the alleged parent and an unknown, randomly selected individual are the biological parents.

$\mathrm{P}(\mathrm{C}|U_1U_2)$ is the probability of observing a child with the tested genotype if two unknown, randomly selected people are the biological parents.

The Parentage Index (PI) is the likelihood ratio calculated by dividing the two probabilities:

$$\frac{P(C|AP, U)}{P(C|U_1, U_2)}$$

Table 4 – Parentage Index for Autosomal Mutation Formula

	Child (C)	Alleged Parent (AP)	Parentage Index (PI)	Probability of Exclusion (PE)[6]
1.	A	B	$\frac{\mu}{\bar{A}}$	$(1-a)^2$
2.	A	BC	$\frac{\mu}{\bar{A}}$	$(1-a)^2$
3.	AB	CD	$\frac{\mu}{\bar{A}}$	$[1-(a+b)]^2$
4.	AB	C	$\frac{\mu}{\bar{A}}$	$[1-(a+b)]^2$

Where μ is the mutation rate and Ā is the mean power of exclusion.

Table 5 – Kinship Coefficients

Relationship	Notation	ϕ_2	ϕ_1	ϕ_0
Self (MZ twin)	MZ	1	0	0
Parent-Offspring	PO	0	1	0
Full Sib	FS	1/4	1/2	1/4
Half-Sib[1], Uncle/Aunt-Nephew/Niece, Grandparent-Grandchild	HS	0	1/2	1/2
Half-Sib whose parents are sibs	HS-S	1/8	1/2	3/8
Half-Sib whose parents are half-sibs	HS-HS	1/16	1/2	7/16
First cousin	1C	0	1/4	3/4
Double first cousin	D	1/16	3/8	9/16
Unrelated	U	0	0	1

Where $Ø_0$ is the probability that the locus genotypes of the two individuals share no allele by descent for the specified kinship; $Ø_1$ is the probability that the locus genotypes of the two individuals share one allele by descent for the specified kinship; $Ø_2$ is the probability that the locus genotypes of the two individuals share two alleles by descent for the specified kinship.

Table 6 – Kinship Index Formulas

#	G_x (Ref)	G_y (E)	Conditional Probability of G_y given G_x under		
			I (2 alleles IDB)	T (1 allele IDB)	O (0 allele IBD)
			$P_2(X,Y)$	$P_1(X,Y)$	$P_0(X,Y)$
1	A	A	1	a	a^2
2	A	B	0	0	b^2
3	A	AB	0	b	$2ab$
4	A	BC	0	0	$2bc$
5	AB	A	0	$\frac{a}{2}$	a^2
	AB	B		$\frac{b}{2}$	b^2
6	AB	C	0	0	c^2
7	AB	AB	1	$\frac{a+b}{2}$	$2ab$
8	AB	AC	0	$\frac{c}{2}$	$2ac$
	AB	BC		$\frac{c}{2}$	$2bc$
9	AB	CD	0	0	$2cd$

Where:

- $P_2(X,Y)$ = probability of G_y (reference) given G_x (evidence) with 2 of their alleles identical by descent (IBD)
- $P_1(X,Y)$ = probability of G_y (reference) given G_x (evidence) with 1 of their alleles IBD
- $P_0(X,Y)$ = probability of G_y (reference) given G_x (evidence) with 0 of their alleles IBD

The probability of observing the locus genotype Gy (reference) given Gx (evidence) for a specified kinship is: P(kinship) + [$P_2(X,Y) \times Ø_2$] + [$P_1((X,Y) \times Ø_1$] + [$P_0((X,Y) \times Ø_0$]

74. APPENDIX B: STRMIX V2.8 SETTINGS

Run Settings

MCMC	
Number of Chains:	8
Burn-in Accepts (per chain):	10,000
Post Burn-in Accepts (per chain):	50,000
Random Walk SD:	0.005
Post Burn-in Shortlist:	9
Extended Output:	Unchecked

MX PRIORS	
Use Mx Priors:	Unchecked

PERFORMANCE	
Number of Threads:	*
Low Memory Mode:	Unchecked
Seed:	Checked

GELMAN-RUBIN	
Auto-Continue on GR:	Checked
Gelman-Rubin Threshold:	1.2
Extra Accepts:	10,000

CONTRIBUTOR RANGE (varNOC)	
Hyper-Rectangle Percent Accepts	2.5

*****This should autofill during STRmix installation and is set equal to the number of computer processors.

Kit Settings

GENERAL	
VARIANCE	
Allelic Variance:	8.333, 0.982
Locus Amplification Variance:	0.011
Minimum Variance Factor:	0.5
Variance Minimization Parameter:	1,000
DROP-IN	
Drop-in Cap:	120
Drop-in Rate Parameter:	0.0029
Drop-in Distribution Parameters:	Checked
ADDITIONAL THRESHOLDS	
Maximum Degradation:	0.01
Degradation Start Point:	Checked
Saturation Threshold:	30,000

STUTTERS	
BACK	
Max Stutter Ratio:	0.3
Variance:	1.514, 10.038
FORWARD	
Max Stutter Ratio:	0.15
Variance:	1.883, 5.271
DOUBLE BACK	
Max Stutter Ratio:	0.1
Variance:	2.313, 3.509
HALF BACK	
Max Stutter Ratio:	0.15
Variance:	1.627, 7.094
6bp BACK	
Max Stutter Ratio:	0.05
Variance:	2.171, 2.816

LOCI									
Locus	Gender?	Repeat Length	Ignore?	Detection Threshold	Stutter				
					Back	Forward	Double Back	Half Back	6BP Back
Amel	•								
D3S1358		4		70	•	•	•		
D1S1656		4		70	•	•	•	•	
D2S441		4		70	•	•	•		
D10S1248		4		70	•	•	•		
D13S317		4		70	•	•	•		
Penta E		5		70	•	•	•		
D16S539		4		70	•	•	•		
D18S51		4		70	•	•	•	•	
D2S1338		4		70	•	•	•		
CSF1P0		4		70	•	•	•		
Penta D		5		70	•	•			
TH01		4		70	•	•	•		
vWA		4		70	•	•	•		
D21S11		4		70	•	•	•		
D7S820		4		70	•	•	•	•	
D5S818		4		70	•	•	•		
TPOX		4		70	•	•	•		
D8S1179		4		70	•	•	•		
D12S391		4		70	•	•	•		
D19S433		4		70	•	•	•		
SE33		4		70	•	•	•	•	•
D22S1045		3		70	•	•	•		
DYS391		4	•	70					
FGA		4		70	•	•	•		
DYS576		4	•	70					
DYS570		4	•	70					

LR Settings

POPULATIONS
FBI_extended_Cauc
FBI_extended_AfAm
FBI_extended_SW_Hisp
SUB-SOURCE LR
Assign Sub-Source LR: Checked

SAMPLING VARIATION	
Calculate HPD	Checked
MCMC Uncertainty	Checked
Allele Frequency Uncertainty	Checked
Number of HPD Iterations	1,000
Probability Interval Quantile	99
Probability Interval Sides	1

Database Search Settings

Minimum LR	5000
Population For Search	NIST1036_Combined
Extended Output	Unchecked
Type of Search	Standard
FST For Search	0.0b(1.0, 1.0)
Assign Sub-Source LR	Checked
Priors Method	Stratify
Auto Database Search	Checked