MNPD Crime Laboratory

Forensic Biology Technical Procedures Manual

Metropolitan Government of Nashville & Davidson County

Police Department

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1.5.1.8 After examination is complete, close the LSV software and restart the computer. After restarting, lock the computer and turn off the LSV base.

1.5.2 Positioning the LSV Head

1.5.2.1 The LSV head can be positioned in three ways: pitch, yaw, and roll.

1.5.2.2 To adjust the pitch, locate the silver Pitch Home Locking Pin on the left side of the LSV head. Pull the pin out and rotate it 90° to unlock the head for adjusting. Position the head up or down as desired and lock it into place using the LSV Head Positioning Lock handle, located on the right side of the head.

Figure 16: Pitch Home Lock Release button

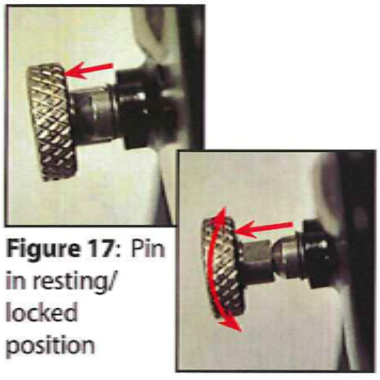


Figure 17: Pin in resting/ locked position

Figure 18: Pin pulled out and rotated 90°

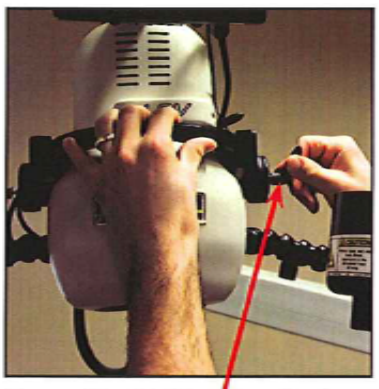


Figure 19: LSV Head Positioning Lock Handle

1.5.2.3

1.5.2.4 To adjust the yaw, locate the Yaw Lock on the right side of the LSV head. Loosen the lock to allow side-to-side movement. Position the head left or right as desired and tighten the Yaw tension handle.

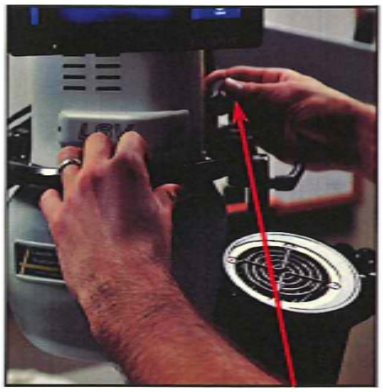


Figure 20: Yaw Tension Handle

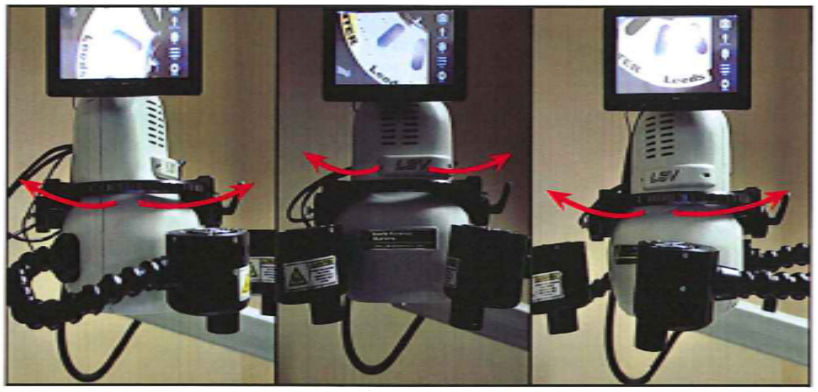


Figure 21: Adjust LSV head yaw left-to-right as desired.

1.5.2.5

1.5.2.6 To adjust the roll, locate the silver Roll Home Locking Pin located on the back of the black LSV head frame. Pull the pin out and rotate it 90° to release the head for adjustment. Turn the head clockwise or counterclockwise to desired position and lock in place using the LSV Head Positioning Lock handle. This handle can be turned clockwise to tighten and counterclockwise to loosen.

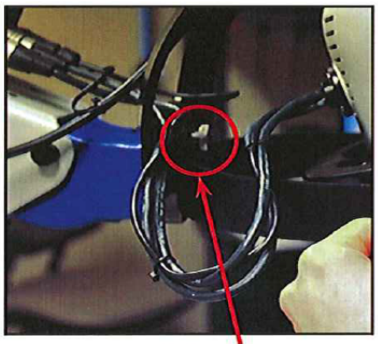


Figure 22: Roll Home Lock Release button

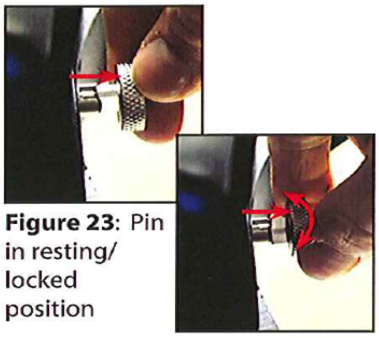


Figure 23: Pin in resting/ locked position

Figure 24: Pin pulled out and rotated 90°

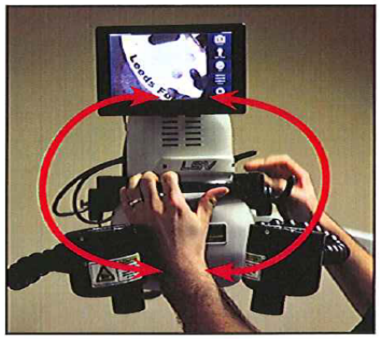


Figure 25: Adjust Roll of LSV Head to desired position

Figure 26: Adjust Roll of LSV Head to desired position

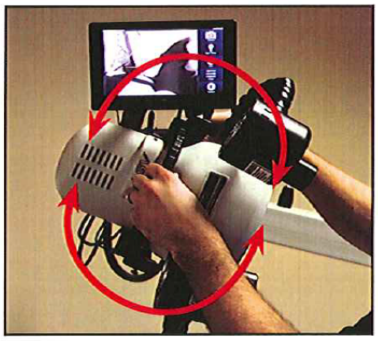


Figure 27: Adjust Roll of LSV Head to desired position

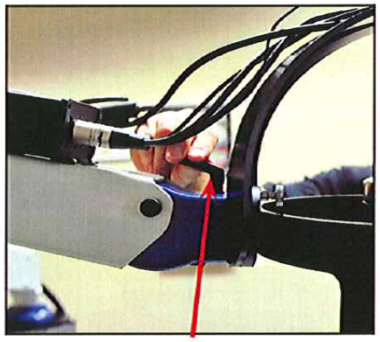


Figure 28: LSV Head Positioning Lock Handle

1.5.2.7

1.5.3 Light and Filter Controls

1.5.3.1 The LSV has seven LED light settings, ranging from 365 nm to 850 nm wavelengths, and a five-position filter cassette, with filters ranging from 400 nm (clear) to 830 nm. This creates multiple light and filter options for viewing evidence, each of which will cause potential stains to look different. Each background material (ex. black t-shirt) and suspected stain type will need to be considered when choosing a light/filter combination.

1.5.3.2 Table 1 displays recommended light and filter settings, along with interpretation guidelines at the various settings.

1.5.3.2.1 **Table 1: Recommended Wavelength/Filter Settings**.

Body Fluid	Type of Fabric	Wavelength	Filter	Positive Result	Negative Result
Blood	White/Light-colored	White/IR	Clear/ IR:830nm	Absorbance observed	No absorbance observed
Blood	Dark-colored, denim, and thick	IR	IR:830nm	Absorbance observed	No absorbance observed
Semen, Saliva, Sweat, and Vaginal Secretions	All	Blue	Orange	Fluorescence observed	No fluorescence observed

1.5.4 Camera Controls

1.5.4.1 There are two modes available in the Camera tab: Manual Exposure and Auto Exposure. In Manual Exposure mode, the LSV's shutter time is controlled by the user. In Auto Exposure mode, the LSV's software establishes the optimum exposure setting, allowing the user to control "Brightness" rather than "Shutter Time". In both modes, the "Gain" slider can be used to optimize frame rate. The White Balance button averages a whole image white balance each time it is clicked.

1.5.5 Lens Controls

1.5.5.1 Focus and Zoom are controlled in this tab, along with Aperture and Diopter Lens. Aperture controls how much light is allowed through the rear focal plane of the zoom optic. It also affects contrast, resolving power, and depth of focus. Diopter lenses allow for greater resolution at different working distances. The +3.5 diopter lens optimizes working distances between approximately 7 and 9 inches. The +2 diopter lens optimizes working distances between approximately 12 and 18 inches. The +1 diopter lens optimizes working distances between approximately 19 and 38 inches. With no diopters engaged, the LSV has a parfocal zoom with a working distance of approximately 40 inches and above.

1.5.5.2 **NOTE**: Light, filter, camera, and lens controls will need to be adjusted to maximize imaging for each piece of evidence. Presets, which allow all settings to be saved, can be made for classes of evidence. Refer to Table 2 for current MNPD presets. To save a preset,

click “Save” under the Preset dropdown menu. Enter the name for the preset, check the boxes beside the options desired, and click “OK”.

1.5.5.2.1 **Table 2: MNPD Presets**.

LSV Preset Names	Light	Filter	Filter Color
Fluorescence	Blue-455	4-550nm	Orange
Fluorescence (2) Violet light	Violet-405	4-550nm	Orange
Alt. Fluorescence-Blue light with 610nm Filter	Blue-455	2-610 nm	Red
Fluorescence (Dark Fabrics)	Blue-455	550-700nm	Orange
Infrared	IR-850	1-830nm	IR
Blood Absorbtion	Violet-400	2-610nm	Red
Blood Absorbtion 2	Violet-400	5-400nm	Clear
Visible	White-5600	5-400nm	Clear
White Balance Calibration	White-5600	5-400nm	Clear

1.5.6 LSV Screening

1.5.6.1 Examine the surface of the evidence, using a permanent marker to circle or otherwise indicate any stains or group of stains found. Capture an image of the circled stain(s). The LSV software allows annotation directly on the image to allow the recording of the analyst’s initials, incident/Lab #, item/stain #, light and filter used, etc.. Below is a list of the options found on the Annotation and Measurement Toolbar.

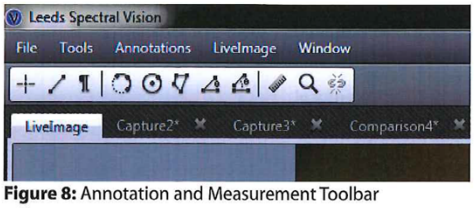


Figure 8: Annotation and Measurement Toolbar

Icon	Name	Function
	Crosshair	Insert a crosshair, defining a point of interest, into the image.
	Line	Insert a line between two points.
	Text	Insert a text window.
	Circle – Three Point	Insert a circle, based on three identified points on the circles circumference.
	Circle – Two Point	Insert a circle, based on the center of the circle and the radius.
	Polygon	Insert a polygon with a user defined number of points marking the outside perimeter.
	Angle – Three Point	Insert an angle based on a point on one arm, definition of the vertex, and a point on the second arm.
	Angle – Four Point	Insert an angle based on two points on the line of each arm.
	Calibration Line	Insert a line of known distance. This will calibrate all other measurements in the image as well. The line length must be defined and applied for calibrated measurements.
	Magnify	Digital zoom on the image.
	Link Images	Links two compared images so that when the field of view of one image is changed (i.e. Increased zoom or moved to display a different region of interest) that other image will change in the same way.

1.5.6.2 7

1.5.6.3 **Capturing, Saving and Exporting Images**.

1.5.6.3.1 In the upper right corner of the screen, click the “Capture” button, and click on the newly created Capture tab. In the File dropdown menu, click “Save As” to save the image and associated metadata. In the pop-up box, navigate to the destination folder and appropriately name the saved image. A metadata text file with all image settings will automatically be generated and saved with the image file. Then, click either “Export Raw Image” or “Export Annotated Image”. Save it as previously stated.

1.5.6.4 **Comparing Images**.

1.5.6.4.1 Live images and/or saved images can be viewed and saved in the same window for side-by-side comparisons by clicking on “Compare To” under the Tools dropdown

menu. Choose the image to be compared in the dropdown menu and click "OK". Click on the newly created image tab with the two images side by side. This new image can be saved and exported as described above.

1.5.6.5 **Applying Settings**.

1.5.6.5.1 To use the same settings as a previously captured image, open the previously captured image and click the "Apply Settings" button on the Image Data panel located on the right side panel. The live image will now have all the settings saved in the metadata file of the previously captured image.

1.6 UltraLite ALS® Turbo Procedure

1.6.1 Preparation for UltraLite ALS® Turbo Screening

1.6.1.1 Clean the tabletop with a bleach solution. Butcher paper may then be used to line the tabletop. Place the evidence to be screened on tabletop.

1.6.1.2 Take the UltraLite ALS® Turbo out of carrying case. Attach the desired Head Assembly to the UltraLite ALS® Turbo body. Insert the head in a straight and slow manner to avoid damaging the contact pins. Hold the head against the handle and rotate the locking ring on the handle until it is hand tight. DO NOT TWIST THE HEAD AND DO NOT OVERTIGHTEN. To remove the head, rotate the locking ring on the handle until the head becomes loose. Pull the head straight away from the handle.

1.6.1.3 Insert a battery pack into the bottom of the handle. The UltraLite ALS® Turbo features an energy conserving "sleep mode." After five minutes of inactivity, the unit will automatically shut off the indicator lights and enter this mode. If the unit is in sleep mode, press the on/off button once to "wake up" the unit. Press the on/off button once again to activate the light.

1.6.1.4 To increase the power output to the desired setting, press the Power Setting button on the top of the unit. The power increases from setting 1 to 2 to 3 to 4, then cycles back to 1. The green lights on the neck of the unit indicate the power setting that is currently in use. Light settings may need to be adjusted to maximize imaging for each piece of evidence.

1.6.1.5 Press the on/off button to deactivate the light.

1.6.1.6

1.6.1.7 Adjust the room lighting as necessary with the dimmer switch located beside the door.

1.6.1.8 NOTE: The yellow light on the neck will illuminate when the battery pack has 20% of its full charge remaining. Actual time remaining will vary, depending on which Head Assembly is in use.

1.6.1.9 Table 2: Recommended Wavelength/Filter Settings

LED Head Assembly	Body Fluid	Wavelength (nm)	Glasses	Positive Results	Negative Result
BMT	Semen, saliva, sweat, and vaginal secretions	450 nm	Amber	Fluorescence observed	No fluorescence observed
UV	Semen, saliva, sweat, and vaginal secretions	400 nm	Amber, Red, Yellow	Fluorescence observed	No fluorescence observed
IR	Blood	850 nm	Infrared detection cameras and other devices	Absorbance observed	No absorbance observed

1.6.1.10 **Charging the Battery Pack**

1.6.1.10.1 Plug the Battery Pack Charger into the wall and insert the cord jack into the side of the charger. Insert a battery pack into one of the battery docks. Make sure the battery is completely inserted and snaps in place.

1.6.1.10.2 The Battery Pack Charger features a logic circuit that will determine if a battery pack is in place and if it needs to be charged. If two battery packs are in place and both need charging, the unit will fully charge both batteries simultaneously. If all packs in the unit are fully charged, it will shut off until the charge falls below full. Each row of colored lights indicates the amount of charge each battery currently has, relative to a full charge.

1.6.2 UltraLite ALS® Turbo Screening

1.6.2.1 Examine the surface of the evidence, using a permanent marker to circle or otherwise indicate any stains or group of stains found.

1.6.2.2 After examination is complete, remove the battery pack from the UltraLite ALS® Turbo to conserve the life of the battery. To remove the battery pack, squeeze both tabs on the sides of the item, then pull straight out.

1.6.2.3 Store the UltraLite ALS® Turbo components in the travel case provided when not in use.

1.7 Limitations

1.7.1 The LSV and UltraLite ALS® Turbo are intended to identify areas for further testing, not make a definitive identification of stain type. This is because many other substances, such as bleach or brown paint, will mimic the appearance of body fluids under an ALS. Other screening techniques, such as AP mapping or the testing of general swabbings, may be used in conjunction with or in place of the LSV or UltraLite ALS® Turbo for difficult fabrics.

1.7.2 The MNPD-CL Forensic Biology Unit does not currently have the appropriate IR detection camera to utilize the IR Head Assembly for the UltraLite ALS® Turbo.

1.7.3 The UltraLite ALS® Turbo does not have image capturing capabilities to photograph the stains under the various light and filter settings.

1.8 Safety

1.8.1 Personal protective equipment must be used at all times. Protective goggles may be worn when using the ultraviolet (UV) light settings.

1.8.2 The LSV/Imaging unit is portable, but moving it requires a minimum of two people. Prior to moving the LSV/Imaging arm, collapse the arm, ensure that the magnetic lock is secure, and strap down the arm with the heavy-duty Velcro strap. Unplug and retract the power

cord into the LSV base. Use the orange wheels located on the caster feet to unlock the wheels. Remember to lock the caster feet again when moving is complete.

1.8.3 The output intensity of the UltraLite ALS® Turbo solid-state emitters is strong enough to present the risk of injury to eye and skin tissue. Do not point the unit directly at the eyes or skin.

1.8.4 Use only accessories and power sources that are supplied with and designed specifically for the UltraLite ALS® Turbo. Do not allow moisture to collect on or enter the unit. Do not immerse. If the unit is used in humid environments, store in a warm, dry place between use to allow moisture to evaporate.

1.9 References

1.9.1 Performance Check of Leeds Spectral Vision (LSV) for Aiding Body Fluid Detection, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, June 2014

1.9.2 Performance Check of Leeds Spectral Vision System with adjusted LSV head and Leeds Software v4.0, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, June 2017

1.9.3 Leeds Spectral Vision System Instruction Manual (2016). Leeds Spectral Vision System. Leeds Forensics, Minneapolis, MN.

1.9.4 Leeds Vision Software Guide, Version 4.0 (2014). Leeds Spectral Vision System. Leeds Forensics, Minneapolis, MN.

1.9.5 Leeds Spectral Vision System Imaging Guide (2015). Leeds Spectral Vision System. Leeds Forensics, Minneapolis, MN.

1.9.6 UltraLite ALS® Turbo Operator's Manual (2011). UltraLite ALS® Turbo. CAO Group, Inc, West Jordan, UT.

1.9.7 Validation of UltraLite ALS® Turbo, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, January 2023. QMS WF 83525.

2. Phenolphthalein Test (Kastle-Meyer Test)

2.1 Scope

2.1.1 To describe the process by which evidence is screened for the potential presence of blood.

2.2 Equipment/Materials/Reagents

2.2.1 Hydrogen Peroxide 3% or 30% (dilute to 3% with Ultrapure water)

2.2.2 Microcentrifuge tubes (1.5mL or 2mL)

2.2.3 Kastle-Meyer Stock Solution (with zinc)

2.2.4 Ethanol

2.2.5 Amber dropper bottles

2.2.6 Sterile swabs

2.3 Standards and Controls

2.3.1 On each day the test will be utilized, a positive control (known blood sample) and a negative control (reagents only) must be tested, and the expected results must be obtained.

2.3.2 The results of these controls must be documented in the casefile.

2.4 Sample Selection

2.4.1 Refer to the section Evidence Handling/Blood of the MNPD-CL Forensic Biology Quality Manual.

2.5 Procedure

2.5.1 A small cutting of stain or swabbing of a suspected bloodstain is placed into a microcentrifuge tube.

2.5.2 Two to three drops of ethanol are placed onto the stain.

2.5.3 Two to three drops of phenolphthalein (Kastle-Meyer Reagent Stock Solution) are added to the stain.

2.5.4 Wait a few seconds to ensure that no color develops at this stage and then add two to three drops of 3% Hydrogen Peroxide.

2.5.5 Interpretation:

2.5.5.1 Positive: A pink color change up to 30 seconds after addition of hydrogen peroxide.

2.5.5.2 Negative: No observed color change or a color change after 30 seconds.

2.5.5.3 Inconclusive: A pink color change prior to the addition of hydrogen peroxide.

2.5.5.3.1 An inconclusive result is not exclusive to the above observation as it could also be reported due to other circumstances (e.g., unable to observe color change to pink due to substrate color).

2.6 Limitations

2.6.1 The Kastle-Meyer test will give false positive results in the presence of some plant peroxidases, such as horseradish. The Kastle-Meyer test is also not specific to human blood and will react with meat and food products containing blood. As such, this test will not be considered a confirmatory test for the presence of human blood.

2.6.2 Chemical oxidants such as copper and nickel salts may cause the Kastle-Meyer reagent to turn pink prior to the addition of hydrogen peroxide; therefore, it is important to wait a few seconds prior to adding hydrogen peroxide during testing.

2.6.3 Certain factors may also prevent blood from producing a positive reaction. Limitations based on the sensitivity of the test may produce negative results when the blood is present at low concentrations. Depending on the substrate on which the stain is deposited, negative results were produced when blood was diluted greater than 1:6000 on filter paper and greater than 1:25000 on a swab according to an internal laboratory study. Washing, rain, heat, and time can reduce the concentration of blood.

2.6.4 Sensitivity limitations are not the only factors that can prevent a positive reaction. Studies have also shown that reducing agents can also interfere with the reaction involved in the Kastle-Meyer test. If reducing agents are present on the item in which the blood is deposited (i.e., metals) or the bloodstain comes into contact with a product with high reduction strength (i.e., certain detergents or foods), interference may occur.

2.7 Safety

2.7.1 Phenolphthalein stock solution is a skin and eye irritant. Hydrogen Peroxide is corrosive. Caution should be used when handling these chemicals and personal protective equipment should be used at all times.

2.7.2 Zinc's reaction with water or moisture in the air produces hydrogen which can react explosively with oxygen. Therefore, zinc should not be discarded in the wastebasket.

2.8 References

2.8.1 Performance Check of Phenolphthalein in the Presumptive Test of Blood, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, June 2014

2.8.2 Tobe, et al. Evaluation of Six Presumptive Tests for Blood, Their Specificity, Sensitivity, and Effect on High Molecular-Weight DNA, J Forensic Sci, January 2007, Vol. 52, No. 1, pg 102-109

2.8.3 Glaister, John. "The Kastle-Meyer Test for the Detection of Blood. Considered from the Medico-Legal Aspect." National Center for Biotechnical Information. www.ncbi.nlm.nih.gov. British Medical Journal, 10 Apr 1926.

2.8.4 Gaensslen, R.E. Sourcebook in Forensic Serology, Immunology, and Biochemistry, Unit II: Identification of Blood: 1989 Update (NCJ 160880), pp. 103-105.

2.8.5 Castello Ponce, Ana et al. Critical Revision of Presumptive Tests for Bloodstains, FBI Forensic Science Communications, July 1999, Volume 1, No. 2, https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/july1999/ponce.htm

3. HemaTrace Blood Test

3.1 Scope

3.1.1 To describe the process by which an evidentiary stain is identified as blood with the HemaTrace test.

3.2 Equipment/Materials/Reagents

3.2.1 Test device (individually packaged)

3.2.2 HemaTrace Extraction Buffer vial

3.2.3 Pipette and pipette tips

3.2.4 Scalpel/scissors

3.2.5 Timer

3.2.6 Transfer pipettes

3.3 Standards and Controls

3.3.1 In addition to lot number quality control testing, on each day that the test is utilized, a positive control (known human blood) and a negative control (extraction buffer only) must be tested, and the expected results must be obtained. The result of the daily testing must be recorded in the casefile. The control line in the control "C" area can be considered an internal procedural control.

3.4 Sample Selection

3.4.1 Refer to The Evidence Handling/Blood/Evidentiary Bloodstains Section of the MNPD-CL Forensic Biology Quality Manual.

3.5 Procedure

3.5.1 A small cutting or swabbing of a suspected bloodstain is placed directly into a labeled HemaTrace extraction buffer vial.

3.5.2 Allow the sample to incubate at room temperature for 30 minutes. Sample may be left to incubate overnight (no longer than 24 hours).

3.5.3 Ensure the vial cap is tight and gently mix the contents for at least 10 seconds without causing the buffer to foam.

3.5.4 Verify the lot number of HemaTrace cards that will be used. Depending on which method is instructed by the manufacturer, add the following to the "S" sample well on the test device using the supplied dropper or a pipette:

3.5.4.1 ~2 drops, or ~80uL

3.5.4.2 ~6-7 drops, or ~200uL

3.5.5 Results can be read at 10 minutes. The minimum time in a positive result is the time at which both lines appear. For negative results, wait the full 10 minutes.

3.6 Interpretation

3.6.1 Positive: A positive result is indicated by two pink lines, one each in the "T" (test) area and in the "C" (control) area.

3.6.2 Negative: A negative result is indicated by a single pink line in the "C" (control) area. No line will be observed in the "T" (test) area.

3.6.3 Inconclusive: Regardless of the presence or absence of a line in the "T" (test) area, valid results will only be recorded if a pink line is present in the "C" (control) area. If no pink line is visible in the "C" area, the test should be repeated.

3.7 Limitations

3.7.1 Low concentration of blood may produce negative results. During an internal laboratory study, negative results were produced when blood was diluted greater than 1:1000. Washing, rain, heat, and time can reduce the concentration of blood.

3.7.2 The results of this test should not be read after 10 minutes since non-specific reactions may occur and can result in false positives.

3.7.3 Positive results may be obtained from whole blood from the domestic ferret and higher primates (Anthropoidea). Since the possibility of encountering a higher primate or ferret blood in routine casework is minimal and can be considered on a case-by-case basis, the fact that the kit cross reacts with these animals is not of great concern. If any doubt exists, the results can be confirmed by DNA analysis.

3.7.4 False negative results can be produced by "High Dose Hook Effect" when large amounts of hemoglobin are present in a sample. An approximate guide for the hook effect is the color of the extract solution. The darker red, the greater the chance of a hook effect occurring. Ideally, for fresh bloodstains, the extract solution should be straw-colored. In

cases where the effect is strongly suspected, the extract may be retested using a 1:10 and/or a 1:100-fold dilution.

3.7.4.1 This test is not specific to human blood and is only considered a confirmatory blood test.

3.8 Safety

3.8.1 Caution should be used when handling kit reagents. Personal protective equipment should be used at all times.

3.9 References

3.9.1 ABAcard HemaTrace Technical Information Sheet, Catalog # 708424. Abacus Diagnostics, rev March 2020.

3.9.2 ABAcard HemaTrace Technical Information Sheet, Catalog # 708424. Abacus Diagnostics, rev December 2022.

3.9.3 ABAcard Hematrace Sensitivity Study, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, January 2023.

3.9.4 RSID Blood and ABAcard Hematrace Test Comparison Study, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, July 2014.

3.9.5 Validation Study of the Abacus Diagnostics ABAcard HemaTrace Membrane Test for the Forensic Identification of Human Blood. S. Johnston, J. Newman, R. Frappier. Canadian Society of Forensic Science, Vol 36, No 3 (2003), pg 173-183.

4. Acid Phosphatase Mapping Test

4.1 Scope

4.1.1 To describe the process by which evidence is preliminarily screened for the potential presence of semen stains.

4.2 Equipment/Materials/Reagents

4.2.1 AP Spray Working Solution (in spray bottle)

4.2.2 Filter paper

4.2.3 Marker/Sharpie

4.2.4 Autoclaved Ultrapure water

4.2.5 Timer

4.2.6 Fume hood

4.3 Standards and Controls

4.3.1 On each day the test will be utilized, a positive control (known semen sample) and a negative control (filter paper) must be tested, and the expected results must be obtained and documented in the casefile.

4.3.2 If the expected results are not obtained, the spray should be discarded and prepared again. The new spray will be tested against the positive and negative controls before being utilized in casework.

4.3.3 Note: Acid phosphatase is known to degrade after being deposited. Moisture and heat are the most deleterious factors, so known semen samples will be dried and stored at room temperature in a dark environment.

4.4 Sample Selection

4.4.1 Refer to The Evidence Handling/Suspected Semen Section of the MNPD-CL Forensic Biology Quality Manual.

4.5 Procedure

4.5.1 Obtain a piece of filter paper that is sized appropriately for the item being tested.

4.5.2 Lay the filter paper on the item. Lines may be drawn on the paper and garment so that the orientation of the paper to the garment can be ascertained once the test is complete.

4.5.3 Wet the filter paper lightly with autoclaved ultrapure water.

4.5.4 Alternatively, when testing swabs, first wet the filter paper lightly with autoclaved ultrapure water. Then wrap the moistened filter paper around the swab tip.

4.5.5 Press the paper into the item for 1-2 minutes.

4.5.6 Remove the filter paper from the evidence. Place the filter paper in a fume hood and spray with the AP Spray Working Solution. Make sure to coat the paper evenly with the spray.

4.5.7 Results should be observed for up to 3 minutes.

4.5.7.1 If acid phosphatase is present at significant levels, a pink/purple color will appear.

4.6 Interpretation:

4.6.1 Positive: A pink/purple color appears within 3 minutes.

4.6.2 Negative: No color change appears within 3 minutes.

4.7 Limitations

4.7.1 Other bodily fluids, such as vaginal secretions and male urine, as well as bacteria, fungi, and some feminine hygiene products are known to produce a positive reaction utilizing this test. As such, this test will only be considered a presumptive test for the presence of semen.

4.7.2 Acid phosphatase is known to degrade after being deposited. Therefore, low level samples or samples with a long time frame between deposit and collection may be degraded beyond the limit of detection.

4.8 Safety

4.8.1 AP Spray Working Solution contains α-Naphthyl phosphate and Fast Blue B, which emit toxic fumes when exposed to heat. Caution should be used when handling these chemicals. All testing should be conducted in a fume hood and personal protective equipment should be used at all times.

4.9 References

4.9.1 Performance Check of Acid Phosphatase Tests for the Presence of Semen, Metro Nashville Police Department – Crime Laboratory Forensic Biology Unit, September 2014.

4.9.2 A Greenfield, M.A. Sloan, Identification of Biological Fluids and Stains, in: S.H. James, J.J. Nordby (Eds.), Forensic Science: An Introduction to Scientific and Investigative Techniques, CRC Press, Boca Raton, 2003, pp. 203-220.

4.9.3 E.L. Jones Jr., The Identification of Semen and Other Body Fluids, in R. Saferstein (Ed.), Forensic Science Handbook, Vol. II, Prentice Hall, Upper Saddle River, NJ, 2005, pp. 329-382.

4.9.4 Lewis, J., S. Jones, F. Baxter, A. Siemieniuk, and R. Talbot. "The Fallacy of the Two-minute Acid Phosphatase Cut off." Science & Justice 52.2 (2012): 76-80.

4.9.5 Redhead, Paul, and Melanie K. Brown. "The Acid Phosphatase Test Two Minute Cut-off: An Insufficient Time to Detect Some Semen Stains." Science & Justice 53.2 (2013): 187-191.

5. Microscopic Examination for Sperm

5.1 Kernechtrot-Picroindigocarmine (KPIC) Staining

5.1.1 Scope

5.1.1.1 To describe the process by which a chemical stain is utilized to make the observation of potential spermatozoa more distinct.

5.1.2 Equipment/Materials/Reagents

5.1.2.1 Autoclaved Ultrapure Water

5.1.2.2 Microscope Slide

5.1.2.3 Glass Coverslips

5.1.2.4 Cytoseal or other mounting medium

5.1.2.5 100% Ethanol

5.1.2.6 Forceps

5.1.2.7 Scalpel/Scissors

5.1.2.8 Hot Plate

5.1.2.9 Picroindigocarmine Stain

5.1.2.10 Nuclear Fast Red Stain

5.1.3 Standards and Controls

5.1.3.1 New lots of Nuclear Fast Red and Picroindigocarmine stains will be tested against a known semen sample prior to use in casework. The reagents will be deemed acceptable for use if Nuclear Fast Red stains nuclear material red and Picroindigocarmine stains non-nuclear material green.

5.1.4 Sample Selection

5.1.4.1 Refer to the Evidence Handling/Suspected Semen section of the MNPD-CL Forensic Biology Quality Manual.

5.1.5 Procedure

5.1.5.1 Pre-heat a hot plate by setting the dial to “2”.

5.1.5.2 If the item being examined is an absorbent material, such as fabric, excise a small piece. For non-absorbing materials, collect the sample onto a sterile Copan swab. Copan swabs received in sexual assault kits will be applied directly to the microscope slides.

5.1.5.3 Wipe the microscope slide well with 100% ethanol and allow to fully dry before applying the sample to the slide.

5.1.5.4 Add 1-2 drops of autoclaved ultrapure water to a sampled area on the labeled (case # and sample ID #, initials, and date) microscope slide. Smear the cutting/swab in the water gently but firmly for 20 seconds, using forceps or scissors to grip the cutting. Cutting/swab may be used for p30 or DNA testing according to workflow in the MNPD-CL Forensic Biology Quality Manual/Evidence Handling/Suspected Semen section.

5.1.5.5 Place the slide on a pre-heated hot plate set to "2" for 20 minutes to allow the cellular material to heat fix to the slide.

5.1.5.6 After 20 minutes, remove the slide from the hot plate, and place it on the slide rack over the sink.

5.1.5.6.1 Turn off the hot plate.

5.1.5.7 Add enough Nuclear Fast Red stain to cover the cellular material on the slide (approx. 2 drops).

5.1.5.8 After 15 minutes, gently rinse the slide with autoclaved ultrapure water in a squeeze bottle.

5.1.5.9 Add enough Picroindigocarmine stain to cover the cellular material on the slide (approx. 2 drops).

5.1.5.10 After 15 seconds, gently rinse the slide with 100% ethanol in a squeeze bottle.

5.1.5.11 Remove the slide from the slide rack over the sink and place on a clean surface.

5.1.5.12 Once the ethanol has fully evaporated, add mounting medium to the stained smear and cover with a glass coverslip, being careful to minimize bubbles. Allow mounting medium to dry before placing slide on microscope.

5.1.6 Limitations

5.1.6.1 Nuclear Fast Red and Picroindigocarmine stain other cellular material along with spermatozoa. The slide must be carefully looked at to distinguish sperm cells from white blood cells, yeast, epithelial cells, etc. Negative slides will be reviewed by a second reader. In the event that a concordant conclusion cannot be drawn from both the analyst and the second reader, or both individuals render the result inconclusive due to degradation,

staining, and/or an obscurity that overlaps a single possible sperm, the microscopic examination will be reported inconclusive.

5.1.6.2 In addition, the chemicals used to stain will stain sperm from other species. However, the morphology of the sperm cells from other species is different and can be distinguished from human spermatozoa. Therefore, this protocol will be considered a confirmatory test for spermatozoa.

5.1.7 Safety

5.1.7.1 Picroindigocarmine contains picric acid, which is explosive when dry. Do not allow solution to dry out. Both stains are irritants and can be toxic. Caution should be used when handling these chemicals. Personal protective equipment should be worn at all times.

5.1.8 References

5.1.8.1 Performance Check of KPIC Staining for the Visualization of Sperm, MNPD-CL Forensic Biology Unit, September 2014.

5.1.8.2 Allery, J.P. et al. Cytological Detection of Spermatozoa: Comparison of Three Staining Methods. J Forensic Sci 2001; 46(2) pp.349-351.

5.1.8.3 Gaennslen, R.E. Sourcebook in Forensic Serology, Immunology, and Biochemistry. U.S. Dept. of Justice, National Institute of Justice, 1983. 149-155. Print.

5.2 Kohler Illumination

5.2.1 Scope

5.2.1.1 To describe the process by which even light distribution is achieved on the light microscope.

5.2.2 Equipment/Materials/Reagents

5.2.2.1 Compound Microscope

5.2.2.2 Microscope Slide

5.2.3 Procedure

5.2.3.1 Place a microscope slide onto the microscope stage and secure it using the specimen holders.

5.2.3.2 Turn the microscope on and adjust the light source to half of the potential brightness.

5.2.3.3 Using the 10X objective lens, focus the specimen using the coarse adjustment knob.

5.2.3.4 To adjust the eyepiece, close the right eye. Use the fine adjustment knob to sharpen the image. Then, close the left eye. Turn the Diopter ring located on the right eyepiece clockwise or counterclockwise to bring the specimen into focus.

5.2.3.5 Close the field diaphragm ring and the condenser aperture diaphragm ring. A small circle of light should be visible, which needs to be centered in the field of view. Adjust the condenser screws slowly to center the circle of light. If the circle of light is not visible, open the field diaphragm ring until the circle of light is just visible. Then, adjust the condenser screws until the light is centered and then close the field diaphragm ring.

5.2.3.6 Adjust the condenser focus knob until the circle of light is sharp.

5.2.3.7 Remove the left eyepiece. Look down the eyepiece cylinder. Open the condenser aperture diaphragm ring slowly until the circle of light fills ¾ of the field. Replace the eyepiece and record the setting for the diaphragm.

5.2.4 References

5.2.4.1 Silverberg, A. “Easy Kohler Illumination Method.” Trent University. Retrieved from www.trentu.ca, Nov. 2013.

5.2.4.2 Leica DM2000, DM2000 LED, DM2500, DM3000, DM3000 LED Operating Manual (2005). DM2000 LED Light Microscope. Leica Microsystems, Wetzlar, Germany.

5.3 Microscopic Examination for Spermatozoa

5.3.1 Scope

5.3.1.1 To describe the process by which chemically stained microscope slides are examined for the potential presence of spermatozoa.

5.3.2 Equipment/Materials/Reagents

5.3.2.1 Compound Microscope

5.3.2.2 Microscope Slide

5.3.3 Procedure

5.3.3.1 Place the slide on the stage of the microscope and examine the stained area using 400X magnification.

5.3.3.2 Start at one end of the stained area and proceed in a grid search fashion until the entire slide is examined. If the presence of spermatozoa is readily apparent, the entire slide does not need to be examined. The sperm head count shall be noted in the case notes as "20+". In the instance of a sample with 20 sperm heads or less, the exact sperm head count shall be noted in the case notes.

5.3.3.2.1 Note: If prone to motion sickness, it is recommended to examine the slide up and down instead of side to side.

5.3.3.3 At a minimum, it is required to capture the image of at least one sperm head to confirm the presence of semen. Refer to Section 5.4.3.2 for instructions on capturing an image.

5.3.4 Limitations

5.3.4.1 Refer to 5.1.6 of the MNPD-CL Technical Procedures Manual.

5.3.5 References

5.3.5.1 Performance Check of KPIC Staining for the Visualization of Sperm, MNPD-CL Forensic Biology Unit, September 2014.

5.3.5.2 Allery, J.P. et al. Cytological Detection of Spermatozoa: Comparison of Three Staining Methods. J Forensic Sci 2001; 46(2) pp.349-351.

5.3.5.3 Gaennslen, R.E. Sourcebook in Forensic Serology, Immunology, and Biochemistry. U.S. Dept. of Justice, National Institute of Justice, 1983. 149-155. Print.

5.4 Leica Application Suite Software (LAS)

5.4.1 Scope

5.4.1.1 To describe the functionality of the Leica Application Suite Software v4.4/4.13 and describe the process of using the software when viewing evidence under the microscope.

5.4.2 Equipment/Materials/Reagents

5.4.2.1 Leica Application Suite Software v4.4/4.13

5.4.2.2 Microscope

5.4.3 Procedure

5.4.3.1 Functions of Software

5.4.3.1.1 For all buttons on the software, once clicked the button will turn red to indicate the function is active. If the button is black, the function is off.

5.4.3.1.2 CAMERA TOOLBOX: (Left to right)

5.4.3.1.2.1 **Automatic Exposure:** Proper exposure is maintained but the user can adjust the brightness and any hardware changes that could affect the exposure are compensated for automatically.

5.4.3.1.2.2 **Exposure Adjust Tab**:

5.4.3.1.2.2.1 **Brightness:** How light or how dark each color in the image is. Use small increases in brightness to help differentiate between colors; too much and detail begins to disappear.

5.4.3.1.2.2.2 **Saturation**: Determines the amount of each color that is present. Use this function to achieve color subtlety in the image.

5.4.3.1.2.2.3 **Gamma**: A value applied to color levels to compensate for different ways in which the image is viewed. Use this function to achieve a contrast 'match' to the specimen.

5.4.3.1.2.2.4 **Gain**: Only active when Automatic Exposure is disabled. A function for changing the brilliance of an image without changing the exposure. Start with a Gain value of 1.0 and gradually increase the value. Too high a Gain setting will 'bleach' the image, causing a loss of fine detail and may introduce 'noise'.

5.4.3.1.2.3 **Automatic White Balance:** All of the neutral tones – white through grey to black – are adjusted to remove any 'color' content to maintain a clean, well-defined image.

5.4.3.1.2.3.1 **NOTE**: If the image is too dark or too light Automatic White Balance may fail and an error message displayed. It may be possible to lighten or darken the image with Exposure Adjust controls or change the lighting conditions at the microscope.

5.4.3.1.2.4 **Easy Camera Control**: If the specimen is evenly and well lit, focus and contrast are acceptable, then the Easy Camera Control tool allows users to 'fine tune' the image and achieve even better results.

5.4.3.1.2.4.1 **Twain Interface**: Can be accessed by right-clicking on the easy button to display a drop-down menu. The Twain Interface shows the camera data and basic exposure settings on a single, compact display.

5.4.3.1.2.5 **Camera and Microscope Linking**: This function is Not Applicable to our microscopes and camera setups.

5.4.3.1.2.6 **Leica High Dynamic Range (HDR)**: Automatically captures a number of images each at different exposure, and then combining them digitally into a single image that balances the contrast range.

5.4.3.1.2.6.1 **Averaging**: Can be accessed by right-clicking on the HDR button to display a drop-down menu. This setting averages multiple exposures to reduce noise without reducing detail.

5.4.3.1.2.7 **Show Under/Over Exposure**: Gives a fast indication of those areas of the image that are not exposed properly.

5.4.3.1.2.8 **Camera Configuration**: Saves Input Settings that users load to use them on another occasion by selecting from a drop-down menu.

5.4.3.1.2.8.1 **NOTE**: When the viewer opens, the configuration defaults to the Last Used settings.

5.4.3.1.2.9 **Shading Configurations**: This function is Not Applicable to our microscopes and camera setups.

5.4.3.1.2.10 **Predefined Camera Setups**: Settings for the most common microscope contrast methods that you can quickly select and use.

5.4.3.1.2.11 **Reset Camera**: Provides a quick way of restoring the camera to factory default settings.

5.4.3.1.3 SIDE TOOL BAR: (Top to bottom)

5.4.3.1.3.1 **Scale Bar and Annotations**: Shows options for annotating an image. Basic annotations include image name, description, date and time, drawing a line, and editing the color and background of the text.

5.4.3.1.3.2 **Floating Navigator**: Click to enable Navigator to file folders.

5.4.3.1.3.3 **Panning**: Examine areas of images that extend beyond the viewer edges into the display area.

5.4.3.1.3.4 **Zoom In and Out**: In the displayed area

5.4.3.1.3.5 **Fit image to the Viewer area**: Causes the image to expand to the full size of the viewer.

5.4.3.1.3.6 **Display at Original Size**: Displays the image at its captured size.

5.4.3.1.3.7 **Form Data**: Shows the images associated metadata in a dock-able window.

5.4.3.1.3.8 **Show/Hide Image**: Displays or removes image from view.

5.4.3.1.3.9 **Show/Hide Data Grid**: Displays or removes image data grid.

5.4.3.1.3.10 **Show/Hide Thumbnail Gallery**: Displays or removes a gallery of captured images.

5.4.3.1.3.11 **Record Details**: Shows all the associated details about captured image.

5.4.3.1.3.12 **Select Visible Fields**: Allows user to select which details will be displayed when Record Details button is selected.

5.4.3.1.3.13 **Viewer Options**: Not Applicable with our microscope/camera setups

5.4.3.1.3.14 **Save Image**: Allows you to save the image.

5.4.3.2 Microscopes

5.4.3.2.1 Ensure the microscope is turned on. Pull the plunger, located under the eyepieces, out to allow viewing on the computer monitor.

5.4.3.2.2 Open the LAS v4.4/4.13 software.

5.4.3.2.3 Begin on the Acquire Workflow Tab and using the 400X magnification, bring the specimen into focus on the screen.

5.4.3.2.4 Using the software functions (refer to 5.4.3.1), white balance the image or select Auto Exposure. Fine tune the image as necessary.

5.4.3.2.5 Once it is determined that an image needs to be captured, ensure microscope is set to 40X objective lens and specimen is in focus on the screen.

5.4.3.2.6 Click Acquire Image to take a photo. Hit the Export button on the right-side panel. In the resulting dialog box, choose the folder to save the image, rename the image with its item number, and change the file type to save as a jpg.

5.4.4 References

5.4.4.1 Leica Application Suite LAS User Manual (2013). DM2000 LED Light Microscope. Leica Microsystems, Wetzlar, Germany.

5.4.4.2 Leica LED 1000 Operating Instruction (2009). DM2000 LED Light Microscope. Leica Microsystems, Wetzlar, Germany.

6. ABAcard® p30 Test

6.1 Scope

6.1.1 To describe a process by which evidence is screened for the potential presence of seminal fluid.

6.2 Equipment/Materials/Reagents

6.2.1 Test device (individually packaged)

6.2.2 p30 Buffer

6.2.3 Thermomixer

6.2.4 Transfer pipette (included in kit)

6.2.5 Timer

6.2.6 Scalpel/scissors

6.2.7 Sample tube (1.5mL or 2.0mL)

6.3 Standards and Controls

6.3.1 In addition to lot number quality control testing, each lot of ABAcards® p30 must be tested using a positive control (known semen standard) and a negative control (p30 buffer) prior to use in casework each day. The result of the daily testing must be recorded in the casefile.

6.3.2 The control line in the control "C" area can be considered an internal procedural control.

6.4 Procedure

6.4.1 Place sample for testing into a sample tube.

6.4.2 If a full swab is used, add 500µl of p30 buffer and allow to incubate at room temperature for 30 to 120 minutes on a thermomixer at room temperature (23°C). If using a partial swab, add 250µl of p30 buffer and allow to incubate at room temperature for 30 to 120 minutes on a thermomixer at room temperature (23°C).

6.4.3 Pipette 80ul (~2 drops with transfer pipette) of supernatant into the sample well "S" on the test device.

6.4.4 Read results at 10 minutes.

6.4.5 Interpretation:

6.4.5.1 Positive: A positive result is indicated by a pink line in the "T" (test) and "C" (control) regions.

6.4.5.2 Negative: A negative result is indicated by a single pink line in the "C" (control) region. No line will be observed in the "T" (test) region.

6.4.5.3 Inconclusive: Regardless of the presence or absence of a line in the "T" (test) region, valid results will only be recorded if a pink line is present in the "C" (control) region. If no pink line is visible in this region, the test should be repeated if sufficient sample remains.

6.5 Limitations

6.5.1 False negative results may be obtained with the ABAcard® p30 test due to "High Dose Hook Effect". The high dose hook effect occurs when there are very high concentrations of p30 in a sample. If a high concentration of p30 is suspected, a 1:10 to 1:10,000 dilution of the sample can be created and retested to confirm the test result.

6.5.2 The results of this test should not be read after 10 minutes since non- specific reactions may occur and may result in false positives.

6.5.3 Prostate specific antigen has been reported in lower concentrations in other bodily fluids such as breast milk, amniotic fluid, and male urine.

6.5.4 Non-biological fluids such as feminine washes, energy drinks, and samples that alter the pH of the test have been shown to produce positive results.

6.6 Safety

6.6.1 Caution should be used when handling kit reagents. Personal protective equipment should be used at all times. Kit reagents contain sodium azide as a preservative which may react with lead or copper in plumbing. Upon disposal, always flush with large volumes of water to prevent build up.

6.7 References

6.7.1 ABAcards® p30 Validation, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, September 2014

6.7.2 ABAcards® p30 Non-biological Fluids Performance Check, Metro Nashville Police Department Crime Laboratory Forensic Biology Unit, September 2014

6.7.3 Abacus Diagnostics, Inc. ABAcard® p30 Test for The Forensic Identification of Semen: Technical Information Sheet. Updated 3/03.

6.7.4 Hobbs, Marcia M et al. Vaginal swab specimen processing methods influence performance of rapid semen detection tests: A cautionary tale. Contraception, September 2010, Vol. 82, No. 3: 291-295.

6.7.5 SERATEC. PSA in Body Fluids: An Overview for Users of the SERATEC PSA SEMIQUANT Tests.

6.7.6 DiFrancesco, J. Re-evaluation Seratec PSA Semiquant Test at the USACIL. AFFS presentation slides.

7. Sample Preparation

7.1 Removal of Hair or Tissue from Mounted Slides

7.1.1 Scope

7.1.1.1 To describe the process of removing hair or tissue from mounted slides.

7.1.2 Equipment/Materials/Reagents

7.1.2.1 Forceps

7.1.2.2 Scalpel/Scribe

7.1.2.3 Xylene

7.1.2.4 Ethanol

7.1.2.5 Ultrapure water

7.1.2.6 1.5mL Microcentrifuge tube

7.1.3 Procedure

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

7.1.3.2 The coverslip may be removed by carefully pipetting xylene around the edges of the coverslip. If the coverslip does not loosen and come off, the entire slide can be covered with xylene for one or more hours until the coverslip has loosened.

7.1.3.3 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.

7.1.3.4 Carefully remove the hair (or tissue), or scrape the semen smear, and place in a 1.5mL tube.

7.1.3.5 Wash in 500µL xylene to remove excess mounting medium.

7.1.3.6 Follow with a wash of 500µL ethanol and a final rinse of autoclaved ultrapure water.

7.1.4 Safety

7.1.4.1 Caution should be used when handling glass. Personal protective equipment should be worn at all times. Xylene is a chemical irritant and flammable; use caution when handling and avoid exposure to open flame. Use in a fume hood when possible.

7.1.5 References

7.1.5.1 Washington State Patrol Crime Laboratory. STR Analysis Procedures. Recovering Slide-Mounted Hairs or Semen Smears.

7.2 Whole Blood Specimens

7.2.1 Scope

7.2.1.1 To describe the process for preparation of whole blood liquid specimens for DNA testing.

7.2.2 Equipment/Materials/Reagents

7.2.2.1 Whole blood specimen in purple top vacutainer tube (PPT)

7.2.2.2 Bloodstain card

7.2.2.3 Decontaminant

7.2.2.4 Laboratory wipes

7.2.2.5 Ink pen or marker

7.2.2.6 Disposable pipette or pipette with filter tip

7.2.3 Procedure

7.2.3.1 Document the packaging, conditions, and any identifying information on the blood tube.

7.2.3.2 At minimum, label the bloodstain card with:

7.2.3.2.1 Name of source

7.2.3.2.2 Incident or Lab number

7.2.3.2.3 Item number

7.2.3.2.4 Date card was spotted

7.2.3.2.5 Preparer's initials

7.2.3.3 Mix the blood by gently inverting the tube several times.

7.2.3.3.1 CAUTION! Ensure the stopper does not dislodge from the top of the tube.

7.2.3.4 Use a wipe to uncap the blood tube.

7.2.3.5 Use a disposable pipette, or pipette with filter tip, to transfer the blood from the tube to the appropriately labeled bloodstain card. Discard any excess blood remaining in the pipette tip into the original blood tube.

7.2.3.5.1 CAUTION! Do not over-saturate the card.

7.2.3.6 Allow the bloodstain card to **COMPLETELY AIR DRY** prior to packaging and sealing in an appropriately labeled package.

7.2.4 Safety

7.2.4.1 Perform the procedure in a well-ventilated area, preferably in the hood designated for the processing of known samples. If a barrier, such as a sash, does not separate the preparer from the blood, a face shield should be worn. Adhere to the general safety precautions outlined in Evidence Handling/ General Safety Section of the MNPD-CL Forensic Biology Quality Manual.

7.2.5 References

7.2.5.1 Evidence Handling/ General Safety Section of the MNPD-CL Forensic Biology Quality Manual

7.3 Liquid Samples, including oral rinses and condoms containing liquid

7.3.1 Scope

7.3.1.1 To describe the process for preparation of liquid samples for Serology and/or DNA testing.

7.3.2 Equipment/Materials/Reagents

7.3.2.1 Vortex

7.3.2.2 Decontaminant

7.3.2.3 Laboratory wipes

7.3.2.4 Sterile nylon flocked swab(s)

7.3.2.5 Sterile cotton swab(s) (if flocked swabs are unavailable)

7.3.3 Procedure

7.3.3.1 Document the packaging, conditions, and any identifying information on the item.

7.3.3.2 For oral rinses, centrifuge and /or allow cellular material to settle to the bottom of the tube or container. Discard the supernatant and collect the sediment on multiple swabs.

7.3.3.3 For condoms containing a liquid sample, preserve the liquid on multiple swabs.

7.3.3.4 If ample amount of sediment or liquid is present, collect on at least four swabs. If desired, pipette a portion of the sediment or liquid to a labeled microscope slide and proceed with slide preparation as outlined in Section 5.0 Microscopic Examination for Sperm.

7.3.3.5 Allow the swabs to **COMPLETELY AIR DRY** prior to packaging and sealing in an appropriately labeled package, or proceed using the process described in **Decision Tree for Semen/Sperm Screening Sexual Assault Kits** found in The Evidence Handling/Suspected Semen Section of the MNPD-CL Forensic Biology Quality Manual.

7.3.4 Safety

7.3.4.1 Adhere to the general safety precautions outlined in Evidence Handling/General Safety Section of the MNPD-CL Forensic Biology Quality Manual.

7.3.5 References

7.3.5.1 Virginia Department of Forensic Science. Forensic Biology Section, Screening and Collection for DNA Analysis. Semen Analysis.

7.4 Cutting Items for Re-extraction

7.4.1 Scope

7.4.1.1 To describe the process for re-cutting items for DNA re-extraction testing.

7.4.2 Equipment/Materials/Reagents

7.4.2.1 Scalpels/scissors/tweezers

7.4.2.2 Decontaminant

7.4.2.3 Laboratory wipes

7.4.2.4 Ink pen or marker

7.4.2.5 Sterile tube(s)

7.4.2.6 Re-extracted Sample Checklist

7.4.3 Procedure

7.4.3.1 Document notes on previously created serology sheet or on a new form.

7.4.3.1.1 Note: Additions to previously created serology sheet must be clear as to what actions were performed on what date.

7.4.3.2 Open the Re-extracted Sample Checklist.

7.4.3.3 Re-cut/swab the item and place in sterile labeled tube.

7.4.3.4 Once all steps have been completed, sign the analyst box on the Re-extracted Sample Checklist and include the form with the casefile.

8. Non-Differential Extraction Procedures

8.1 EZ1/2 Trace Protocol

8.1.1 Scope

8.1.1.1 To describe the process of robotic DNA extraction of questioned or known DNA samples using the EZ1 Advanced XL and the EZ2 Connect Fx.

8.1.2 Equipment/Materials/Reagents

8.1.2.1 EZ1 Advanced XL

8.1.2.2 EZ2 Connect Fx

8.1.2.3 EZ1/2 DNA Investigator Kit

8.1.2.4 G2 Buffer

8.1.2.5 Proteinase K

8.1.2.6 Thermomixer w/2.0mL block

8.1.2.7 Scissors

8.1.2.8 Lyse and Spin Basket Kit

8.1.2.9 Vortex

8.1.2.10 Centrifuge

8.1.2.11 Qiagen Pipette Tips

8.1.2.12 Qiagen Pipette Tip Holder

8.1.2.13 1.5mL Qiagen Elution Tubes

8.1.2.14 Autoclaved Water

8.1.2.15 Pipettes

8.1.3 Standards and Controls

8.1.3.1 See Reagents/Supplies of the FB Quality Manual for reagents that require QC prior to use.

8.1.3.2 At least one reagent blank will be run with each extraction batch. A reagent blank is used to test all of the reagents used throughout the DNA process for potential extraneous DNA. It is subjected to the same processes as the DNA samples; however, no DNA is present.

8.1.3.3 An extraction batch is defined as a group of samples that are concurrently (i.e., simultaneously or sequentially) subjected to the same processes on the same instrument model and that are associated with the same reagent blank.

8.1.4 Calibration

8.1.4.1 The thermomixers shall be calibrated once per year to ensure they are achieving the proper temperature range.

8.1.4.2 The EZ1/2 instruments shall undergo annual preventative maintenance followed by a performance check to ensure the instruments are performing properly, prior to running casework samples.

8.1.4.3 Refer to the maintenance and calibration protocols of the MNPD-CL Forensic Biology Quality Manual.

8.1.5 Procedure

8.1.5.1 Excise an appropriately sized sample and place it into a clean, labeled Lyse and Spin Basket tube.

8.1.5.2 Prepare a master mix of diluted G2 Buffer and Proteinase K. The mix is prepared using 480µL of the diluted G2 Buffer (1:2 dilution with water: 240µL of G2 Buffer and 240µL of Autoclaved Water) and 20µL Proteinase K per sample. The number of samples calculated may be increased to ensure enough master mix is prepared.

8.1.5.3 Aliquot 500µL of the master mix into each of the Lyse & Spin baskets.

8.1.5.4 Mix the sample by vortexing for 5-15 seconds.

8.1.5.5 Incubate the sample at 56°C for 1-2 hours. While incubating, shake the sample at 900 rpm.

8.1.5.6 Centrifuge the sample for 1 min at 10,000x RCF. Make sure all the liquid has passed through the membrane into the collection tube. Additional spin(s) may be necessary and can be carried out up to 20,000x RCF. After all liquid has passed through the membrane, using clean scissors, cut the hinge of the microcentrifuge tube (with the spin basket still in place and the lid closed).

8.1.5.6.1 If the liquid has not passed through the membrane after a 20,000x RCF spin, the sample (i.e., the substrate, the liquid within the basket and the passthrough within the tube) may be transferred to a new Lyse & Spin Basket kit. This step must be witnessed by a qualified individual and documented on the batch witness sheet. Utensils used to transfer the substrate must be sterilized before and after use (e.g., cleaned with 10% bleach followed by a 70% - 100% ethanol wiping).

8.1.5.7 Turn on the EZ1/2.

8.1.5.7.1 Load the EZ1/2 DNA Investigator Cartridge into the appropriate position on the EZ1/2 cartridge rack.

8.1.5.7.1.1 Prior to loading the cartridges into the EZ1/2 the analyst must check the following:

8.1.5.7.1.1.1 The analyst must check the cartridge clam shell to ensure that cartridges are intended to be used with the DNA Investigator protocol.

8.1.5.7.1.1.2 The analyst must check the sticker on the held end of the cartridge to ensure that cartridges are intended to be used with the DNA Investigator protocol.

8.1.5.7.1.1.2.1 The analyst must compare each individual cartridge wells to the ground truth cartridge photo to ensure the cartridge is filled properly.

8.1.5.7.2 EZ1 Advanced XL

8.1.5.7.2.1 Load the Qiagen pipette tip and pipette tip holder into the appropriate position on row 2 of the deck of the EZ1.

8.1.5.7.2.2 Load a clean labeled Qiagen 1.5mL elution tube into the appropriate position on row 1 of the deck of the EZ1.

8.1.5.7.2.2.1 **NOTE**: If two extracts from the same sample are being combined, the previously labeled elution tube containing the original extract may be placed in the elution tube position.

8.1.5.7.2.3 Remove the lid and spin basket and discard them if necessary. Load the sample tube into the appropriate position on row 4 of the deck of the EZ1.

8.1.5.7.2.4 Run the Trace Protocol. Select to elute in TE buffer. Elute in 50, 100, or 200µL depending on sample type.

8.1.5.7.2.5 Start run.

8.1.5.7.3 EZ2 Connect Fx

8.1.5.7.3.1 Load the Qiagen pipette tip and pipette tip holder into the appropriate position on row C of the deck of the EZ2.

8.1.5.7.3.2 Load a clean labeled Qiagen 1.5mL elution tube into the appropriate position on row D of the deck of the EZ2.

8.1.5.7.3.2.1 **NOTE**: If two extracts from the same sample are being combined, the previously labeled elution tube containing the original extract may be placed in the elution tube position.

8.1.5.7.3.3 Remove the lid and spin basket and discard them if necessary. Load the sample tube into the appropriate position on row A of the deck of the EZ2.

8.1.5.7.3.4 Run the DNA Investigator Trace Protocol. For extracts contained in a 1.5mL flip cap tube, run the DNA Investigator Trace Custom Protocol.

8.1.5.7.3.4.1 On the screen, log in with the username (i.e., Admin) only.

8.1.5.7.3.4.2 Select DNA in the applications field.

8.1.5.7.3.4.3 Select DNA Investigator Kit.

8.1.5.7.3.4.4 In the protocol field, tap DNA Investigator Trace or DNA Investigator Trace Custom.

8.1.5.7.3.4.5 In the define parameters field, select TE from the drop down, for the rack type select tip rack, and select 50uL for the elution volume.

8.1.5.7.3.4.6 Select "generate missing sample ID's" and tap next. Select all channels being used on the screen. Select "skip load check" and the protocol will begin running.

8.1.5.7.3.5 Start run.

8.1.5.7.3.6 If the run is aborted, use the Appendix: EZ2 Sample Recovery Procedure.

8.1.6 Limitations

8.1.6.1 **CAUTION**: Certain porous materials interfere with bead extraction processes, preventing the recovery of DNA from a sample.

8.1.7 Safety

8.1.7.1 Buffers in the reagent cartridges contain guanidine salts, which can form highly reactive compounds when combined with bleach. Do not use bleach or allow bleach to contact reagent waste. The designated Qiagen waste biohazard container must be used when disposing of the reagent cartridges. Caution should be used when handling chemicals and personal protective equipment should be used at all times.

8.1.8 References

8.1.8.1 EZ1 DNA Investigator Handbook (2009). EZ1 DNA Investigator Kit. Qiagen, Hilden, Germany.

8.1.8.2 EZ1 Advanced XL User Manual (2009). EZ1 Advanced XL DNA Extraction Robot. Qiagen, Hilden, Germany.

8.1.8.3 EZ2 Connect and EZ2 Connect Fx User Manual, Qiagen, Hilden, Germany, March 2024

8.1.8.4 EZ2 Connect Fx Recovery Procedure Instruction Manual, Qiagen, Hilden, Germany, June 2023

8.1.8.5 DNA Extraction with QIAGEN EZ1® DNA Investigator Kit and EZ1® Advanced XL (July 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

8.1.8.6 Developmental Validation for QIAGEN EZ1® Robotic Platform and DNA Investigator® Silica-based Extraction Chemistry (October 2013). Qiagen.

8.1.8.7 EZ1® Validation Guide for the EZ1 DNA Investigator Kit, BioRobot® EZ1, EZ1 Advanced and EZ1 Advanced XL (June 2009). Qiagen.

8.1.8.8 QMS WF 108903 – EZ2 Connect Fx Performance Check

8.1.8.9 Phillips, Kirsty, Nicola McCallum, and Lindsey Welch. "A Comparison of Methods for Forensic DNA Extraction: Chelex-1001 and TheQIAGEN DNA Investigator Kit (manual and Automated)." Forensic Science International: Genetics (2012): 282-85.

9. Differential Extraction Procedures

9.1 Automated Separation Method

9.1.1 Scope

9.1.1.1 To describe the process of extracting DNA from evidence samples suspected to contain semen utilizing an automated separation of the "sperm and non-sperm fractions" via the QIAcube. "Sperm and non-sperm fractions" are placed in quotations because these titles (i.e., sperm fraction and non-sperm fraction) are theoretical. The differential extraction does not determine if sperm is present. The "sperm fraction" is also termed the E1 (i.e., extract 1) and the "non-sperm fraction" is also termed the E2 (i.e., extract 2).

9.1.2 Equipment/Materials/Reagents

9.1.2.1 EZ1 Advanced XL

9.1.2.2 EZ2 Connect Fx

9.1.2.3 EZ1/2 DNA Investigator Kit

9.1.2.4 QIAcube HID Differential Washing Station

9.1.2.5 Thermomixer

9.1.2.6 Scissors

9.1.2.7 Buffer ATL Working Solution

9.1.2.8 Proteinase K

9.1.2.9 Qiagen disposable 30mL reagent bottles

9.1.2.10 1000µL Qiagen wide-bore filter tips

9.1.2.11 Qiagen Rotor Adapter and Holder

9.1.2.12 1.5mL Qiagen sample tube

9.1.2.13 2.0mL Qiagen sample tube

9.1.2.14 Shaker Rack Plugs

9.1.2.15 Screw caps

9.1.2.16 G2 Buffer

9.1.2.17 Vortex

9.1.2.18 DTT

9.1.2.19 Autoclaved Water

9.1.2.20 Tweezers

9.1.2.21 Promega Spin Baskets

9.1.2.22 Centrifuge

9.1.2.23 Qiagen Pipette Tips

9.1.2.24 Qiagen Pipette Tip Holder

9.1.2.25 1.5mL Qiagen Elution Tubes

9.1.2.26 Pipettes

9.1.3 Standards and Controls

9.1.3.1 See Reagents/Supplies of the FB Quality Manual for reagents that require QC prior to use.

9.1.3.2 At least one reagent blank will be run with each extraction batch. A reagent blank is used to test all of the reagents used throughout the DNA process for potential extraneous DNA. It is subjected to the same processes as the DNA samples; however, no DNA is present.

9.1.3.3 An extraction batch is defined as a group of samples that are concurrently (i.e., simultaneously or sequentially) subjected to the same processes on the same instrument model and that are associated with the same reagent blank.

9.1.4 Calibration

9.1.4.1 The thermomixers shall be calibrated once per year to ensure they are achieving the proper temperature range.

9.1.4.2 The EZ1/2 instruments shall undergo annual preventative maintenance followed by a performance check to ensure the instruments are performing properly, prior to running casework samples.

9.1.4.3 Refer to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual.

9.1.4.4 The QIAcube instruments shall undergo annual preventative maintenance followed by a performance check to ensure the instruments are performing properly, prior to running casework samples.

9.1.5 Procedure

9.1.5.1 Place sample cutting or swab head(s) into a clean, labeled 1.5mL Qiagen sample tube.

9.1.5.2 Prepare a master mix of diluted ATL and Proteinase K. The mix is prepared using 480µL of the diluted ATL and 20µL Proteinase K per sample. The number of samples calculated may be increased to ensure enough master mix is prepared.

9.1.5.3 Aliquot 500µL of the master mix into each of the extraction tubes.

9.1.5.4 Mix the sample by vortexing for 5-15 seconds. Briefly centrifuge the sample to collect all the liquid into the tube.

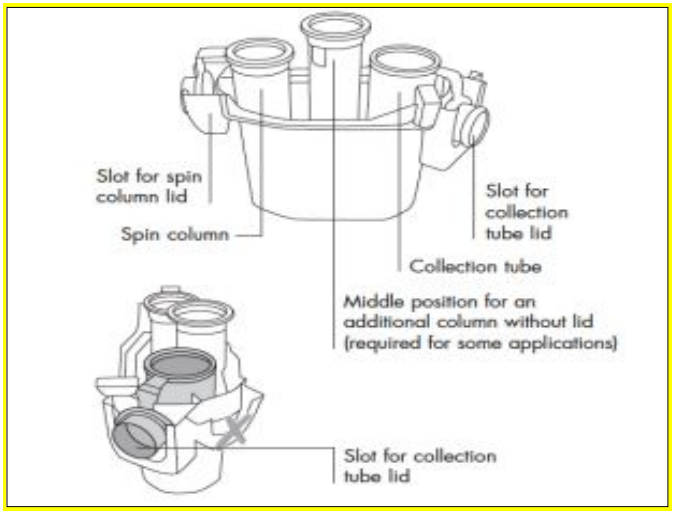
9.1.5.5 Incubate the sample at 56°C for 1.5-2 hours. While incubating, shake the sample at 900rpm.

9.1.5.6 Turn on and clean the QIAcube instrument.

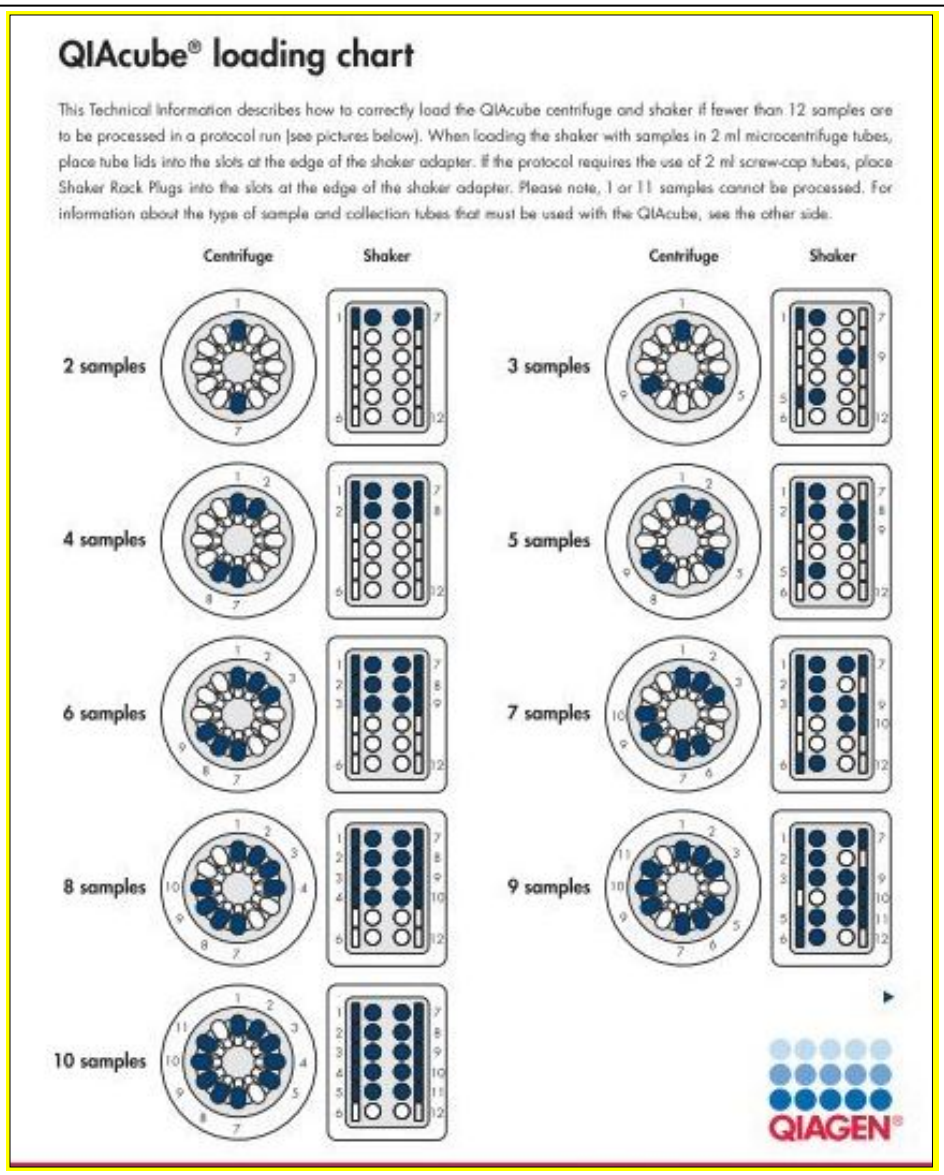
9.1.5.7 After incubation, pulse spin the sample to collect all the liquid. Place the cutting/swab from the sample into a clean spin basket using clean tweezers. Return the spin basket to the tube and centrifuge the basket and tube for 5 minutes at 10,000 x RCF. Discard the basket with the cutting/swab inside.

9.1.5.8 Place the 1.5mL Qiagen sample tube in position 3 (noted as collection tube in picture below) of the QIAcube rotor adapter.

9.1.5.8.1



9.1.5.9 Place the rotor adapter in the proper position of the centrifuge. Refer to the QIAcube loading chart.



9.1.5.9.1

9.1.5.10 Place a clean, labeled 2.0mL Qiagen sample tube into the proper position on the shaker of the QIAcube. Refer to the QIAcube loading chart. Insert shaker rack plugs into the corresponding slots at the edges of the shaker rack.

9.1.5.11 Fill both tip racks of the QIAcube with 1000µL Qiagen wide-bore filter tips.

9.1.5.12 Place a 30mL reagent bottle with at least 2.0mL of G2 Buffer per sample into position 1 of the reagent bottle rack of the QIAcube.

9.1.5.13 Place a 30mL reagent bottle with at least 1.0mL of autoclaved water per sample into position 2 of the reagent bottle rack of the QIAcube.

9.1.5.14 Close the QIAcube lid and start the “Separate and Lyse 12A Mod” Protocol on the QIAcube. To start the protocol, select “DNA” > select “Pipetting” > select “Epithelial and

Sperm Cell" > select "Separate and Lyse 12A Mod". Then, follow prompts to begin the run by selecting "Start".

9.1.5.15 When the protocol is finished, remove the 2.0mL Qiagen sample tube from the shaker. This is the non-sperm (epithelial) fraction. The "non-sperm fraction" can then be processed following the steps outlined in the Procedure section of the Non-Differential Extraction method described in this manual, beginning with turning on the EZ1/2.

9.1.5.16 Refill the tip racks with 1000µL Qiagen wide-bore filter tips and start the "Separate and Lyse 12B Mod" Protocol on the QIAcube by following the above steps. When the protocol is finished, remove the 1.5 mL Qiagen sample tube from position 3 of the QIAcube rotor adapter. Wipe the outside of the tubes with 10% bleach then 70% - 100% ethanol. This is the "sperm fraction".

9.1.5.17 Prepare a master mix for the "sperm fraction" of G2 Buffer, Proteinase K, and DTT. The mix is prepared using 160µL G2 Buffer, 10µL Proteinase K, and 40µL DTT per sample. The number of samples calculated may be increased to ensure enough master mix is prepared.

9.1.5.18 Aliquot 210µL of the master mix into each of the "sperm fraction" tubes.

9.1.5.19 Mix the sample by vortexing for 5-15 seconds. Briefly centrifuge the sample to collect all the liquid into the tube.

9.1.5.20 Incubate the sample at 70°C for 10 min while shaking at 900rpm. After incubation, vortex the sample for 10 seconds, pulse spin to collect the liquid, and remove the lid by cutting the hinge using clean scissors.

9.1.5.21 The "sperm fraction" can then be processed following the steps outlined in the Procedure section of the Non-Differential Extraction method described in this manual, beginning with turning on the EZ1/2.

9.1.6 Limitations

9.1.6.1 This procedure should not be used for extraction of DNA from samples in which no semen is suspected. For extracting DNA from samples in which no semen is suspected, refer to the Non-Differential Extraction Procedures of this manual.

9.1.7 Safety

9.1.7.1 Buffers in the reagent cartridges contain guanidine salts, which can form highly reactive compounds when combined with bleach. Do not use bleach or allow bleach to contact

reagent waste. The designated Qiagen waste biohazard container must be used when disposing of the reagent cartridges. Caution should be used when handling chemicals and personal protective equipment should be used at all times.

9.1.8 References

9.1.8.1 Developmental Validation of QIAcubeTM Automated Platform and Differential Wash Protocol for Forensic DNA Testing Laboratories (October 2013). Qiagen.

9.1.8.2 EZ1 DNA Investigator Handbook (2009). EZ1 DNA Investigator Kit. Qiagen, Hilden, Germany.

9.1.8.3 EZ1 DNA Investigator Handbook (July 2014). EZ1 DNA Investigator Kit. Qiagen, Hilden, Germany.

9.1.8.4 EZ1 Advanced XL User Manual (2009). EZ1 Advanced XL DNA Extraction Robot. Qiagen, Hilden, Germany.

9.1.8.5 EZ2 Connect and EZ2 Connect Fx User Manual, Qiagen, Hilden, Germany, March 2024

9.1.8.6 EZ2 Connect Fx Recovery Procedure Instruction Manual, Qiagen, Hilden, Germany, June 2023

9.1.8.7 QMS WF 108903 – EZ2 Connect Fx Performance Check

9.1.8.8 QIAcube User Manual (2008). QIAcube HID Differential Washing Station. Qiagen, Hilden, Germany.

9.1.8.9 Differential Processing of Semen Samples using the QIAcube® Differential Washing Station Validation Report (August 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

9.2 Manual Separation Method

9.2.1 Scope

9.2.1.1 To describe the process of extracting DNA from semen stains utilizing a manual separation of the “sperm and non-sperm fractions”. This method is intended for use only if the QIAcube is unavailable for use.

9.2.2 Equipment/Materials/Reagents

9.2.2.1 EZ1 Advanced XL DNA Extraction Robot

9.2.2.2 EZ2 Connect Fx

9.2.2.3 EZ1/2 DNA Investigator Kit

9.2.2.4 Thermomixer

9.2.2.5 Scissors

9.2.2.6 Buffer ATL Working Solution

9.2.2.7 Proteinase K

9.2.2.8 G2 Buffer

9.2.2.9 DTT

9.2.2.10 Autoclaved Water

9.2.2.11 Promega Spin Basket

9.2.2.12 1.5mL Qiagen Sample Tube

9.2.2.13 Tweezers

9.2.2.14 Vortex

9.2.2.15 Centrifuge

9.2.2.16 Pipettes

9.2.3 Standards and Controls

9.2.3.1 See Reagents/Supplies of the FB Quality Manual for reagents that require QC prior to use.

9.2.3.2 At least one reagent blank will be run with each extraction batch. A reagent blank is used to test all of the reagents used throughout the DNA process for potential extraneous DNA. It is subjected to the same processes as the DNA samples; however, no DNA is present.

9.2.3.3 An extraction batch is defined as a group of samples that are concurrently (i.e., simultaneously or sequentially) subjected to the same processes on the same instrument model and that are associated with the same reagent blank.

9.2.4 Calibration

9.2.4.1 The thermomixers shall be calibrated once per year to ensure they are achieving the proper temperature range.

9.2.4.2 The EZ1/2 instruments shall undergo annual preventative maintenance followed by a performance check to ensure the instruments are performing properly, prior to running casework samples.

9.2.4.3 Refer to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual.

9.2.5 Procedure

9.2.5.1 Place sample cutting or Copan swab head(s) into a clean, labeled 1.5mL Qiagen sample tube.

9.2.5.2 Prepare a master mix of diluted Buffer ATL Working Solution and Proteinase K. The mix is prepared using 480µL of the diluted ATL Buffer and 20µL Proteinase K per sample. The number of samples calculated may be increased to ensure enough master mix is prepared.

9.2.5.3 Aliquot 500µL of the master mix into each of the extraction tubes.

9.2.5.4 Mix the sample by vortexing for 5-15 seconds. Briefly centrifuge the sample to collect all the liquid into the tube.

9.2.5.5 Incubate the sample at 56°C for 1.5-2 hours. While incubating, shake the sample at 900 rpm.

9.2.5.6 After incubation, pulse spin the sample to collect all the liquid. Place the cutting/swab from the sample in a clean spin basket. Centrifuge basket and tube for 5 minutes at 10,000x RCF. Discard the basket with the sample cutting/swab inside.

9.2.5.7 Centrifuge the sample for 5 minutes at 10,000x RCF. Transfer the supernatant to a new clean, labeled 1.5mL microcentrifuge tube. This is the "non-sperm (epithelial) fraction".

9.2.5.8 The "non-sperm fraction" can then be processed following the steps outlined in the Procedure section of the Non-Differential Extraction method described in this manual, beginning with turning on the EZ1/2.

9.2.5.9 Add 500µL G2 Buffer to the pellet.

9.2.5.10 Mix the sample by vortexing for 5-15 seconds. Centrifuge the sample for 5 minutes at 10,000x RCF.

9.2.5.11 Carefully remove the supernatant (without disturbing the pellet) and discard it.

9.2.5.12 Repeat the wash step with G2 Buffer two additional times.

9.2.5.13 Add 700µL autoclaved water to the sample.

9.2.5.14 Mix the sample by vortexing for 5-15 seconds. Centrifuge the sample for 5 minutes at 10,000x RCF.

9.2.5.15 Remove all the supernatant except approximately 50uL (without disturbing the pellet) and discard it. This is the "sperm fraction".

9.2.5.16 Prepare a master mix for the "sperm fraction" of G2 Buffer, Proteinase K, and DTT. The mix is prepared using 160µL G2 Buffer, 10µL Proteinase K, and 40µL DTT per sample. The number of samples calculated may be increased to ensure enough master mix is prepared.

9.2.5.17 Aliquot 210µL of the master mix into each of the "sperm fraction" tubes.

9.2.5.18 Mix the sample by vortexing for 5-15 seconds. Briefly centrifuge the sample to collect all the liquid into the tube.

9.2.5.19 Incubate the sample at 70°C for 10 min while shaking at 900rpm. After incubation, vortex the sample for 10 seconds, pulse spin to collect the liquid, and remove the lid by cutting the hinge using clean scissors.

9.2.5.20 The "sperm fraction" can then be processed following the steps outlined in the Procedure section of the Non-Differential Extraction method described in this manual, beginning with turning on the EZ1/2.

9.2.6 Limitations

9.2.6.1 This procedure should not be used for extraction of DNA from samples in which no semen is suspected. For extracting DNA from samples in which no semen is suspected, refer to the Non-Differential Extraction Procedures of this manual .

9.2.7 Safety

9.2.7.1 Buffers in the reagent cartridges contain guanidine salts, which can form highly reactive compounds when combined with bleach. Do not use bleach or allow bleach to contact reagent waste.

9.2.7.2 **CAUTION** should be used when handling chemicals and personal protective equipment should be used at all times.

9.2.8 References

9.2.8.1 EZ1 DNA Investigator Handbook (2009). EZ1 DNA Investigator Kit. Qiagen, Hilden, Germany.

9.2.8.2 EZ1 DNA Investigator Handbook (July 2014). EZ1 DNA Investigator Kit. Qiagen, Hilden, Germany.

9.2.8.3 EZ1 Advanced XL User Manual (2009). EZ1 Advanced XL DNA Extraction Robot. Qiagen, Hilden, Germany.

9.2.8.4 EZ2 Connect and EZ2 Connect Fx User Manual, Qiagen, Hilden, Germany, March 2024

9.2.8.5 EZ2 Connect Fx Recovery Procedure Instruction Manual, Qiagen, Hilden, Germany, June 2023

9.2.8.6 QMS WF 108903 – EZ2 Connect Fx Performance Check

9.2.8.7 Differential Processing of Semen Samples using the QIAcube Differential Washing Station Validation Report (August 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

10. Quantitation

10.1 Setup Protocol

10.1.1 Scope

10.1.1.1 To describe the manual preparation of DNA samples and standards for quantitation using the Plexor® HY System.

10.1.2 Equipment/Materials/Reagents

10.1.2.1 Plexor HY System Kit

10.1.2.1.1 Plexor® HY 2X Master Mix

10.1.2.1.2 Plexor® HY 20X Primer/IPC Mix

10.1.2.1.3 Plexor® HY Male Genomic DNA Standard, 50ng/µl

10.1.2.1.4 Water, Amplification Grade

10.1.2.2 TE Buffer

10.1.2.3 1.5mL Microcentrifuge tubes

10.1.2.4 1.5 mL low adhesion tubes

10.1.2.5 96-well optical-grade PCR plate

10.1.2.6 Optical adhesive cover and plate cover applicator

10.1.2.7 Optical Support Base

10.1.2.8 7500 Real-Time PCR System

10.1.2.9 QIAgility

10.1.2.10 QIAgility Software v4.18.1

10.1.2.11 Vortex

10.1.2.12 Centrifuge

10.1.2.13 Pipettes

10.1.3 Standards and Controls

10.1.3.1 A standard curve is generated using DNA standards in the range of 3.2pg/µl to 50ng/µl. Plexor HY Male Genomic DNA Standard at an undiluted concentration of 50ng/µl is used to prepare a serial dilution of the remaining DNA standards. Each standard point will be run in duplicate.

10.1.3.2 Creating the standards:

10.1.3.2.1 Upon first use of each standard tube, thaw and vortex the Plexor HY Male Genomic DNA Standard at high speed for 10sec. Do not centrifuge standard after vortexing.

10.1.3.2.2 Prepare serial dilutions for the Plexor HY standards as indicated below using 1.5mL low adhesion tubes. Vortex each dilution for 10sec before removing an aliquot. Do not centrifuge standards after vortexing. It is recommended to change gloves after handling the first two concentration points of standards.

Standard	Concentration	Volume of DNA	Volume of TE
Standard 1	50ng/ul	Use neat standard	0ul
Standard 2	10ng/ul	10µl of neat standard	40ul
Standard 3	2ng/ul	10µl of 10ng/µl dilution	40ul
Standard 4	0.4ng/ul	10µl of 2ng/µl dilution	40ul
Standard 5	0.08ng/ul	10µl of 0.4ng/µl dilution	40ul
Standard 6	0.016ng/ul	10µl of 0.08ng/µl dilution	40ul
Standard 7	0.0032ng/ul	10µl of 0.016ng/µl dilution	40ul

10.1.3.2.3 Fresh standards should be made each day and stored at 2-8°C when not in use. Do not refreeze DNA standards.

10.1.4 Calibration

10.1.4.1 Each 7500 shall undergo annual preventative maintenance followed by a performance check to ensure the instruments are performing properly, prior to running casework samples.

10.1.4.2 Refer to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual.

10.1.5 Procedure

10.1.5.1 Thaw Plexor HY kit components at room temperature.

10.1.5.2 Vortex Master Mix and Primer Mix for 10sec. Tap tube to remove any reaction mix from the top of the tube. Do not centrifuge kit components or reaction mix after vortexing.

10.1.5.3 Determine the number of reactions to be set up, including standards. Also, add enough for at least 4 additional reactions.

10.1.5.4 Prepare reaction mix:

Component	Volume (Per Reaction)
Plexor HY 2X Master Mix	10µl
Water, Amplification Grade	7µl
Plexor HY 20X Primer/IPC Mix	1µl
FINAL VOLUME	18µl

10.1.5.5 Proceed to the Manual Plating or QIAgility Plating quantitation procedure.

10.1.5.6 Manual Plating

10.1.5.6.1 Vortex prepared reaction mix for 10secs. Tap tube to remove any reaction mix from the top of the tube.

10.1.5.6.2 Add 18µl reaction mix to each well of an optical-grade PCR plate as determined by the plate setup sheet. The reaction plate should be seated in an optical support base and the bottom of the wells should not be touching the benchtop.

10.1.5.6.3 Following the addition of master mix and prior to the addition of sample, check to ensure that master mix has been added to all appropriate wells.

10.1.5.6.4 Vortex and spin down samples.

10.1.5.6.5 Add 2µl of DNA standards or unknown samples to reaction mix as determined by the plate setup sheet. DNA standards must be run in duplicate.

10.1.5.6.6 Seal plate with optical adhesive cover using plate cover applicator.

10.1.5.6.7 Centrifuge plate briefly to collect contents at bottom of wells and eliminate bubbles.

10.1.5.6.8 Proceed to Section 10.2 7500 Instrument Setup.

10.1.5.7 *QIAgility Plating*

10.1.5.7.1 Preparation Using the DNA Batch Workbook

10.1.5.7.1.1 Upon entering sample information into the DNA Batch Workbook, the “Quant” tab will populate with samples entered into the “Master List” tab. Save the information in the

"Quant QIA" tab as a .txt file (tab delimited). (Instruction will be listed at the top of the excel sheet in this tab.)

10.1.5.7.1.2 Reagent volumes needed for the quantitation set up will be calculated and listed in the Quant Set-up of the DNA Batch Workbook. (Note: add three additional samples to the ones listed on the sheet. Do this by leaving "0" in three of the remaining wells. Remove the "0"'s from any unused wells.)

10.1.5.7.2 QIAgility Instructions

10.1.5.7.2.1 Vortex prepared reaction mix for 10secs. Tap tube to remove any reaction mix from the top of the tube.

10.1.5.7.2.2 Turn on computer.

10.1.5.7.2.3 Turn on instrument.

10.1.5.7.2.4 Launch QIAgility Software v4.18.1. The QIAgility instrument lid must be closed for the software and hardware to initialize upon start-up and for a run to proceed.

10.1.5.7.2.5 Place prepared Plexor HY Reaction Mix into position G on the Reagent Block (R1) of the QIAgility. Using prepared quantitation standards, load the standards into positions I through O on the Reagent Block (R1).

10.1.5.7.2.6 Load sample tubes into the 4 x 8-well sample racks in the Sample Block (A2).

10.1.5.7.2.7 Place a 96-well optical plate in Reaction Block (C1).

10.1.5.7.2.8 Open the Quant protocol by going to "File" at the top left of the software screen, click "Open," and find the Quant protocol.

10.1.5.7.2.9 In the software, select the sample rack position A2. Click on the "Import" button and import the .txt file generated for your run.

10.1.5.7.2.10 Click on the green start arrow on the toolbar or select "Control/Start".

10.1.5.7.2.11 Check the pre-run report to verify the location and amount/volume of consumables and liquids that are required on the worktable for completion of the loaded run file.

10.1.5.7.2.12 The pre-run "Checklist" dialog box will appear. If no warnings or errors are listed, select the boxes to continue and click "OK" to start the run. If errors are listed, user intervention is required at this step.

10.1.5.7.2.12.1 **NOTE**: Care must be taken when opening the QIAgility lid during a run. It takes up to 10 seconds for the instrument to complete its current movement and for the pause to take effect.

10.1.5.7.2.13 Follow the prompted action pop-up windows during the duration of the Quant protocol.

10.1.5.7.2.14 Upon completion of the run, a "Post-run report" will appear. Save the "Post-run report" for the quant set up for the case file.

10.1.5.7.2.15 Remove the optical-grade PCR reaction plate from the QIAgility deck.

10.1.5.7.2.16 Seal plate with optical adhesive cover using plate cover applicator.

10.1.5.7.2.17 Centrifuge plate briefly to collect contents at bottom of wells and eliminate bubbles.

10.1.5.7.2.18 Proceed to Section 10.2 7500 Instrument Setup.

10.1.6 Limitations

10.1.6.1 Plexor HY Male Genomic DNA Standard may form aggregates or concentration gradients when frozen, which may result in sampling error. Ensure the standard is thawed at room temperature and vortex prior to use.

10.1.6.2 It is critical that the same reaction mix is used for the entire run. Ensure enough reaction mix is prepared to account for pipetting error.

10.1.6.3 Protect the reaction plate from extended light exposure or elevated temperature before cycling.

10.1.7 Safety

10.1.7.1 Caution should be used when handling chemicals and personal protective equipment should be used at all times.

10.1.7.2 The robotic arm of the QIAgility instrument moves during position calibration while the instrument lid is raised. Never click any buttons while parts of your body are within the instrument workspace.

10.1.8 References

10.1.8.1 Applied Biosystems. 2006. Validation Using SDS Software Version 1.2.3 on the Applied Biosystems 7500 Real-Time PCR System and the ABI PRISM® 7000 Sequence Detection System: User Bulletin.

10.1.8.2 Krenke, Benjamin E., et al. Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA: Developmental Validation of the Plexor® HY System. Promega Corporation, 2007.

10.1.8.3 Plexor HY Technical Manual (May 2013). Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems. Promeg, USA.

10.1.8.4 Internal Validation with Plexor® HY and 7500 Real-Time PCR System: (October 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

10.1.8.5 Internal Validation with QIAgility: : (2015), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

10.1.8.6 QIAgility® User's Manual (June 2013). QIAGEN.

10.1.8.7 Washington State Patrol Casework STR Analysis Procedures Crime Laboratory Division. August 2013.

10.2 7500 Instrument Setup

10.2.1 Scope

10.2.1.1 To describe the process of setting up the Applied Biosystems 7500 instrument software and creating a template.

10.2.2 Equipment/Materials/Reagents

10.2.2.1 Applied Biosystems 7500

10.2.2.2 HID Real-Time PCR Analysis Software v1.2

10.2.2.3 Centrifuge

10.2.2.4 Optical Adhesive Cover

10.2.2.5 Plate Cover Applicator

10.2.3 Procedure

10.2.3.1 Turn on the 7500 instrument.

10.2.3.2 Open the HID software. Log in with your initials or log in as a guest.

10.2.3.3 Open the 7500 instrument and place the plate with well A1 in the upper left corner.

10.2.3.4 Select "Custom Assays", then File > New Experiment > From Template.

10.2.3.5 Import the Plexor HY template:

10.2.3.5.1 This PC > OS (C:) > Applied Biosystems > 7500 > Config > Templates > Plexor HY

10.2.3.6 Change the experiment name to match your Quant plate name.

10.2.3.7 Under the "Setup" > "Plate Set Up" heading:

10.2.3.7.1 Select the "Assign Targets and Samples" tab

10.2.3.7.1.1 Highlight wells containing DNA.

10.2.3.7.1.2 Select all targets (auto, IPC, PassRef, and Y)

10.2.3.8 Click Start Run, select "ok", then save to experiments folder on C: drive.

10.2.3.9 After the run is completed, remove the plate and discard it in the biohazard waste.

10.2.3.10 Export run data following the steps outlined in section 10.3.3. Once data is exported, turn off the instrument and close the software.

10.2.3.11 For data analysis and data interpretation, see sections 10.4 and 10.5, respectively.

10.2.3.12 If, for some reason, the plate was loaded backwards (well A1 in lower right corner) or a well was missed when assigning targets, the run does not need to be redone. Return to the "Assign Targets and Samples" tab under the "Setup" > "Plate Set Up" heading. Clear all incorrectly assigned wells and associated targets. Then, select correct wells that contained DNA and subsequently assign targets.

10.2.3.12.1 IMPORTANT: Reanalyze the run by selecting the green "Analyze" button under the "Analysis" heading. Save the run and continue exporting the run data by following the steps outlined in section 10.3.3.

10.2.4 References

10.2.4.1 Applied Biosystems. 2006. Validation Using SDS Software Version 1.2.3 on the Applied Biosystems 7500 Real-Time PCR System and the ABI PRISM® 7000 Sequence Detection System: User Bulletin.

10.2.4.2 Krenke, Benjamin E., et al. Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA: Developmental Validation of the Plexor® HY System. Promega Corporation, 2007.

10.2.4.3 Plexor HY Technical Manual (May 2013). Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems. Promega, USA.

10.2.4.4 Internal Validation with Plexor® HY and 7500 Real-Time PCR System: (October 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

10.3 7500 Instrument Data Export into the Plexor® Analysis Software

10.3.1 Scope

10.3.1.1 To describe the process of exporting the data generated by the Applied Biosystems 7500 instrument into the Plexor Analysis Software.

10.3.2 Equipment/Materials/Reagents

10.3.2.1 Applied Biosystems 7500

10.3.2.2 HID Real-Time PCR Analysis Software v1.3

10.3.2.3 Plexor® Analysis Software (forensic release) v1.5.6.7

10.3.3 Procedure

10.3.3.1 Export from HID Software v1.3:

10.3.3.1.1 Click Analyze on the 7500 software. Then select "Export" from the top toolbar.

10.3.3.1.2 In the "Export Data" window, select the following "Export Properties":

10.3.3.1.2.1 Ensure that "Multicomponent Data" is selected.

10.3.3.1.2.2 File Type: Select .xls.

10.3.3.1.2.3 Specify the appropriate export file name & file location.

10.3.3.1.2.3.1 G drive (G:) > Instrument-DNA > Run Files > 7500 Real Time PCR > *Name of Instrument* > Year > Month

10.3.3.1.2.4 Select "Start Export".

10.3.3.1.3 Close HID Software and turn off 7500 instrument.

10.3.3.2 Import into Plexor Analysis Software v1.5.6.7:

10.3.3.2.1 Launch Plexor Analysis Software v1.5.6.7.

10.3.3.2.2 In the File menu, ensure "Set Passive Reference On Import" is selected. With HID software, the IC5 passive reference data is imported separately, and the normalization is applied within Plexor® Analysis Software.

10.3.3.2.3 In the File menu, select "Import New Run" or select the icon. 

10.3.3.2.4 Ensure "Applied Biosystems 7500 HID" is selected as the instrument type.

10.3.3.2.5 Ensure the target names and each of the dyes are assigned as follows:

10.3.3.2.5.1 Autosomal (FL) – Amp & Melt boxes selected

10.3.3.2.5.2 Y(CO560) – Amp & Melt boxes selected

10.3.3.2.5.3 IPC (CR610) – Amp & Melt boxes selected

10.3.3.2.5.4 Passive Reference (IC5) – Pass. Ref box is selected (this is the only box selected)

10.3.3.2.6 Select "Next".

10.3.3.2.7 Enter information specific to your run in the Run Info screen, Step 2. "Operator Name" is required. Details such as the date, notes, title, name of the person performing the experiment, etc., can also be entered. These fields are optional.

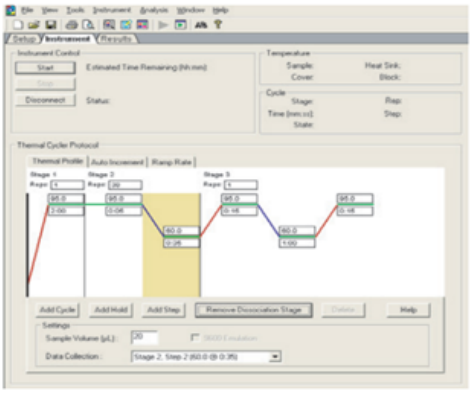
10.3.3.2.8 Select "Next".

10.3.3.2.9 In Step 3, use the File Import screen to specify the appropriate HID data file.

10.3.3.2.10 Ensure the number of amplification cycles is set to 38 and the range of melt temperature is set to 60 to 95.

10.3.3.2.11 Ensure the cycling parameters noted below are used when running samples on the 7500 RT PCR instrument utilizing Plexor HY.

Step	Temperature	Time	Number of Cycles
Initial denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	38 cycles
Annealing and extension:	60°C	35 seconds	
Melt temperature curve:	Use the default "Dissociation Function" settings.		



10.3.3.2.11.1

10.3.3.2.12 In the Advanced Options box, ensure that the Plexor Run Template is selected under Run Template. If not, click Edit, then Import, and navigate to the Template located in the G-drive with the following pathway: G:\Instrument-DNA\Run Files\Plexor software and template.

10.3.3.2.13 Select "Finish" to complete the data import and open the Analysis Desktop.

10.3.4 References

10.3.4.1 Applied Biosystems. 2006. Validation Using SDS Software Version 1.2.3 on the Applied Biosystems 7500 Real-Time PCR System and the ABI PRISM® 7000 Sequence Detection System: User Bulletin.

10.3.4.2 Krenke, Benjamin E., et al. Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA: Developmental Validation of the Plexor® HY System. Promega Corporation, 2007.

10.3.4.3 Plexor HY Technical Manual (May 2013). Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems. Promeg, USA.

10.3.4.4 Internal Validation with Plexor® HY and 7500 Real-Time PCR System: (October 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

10.4 Plexor® HY and 7500 Instrument Data Analysis

10.4.1 Scope

10.4.1.1 To describe the analysis process and interpretation of the data by the Plexor® Analysis Software v1.5.6.7.

10.4.2 Equipment/Materials/Reagents

10.4.2.1 Plexor® Analysis Software (forensic release) v1.5.6.7

10.4.3 Procedure

10.4.3.1 After data import is complete, the PCR curves tab is displayed in the Analysis Desktop.

10.4.3.2 If the Plexor Run Template was not selected on import, the DNA standards must be defined:

10.4.3.2.1 Using the well selector, highlight wells that contain the DNA standards

10.4.3.2.2 Select Create Dilution Series icon.

10.4.3.2.3 Confirm that the series selected is a “Vertical Series” and the series is “Decreasing”. Enter 50 for the starting concentration and 5 for the dilution factor.

10.4.3.3 <u>Assign or edit sample names:</u>

10.4.3.3.1 Select the Sample IDs tab, select each well and enter the desired sample name. Repeat to enter the sample names for the other wells.

10.4.3.3.2 Samples with the same name will have their DNA quantities averaged in the Forensics Report created by the software.

10.4.3.3.2.1 **NOTE**: Samples cannot be exported from the forensics report that have the same name.

10.4.3.4 Alternatively, sample names may be copied from a Microsoft Excel spreadsheet. Highlight the sample names on the spreadsheet and select Copy. In the Edit menu of the Plexor Analysis Software, select Paste Sample Names from Template. If copying sample names, the layout of the sample names in the spreadsheet must be the same as the layout of the samples within the PCR plate.

10.4.3.5 Once complete, click “Accept Changes”.

10.4.3.6 Adjust the Expected Target Melt Temperature:

10.4.3.7 Select the PCR Curves tab

10.4.3.8 Select all wells that contain the DNA standards.

10.4.3.9 The Tm for each selected sample will be displayed. The expected melt temperature range must be adjusted for each dye. The average expected target melt temperatures are as follows:

10.4.3.9.1 FL 79-81°C

10.4.3.9.2 CO560 81-83°C

10.4.3.9.3 CR610 79-81°C

10.4.3.10 For some samples the IPC (CR610) may fall outside of this range by as much as 2°C. The amplification data, the Cq values in particular, are the primary means of analyzing the IPC data.

10.4.3.11 In the melt curves window of FL tab, drag the expected target melt temperature line to the midpoint of the melt curves. Alternatively, double-click on the line, and enter the desired temperature.

10.4.3.12 Repeat for CO560 and CR610 tabs.

10.4.3.13 Generating a Standard Curve:

10.4.3.13.1 In the autosomal channel (FL tab), select all samples and DNA standards.

10.4.3.13.2 Select the Create a Standard Curve icon at the top of the screen to generate a standard curve and determine DNA concentrations of the unknowns based on the standard curve. Alternatively, “Add Standard Curve” can be selected from the Edit dropdown.

10.4.3.13.3 Repeat this for the Y channel (CO560 tab)

10.4.3.13.4 Select the Standard Curves tab to view the standard curves. The slope, Y-intercept, R^2 value, and efficiency are displayed on the graph along with the samples that do not have a valid Cq value (meaning they did not cross the amplification threshold).

10.4.3.14 Run Quality Determination:

10.4.3.14.1 The standard curve for the autosomal target (FL) has an average slope in the range of -3.2 to -4.0 (rounding allowed).

10.4.3.14.2 The standard curve for the Y-chromosomal target (CO560) has an average slope in the range of -3.0 to -3.6 (rounding allowed).

10.4.3.14.3 The R^2 value will be evaluated and must be ≥ 0.99.

10.4.3.14.4 If the slope value is outside of allowable autosomal and/or Y-chromosomal range and/or the R^2 values are <0.99, standards can be omitted as follows:

10.4.3.14.4.1 The sample type for the 0.0032ng/µl standard point (one or both replicates) may be changed from standard to unknown. This will automatically be incorporated into the standard curves and may improve the slope value(s) and/or R^2 value.

10.4.3.14.4.1.1 One additional replicate point may be changed from standard to unknown to achieve the appropriate slope value(s) and/or R^2 value.

10.4.3.14.4.2 If the appropriate slope value(s) and/or R^2 value cannot be achieved, quantitation of the samples must be repeated.

10.4.3.14.4.3 To remove a point from the standard curve

10.4.3.14.4.3.1 PCR Curves

10.4.3.14.4.3.1.1 Click the standard in the bottom of the screen that you would like removed.

10.4.3.14.4.3.1.2 Select the UNK symbol at the top of the page.

10.4.3.14.5 If the y-intercept changes significantly from run to run without a change in the slope value(s) or R^2 value of the standard curve, then this suggests the DNA standard was not sufficiently mixed before use or has degraded.

10.4.3.15 <u>Generating a Forensic Report:</u>

10.4.3.15.1 Navigate to the “Forensics” tab at the top of the Plexor Analysis Software and select “Set Normalization and IPC Parameters”. No changes are necessary to the resulting pop-up window. Select “OK”.

10.4.3.15.2 A new tab labeled “Forensics” will populate under the “Reports” tab for your data. This Forensics report contains the data that will be used downstream.

10.4.3.15.3 Under Reports > Forensics

10.4.3.15.3.1 Click the location header to order the wells as follows: 1st set of standards, 2nd set of standards, samples.

10.4.3.15.3.2 Select and copy all wells

10.4.3.15.4 Paste special into the Quant Results sheet of the DNA Batch Workbook

10.4.3.15.4.1 Wrap text in cells containing “Sample Type” and “[Auto] ng/µL”

10.4.3.15.4.2 Change from scientific notation to general in the cells under "[Auto] ng/µL" and "[Y] ng/µL"

10.4.3.15.5 In the Plexor Software

10.4.3.15.5.1 File > Save Analysis File as New (.aan)

10.4.3.15.5.2 G Drive (G:) > Instrument- DNA > Plexor Projects > *Year* > Project

10.4.4 Limitations

10.4.4.1 See 10.5 Plexor HY Interpretation for additional information.

10.4.5 References

10.4.5.1 Applied Biosystems. 2006. Validation Using SDS Software Version 1.2.3 on the Applied Biosystems 7500 Real-Time PCR System and the ABI PRISM® 7000 Sequence Detection System: User Bulletin.

10.4.5.2 Krenke, Benjamin E., et al. Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA: Developmental Validation of the Plexor® HY System. Promega Corporation, 2007.

10.4.5.3 Plexor HY Technical Manual (May 2013). Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems. Promega, USA.

10.4.5.4 Internal Validation with Plexor® HY and 7500 Real-Time PCR System: (October 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

10.5 Plexor® HY Interpretation

10.5.1 Scope

10.5.1.1 To define the interpretation of samples after quantitation analysis and some limitations of the Plexor HY system data.

10.5.2 Standards and Controls

10.5.2.1 If the quantitation fails, resulting in samples being reprocessed, reagent blanks must also be reprocessed.

10.5.3 Procedure

10.5.3.1 Data from samples are compared to data from DNA standards of similar autosomal concentration values. The IPC amplification is designed to be the most inhibition-sensitive or least robust amplification in the triplex.

10.5.3.2 To determine quality of each quantitation value, look at the autosomal concentration in ng/µl of sample and find the IPC Cq for this sample. Then compare this IPC Cq to the value of the IPC Cq for the DNA standard that has the closest autosomal concentration in ng/µl.

10.5.3.2.1 If these values are similar, the sample quantitation is performing as expected with no indications of inhibition.

10.5.3.2.2 This may also be identified by looking at the Forensics report. If it indicates "Check IPC", this may indicate inhibition in a sample of possibly limited quantity. DNA concentration results for inhibited samples should be interpreted with caution.

10.5.3.2.2.1 Extremely high concentrations of human genomic DNA (>10 ng/µL) may result in a high IPC Cq and cause the "Check IPC" indicator to flag. It is unlikely that PCR inhibitors are present, and the sample may be carried straight to amplification.

10.5.3.2.3 If the IPC for a sample does not cross the threshold and inhibition is suspected, dilutions will be created and re-quanted to correct the IPC. The dilution series for the re-quant set up is 1:2, 1:4, or both (1:2 – 1ul of extract and 1ul of TE buffer; 1:4 – 1ul of extract and 3ul of TE buffer).

10.5.3.2.4 If the IPC for a reagent blank does not cross the threshold, the reagent blank must be re-quantified. If the reagent blank must be re-quantified, it must be re-quantified neat (i.e., not diluted).

10.5.3.3 The amplification target must result in at least 10pg of DNA to proceed with autosomal amplification.

10.5.3.4 Samples being processed using Y-screening will be analyzed prior to proceeding to amplification.

10.5.3.4.1 If an extracted evidence sample being Y-screened has a Y quantitation value of 0ng/µl or N/A as displayed on the Forensic Report, it may not be further processed and may be stopped at quant.

10.5.3.4.2 It may be necessary to evaluate Auto/Y ratio to determine if the samples should be further processed or may be stopped at quant. Due to multi-copy target regions, there may be some variation observed in the Auto/Y ratio of a single male sample. See the below chart for various ranges of Auto/Y ratio (based on internal validation):

Comparable autosomal male profiles obtained	May produce comparable autosomal male profiles	Will not be forwarded for autosomal amplification
Less than or equal to 20/1	Greater than 20/1 less than or equal to 100/1	Greater than 100/1

10.5.3.4.3 The above chart may be used in deciding which samples to forward for amplification. For cases associated with a single assailant and no consensual sex partner within 5 days, one sample in the less than or equal to 20/1 range may be forwarded for autosomal amplification when available. If no samples exhibit a 20/1 ratio, all samples less than or equal to 100/1 should be forwarded for autosomal amplification. For cases associated with multiple assailants, a consensual sex partner within 5 days of the assault, or an unknown number of assailants, all samples exhibiting a less than or equal to 100/1 ratio should be forwarded for autosomal amplification. When the Auto/Y ratio for a sample is greater than 100/1, the sample will not be considered for autosomal STR amplification.

10.5.3.4.4 When a sample exhibits a Y melt that does not cross the threshold, processing of the sample will routinely stop at quantitation due to the limited quantitation data (see QMS ID 26012). The sample will be reported stating no determinations will be made regarding the presence of male DNA in the sample.

10.5.3.5 When all evidence in a case stops at the quantitation step, associated reference samples may also be stopped, provided that the samples are not eligible for the DNA database. The report will reflect that amplifiable DNA was obtained, but that no further testing was conducted.

10.5.4 Limitations

10.5.4.1 Plexor HY is limited in its ability to indicate the quality of the DNA. Low-level inhibition may not be indicated in the quantitation. Plexor HY does not possess an indicator for degradation. The quantitative system allows for an estimate of the amount of DNA present in the extract. Validation data showed a 2-3-fold variance in the quantitation data from the same sample.

10.5.5 Safety

10.5.5.1 Caution should be used when handling chemicals and personal protective equipment should be used at all times.

10.5.6 References

10.5.6.1 Applied Biosystems. 2006. Validation Using SDS Software Version 1.2.3 on the Applied Biosystems 7500 Real-Time PCR System and the ABI PRISM® 7000 Sequence Detection System: User Bulletin.

10.5.6.2 Krenke, Benjamin E., et al. Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA: Developmental Validation of the Plexor® HY System. Promega Corporation, 2007.

10.5.6.3 Plexor HY Technical Manual (May 2013). Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems. Promeg, USA.

10.5.6.4 Internal Validation with Plexor® HY and 7500 Real-Time PCR System: (October 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

11. PowerPlex Fusion System

11.1 Extracted Samples

11.1.1 Scope

11.1.1.1 To describe the process by which casework samples that have been previously extracted are amplified at the following loci utilizing the PowerPlex Fusion System: D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, DYS391, D8S1179, D12S391, D19S433, FGA, D22S1045, and the gender marker Amelogenin.

11.1.2 Equipment/Materials/Reagents

11.1.2.1 PowerPlex Fusion System

11.1.2.1.1 PowerPlex® Fusion 5X Master Mix

11.1.2.1.2 PowerPlex® Fusion 5X Primer Pair Mix

11.1.2.1.3 2800M Control DNA, 10ng/µl

11.1.2.1.4 Water, Amplification Grade

11.1.2.2 1.5mL microcentrifuge tube

11.1.2.3 96-well reaction plate or 0.2mL amplification tubes

11.1.2.4 Vortex

11.1.2.5 Centrifuge

11.1.2.6 TE Buffer

11.1.2.7 8-Strip Caps

11.1.2.8 Veriti Thermal Cyclers

11.1.2.9 QIAgility

11.1.2.10 QIAgility Software v4.18.1

11.1.2.11 Pipettes

11.1.2.12 Pipette Tips

11.1.2.13 2800M Control DNA, 10ng/µl(purchased separately from PowerPlex Fusion System)

11.1.3 Standards and Controls

11.1.3.1 Positive and negative amplification controls must be co-amplified with each batch of samples. For the positive control, the 2800M Control DNA will be utilized. After its initial thaw, the 2800M Control DNA will be stored at 2-8°C. The diluted positive control should

be made daily and stored at 2-8°C. The same lot of TE buffer that was used to dilute the samples will be used as the negative control.

11.1.3.2 At a minimum, one reagent blank must be co-amplified with each extraction batch. Reagent blanks must be amplified using the same testing kit, instrument model and concentration conditions as required by the sample(s) containing the least amount of DNA.

11.1.3.2.1 If multiple reagent blanks are present for each extraction batch, then amplify the reagent blank that demonstrates the highest quantified DNA concentration. If both reagent blanks demonstrate the same value, then either blank may be amplified.

11.1.3.3 When all samples in the batch stop at quantitation, the blank may be stopped at quant if all samples result in N/A. If any sample in the batch produces a quantitation value (autosomal and/or Y), the blank must be carried through to amplification and analysis.

11.1.3.4 Refer to Section 13.2.3 of the MNPD-CL Forensic Biology Technical Procedures Manual for the criteria of passing controls.

11.1.4 Calibration

11.1.4.1 The Veriti thermal cyclers must be calibrated annually. Refer to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual.

11.1.4.2 Each lot of Fusion kits will be quality control tested before being used in casework. Refer to Reagent Quality Control of the MNPD-CL Forensic Biology Quality Manual.

11.1.5 Procedure

11.1.5.1 Obtain and thaw the PowerPlex Fusion System at room temperature.

11.1.5.2 Normalization

11.1.5.2.1 Thaw, vortex, and pulse spin samples.

11.1.5.2.2 Proceed to Manual or QIAgility normalization procedure.

11.1.5.2.3 Manual

11.1.5.2.3.1 The 2800M positive control DNA and some samples will need to be diluted to achieve the appropriate input target value for the amplification reaction. TE buffer will be used to dilute the positive control and samples being amplified. For all casework samples and controls the target values should not initially exceed 1.0ng. Upon reamplification a target value up to 2.0ng is allowed.

11.1.5.2.3.1.1 The following is an example of a normalization calculation. The example calculation assumes that only 1µl of quantified extract is used, the amplification target is 0.5ng, and 15µl of the diluted extract will be added to the amplification reaction. The DNA Batch Workbook calculates normalizations in the manner described in this example. Note: calculated values are approximate and can be rounded to the nearest microliter.

11.1.5.2.3.1.1.1 To obtain the desired dilution concentration (i.e., target dilution) the desired amplification target (0.5ng) is divided by the desired amplification input volume (15µl).

11.1.5.2.3.1.1.1.1 Target dilution = 0.5ng/15µl = 0.0333ng/µl

11.1.5.2.3.1.1.2 To obtain the volume of TE needed to dilute the quantified extract, divide the extract's quantitation value (ng/µl) by the target dilution and then subtract the input volume of quantified extract.

11.1.5.2.3.1.1.2.1 Volume of TE = (*XYZ* ng/µl / 0.0333ng/µl) - 1µl. Ensure that the total volume is a minimum of 20µl.

11.1.5.2.3.1.1.3 In a dilution tube (should use a sterile tube that will house the necessary volumes), add 1µl of quantified extract and the calculated volume of TE. 15µl of this dilution will be added to the amplification reaction. If the total volume is less than 15µl, then multiply the volume of TE needed and the extract volume by the same factor until they add up to more than 15µl. For example, if the total volume is 5µl (1µl extract and 4µl TE buffer), multiply both values by 4 (4µl extract and 16µl TE buffer for a total of 20µl).

11.1.5.2.3.2 Proceed to the Amplification Set-up procedure.

11.1.5.2.4 QIAgility

11.1.5.2.4.1 Preparation Using the DNA Batch Workbook

11.1.5.2.4.1.1 Upon entering sample and quantification results information into the DNA Batch Workbook, the "Norm-QIA" tab will populate. Save the information in the "Norm-QIA" tab as a .txt file (tab delimited). (Instruction will be listed at the top of the excel sheet in this tab.)

11.1.5.2.5 QIAgility Instrument

11.1.5.2.5.1 **NOTE**: The QIAgility can only normalize samples ranging from 0.05 ng/µl to 10ng/µl. If a sample is greater than 10ng/µl, the sample can be diluted and placed on the QIAgility with the calculated amount noted as the sample's DNA concentration on the.txt file.

11.1.5.2.5.2 Turn on computer.

11.1.5.2.5.3 Turn on instrument.

11.1.5.2.5.4 Launch QIAgility Software v4.15.1. The QIAgility instrument lid must be closed for the software and hardware to initialize upon start-up and for a run to proceed.

11.1.5.2.5.5 Open the Normalization protocol on the QIAgility by going to "File" at the top left of the software screen, click "Open," and find the "Normalization" protocol.

11.1.5.2.5.6 In the software, select the sample rack position C2. Click on the "Import" button and import the .txt file generated for your run. Then find the "Banks" section and select number "2" in the "Banks Specified in Column" option. Then click "Finished" button.

11.1.5.2.5.7 Load sample tubes into the 4 x 8-well sample racks in the Sample Block (C2).

11.1.5.2.5.8 Select the sample rack position (A2). Select the normalize samples Diluent Bank 2 module and click the edit button. Change the "First Well Number" to the beginning well number where the samples of Bank 2 begin on sample rack (C2). Then do this again for the remaining sample banks.

11.1.5.2.5.9 Load labeled empty 1.5mL flip cap tubes (in the same order as the sample tubes) into the 4 x 8-well sample racks in the sample rack position (A2).

11.1.5.2.5.10 Move the cursor over to Mix Plate (M1) position (A). A pop-up box will appear listing the amount of diluent that is needed for the run. Add this amount of diluent (TE Buffer) to a Qiagen 5mL diluent tube and place it on the Mix Plate (M1) at position (A).

11.1.5.2.5.11 Click on the green start arrow on the toolbar or select "Control/Start".

11.1.5.2.5.12 Check the pre-run report to verify the location and amount/volume of consumables and liquids that are required on the worktable for completion of the loaded run file.

11.1.5.2.5.13 The pre-run "Checklist" dialog box will appear. If no warnings or errors are listed, select the boxes to continue and click "OK" to start the run. If errors are listed, user intervention is required at this step.

11.1.5.2.5.14 Follow any prompted action pop-up windows during the duration of the normalization protocol.

11.1.5.2.5.15 **NOTE**: Care must be taken when opening the QIAgility lid during a run. It takes up to 10 seconds for the instrument to complete its current movement and for the pause to take effect.

11.1.5.2.5.16 Upon completion of the run, a "Post-run report" will appear. Save the "Post-run report" for the normalization set up for the case file.

11.1.5.2.5.17 Remove the now normalized samples on sample rack position (A2) and proceed to the Amplification Set-up procedure.

11.1.5.3 Amplification Set-up

11.1.5.3.1 Vortex the 5X Master Mix and 5X Primer Pair Mix for 5-15 seconds. Tap tubes to remove any reaction mix from the top of the tubes. Do not centrifuge after vortexing.

11.1.5.3.2 Prepare the amplification reaction mix in a clean 1.5mL or 2.0mL microcentrifuge tube. To prepare the reaction mix, count the number of samples to be amplified (including positive and negative controls). Add four to this total count for pipetting error. Multiply this value by five. The resulting value will be the volume (µl) of 5X Master Mix and 5X Primer Mix needed for the reaction mix. Add the calculated volumes of 5X Master Mix and 5X Primer Mix to the tube.

11.1.5.3.3 Vortex the amplification reaction mix. Tap tube to remove any reaction mix from the top of the tube. Do not centrifuge after vortexing.

11.1.5.3.4 Proceed to the Manual or QIAgility amplification procedure.

11.1.5.3.4.1Manual

11.1.5.3.4.1.1 Dispense 10µl of the reaction mix into the appropriate wells of a 96-well reaction plate, skipping wells assigned for Ladders. 0.2mL amplification tubes may also be used.

11.1.5.3.4.1.2 Following the addition of reaction mix and prior to the addition of sample, check to ensure that reaction mix has been added to all appropriate wells.

11.1.5.3.4.1.3 Add 15µl of TE (negative control) or diluted positive control to the appropriate well/tube.

11.1.5.3.4.1.4 Add 15µl of DNA extract or normalized DNA extract dilution to the appropriate wells/tubes.

11.1.5.3.4.1.5 Cap each column of the plate with strip caps as each column is completed. If using tubes, only one tube should be open at a time.

11.1.5.3.4.1.6 Proceed to Preparation of Plate for PCR.

11.1.5.3.4.2 QIAgility

11.1.5.3.4.2.1 Preparation Using the DNA Batch Workbook

11.1.5.3.4.2.1.1 Upon entering sample information into the DNA Batch Workbook, open the "Amp-QIA" tab. Save the information in the "Amp-QIA" tab as a .txt file (tab delimited). (Instruction will be listed at the top of the excel sheet in this tab.)

11.1.5.3.4.2.2 QIAgility Instrument

11.1.5.3.4.2.2.1 Turn on computer.

11.1.5.3.4.2.2.2 Turn on instrument.

11.1.5.3.4.2.2.3 Launch QIAgility Software v4.15.1. The QIAgility instrument lid must be closed for the software and hardware to initialize upon start-up and for a run to proceed.

11.1.5.3.4.2.2.4 Place prepared PowerPlex Fusion Reaction Mix into position F on the Reagent Block (R1) of the QIAgility. Place a 1.5ml flip cap tube with water in the "Ladder Blank" position H on Reagent Block (R1).

11.1.5.3.4.2.2.5 Load sample tubes into the 4 x 8-well sample racks in the Sample Block (A2)

11.1.5.3.4.2.2.6 Place a 96-well optical plate in Reaction Block (C1).

11.1.5.3.4.2.2.7 Open the Amplification protocol by going to "File" at the top left of the software screen, click "Open," and find the "Amp (15)" protocol.

11.1.5.3.4.2.2.8 In the software, select the sample rack position A2. Click on the "Import" button and import the .txt file generated for your run.

11.1.5.3.4.2.2.9 Click on the green start arrow on the toolbar or select "Control/Start".

11.1.5.3.4.2.2.10 Check the pre-run report to verify the location and amount/volume of consumables and liquids that are required on the worktable for completion of the loaded run file.

11.1.5.3.4.2.2.11 The pre-run "Checklist" dialog box will appear. If no warnings or errors are listed, select the boxes to continue and click "OK" to start the run. If errors are listed, user intervention is required at this step.

11.1.5.3.4.2.2.12 Follow any prompted action pop-up windows during the duration of the protocol.

11.1.5.3.4.2.2.13 **NOTE**: Care must be taken when opening the QIAgility lid during a run. It takes up to 10 seconds for the instrument to complete its current movement and for the pause to take effect.

11.1.5.3.4.2.2.14 Upon completion of the run, a "Post-run report" will appear. Save the "Post-run report" for the amplification set up for the case file.

11.1.5.3.4.2.2.15 Proceed to the Preparation of Plate for PCR.

11.1.5.4 Preparation of Plate for PCR

11.1.5.4.1 Cap each column in the plate with sample in it with the 8-strip cap or cap each tube.

11.1.5.4.2 Briefly centrifuge the plate.

11.1.5.5 PCR

11.1.5.5.1 Log on to the thermal cycler using the following credentials:

11.1.5.5.1.1 Username: scientist

11.1.5.5.1.2 Password: scientist1

11.1.5.5.2 Load the samples onto the Veriti thermal cycler and close the lid. Select the "PowerPlex Fusion 5C" method. Verify the program is set to 9700 Max mode and is as follows:

11.1.5.5.2.1 96°C for 1 min

11.1.5.5.2.2 Then, 30 cycles of:

11.1.5.5.2.2.1 94°C for 10 sec

11.1.5.5.2.2.2 59°C for 1 min

11.1.5.5.2.2.3 72°C for 30 sec

11.1.5.5.2.3 Then,

11.1.5.5.2.3.1 60°C for 10 min

11.1.5.5.2.3.2 4°C soak

11.1.5.5.3 Start the run.

11.1.5.5.4 Once the program is complete, the samples are ready for capillary electrophoresis or can be stored at -10°C to -25°C for future use.

11.1.5.5.5 The sequence of this procedure is not absolute. An analyst may choose to prepare the sample dilutions prior to preparing the amplification reaction mix or vice versa. An analyst may also choose to add TE buffer to the amplification tube or plate prior to dispensing the reaction mix.

11.1.6 Limitations

11.1.6.1 If necessary, the amplification input volume of DNA can be adjusted. If this is necessary, TE must be used to so that the total amplification input volume of DNA and TE is 15ul. Prior approval must be obtained by the DNA Technical Leader in order to adjust the input volume.

11.1.6.2 This procedure is not optimized for direct amplification of known standards that have not been previously extracted.

11.1.7 Safety

11.1.7.1 Caution should be used when handling chemicals and personal protective equipment should be used at all times.

11.1.7.2 The robotic arm of the QIAgility instrument moves during position calibration while the instrument lid is raised. Never click any buttons while parts of your body are within the instrument workspace.

11.1.8 References

11.1.8.1 Developmental Validation of the PowerPlex® Fusion System for Analysis of Casework and Reference Samples: A 24-locus Multiplex for New Database Standards (2014). Forensic Science International: Genetics, web.

11.1.8.2 Internal Validation of Promega PowerPlex® Fusion using a Veriti

11.1.8.3 Thermal Cycler and 3500 Series Genetic Analyzer (August 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

11.1.8.4 PowerPlex Fusion System Technical Manual (2012). Instructions for use of Products DC2402 and DC2408. Promega, Madison, WI.

11.1.8.5 Internal Validation with QIAgility: (2015), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

11.1.8.6 QIAgility® User's Manual (June 2013). QIAGEN.

11.2 Direct Amplification of Swabs

11.2.1 Scope

11.2.1.1 To describe the process by which swab samples undergo direct amplification at the following loci utilizing the PowerPlex Fusion System: D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA,

D21S11, D7S820, D5S818, TPOX, DYS391, D8S1179, D12S391, D19S433, FGA, D22S1045, and the gender marker Amelogenin.

11.2.2 Equipment/Materials/Reagents

11.2.2.1 PowerPlex® Fusion System

11.2.2.2 SwabSolution™ Reagent

11.2.2.3 1.5mL microcentrifuge tubes

11.2.2.4 96-well reaction plate or 0.2mL amplification tubes

11.2.2.5 Vortex

11.2.2.6 Centrifuge

11.2.2.7 Amplification grade water

11.2.2.8 Heat block

11.2.2.9 8-Strip Caps

11.2.2.10 Veriti Thermal Cyclers

11.2.3 Standards and Controls

11.2.3.1 Positive and negative amplification controls must be co-amplified with each batch of samples. For the positive control, the 2800M Control DNA that is supplied with the Fusion System will be utilized. The same lot of amplification grade water used in the master mix will be used as the negative control.

11.2.3.2 At a minimum, one reagent blank must be co-amplified with each extraction batch.

11.2.3.3 Refer to Section 13.2.3 of the MNPD-CL Forensic Biology Technical Procedures Manual for the criteria of passing controls.

11.2.4 Performance Checks

11.2.4.1 The Veriti thermal cyclers are performance checked annually. Refer to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual.

11.2.4.2 Each lot of Fusion kits and SwabSolution™ Reagent will be performance checked (i.e., quality control tested) before being used in casework. Refer to Reagent Quality Control of the MNPD-CL Forensic Biology Quality Manual.

11.2.5 Procedure

11.2.5.1 Set heat block to 70ºC and allow it to heat to temperature.

11.2.5.2 Place each buccal swab head in a separate 1.5mL tube. Add 1mL of SwabSolution™ Reagent to each buccal swab head. Place tubes in heat block and incubate samples at 70ºC for 30 minutes.

11.2.5.2.1 **Note**: There is no need to vortex the tubes after addition of the SwabSolution™ Reagent, prior to or after the incubation.

11.2.5.3 Obtain and thaw the PowerPlex Fusion System at room temperature.

11.2.5.4 Vortex the 5X Master Mix and 5X Primer Pair Mix for 15 seconds.

11.2.5.5 Prepare the amplification master mix in a clean 1.5mL microcentrifuge tube. Count the number of samples to be amplified (including positive and negative control) and add four for pipetting error. For this number, add 13µl amplification grade water, 5µl of the 5X Master Mix and 5µl of the 5X Primer Mix to the tube.

11.2.5.6 Vortex the amplification master mix. Dispense 23µl of the master mix into the appropriate well of a 96-well reaction plate or the appropriately labeled 0.2mL amplification tube for each sample.

11.2.5.7 Dispense 2µl of swab extract for each sample into the appropriate well or tube containing 23µl of master mix.

11.2.5.8 For the positive control, vortex the tube of 2800M DNA, then dilute to the appropriate target (total target of 1-2 ng) using amplification grade water.

11.2.5.9 For the negative control, add 2µl of amplification grade water to the appropriate well or tube containing 23µl of master mix.

11.2.5.10 Cap each column in the plate with sample in it with the 8-strip cap or cap each tube.

11.2.5.11 Briefly centrifuge the plate.

11.2.5.12 Load the samples onto the Veriti thermal cycler, close the lid, and start the Fusion program for direct amplification samples. Verify the program is set to 9700 Max mode and is as follows:

11.2.5.12.1 96°C for 1 min

11.2.5.12.2 Then, 27 cycles of:

11.2.5.12.2.1 94°C for 10 sec

11.2.5.12.2.2 59°C for 1 min

11.2.5.12.2.3 72°C for 30 sec

11.2.5.12.3 Then,

11.2.5.12.3.1 60°C for 20 min

11.2.5.12.3.2 4°C soak

11.2.5.13 Once the program is complete, the samples are ready for capillary electrophoresis or can be stored at -10°C to -25°C for future use.

11.2.6 Calculations

11.2.6.1 The 2800M positive control DNA will need to be diluted to achieve the appropriate input target value for the amplification reaction. Use these calculations for determining the dilution factor of the 2800M to be amplified:

11.2.6.1.1 For a 2ng target dilution, quant value (ng/µl)/1 = total volume in µl.

11.2.6.1.2 Total volume – 1µl undiluted 2800M = volume of amplification grade water needed.

11.2.7 Limitations

11.2.7.1 This procedure is not optimized for amplification of extracted samples. Refer to Section 11.1.5 for that procedure.

11.2.8 Safety

11.2.8.1 Caution should be used when handling chemicals and personal protective equipment should be used at all times.

11.2.9 References

11.2.9.1 Developmental Validation of the PowerPlex® Fusion System for Analysis of Casework and Reference Samples: A 24-locus Multiplex for New Database Standards (2014). Forensic Science International: Genetics, web.

11.2.9.2 Internal Validation of Direct Amplification of Swabs using the Promega PowerPlex® Fusion System (2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

11.2.9.3 PowerPlex® Fusion System Technical Manual (2012). Instructions for use of Products DC2402 and DC2408. Promega, Madison, WI.

11.2.9.4 SwabSolution™ Kit Technical Manual (2013). Instructions for use of Product DC8271. Promega, Madison, WI.

12. Capillary Electrophoresis Protocols

12.1 Scope

12.1.1 To describe the process of separating DNA fragments via capillary electrophoresis utilizing the Applied Biosystems 3500 Genetic Analyzer.

12.2 Equipment/Materials/Reagents

12.2.1 Applied Biosystems 3500 Series Genetic Analyzer

12.2.2 Applied Biosystems 3500 Series Genetic Analyzer Data Collection Software version 4.0.1

12.2.3 Vortex

12.2.4 Centrifuge

12.2.5 Microcentrifuge tubes

12.2.6 Pipettes

12.2.7 Pipette tips

12.2.8 Hi-Di formamide

12.2.9 WEN Internal Lane Standard 500 (ILS)

12.2.10 POP-4 polymer

12.2.11 Anode buffer container

12.2.12 Cathode buffer container

12.2.13 3500 series 96-well plate base/retainer set

12.2.14 96-well plate

12.2.15 96-well plate septa

12.2.16 PowerPlex Fusion Allelic Ladder

12.3 Standards and Controls

12.3.1 The WEN Internal Lane Standard 500 (ILS) will be co-injected with every sample/allelic ladder. The ILS contains DNA fragments of known sizes that are used to size allelic ladder(s) which are then compared to the PCR products of the samples that have also been sized in base pairs with their own ILS to allow allele designation.

12.3.2 At a minimum, one allelic ladder must be injected for each 96-well plate. It is recommended that an allelic ladder be run every other injection to compensate for

potential migration differences. The allelic ladder is used to assign allele calls to the samples.

12.4 Calibration

12.4.1 The Applied Biosystems 3500 series Genetic Analyzer must undergo annual maintenance. Refer to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual for the maintenance and calibration procedures. When the annual maintenance is complete, the instrument will be performance checked prior to running casework samples.

12.4.2 All reagent components used in capillary electrophoresis will be quality control checked prior to being used in casework. Refer to Reagent Quality Control of the MNPD-CL Forensic Biology Quality Manual.

12.5 Procedure

12.5.1 Turn on the heat block and pre-heat to 95°C.

12.5.2 If not previously done, thaw the ILS and allelic ladder(s) to room temperature. Also, thaw an aliquot of Hi-Di formamide to room temperature.

12.5.3 Vortex the ILS, ladders, and formamide for 5-15 seconds.

12.5.4 To prepare a master mix of the ILS and formamide, count the number of samples to be injected (including allelic ladders, reagent blanks, and positive and negative amplification controls) and add four for pipetting error.

12.5.4.1 To obtain the necessary volume of the ILS, multiply the total count by the volume of ILS (0.5 – 1.0 µL of ILS per sample).

12.5.4.2 To obtain the necessary volume of formamide, multiply the total count by 10 (10 µL of formamide per sample).

12.5.4.3 Add the calculated volumes of formamide and ILS in a 1.5mL microcentrifuge tube.

12.5.5 Vortex the master mix for 5-15 seconds.

12.5.6 Proceed to the Manual Plating or QIAgility Plating CE procedure.

12.5.7 Manual Plating

12.5.7.1 Dispense 10µL of the master mix into the appropriate wells of a labeled 96-well plate. If a column on the plate has at least one sample being injected, then dispense the master

mix into all eight wells. Wells that do not contain a sample can be filled with 10ul of formamide.

12.5.7.2 Add 1µL of the previously amplified samples or allelic ladder to the appropriate wells of the 96-well plate.

12.5.7.3 **NOTE**: Amplified product may be diluted in ultrapure water when data generated exhibits oversaturation and/or pull-up. 1µL of the dilution should be added to the 96-well plate prepared for CE.

12.5.7.4 Proceed to Prepare the plate for placement on the CE instrument.

12.5.8 QIAgility Plating

12.5.8.1 Preparation Using the DNA Batch Workbook

12.5.8.1.1 In the DNA Batch Workbook open the "CE- QIA" tab. Save the information in the Amp Set-Up 1 tab or Amp Set-Up 2 tab as a .txt file (tab delimited). (Instruction will be listed at the top of the Excel sheet in this tab.)

12.5.8.1.2 **Note**: To combine amplification plates on a CE plate, separate .txt files must be saved for each, with one named Plate1 (Ex. "Fusion_Plate1_Bank1") and the other named Plate2 (Ex. "Fusion_Plate2_Bank1.")

12.5.8.1.2.1 The "Plate1" or "Plate 2" designation denotes which of the two plate sample areas on the QIAgility work deck is being utilized.

12.5.8.2 QIAgility Instructions

12.5.8.2.1 Turn on computer.

12.5.8.2.2 Turn on instrument.

12.5.8.2.3 Launch QIAgility Software v4.15.1. The QIAgility instrument lid must be closed for the software and hardware to initialize upon start-up and for a run to proceed.

12.5.8.2.4 Place prepared formamide and ILS Mix into position N on the Reagent Block (R1) of the QIAgility. Place the Powerplex Fusion Ladder into position P on Reagent Block (R1).

12.5.8.2.5 Load an amplification plate or samples into the Sample Block (B1) or (C1).

12.5.8.2.6 Place a 96-well optical plate in Reaction Block (B2).

12.5.8.2.7 Open the CE setup protocol by going to "File" at the top left of the software screen, click "Open," and find the "Post Setup-consolidated" protocol.

12.5.8.2.8 In the software, select the sample rack position (B1) or (C1). Click on the "Import" button and import the .txt file generated for your run.

12.5.8.2.8.1 **Note**: This is where the user will designate "Plate1" and "Plate2." Therefore, if the user designates a set of samples for "Plate1" and imports those to sample rack position (B1), then samples for "Plate2" must be imported to sample rack position (C1).

12.5.8.2.9 Click on the green start arrow on the toolbar or select "Control/Start".

12.5.8.2.10 Check the pre-run report to verify the location and amount/volume of consumables and liquids that are required on the worktable for completion of the loaded run file.

12.5.8.2.11 The pre-run "Checklist" dialog box will appear. If no warnings or errors are listed, select the boxes to continue and click "OK" to start the run. If errors are listed, user intervention is required at this step.

12.5.8.2.12 Follow any prompted action pop-up windows during the duration of the protocol.

12.5.8.2.13 Care must be taken when opening the QIAgility lid during a run. It takes up to 10 seconds for the instrument to complete its current movement and for the pause to take effect.

12.5.8.2.14 Upon completion of the run, a "Post-run report" will appear. Save the "Post-run report" for the casefile.

12.5.8.2.15 Remove the 96-well optical plate in Reaction Block (B2) and proceed to Prepare the plate for placement on the CE instrument.

12.5.9 Prepare the plate for placement on the CE instrument

12.5.9.1 Place a 96-well plate septa onto the plate, ensuring that the wells are aligned with the holes in the septa.

12.5.9.2 Centrifuge the plate briefly to force the contents to the bottom of the wells and to remove bubbles.

12.5.9.3 Denature samples on the heat block at 95°C for 3 minutes, and then immediately chill on crushed ice, a freezer plate block, or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

12.5.10 CE

12.5.10.1 Perform a shutdown and restart on the first use of the day.

12.5.10.2 Turn off the computer.

12.5.10.3 Turn off the instrument.

12.5.10.4 After one minute, turn on the computer, but do not log in.

12.5.10.5 Turn on the instrument and wait for the green light to stop flashing.

12.5.10.6 Log into the computer (Username: Administrator / Password: Administrator).

12.5.10.7 Wait for all connections to be made indicated by a green checkmark in the task bar.

12.5.10.8 Launch the Applied Biosystems 3500 Series Genetic Analyzer Data Collection Software v4.0.1. Login to the software using the following credentials:

12.5.10.8.1 Username: Scientist

12.5.10.8.2 Password: Scientist1

12.5.10.9 Ensure that the polymer, cathode buffer container, and anode buffer container contain enough reagents to complete all injections and are within the validated expiration period. Also, verify that the necessary maintenance procedures have been completed according to the Maintenance and Calibration Protocols of the MNPD-CL Forensic Biology Quality Manual.

12.5.10.9.1 Optional: pre-heat the oven.

Reagent	Allowance on 3500		
Polymer	≤ 14 days	≤ 384 samples	Expiration Date
Anode Buffer	≤ 14 days	≤ 240 injections	Expiration Date
Cathode Buffer	≤ 14 days	≤ 240 injections	Expiration Date
Capillary Array	≤ 120 injections		

12.5.10.10 Place the plate into a 3500 Series plate retainer/base set. Load the plate onto the instrument with the notched corner of the plate aligned with the notched corner of the autosampler tray.

12.5.10.11 From the Dashboard click the “Create New Plate” button. Enter the plate name. Select “96” for Number of Wells, “HID” for Plate Type, “36” cm for Capillary Length, and “POP4” for Polymer. At the bottom of the page, click Assign Plate Contents.

12.5.10.12 Proceed to Manual Sample Entry or DNA Batch Workbook Import.

12.5.10.13 Manual Sample Entry

12.5.10.13.1 Type each sample or control name in the appropriate well. Click on Add from Library under Assays and select “PowerPlex Fusion WENILS 5C Casework Long”.

12.5.10.13.2 Click on Add from Library under File Name Convention and select “MNPD”.

12.5.10.13.3 Click on Add from Library under Results Group and select “MNPD Fusion”.

12.5.10.13.4 To assign the assay, file name convention, and results group to the plate, select all wells being used and check the boxes beside each option just imported from the libraries.

12.5.10.13.5 Click the Table View tab.

12.5.10.13.6 For each control, select the appropriate control designation in the dropdown menu under sample type.

12.5.10.13.7 Proceed to the *Begin the run* section.

12.5.10.14 DNA Batch Workbook Import

12.5.10.14.1 In the data collection software, import the plate's Text Tab Delimited file from the DNA Batch Workbook (Created in the "3500-template" tab. Instructions will be listed at the top of the Excel sheet in this tab. Save the file to a USB drive, and transfer USB to instrument computer.) Under "Assign Plate Contents", select "Import"

12.5.10.14.2 Navigate to the .txt file in the USB drive and select it. Enter or verify the plate name. Ensure the plate contents have populated correctly from the import file and that the Assay "PowerPlex Fusion WENILS 5C Casework Long", File Name Convention "MNPD", and Results Group "MNPD Fusion" have been selected for each sample well in the run. If desired, update the sample type for controls (i.e., allelic ladder, positive control). Sample types can be edited in GeneMapper as well.

12.5.10.14.3 Proceed to the *Begin the run* section.

12.5.10.15 Begin the run

12.5.10.15.1 Click the "Link for Run" button at the bottom of the page and then click "OK". In the "Load Plates for Run" screen, ensure that the Run File Name of the plate is associated with the correct side of the autosampler tray.

12.5.10.15.2 Click "Create Injection List" and a "Preview Run Screen" will appear with a list of injections and the plate layout on the right-hand side. Review the injection list and sample order (may be altered if necessary).

12.5.10.15.3 Click "Start Run" at the bottom of the screen.

12.5.10.15.4 When the run is finished, go to the Data shortcut on the desktop of the 3500 computer. Copy the Run File with your data to a USB drive. Take the USB to the other networked computer and copy the data to the G drive (G:\Instrument – DNA\Run

Files\3500 Genetic Analyzer\Joker). Save under the specific year and month associated with the run.

12.6 Limitations

12.6.1 Repeated freeze/thaw cycles should be avoided with formamide as this may cause the product to break down into formic acid and formate. Formate ions migrate preferentially into the capillary during electrokinetic injection causing loss of signal intensity.

12.7 Safety

12.7.1 Formamide is a known teratogen. Work should be conducted inside a safety enclosure when handling this reagent. Personal protective equipment should be worn at all times. Formamide should be disposed of in the biohazard waste.

12.7.2 The robotic arm of the QIAgility instrument moves during position calibration while the instrument lid is raised. Never click any buttons while parts of your body are within the instrument workspace.

12.8 References

12.8.1 Applied Biosystems 3500/3500xL Genetic Analyzer User Guide (2010). Applied Biosystems, Foster City, CA.

12.8.2 Applied Biosystems. 2011. Applied Biosystems 3500/3500xL Genetic Analyzer: User Bulletin.

12.8.3 Internal Validation of Promega PowerPlex® Fusion using a Veriti Thermal Cycler and 3500 Series Genetic Analyzer (August 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

12.8.4 PowerPlex Fusion System Technical Manual (2012). Instructions for use of Products DC2402 and DC2408. Promega, Madison, WI.

12.8.5 Internal Validation with QIAgility: : (2015), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

12.8.6 QIAgility® User's Manual (June 2013). QIAGEN.

13. Data Analysis

13.1 GeneMapper® ID-X Software Setup and Functionality

13.1.1 Scope

13.1.1.1 To describe the importing of raw data obtained from the Applied Biosystems 3500 Genetic Analyzer using the appropriate version of the GeneMapper® ID-X Software.

13.1.2 Equipment/Materials/Reagents

13.1.2.1 GeneMapper® ID-X Software v1.6

13.1.3 Standards and Controls

13.1.3.1 Each folder in a project must contain at least one allelic ladder for proper genotyping.

13.1.4 Procedure

13.1.4.1 Double-click  (GeneMapper® ID-X) on the desktop to launch the software.

13.1.4.2 In the Login to GeneMapper® ID-X dialog box, enter User Name and Password, then click OK.

13.1.4.3 Select File, then New Project.

13.1.4.4 In the new Project window, click  (Add Samples to Project).

13.1.4.5 Browse to the location of the run files (This PC > Crime Lab Backup (G:) > Run Files > 3500 Genetic Analyzer > Joker > Year > Month). Highlight desired files/CE folder, then select “Add To List” followed by “Add”.

13.1.4.6 If not done during CE setup, in the Sample Type column, use the drop-down menu to select Allelic Ladder, Sample, Positive Control, or Negative Control as appropriate for each sample.

13.1.4.6.1 Every folder in the project must contain at least one allelic ladder for proper genotyping.

13.1.4.7 In the Analysis Method column, select “2026 PowerPlex Fusion MNPD”.

13.1.4.7.1 Analytical Thresholds:

13.1.4.7.1.1 Blue: 127 RFU

13.1.4.7.1.2 Green: 131 RFU

13.1.4.7.1.3 Yellow: 158 RFU

13.1.4.7.1.4 Red: 153 RFU

13.1.4.7.1.5 Orange: 100 RFU

13.1.4.8 In the Panel column, select “PowerPlex_Fusion_Panels_IDX_v2.0”.

13.1.4.9 Ensure that “WEN_ILS_500_IDX” is selected in the Size Standard column.

13.1.4.10 Highlight “Analysis Method”, “Panel”, and “Size Standard” columns and press “Ctrl + D” to apply selections to all samples.

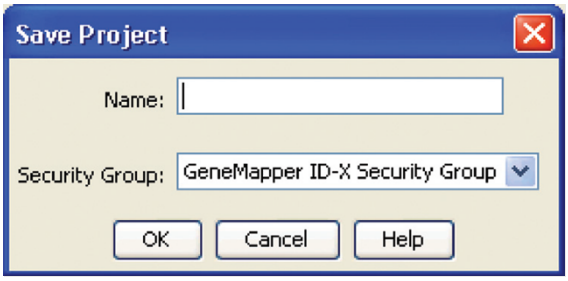
13.1.4.11 In the Custom Control column, select “2800 M” for each positive control.

13.1.4.12 Click (Analyze) to start data analysis.

13.1.4.13 A pop-up window titled “Samples Not Normalized” opens. Click “OK”.

13.1.4.14 If all analysis requirements are met, the “Save Project” dialog box will open.

13.1.4.15 In the “Save Project” dialog box, enter the project name. This should have the same name as the CE plate and CE run. Choose the applicable security group, then select OK.

13.1.4.15.1 

13.1.4.15.1.1 If a “Disk Space Alert” window pops up, select “OK”.

13.1.4.15.2 Analysis will now begin.

13.1.4.15.3 The software identifies any conditions that may prevent analysis or cause unexpected results. The Analysis Summary (ARS) tab will open if at least one sample in the project does not meet one or more analysis requirements.

13.1.4.15.3.1 From the ARS, you may view the samples that do not meet the analysis requirements or continue with analysis by clicking the “Samples” tab.

13.1.4.15.4 Use the table setting “MNPD – Casework Table” when reviewing results.

13.1.4.16 Saving the Project:

13.1.4.16.1 When editing of samples has been completed (refer to Data Analysis and Editing section), click (Save Project).

13.1.5 Limitations

13.1.5.1 Although a global forward stutter percentage is set for the PowerPlex Fusion system in the analysis method, the analyst should note that the forward stutter filter is only applied to tetra repeats.

13.1.5.2 For PowerPlex Fusion data: Sizing of Penta E and DYS391 alleles ≥475 bases will not use Local Southern Method. For Penta E, alleles >24 will be labeled as "OL".

13.1.6 References

13.1.6.1 GeneMapper® ID-X Software Version 1.0 Getting Started Guide (October 2007). Applied Biosystems, Foster City, CA.

13.1.6.2 PowerPlex Fusion System Technical Manual (October 2012). Promega, Madison, WI.

13.2 Data Analysis and Editing

13.2.1 Scope

13.2.1.1 To describe the process of analyzing the raw data obtained from the Applied Biosystems 3500 Genetic Analyzer using the appropriate version of the GeneMapper® ID-X Software.

13.2.2 Equipment/Materials/Reagents

13.2.2.1 GeneMapper® ID-X Software v1.6

13.2.2.2 HID data files from AB 3500

13.2.3 Procedure

13.2.3.1 To begin data analysis, samples must be added to the project to be analyzed.

13.2.3.1.1 Refer to GeneMapper® ID-X Software Setup and Functionality for how to analyze data.

13.2.3.2 Examine the internal size standard (ILS) for all samples and controls included in the project.

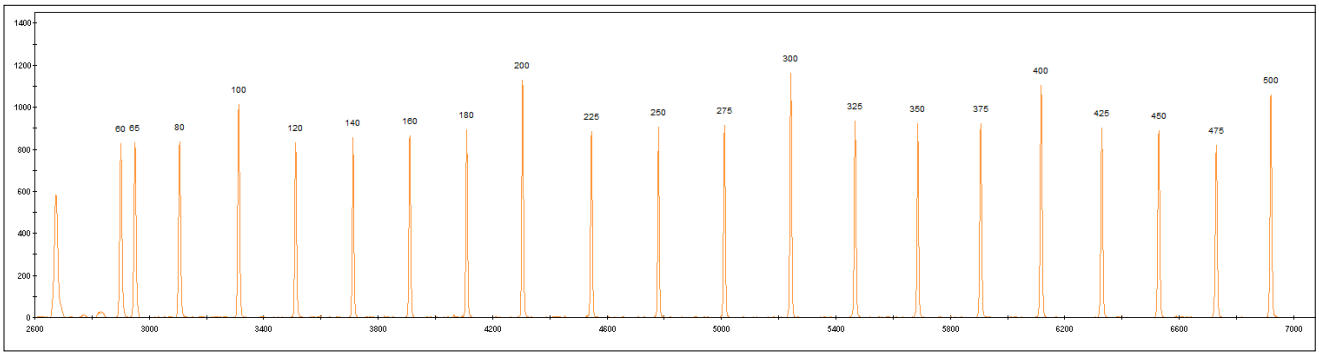
13.2.3.2.1 During peak detection and size-calling, the GeneMapper® ID-X Software matches an observed fragment peak from the size standard run with the sample to a corresponding size in the definition file.

13.2.3.2.2 Click on the Project node for the entire project being analyzed or the individual sample to be examined, then click (Size Match Editor) to view the peak assignments for the size standard peaks in the sample(s).

13.2.3.2.2.1 Alternatively, highlight the sample(s) then click (Display Plots). In the Plot Setting, select "Check LIZ Size Standard" from the drop-down list.

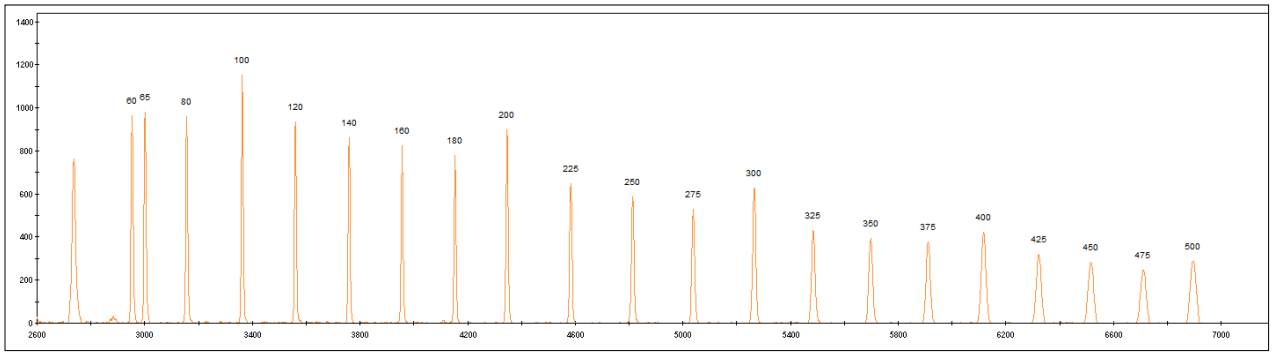
13.2.3.2.3 Check to determine that all peaks for the size standard are detected and labeled properly.

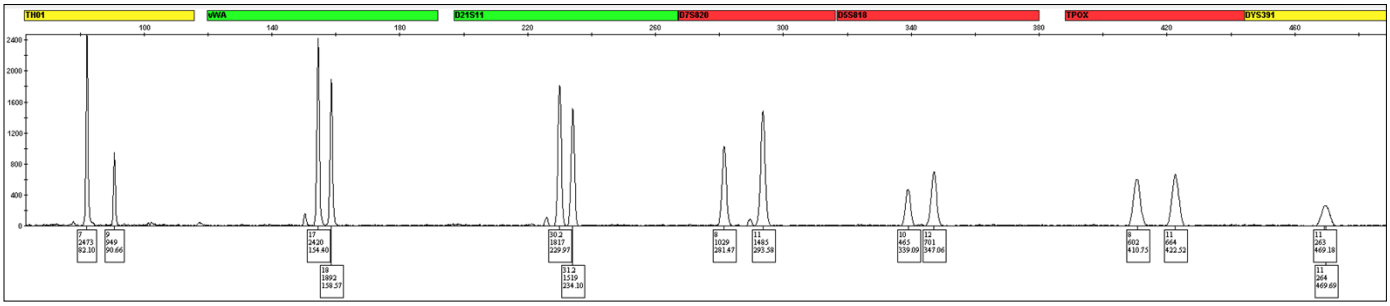
13.2.3.2.3.1 The sizes for the ILS fragments should be labeled as follows: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500 bases.



13.2.3.2.3.2 If peaks are incorrectly labeled, they may be edited by highlighting and right clicking on the peak to be edited in the Size Match Editor window. Select "Add", "Delete", or "Change Peak" as appropriate. Select "Apply" to accept changes. The project will need to be reanalyzed.

13.2.3.2.3.3 If a sample's size standard is sloping to the right (decreasing in peak height and broadening of peaks) the resolution of the sample may be affected. This sample should be carefully reviewed as it may need to be reinjected or replated to obtain quality results. Poor resolution in a sample can appear as broad/wide allele peaks on the right side of the electropherogram.





13.2.3.3 Review the Allelic Ladder quality.

13.2.3.3.1 To accurately genotype samples, the GeneMapper® ID-X Software requires at least one allelic ladder sample per run folder. For a ladder to be used for data analysis, the sizing quality (SQ) and composite genotype quality (CGQ) flags must not be flagged (indicated as green squares).

13.2.3.3.1.1 **NOTE**: If a run folder contains multiple allelic ladder samples, the GeneMapper® ID-X Software calculates bin offsets using an average of all ladders that use the same panel within a run folder. Only allelic ladders processed with the associated samples will be used in the run project.

13.2.3.3.2 If the ladder does not pass, then click (Display Plots). When troubleshooting failed ladders, select "Allelic Ladder Analysis" from Plot Settings drop-down list.

13.2.3.3.3 Verify the ladder has the correctly called alleles for each locus and the peak morphology is characteristic of peaks (i.e., no broadening of peaks).

13.2.3.3.4 If a ladder does not call correctly, change the failed ladder(s) to 'Sample' and re-analyze the project. Only one ladder is required per project.

13.2.3.3.5 Since the software applies an average of the ladders to the samples, it may be necessary to remove outlying ladders by changing them to 'Sample' and re-analyzing the project. Only one ladder is required per project.

13.2.3.3.6 **Note**: If only one ladder is selected, this may lead to off-ladder allele calls in the samples.

13.2.3.4 Review of Positive Controls

13.2.3.4.1 During analysis of the project, the positive control sample should be selected as a custom control matching to 2800M. If the SQ and CGQ Quality Flags indicate passing (green squares), then the positive control has passed and may be used for analysis. If either of these flags does not pass, follow the steps below.

13.2.3.4.1.1 Select the Positive Control sample(s) in the Samples table, then click (Display Plots).

13.2.3.4.1.2 Select "Data Interpretation" from the Plot Setting drop-down list.

13.2.3.4.1.3 Verify that the DNA allelic designations gave the expected results.

STR Locus	2800M
Amelogenin	X, Y
D3S1358	17, 18
D1S1656	12, 13
D2S441	10, 14
D10S1248	13, 15
D13S317	9, 11
Penta E	7, 14
D16S539	9, 13
D18S51	16, 18
D2S1338	22, 25
CSF1PO	12, 12
Penta D	12, 13
TH01	6, 9.3
vWA	16, 19
D21S11	29, 31.2
D7S820	8, 11
D5S818	12, 12
TPOX	11, 11
DYS391	10
D8S1179	14, 15
D12S391	18, 23
D19S433	13, 14
FGA	20, 23
D22S1045	16, 16

13.2.3.4.1.4 A positive control is considered to pass when a full profile is obtained, with no more than 6 locations exhibiting peak height ratios below 60%. If dropout is detected in

the positive control, data may be used with the approval and guidance of the DNA Technical Leader.

13.2.3.4.1.5 If the positive control injected poorly, it can be re-injected or if necessary, the positive control sample can be re-prepared and injected. The original sample injections can be analyzed if upon re-inject/rerun the positive control gives the expected results.

13.2.3.4.1.6 Positive controls that do not meet passing criteria, remain in the project as a "Positive Control". Note failed positive controls in the associated comment box in GeneMapper® ID-X.

13.2.3.5 Review of Negative Controls and Reagent Blanks

13.2.3.5.1 Select the negative control sample(s) or reagent blank(s) in the Samples table, then click (Display Plots). Select the "RB & Neg View" Plot Setting from the Plot Settings drop-down list.

13.2.3.5.1.1 The RB & Neg View Plot Setting must be used to view any reagent blank or negative control samples. This plot setting should include the primer dimer range. Alternatively, the Raw Data tab may be used to ensure primer dimer is present.

13.2.3.5.2 Verify that no called peaks are detected in the negative control or reagent blank.

13.2.3.5.2.1 A negative control or reagent blank that has no called peaks but appears to have allelic activity below the analytical threshold, does not render rework.

13.2.3.5.2.2 A negative control or reagent blank exhibiting a single called drop-in peak, with no allelic activity below the analytical threshold, does not render rework.

13.2.3.5.2.3 A negative control or reagent blank exhibiting a single called drop-in peak, with allelic activity below the analytical threshold, does render rework.

13.2.3.5.2.3.1 The associated sample(s), controls, and reagent blank(s) must be re-injected, re-plated re-amplified, and/or re-extracted to resolve the issue.

13.2.3.5.2.4 A negative control or reagent blank exhibiting more than one called peak renders rework.

13.2.3.5.2.4.1 The associated sample(s), controls, and reagent blank(s) must be re-injected, re-plated re-amplified, and/or re-extracted to resolve the issue.

13.2.3.6 Review of Evidence Sample Data

13.2.3.6.1 After all quality control samples have been reviewed and passed, each of the evidence samples should be reviewed. The GeneMapper® ID-X Software provides information on peak quality (height, base pair size, shape, number) as well as designating alleles.

13.2.3.6.2 Select the evidence sample to review in the Samples table, then click (Display Plots).

13.2.3.6.3 Select "Data Interpretation' from the Plot Settings drop-down list.

13.2.3.6.4 Check for true Off Ladder (OL) alleles in the evidence sample profile. A true OL allele is an allele believed to be a true/real allele and not the result of an artifact.

13.2.3.6.4.1 If a true OL allele is thought to be present, reamplify the sample to confirm the OL allele.

13.2.3.6.5 Check for the presence of allele drop-out.

13.2.3.6.5.1 Drop-out can indicate the need to reamplify the sample at a higher amplification target.

13.2.3.6.5.1.1 If the sample was amplified to its maximum target (i.e., a normalization dilution was not performed on the sample), reamplification will not be helpful. In these instances, the analyst should determine if re-extraction is appropriate.

13.2.3.6.6 Consider data RFUs and the stochastic thresholds (STO).

13.2.3.6.6.1 If data RFUs are at, around, and/or below the stochastic threshold, the sample should be re-amplified at a higher target, if possible. Saturation of the 3500 CCD camera should be considered in this decision.

13.2.3.6.6.2 Blue dye STO = 795 RFU

13.2.3.6.6.3 Green dye STO = 1290 RFU

13.2.3.6.6.4 Yellow dye STO = 1255 RFU

13.2.3.6.6.5 Red dye STO = 760 RFU

13.2.3.7 Review of Casework Reference Sample Data

13.2.3.7.1 Select the reference sample to review in the Samples table, then click (Display Plots).

13.2.3.7.2 If not already done, select 'Data Interpretation' from the Plot Settings drop-down list.

13.2.3.7.3 Check for true Off Ladder (OL) alleles in the reference sample profile. A true OL allele is an allele believed to be a true/real allele and not the result of an artifact.

13.2.3.7.3.1 If a true OL allele is thought to be present, reamplify the sample to confirm the OL allele.

13.2.3.7.4 Check for the presence of allele drop-out.

13.2.3.7.4.1 Drop-out can indicate the need to reamplify the sample at a higher amplification target.

13.2.3.7.5 If there is indication of male DNA in the sample (presence of Y allele at Amelogenin), check for called allele(s) at DYS391.

13.2.3.7.5.1 If there is reason to suspect drop-out at DYS391, reamplify the sample at a higher amplification target.

13.2.3.7.6 Review any apparent homozygous alleles.

13.2.3.7.6.1 If any apparent homozygous allele is < the stochastic threshold, reamplify the sample at a higher amplification target to increase the allele RFU to ≥ the stochastic threshold.

13.2.3.7.6.1.1 If after re-amplifying the reference sample to its maximum target, the apparent homozygous allele(s) remains < the stochastic threshold, the analyst should re-extract the reference sample. If re-extraction is not possible and/or the issue is not resolved after re-extraction and re-processing, the DNA Technical Leader must be consulted to determine the best course of action.

13.2.3.8 Artifacts

13.2.3.8.1 During the review steps previously described, the analyst will also identify and characterize artifacts.

13.2.3.8.1.1 Some called data may not represent actual alleles that originate in the sample. It is therefore necessary, before the STR typing results can be used for comparison purposes, to identify any potential non-allelic peaks (i.e., artifacts). Artifacts may be PCR products (e.g., stutter and incomplete nucleotide addition), instrumental artifacts (e.g., spikes and raised baseline), or instrumental limitations (e.g., incomplete spectral separation resulting in pull-up). Generally, artifacts such as stutter, incomplete nucleotide addition, and pull-up are reproducible; spikes and raised baseline are generally non-reproducible.

13.2.3.8.1.2 If an artifact can be confidently characterized, the sample may be used for analysis. If an artifact cannot be confidently characterized, the data will be further assessed through reanalysis (i.e., re-injected, re-plated, re-amplified, re-quantified, and/or re-extracted).

13.2.3.8.1.2.1 If an allele is modified, re-labeled, and/or deleted within the GeneMapper® ID-X Software this must be documented within the case file.

13.2.3.8.2 Below are examples of artifacts that can be observed in the data:

13.2.3.8.2.1 Stutter:

13.2.3.8.2.1.1 Stutter is believed to be a result of slippage of the polymerase during amplification. This is represented as a small peak, generally one repeat unit lesser than or greater than the true peak.

13.2.3.8.2.1.2 Stutter may also appear as multiples of the repeat unit (e.g., n - 8, n + 8) or portions of the repeat unit (e.g., n – 2, n+2, n-3, n+3).

13.2.3.8.2.1.3 GeneMapper® ID-X Software is programmed to detect and filter (i.e., not call as an allele) n-4 and n+4 stutter peaks using the maximum stutter percentage observed between the MNPD-CL in-house validation and the manufacturer recommendation. These maximum stutter percentages (i.e., stutter ratio thresholds) are defined in the GeneMapper® ID-X Panel Manager.

13.2.3.8.2.1.3.1 The forward stutter (n + 4) ratio threshold is set globally at 4.5% for all loci except D22S1045, which will use the manufacturer forward stutter filter of 8.6%.

13.2.3.8.2.1.4 Manual stutter calculations will also utilize the maximum stutter percentage described in the stutter ratio threshold charts below. Analysts should truncate the calculated value to the tenth decimal place (e.g., 12.89% is considered 12.8%).

13.2.3.8.2.1.4.1 The forward stutter for Penta E and Penta D will be calculated manually as the forward stutter filter is only applied to the tetra repeats. The forward stutter ratio is 4.5% for Penta E and Penta D.

13.2.3.8.2.1.4.2 See the charts below for the stutter ratio thresholds.

13.2.3.8.2.1.5 Combination stutter is defined as a stutter allele resulting from the combination of minus (n-2, n-3, n-4, and/or n-8) and/or plus (n+2, n+3, n+4, and/or n+8) stutter.

13.2.3.8.2.1.5.1 For example, combination stutter could be observed when alleles differ by two repeat units with an allele in between them that differs by one repeat unit (e.g., 12 and 14 alleles with an allele at the 13 position resulting from minus stutter from the 14 allele and plus stutter from the 12 allele). Additionally, combination stutter could result from the combination of different stutter types. For example, a true allele's (e.g., 6) n+8 stutter and a different true allele's (e.g., 9) n-4 stutter could combine (e.g., 8) to create a combined stutter allele. The examples listed

are only examples, and not rules, since combination stutter can be observed in various ways by combining multiple stutter types.

13.2.3.8.2.1.6 If an allele in this combined stutter position does not meet the minus and/or plus stutter thresholds, the following calculation should be performed to determine if the allele can be characterized as combined stutter.

13.2.3.8.2.1.6.1 Terms

13.2.3.8.2.1.6.1.1 Allele A – first allele (12 allele in the example above).

13.2.3.8.2.1.6.1.2 Allele B – possible stutter allele (13 allele in the example above).

13.2.3.8.2.1.6.1.3 Allele C – last allele (14 allele in the example above).

13.2.3.8.2.1.6.2 Formula (□ + □ = □)

13.2.3.8.2.1.6.2.1 X = (RFUs of Allele A * maximum plus stutter % of locus)

13.2.3.8.2.1.6.2.2 Y = (RFUs of Allele C * maximum minus stutter % of locus)

13.2.3.8.2.1.6.2.3 Z = Maximum RFU threshold of Allele B (this value will be truncated)

13.2.3.8.2.1.6.3 If RFU of Allele B ≤ Z

13.2.3.8.2.1.6.3.1 Allele B will be characterized as stutter and must not be used in interpretation and/or comparison.

13.2.3.8.2.1.6.4 If RFU of Allele B > Z

13.2.3.8.2.1.6.4.1 Allele B is considered a true peak.

13.2.3.8.2.1.7 After filtering, if a peak(s) is suspected to be elevated stutter (i.e., a true stutter peak whose ratio has exceeded the threshold) the data will be further assessed through reanalysis (i.e., re-injected, re-plated, re-amplified, re-quantified, and/or re-extracted).

13.2.3.8.2.2 Pull-up:

13.2.3.8.2.2.1 This type of artifact is caused by poor spectral separation of the dye channels and is typically caused by high quantity of input DNA.

13.2.3.8.2.2.2 If pull up peaks are due to poor color separation and not excessive peak height in other channels, then a new spectral calibration may need to be performed, and the sample analyzed using the new spectral calibration.

13.2.3.8.2.3 Incomplete +A nucleotide addition (-A):

13.2.3.8.2.3.1 The PCR process utilizes a polymerase that is optimized to add an additional adenosine nucleotide onto the extended fragment. Excessive input DNA can make

this process less efficient resulting in PCR fragments that are one nucleotide shorter than the true amplicon size (-A).

13.2.3.8.2.3.2 Spikes:

13.2.3.8.2.3.2.1 Spikes are peaks that generally have a sharp, needle-like appearance. Commonly, spikes will be 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.

13.2.3.8.2.3.3 The software uses a proprietary algorithm that detects spikes based on the peak morphology and will automatically flag spikes within the analyzed range. Occasionally, the software may mislabel a spike as an allele.

13.2.3.8.2.4 Dye Blob/Raised Baseline:

13.2.3.8.2.4.1 This type of artifact is generally caused by free dye-labeled primers that fall within the analyzed range. This results in an elevation of the baseline sometimes beyond the analytical threshold but not generally with any defined or identifiable morphological shape.

13.2.3.8.3 The below charts may be used to evaluate the presence of stutter and possible kit artifacts:

	Stutter Peaks				Other Artifacts
Locus	**In-House (Avg+3)**	**Max (if > Avg+3)**	**Manufacturer (if > Avg+3)**	**n-1**	**Additional**
AMEL	n/a	n/a	n/a	X	
D3S1358	12.1%				
D1S1656	13.3% (n-4) 3.5% (n-2)		14.2% (n-4) 3.6% (n-2)		
D2S441	7.7%		9.2%		
D10S1248	11.6%		12.4%		
D13S317	9.6%		9.8%		
Penta E	5.7%		7.6%		
D16S539	11.0%				84 bases (allele 5)
D18S51	16.1%	17.7%			214 bases
D2S1338	14.9%	19.0%			247 bases
CSF1PO	9.9%				
Penta D	3.4%		6.8%		
THO1	5.6%	7.2%			*71-73 and *75-77 bases (*may call as OL)
vWA	13.7%				
D21S11	14.1%	19.7%			
D7S820	10.6%	12.3%			
D5S818	12.5%	17.1%			
TPOX	6.4%				
DYS391	8.5%		8.7%		
D8S1179	11.5%				
D12S391	20.1%				
D19S433	10.6%	11.6%			
FGA	14.0%	15.9%			
D22S1045	18.7% (n-3)				

Dye Channels	**Kit Artifacts**
fluorescein (blue)	62-65, 63-68, and -86 bases
JOE (green)	68-71, 79-80, 214, and 247 bases
TMR-ET (yellow)	58-61, 64-67, and 69-72 bases
CXR-ET (red)	58-65 bases

Additional Stutter Thresholds						
[redacted]	**N-2***	**N+2**	**N-8**	**N+8**	**N-3**	**N+3**
All loci	1.9%	2.6%	2.8%	1.5%	[redacted]	[redacted]
D12S391	[redacted]				2.2%	2.3%
*For N-2, D1S1656 will adhere to percentage in the previous chart.						

13.2.3.8.4 Labeling of Artifacts:

13.2.3.8.4.1 For analysis of sample data, all electropherograms must be visually evaluated by the analyst to ensure that artifacts are not mislabeled. If a peak is mislabeled, the allele call should be edited by the analyst. Care must be taken when evaluating artifacts observed in samples arising from DNA from more than one individual; if necessary, re-injection, re-amplification, re-quantification, or re-extraction may be needed to make a determination of artifact vs. true allele. When a peak has been determined to be a true artifact, the allele should be deleted.

13.2.3.8.4.2 To edit an allele call, select the peak(s) of interest by right clicking on it.

13.2.3.8.4.3 Select Delete Label(s) from the drop-down list.

13.2.3.8.4.3.1 **NOTE**: When multiple peaks are selected at the same time, the changes will be applied to all selected peaks and the same Reason For Change will be applied to all of them.

13.2.3.8.4.3.2 When deleting a software labeled peak, indicate Reason For Change (i.e., ART = artifact).

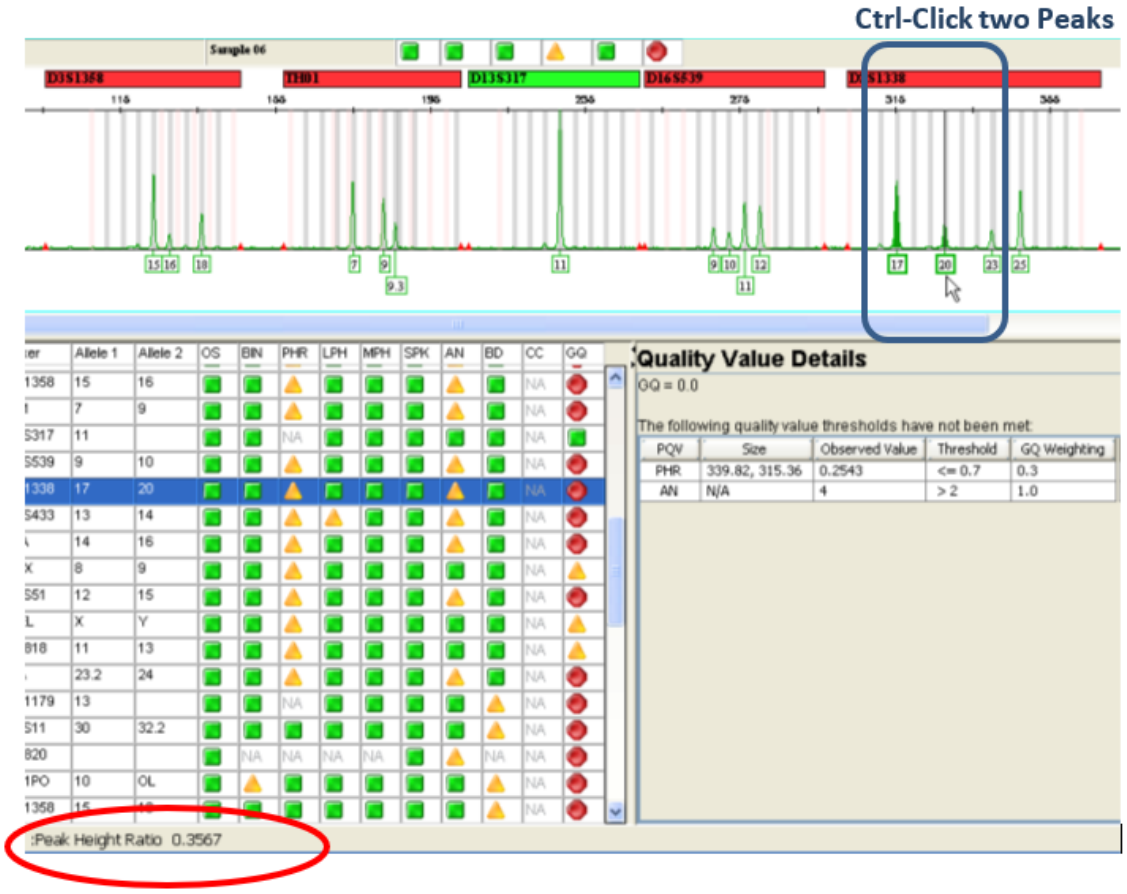
13.2.3.8.4.3.3 The modification, re-labeling, and/or deletion of an allele within the GeneMapper® ID-X Software must be documented within the case file.

13.2.3.9 Peak Height Ratio (PHR):

13.2.3.9.1 The GeneMapper® ID-X Software has a quality flag that can aid in analysis and interpretation of single source and mixed samples based on the peak height ratio of alleles within a locus (PHR PQV). A flag is displayed when there are two alleles present and the ratio between the lower allele height and the higher allele height is

below a certain level (60% set in the Peak Quality tab of the analysis method). The ratio is based on the peak height of the called allele peaks.

13.2.3.9.2 In a mixed source sample, it is also possible to ctrl-click on the two allele peaks within the Sample plot then the software will automatically calculate a peak height ratio (PHR) value for the selected peaks and displays the results in the status bar at the bottom of the Samples plot.



13.2.3.9.2.1

13.2.3.10 Off-scale Data (OS):

13.2.3.10.1 When an excess of DNA is added to the amplification reaction, the result can be PCR product that produces a fluorescent intensity that exceeds the dynamic range for detection by the instrument called off-scale data. This is represented in the software by a bright pink line through the data that exceeds the detection threshold of the instrument (i.e., RFUs ≥ 30,000).

13.2.3.10.2 When data is off-scale, peak heights may not be accurately scaled, interfering with data interpretation.

13.2.3.10.2.1 Evidence samples with data over 30,000 RFUs will be re-run (i.e., diluted and re-amplified at a lower target).

13.2.3.10.2.2 Standard profiles with data over 30,000 RFUs can be utilized if after removing all potential artifacts (if applicable) the standard profile results in loci containing one allele or two alleles, and/or a confirmed tri-allelic pattern. If these criteria are not met, then the standard(s) will be re-run.

13.2.3.10.2.3 Positive controls with data over 30,000 RFUs can be utilized if after removing all potential artifacts (if applicable) the expected positive control profile is obtained. If the expected positive control profile is not obtained, then the positive(s) will be re-run.

13.2.3.11 Off Ladder (OL) Allele Calls:

13.2.3.11.1 If a peak falls outside of one of the defined bins, the software labels it OL (Off Ladder). OL calls may be caused by the presence of a microvariant allele at a particular locus or sample migration anomalies and artifacts.

13.2.3.11.2 After reamplification to confirm the presence of a called OL, allele names may be assigned to these OL peaks based on the number of complete or partial base pair repeat units.

13.2.3.11.2.1 To evaluate an OL allele call in GeneMapper® ID-X, highlight the sample in question and allelic ladder(s) under the Samples tab.

13.2.3.11.2.2 Click  (Display Plots) and zoom into the peak in question. This should also magnify the ladder(s) automatically.

13.2.3.11.2.3 Compare the base pair size of the peak in question (OL) to, at minimum, the surrounding known ladder allele(s) to determine the size of the peak.

13.2.3.11.2.3.1 If the allele is seen to the right of the largest allelic ladder peak of the locus, it will be assigned the type of the largest allele of the allelic ladder with a greater than sign (>).

13.2.3.11.2.3.2 If the allele is seen to the left of the smallest allelic ladder peak of the locus, the allele will be assigned the type of the smallest allele of the allelic ladder with a less than sign (<).

13.2.3.11.2.3.3 If an allele is seen between two loci and either the locus to the right OR left of the peak contains two peaks, the allele will be considered to belong with the locus not containing two peaks. Naming should follow the directions stated above.

13.2.3.11.2.3.4 If an allele is seen between two loci and neither the higher nor lower molecular weight loci contain an allele or two alleles, the base pair size for the allele in question must be assessed. Compare the base pair sizes of the largest/smallest surrounding allelic ladder peaks to determine which is closest in proximity and falls within an appropriate size distance from the locus. Naming should follow the directions stated above.

13.2.3.11.2.3.5 Alleles that fall between two allelic ladder peaks of the same locus are considered microvariant alleles. Microvariant alleles contain a partial repeat and are designated by a decimal followed by the number of bases in the partial repeat (i.e., an FGA 26.2 allele).

13.2.3.11.2.4 Once the size of the peak and appropriate naming for the OL allele have been determined, the peak should be relabeled by right-clicking on the peak, then selecting "Rename Allele Label" from the drop-down list.

13.2.3.11.2.5 In the "Add Custom Allele Label" dialog box, enter the allele name, then click "OK". Enter the reason for the change in the prompt box.

13.2.3.11.2.5.1 In the prompt box, the bp size of the peak should be noted.

13.2.3.11.2.6 For the case file, select the sample containing the microvariant and one ladder. Zoom to the microvariant locus in the "Microvariant" plot setting. Print to PDF and include with the electropherogram of the used data.

13.2.3.12 Review of Raw Data

13.2.3.12.1 To troubleshoot any anomalies, the causes of poor size-calling, and to determine the start and stop points of analysis, it can be beneficial to examine the unseparated raw fluorescent data.

13.2.3.12.2 In the Project window, click + to expand the Samples folder, highlight each sample to view its associated information.

13.2.3.12.3 The Info tab for the selected sample is displayed. Review the sample-specific information presented in this tab, including error messages, run information, and data collection settings.

13.2.3.12.4 Select the Raw Data tab in the content pane to help evaluate any anomalies, the causes of poor size-calling, and to aid in troubleshooting the start and stop points for analysis.

13.2.3.12.5 Select the EPT Data tab in the content pane. The EPT plot is displayed for the selected sample.

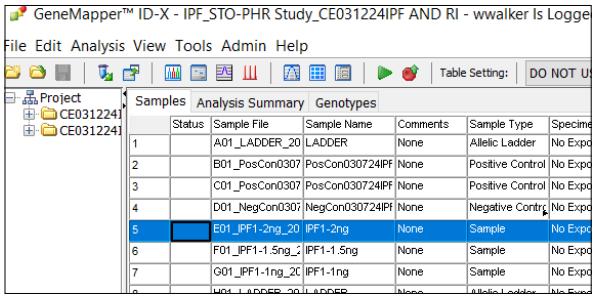
13.2.3.12.6 Select the Project node in the navigation pane to return to the Samples table view.

13.2.3.12.7 To view a sample, highlight the sample then click (Display Plots).

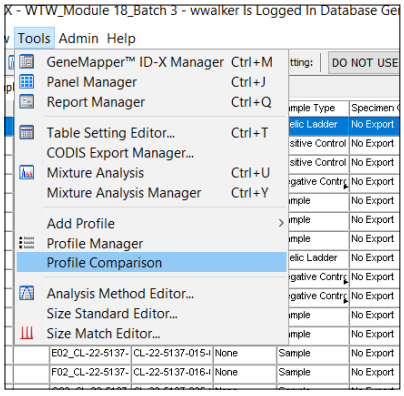
13.2.3.13 GeneMapper™ ID-X Profile Comparison Tool may be used to help assess for possible contamination. The profile comparison tool can be used to compare profiles within a project to each other, to MNPD employee profiles, and to donated profiles (e.g., vendor technician profiles) to assess for possible contamination. Evidence profiles should not be compared to case relevant references prior to interpretation. To utilize the profile comparison tool, perform the following:

13.2.3.13.1 On the project samples table tab select Tools > Profile Comparison

13.2.3.13.1.1



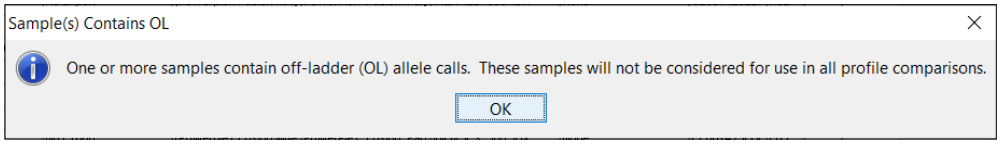
13.2.3.13.1.2



13.2.3.13.1.2.1 If any samples within the project contain OLs a pop-up window will be returned. The sample(s) that contains an OL will not be included in the profile

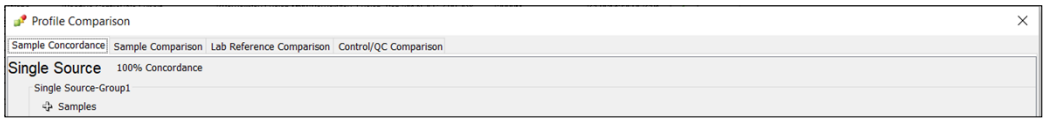
comparisons assessment. OLs must be addressed for the sample(s) to be included in the profile comparison assessment.

13.2.3.13.1.2.1.1



13.2.3.13.2 The profile comparison window will open.

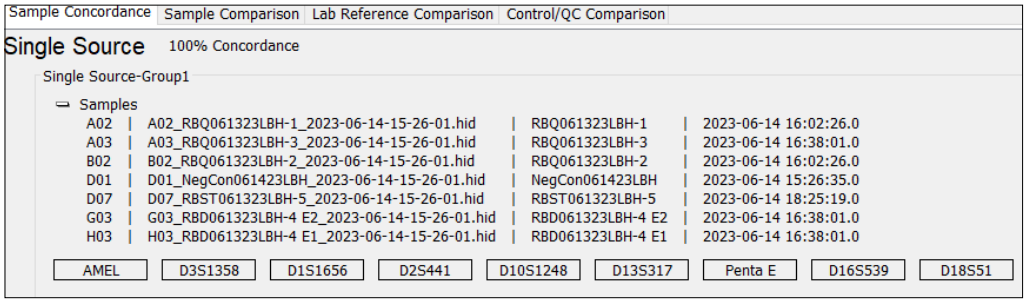
13.2.3.13.2.1



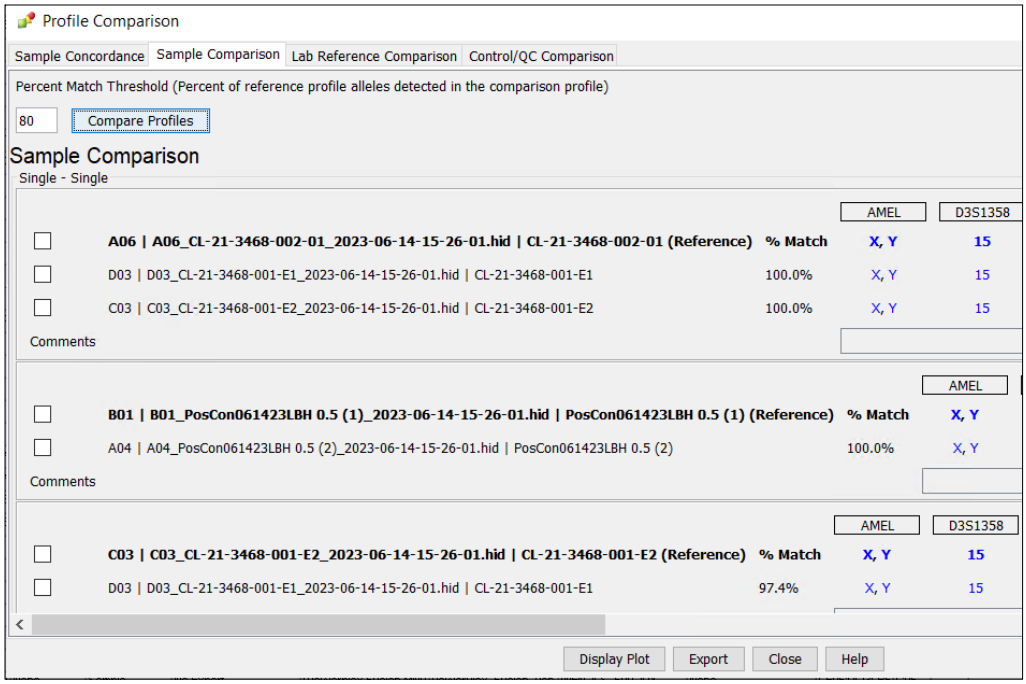
13.2.3.13.2.2 Profile Comparison window tab definitions:

13.2.3.13.2.2.1 Sample Concordance tab automatically shows the user all single source and mixed source samples within the project that are 100% concordant with each other.

13.2.3.13.2.2.1.1

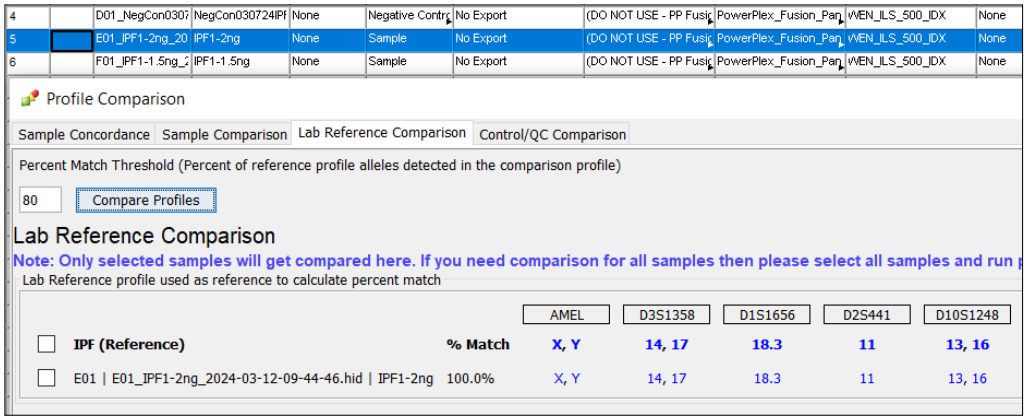


13.2.3.13.2.2.2 Sample Comparison tab* can help users assess if samples within the same project have a unique or a common contributor. Select “Compare Profiles” to run the query.



13.2.3.13.2.2.2.1

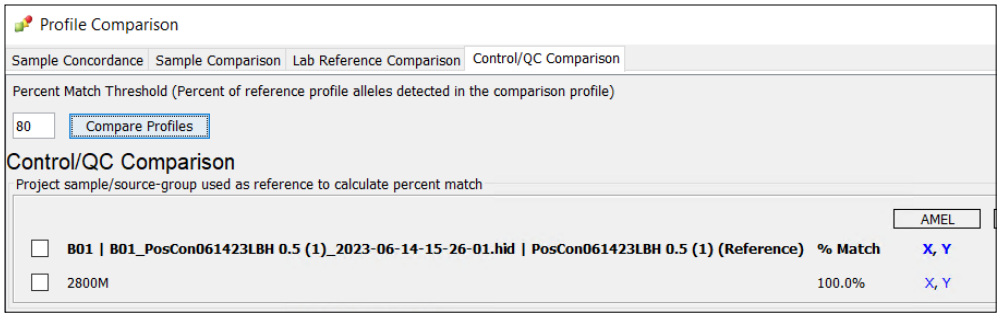
13.2.3.13.2.2.3 Lab Reference Comparison tab* allows users to compare MNPD references (e.g., staff profiles, donated vendor profiles, etc.) to samples within the project. Within the project samples table, the user must select the sample(s) that will be compared to the lab references. Select "Compare Profiles" to run the query.



13.2.3.13.2.2.3.1

13.2.3.13.2.2.4 Control/QC Comparison tab* compares custom controls (i.e., positive control 2800M) to the samples within the project.

13.2.3.13.2.2.4.1



13.2.3.13.2.2.5 *In each tab users can set a percentage match threshold (e.g., 80% as seen in the example pictures). The percent match threshold is the percent of the project sample alleles detected in the comparison profile. Users may see a pop-up window returned when no matches are observed at the set percent match threshold. The threshold can be adjusted, ranging from 50% - 100%.

13.2.3.14 Export of GeneMapper project

13.2.3.14.1 Under the "Tools" tab, select "GeneMapper™ ID-X Manager".

13.2.3.14.2 Enter the associated GeneMapper project name in the "Find Name Containing" field.

13.2.3.14.3 Highlight the project in the list and select "Export...".

13.2.3.14.4 Browse to the designated export location following the path: This PC > Crime Lab Backup (G:) > Instrument-DNA > GM projects > YYYY "Casework" > Month. Save as a ".ser" file.

13.2.3.14.5 Click "Done" once export is complete.

13.2.3.14.6 Project will remain in GeneMapper until review is complete.

13.2.4 Limitations

13.2.4.1 If the GeneMapper project is reanalyzed after edits have been made to allele calls, those edits will be deleted. Therefore, it is important to ensure all QC parameters are met prior to the labeling of artifacts.

13.2.5 References

13.2.5.1 GeneMapper® ID-X Software Version 1.0 Getting Started Guide (October 2007). Applied Biosystems.

13.2.5.2 GeneMapper® ID-X Software Version 1.2 Reference Guide (October 2007). Applied Biosystems.

13.2.5.3 GeneMapper® ID-X Software Version 1.2 (December 2009). Applied Biosystems.

13.2.5.4 GeneMapper® ID-X Software Version 1.3 (October 2011). Applied Biosystems.

13.2.5.5 PowerPlex Fusion System Technical Manual (October 2012). Promega, Madison, WI.

13.2.5.6 GeneMapper® ID-X Software Version 1.4 (December 2012)

14. Data Interpretation

14.1 Scope

14.1.1 To describe the process of analysis and interpretation of casework data for the purpose of comparison, reporting and statistical analysis.

14.1.2 This procedure will be used to evaluate a questioned profile's suitability for comparison to a reference sample. It's not feasible for this procedure to describe all possible case scenarios or data permutations. Analysts may draw upon experience and relevant case information when making an interpretation; however, the analyst must provide justification for interpretations of situations not described in these procedures. Justifications will be clearly and methodically documented in the case file/notes. Deviations from these procedures require prior approval from the DNA Technical Leader.

14.2 Equipment/Materials/Reagents

14.2.1 GeneMapper® ID-X Software v1.6

14.2.2 Traditional Deconvolution excel file

14.2.3 Major Deconvolution excel file

14.3 Sample Data: Interpretability

14.3.1 If data is present at < 5 loci (excluding Amelogenin and DYS391), the data is uninterpretable and will be deemed inconclusive due to limited data. This statement supersedes all exceptions described in this procedure. A profile must have data at ≥ 5 loci to be considered for interpretation with methods described in this procedure.

14.3.2 A questioned profile will be deemed interpretable or uninterpretable prior to comparison to a known profile(s).

14.3.3 If a questioned profile is deemed interpretable, data interpretation will be performed prior to comparison to a known profile(s).

14.3.3.1 An exception is made for data that may contain a reasonably expected contributor. In this instance the analyst may choose to immediately interpret the data assuming the presence of the reasonably expected contributor's known profile (see Assumed Deconvolution section). See guidance below for when an individual may be reasonably expected.

14.3.4 Regardless of a questioned profile's interpretability, if a contributor(s) can be reasonably expected to be found in a questioned profile, a known profile(s) may aid in the data interpretation to determine the foreign profile. A foreign profile can be determined by "backing out" the reasonably expected contributor's profile. This process is further described in the Assumed Deconvolution section. A contributor may be reasonably expected if at least one of the following apply:

14.3.4.1 The evidence sample is an intimate sample from the known individual. An evidence sample is deemed intimate in nature when it originates directly from the donor's body (e.g., fingernail clippings/swabbing, vaginal swabs, oral swabs, or a swabbing from any skin surface). An intimate sample is generally expected to yield DNA from the individual from whom the sample was collected.

14.3.4.2 The evidence sample was obtained from clothing worn by the known individual. The clothing must be collected from the individual's body or identified by the individual as their clothing. Documented communication stating this must be maintained (e.g., MNPD 282 form, phone log, email, evidence label, etc.).

14.3.4.3 The sample was/is collected from an area, location, or item that the known individual is known to have occupied or touched (e.g., vehicle, residence, etc.). Documented communication stating this must be maintained (e.g., MNPD 282 form, phone log, email, evidence label, etc.).

14.3.4.4 The evidence profile originates from an alleged sexual assault case where a consensual partner(s) has been reported. Given the case scenario, the consensual partner may be considered a reasonably expected contributor.

14.3.5 Determining the minimum number of contributors: The number of contributors to the questioned profile will be determined for all genotyping results. This assessment should be made based upon the locus that contains the greatest number of allelic peaks, coupled with the observed peak height ratios and an assessment of the potential for dropout. All three of these factors must be evaluated before determining the number of individuals within a questioned profile. Be aware, phenomena such as tri-alleles, primer binding site mutations, or allele sharing between close relatives can complicate the determination of number of contributors.

14.3.5.1 Number of allelic peaks:

14.3.5.1.1 A questioned profile can be assumed to be single source when each locus displays one or two alleles and no more than one locus has three alleles.

14.3.5.1.2 A questioned profile can be considered a mixture when it contains at least three alleles at more than one locus and/or at least four alleles at one locus.

14.3.5.1.2.1 A mixture may be assumed to be of two individuals if no more than four alleles are present at any one locus.

14.3.5.1.2.2 A mixture may be assumed to be of two individuals if three alleles are present at two or more loci.

14.3.5.1.2.3 A mixture may be assumed to be of three individuals if no more than six alleles are present at any one locus.

14.3.5.1.2.4 A mixture may be assumed to be of three individuals if five alleles are present at two or more loci.

14.3.5.1.2.5 A mixture may be assumed to be from four or more individuals if at least seven alleles are present at any locus.

14.3.5.1.3 Apparent extraneous alleles

14.3.5.1.3.1 There must be at least two extraneous alleles in the questioned profile for the analyst to state the extraneous alleles are equivalent to an additional individual.

14.3.5.1.3.2 Two extraneous alleles moves the number of contributor determination from X individuals to X+1 individuals.

14.3.5.1.3.3 One extraneous allele moves the number of contributor determination from X individuals to at least X individuals.

14.3.5.2 Peak height ratios (PHR):

14.3.5.2.1 The PHR of sister alleles (i.e., heterozygous alleles) is expected to be ≥ 50% for a contributor amplified at a DNA template of ≥ 500pg with PowerPlex Fusion 5C. Due to inhibition, degradation, low DNA template causing stochastic amplification variation, or primer binding site issues, a PHR of ≥ 50% may not always occur. The PHR of sister alleles can be as low as 23% in these instances.

14.3.5.2.1.1 It should be noted that PHRs of < 23% (as low as 8.7%) have been observed for sister alleles. However, a minimum PHR of 23% will be used during interpretation. For more information on this, please see QMS WF 98598.

14.3.5.2.2 If a sample appears to be single source but contains a locus with two peaks where the PHR is < 23%, the sample may be assumed to be from at least one individual. However, this type of questioned profile may be reported as one individual if a reasonably assumed contributor exhibits a full and complete match of each allele at this locus. This full and complete match shows the questioned profile of concern has been produced from a single individual, thus eliminating the uncertainty.

14.3.5.2.2.1 The term "at least" is interpreted to mean that we are assuming there are, at minimum (i.e., at least) X individual(s) in the questioned profile and that there could be more contributors than X, but we can only assume X given the data.

14.3.5.2.3 For a mixture profile, the analyst should ensure PHRs are logically consistent with being from a given number of contributors.

14.3.5.2.3.1 Analysts should assess, given the number of alleles and given the RFU of each allele, how these alleles could combine to be shared. The stochastic threshold discussed below can also assist in this determination. This should be done with scientific reason.

14.3.5.2.3.1.1 For example, it would not be plausible to obtain a mixture of three individuals and expect that each individual has contributed, and could only contribute, one allele at each locus.

14.3.5.3 Potential for dropout:

14.3.5.3.1 The stochastic threshold (STO) will also be used to determine the number of individuals present in a questioned profile. STO is defined as the RFU above which it is reasonable to assume allelic dropout has not occurred. The STOs for PowerPlex Fusion 5C are as follows:

14.3.5.3.1.1 Blue – 795 RFU

14.3.5.3.1.2 Green – 1290 RFU

14.3.5.3.1.3 Yellow – 1255 RFU

14.3.5.3.1.4 Red – 760 RFU

14.3.5.3.2 The STO is related to the certainty an analyst has in the number of individuals within a questioned profile.

14.3.5.3.2.1 The STOs assists in an analyst's evaluation of a questioned profile when determining if all the information is present from a particular individual(s) at a locus and/or the profile in total. If the profile or a contributor within the profile's RFUs are at and/or below the STOs, then the analyst may be uncertain whether all the information from

an individual or individuals is present. The analyst may also be uncertain as to how many contributors comprise this profile, as the profile may be a combination of multiple individuals. However, the analyst should balance this uncertainty given the number of allelic peaks observed, the presence or absence of allelic activity below the AT, and the PHRs of possible genotype combinations.

14.3.5.3.3 If all alleles in a questioned profile are below the STOs, the data will be reamplified to a higher DNA template, if possible, to obtain more information. Ultimately, if all alleles are < STOs the profile will be deemed inconclusive due to limited/complex data.

14.3.5.3.3.1 This includes questioned profiles appearing to be from a single source. This sample will be reported as at least one contributor.

14.3.5.3.3.1.1 This type of questioned profile will not be deemed inconclusive if a reasonably expected contributor exhibits a full and complete match to each allele of this questioned profile. This full and complete match shows the questioned profile of concern has been produced from a single individual, thus eliminating the uncertainty. This questioned sample will be reported as one individual.

14.3.5.3.3.2 This includes questioned mixture profiles that may contain a reasonably expected contributor.

14.3.5.3.3.3 This does not include reference profiles. See the Review of Casework Reference Sample Data instruction in the data analysis section.

14.3.5.4 Analysts may use unlabeled peaks below the AT to assist in their assessment. Generally, the usage of unlabeled peaks below the AT in this assessment is limited to a profile containing data at and/or below the STO.

14.3.5.4.1 For example, if a profile consists of only one or two labeled peaks above the AT, but unlabeled peaks demonstrating good peak morphology are also present below the AT, the analyst may use the presence of these additional peaks to make the determination that the sample is not from a single source. This example would be reported as at least one individual.

14.3.6 How to proceed after determinations have been made:

14.3.6.1 After evaluation, if a set number of contributors for the questioned profile can be determined, proceed to the Sample Data: Options for Interpretation of Questioned Sample section.

14.3.6.2 After evaluation, if the number of contributors in the questioned profile appears to be from definitively *X* number of contributors but contains one extraneous allele, proceed to the Sample Data: Options for Interpretation of Questioned Sample section.

14.3.6.3 After evaluation, if the analyst cannot reasonably assume a set number of contributors for the questioned profile (with the exception of the preceding bullet), the sample will be reported as "at least" the number of contributors observed based upon the maximum allele count, peak height ratios, and potential for drop out. The questioned profile will not be further interpreted. The profile is uninterpretable and will be deemed inconclusive.

14.3.6.3.1 Note: If a questioned profile is deemed inconclusive, but was not amplified to its maximum amplification target, the sample must be re-amplified to its maximum amplification target to obtain more information.

14.3.6.4 Exceptions:

14.3.6.4.1 If the questioned profile was developed from an evidence sample where a contributor may be reasonably expected the analyst should assess if making an assumption would aid in the interpretation of the profile.

14.3.6.4.1.1 If making an assumption would aid in the interpretation of the questioned profile, proceed to the Sample Data: Options for Interpretation of Questioned Sample section.

14.3.6.4.1.2 If making an assumption would not aid in the interpretation of the questioned profile, the profile is uninterpretable and will be deemed inconclusive.

14.3.6.4.2 If the profile contains a visually major component, proceed to the Sample Data: Options for Interpretation of Questioned Sample section.

14.3.6.4.3 No further interpretation will be conducted on a questioned profile of four or more individuals. These profiles are uninterpretable and must be deemed inconclusive.

14.4 Sample Data: Options for Interpretation of Questioned Sample:

The bullets below describe how the analyst may proceed after determining the questioned profile is interpretable. When multiple interpretation options are allowed the analyst may choose the option that best interprets the questioned profile. Documentation of all interpretations, used or unused, must be maintained within the case folder.

14.4.1 One contributor – may proceed to one of the following options:

14.4.1.1 Random Match Probability (RMP) calculation

14.4.1.1.1 A confirmed tri-allelic locus will not be included in the frequency estimate.

14.4.1.2 CODIS entry

14.4.1.3 RMP calculation and CODIS entry

14.4.2 At least one contributor – type 1

14.4.2.1 The dropout and/or potential for dropout did not lead the analyst to deem this questioned profile uninterpretable. This profile may proceed to the following:

14.4.2.1.1 RMP calculation

14.4.2.1.1.1 A confirmed tri-allelic locus will not be included in the frequency estimate.

14.4.2.1.2 CODIS entry

14.4.2.1.3 RMP calculation and CODIS entry

14.4.2.2 The dropout and/or potential for dropout **did** lead the analyst to deem this questioned profile uninterpretable. Apart from the exceptions previously stated regarding reasonably expected contributors, these profiles are uninterpretable and must be deemed inconclusive.

14.4.3 At least one contributor – type 2

14.4.3.1 A questioned profile that appears to be from one contributor but contains one extraneous allele. The analyst will assess the PHR of the possible heterozygous pairs. The locus with the extraneous allele is not considered in the following assessment.

14.4.3.1.1 If any PHRs are < 23% the number of contributors (NOC) will be moved to 2.

14.4.3.1.2 If all PHRs are ≥ 23% this profile should be interpreted using one of the following options. No determinations will be made regarding the extraneous allele.

14.4.3.1.2.1 RMP calculation

14.4.3.1.2.2 CODIS entry

14.4.3.1.2.3 RMP calculation and CODIS entry

14.4.4 Two contributors and at least two contributors – proceed to one of the following options.

As a reminder, the analyst may choose the option that best interprets the questioned profile. Documentation of all interpretations, used or unused, must be maintained within the case folder.

14.4.4.1 Traditional Deconvolution

14.4.4.2 Assumed Deconvolution

14.4.4.3 Major Deconvolution

14.4.5 Three contributors and at least three contributors

14.4.5.1 Major Deconvolution

14.4.5.2 Assumed Deconvolution

14.4.6 **Note**: When performing any deconvolution, the analyst has the discretion to call a locus, loci, or profile as inconclusive. If the chosen deconvolution method does not support this inconclusive determination, the analyst must thoroughly justify this conclusion in the casefile. Stating simply "artifact interference" is not an acceptable justification. The analyst must thoroughly, methodically, and explicitly explain what artifact interference means in relation to the locus, loci, or profile of interest.

14.5 Sample Data: Traditional Deconvolution

14.5.1 **Perform a major evaluation.** See Appendix: Exporting Data and Major Evaluation for workbook instructions. Save the major evaluation file as "*Sample#_MajorEval_Initials".* Save the traditional deconvolution file as "*Sample#_TradDecon_Initials*".

14.5.1.1 If the overall percent contribution ≥ 75% proceed to the resolved method.

14.5.1.2 If the overall percent contribution < 75% proceed to the unrestricted method.

14.5.2 Resolved Method – utilizes the interpretation parameters (see Appendix: Interpretation Parameters) to maximize restrictions to the developed random match probability (RMP). See Appendix: Traditional Deconvolution Workbook for workbook instructions.

14.5.2.1 Resolved – 5 allele locus

14.5.2.1.1 If a locus contains 5 alleles, its locus tab will be blank upon data import. No determinations will be made at a locus containing 5 alleles. If this occurs, the analyst must denote it in the traditional deconvolution workbook and on the electropherogram (e.g., No determinations made at this locus due to 5 alleles).

14.5.2.2 Resolved – 4 allele loci

14.5.2.2.1 All alleles are < STO.

14.5.2.2.1.1 This locus is considered unresolved. Entries will be as described in the unresolved method's 4 allele loci section.

14.5.2.2.2 No option meets the interpretation parameters.

14.5.2.2.2.1 This locus is considered unresolved. Entries will be as described in the unresolved method's 4 allele loci section.

14.5.2.2.3 More than one option meets the interpretation parameters.

14.5.2.2.3.1 This locus is considered unresolved. Entries will be as described in the unresolved method's 4 allele loci section.

14.5.2.2.4 One option meets the interpretation parameters (at least one allele is ≥ STO).

14.5.2.2.4.1 Allele RFU Assessment Table

14.5.2.2.4.1.1 Enter the major genotype that meets parameters (i.e., interpretation parameters) to cells K10 and K11.

14.5.2.2.4.1.2 Enter the minor genotype that meets parameters to cells M10 and M11.

14.5.2.2.4.2 Major Allele Pair(s) – RMP Table

14.5.2.2.4.2.1 Entry will be auto populated. No additional entries will be made.

14.5.2.2.4.3 Minor Allele Pair(s) – RMP Table

14.5.2.2.4.3.1 Entry will be auto populated. No additional entries will be made.

14.5.2.3 **Resolved – 3 allele loci:** How many options meet the interpretation parameters?

14.5.2.3.1 ***All alleles are < STO*** *– if an allele is ≥ STO, proceed with determining how many options meet the interpretation parameters.*

14.5.2.3.1.1 Allele RFU Assessment Table

14.5.2.3.1.1.1 Major – enter the allele with the largest RFU value to cell K10.

14.5.2.3.1.1.2 Minor – enter the allele with the smallest RFU value to cell M10.

14.5.2.3.1.2 Major Allele Pair(s) – RMP Table

14.5.2.3.1.2.1 Enter each allele as "Allele,+".

14.5.2.3.1.3 Minor Allele Pair(s) – RMP Table

14.5.2.3.1.3.1 Enter each allele as "Allele,+".

14.5.2.3.2 ***Resolved – Zero*:** This locus is considered unresolved. Entries will be as described in the unresolved method's 3 allele loci section.

14.5.2.3.3 ***Resolved – One*:** only 1 homozygous grouping option meets parameters where the major proportion is homozygous and the minor proportion is heterozygous *and* all other genotype options have at least one PHR that is < 23%.

14.5.2.3.3.1 Allele RFU Assessment Table

14.5.2.3.3.1.1 Enter the major genotype that meets parameters to cells K10 and K11.

14.5.2.3.3.1.2 Enter the minor genotype that meets parameters to cells M10 and M11.

14.5.2.3.3.2 Major Allele Pair(s) – RMP Table

14.5.2.3.3.2.1 Entry will be auto populated. No additional entries will be made.

14.5.2.3.3.3 Minor Allele Pair(s) – RMP Table

14.5.2.3.3.3.1 Entry will be auto populated. No additional entries will be made.

14.5.2.3.4 ***Resolved – One*****:** if the previously described one option meeting criteria is not met, this locus is considered unresolved. Entries will be as described in the unresolved method's 3 allele loci section.

14.5.2.3.5 ***Resolved – Two*****:** This locus is considered unresolved. Entries will be as described in the unresolved method's 3 allele loci section.

14.5.2.3.6 ***Resolved – Three*****:** 1 homozygote grouping option and 2 heterozygote grouping options meet the parameters. ***Note:*** *If the major evaluation resulted in an overall percent contribution of ≥ 75% but < 76%, this locus will be considered unresolved. Entries will be as described in the unresolved method's 3 allele loci section.*

14.5.2.3.6.1 Allele RFU Assessment Table

14.5.2.3.6.1.1 Enter the major genotype that meets parameters to cells K10 and K11.

14.5.2.3.6.1.2 Enter the consistent minor allele to only cell M10.

14.5.2.3.6.2 Major Allele Pair(s) – RMP Table

14.5.2.3.6.2.1 Entry will be auto populated. No additional entries will be made.

14.5.2.3.6.3 Minor Allele Pair(s) – RMP Table

14.5.2.3.6.3.1 Entry will be auto populated. No additional entries will be made.

14.5.2.3.7 ***Resolved – Three*****:** 2 homozygote grouping options and 1 heterozygote grouping option meets the parameters.

14.5.2.3.7.1 This locus is considered unresolved. Entries will be as described in the unresolved method's 3 allele loci section.

14.5.2.3.8 ***Resolved – ≥ Four*****:** This locus is considered unresolved. Entries will be as described in the unresolved method's 3 allele loci section.

14.5.2.4 Resolved – 2 allele loci

14.5.2.4.1 Resolved – All alleles are < STO

14.5.2.4.1.1 Allele RFU Assessment Table

14.5.2.4.1.1.1 Major – enter the allele with the largest RFU value to cell K10.

14.5.2.4.1.1.2 Minor – enter the allele with the smallest RFU value to cell M10.

14.5.2.4.1.2 Major Allele Pair(s) – RMP Table

14.5.2.4.1.2.1 No entries will be made.

14.5.2.4.1.3 Minor Allele Pair(s) – RMP Table

14.5.2.4.1.3.1 No entries will be made.

14.5.2.4.2 Resolved – At least one of the two alleles is ≥ STO

14.5.2.4.2.1 Allele RFU Assessment Table

14.5.2.4.2.1.1.1 Major – enter the allele with the largest RFU value to cell K10.

14.5.2.4.2.1.1.2 Minor – is the AB (major only) peak height ratio < 23%?

14.5.2.4.2.1.1.2.1 Yes – enter the allele with the smallest RFU value to cell M10

14.5.2.4.2.1.1.2.2 No – no entry for the minor.

14.5.2.4.2.2 Major Allele Pair(s) – RMP Table

14.5.2.4.2.2.1 If both alleles are ≥ STO enter all possible genotype combinations.

14.5.2.4.2.2.1.1 Example: 2 alleles observed all ≥ STO (e.g., 15, 16). Enter each of the following combinations.

14.5.2.4.2.2.1.1.1 --- 15,15

14.5.2.4.2.2.1.1.2 --- 16,16

14.5.2.4.2.2.1.1.3 --- 15,16

14.5.2.4.2.2.2 If one allele is < STO enter the genotype combinations as follows.

14.5.2.4.2.2.2.1 Example: 2 alleles observed with one allele* < STO (e.g., 15*, 16). Enter each of the following combinations.

14.5.2.4.2.2.2.1.1 --- 15,+

14.5.2.4.2.2.2.1.2 --- 16,+

14.5.2.4.2.3 Minor Allele Pair(s) – RMP Table

14.5.2.4.2.3.1 Entry will be auto populated. If entry is not auto populated, no entries will be made for the minor.

14.5.2.5 Resolved – 1 allele loci

14.5.2.5.1 Resolved – Allele ≥ STO

14.5.2.5.1.1 Allele RFU Assessment Table

14.5.2.5.1.1.1 Major – Enter allele to cells K10 and K11.

14.5.2.5.1.1.2 Minor – no entry for the minor.

14.5.2.5.1.2 Major Allele Pair(s) – RMP Table

14.5.2.5.1.2.1 Entry will be auto populated. No additional entries will be made.

14.5.2.5.1.3 Minor Allele Pair(s) – RMP Table

14.5.2.5.1.3.1 No entry for the minor.

14.5.2.5.2 Resolved – Allele < STO

14.5.2.5.2.1 No entries to allele RFU assessment table or the major/minor allele pair(s) – RMP tables.

14.5.3 Unresolved Method – meeting or not meeting the interpretation parameters is ignored. Instead, the unresolved method considers all genotype combinations while still focusing on peak height ratios, proportions, and mixture ratios (MR) to possibly restrict the developed RMP. See Appendix: Traditional Deconvolution Workbook for instructions.

14.5.3.1 Unresolved – 4 allele loci

14.5.3.1.1 Does each genotype combination contain at least one allele pair with a peak height ratio < 23%?

14.5.3.1.2 Yes

14.5.3.1.2.1 No entries will be made to the allele RFU assessment table or RMP tables at this locus. The PHRs suggest (note that Fusion can produce PHRs as low as 8.7% – see QMS WF 98598) the possibility of an additional contributor. Due to this locus, the data will be reported as "at least *X* contributors". For example, if the data was determined to contain 2 contributors, the data will now be reported as at least 2 contributors. The analyst will note that no determinations were made at the locus due to PHRs.

14.5.3.1.2.1.1 If the data was already considered to be at least 2 contributors due to this locus, the at least 2 NOC determination will be maintained. However, if this locus was not involved in the analyst's at least 2 contributors' determination, the NOC will move to 3. If the NOC moves to 3 the interpretation can be stopped and marked as, "Do not use: NOC moved to three contributors." Variations of this statement are acceptable if they convey the same meaning. The analyst may assess the data to determine if a Major Deconvolution can be performed. The analyst may also assess the case to determine if an Assumed Deconvolution is applicable.

14.5.3.1.3 No, all allele pairs of at least one genotype combination have peak height ratios ≥ 23%.

14.5.3.1.3.1 Move forward in instruction.

14.5.3.1.4 Allele RFU Assessment Table

14.5.3.1.4.1 Major – enter the two alleles with the largest RFU values to cell K10 and K11.

14.5.3.1.4.2 Minor – enter the two alleles with the smallest RFU values to cell M10 and M11.

14.5.3.1.5 Major Allele Pair(s) – RMP Table

14.5.3.1.5.1 Enter all remaining genotype combinations. PHRs, proportions and MRs will not be used to restrict genotypes.

14.5.3.1.5.1.1 For example: If the alleles are 10,12,14, and 16 and the 10,12 genotype has been auto populated, the analyst would enter the following genotype combinations.

14.5.3.1.5.1.1.1 --- 10,14

14.5.3.1.5.1.1.2 --- 10,16

14.5.3.1.5.1.1.3 --- 12,14

14.5.3.1.5.1.1.4 --- 12,16

14.5.3.1.5.1.1.5 --- 14,16

14.5.3.1.6 Minor Allele Pair(s) – RMP Table

14.5.3.1.6.1 Enter all remaining genotype combinations. PHRs, proportions and MRs will not be used to restrict genotypes.

14.5.3.1.6.1.1 For example: If the alleles are 10,12,14, and 16 and the 14,16 genotype has been auto populated, the analyst would enter the following genotype combinations.

14.5.3.1.6.1.1.1 --- 10,12

14.5.3.1.6.1.1.2 --- 10,14

14.5.3.1.6.1.1.3 --- 10,16

14.5.3.1.6.1.1.4 --- 12,14

14.5.3.1.6.1.1.5 --- 12,16

14.5.3.2 Unresolved – 3 allele loci

14.5.3.2.1 Does each genotype combination contain at least one allele pair with a peak height ratio < 23%?

14.5.3.2.2 Yes

14.5.3.2.2.1 No entries will be made to the allele RFU assessment table or RMP tables. The PHRs suggest (note that Fusion can produce PHRs as low as 8.7% – see QMS WF 98598) the possibility of an additional contributor. The data will be reported as "at least *X* contributors". For example, if the data was determined to contain 2 contributors, the data will now be reported as at least 2 contributors. The analyst will note that no determinations were made at the locus due to PHRs.

14.5.3.2.2.1.1 If the data was already considered to be at least 2 contributors due to this locus, the at least 2 NOC determination will be maintained. However, if this locus was not involved in the analyst's at least 2 contributors' determination, the NOC will move to 3. If the NOC moves to 3 the interpretation can be stopped and marked as, "Do not use: NOC moved to three contributors." Variations of this statement are acceptable if they convey the same meaning. The analyst may assess the data to determine if a Major Deconvolution can be performed. The analyst may also assess the case to determine if an Assumed Deconvolution is applicable.

14.5.3.2.3 No, all allele pairs of at least one genotype combination have peak height ratios ≥ 23%.

14.5.3.2.3.1 Move forward in instruction.

14.5.3.2.4 Allele RFU Assessment Table

14.5.3.2.4.1 Major – enter the allele with the largest RFU value to cell K10.

14.5.3.2.4.2 Minor – enter the allele with the smallest RFU value to cell M10.

14.5.3.2.5 Major Allele Pair(s) – RMP Table

14.5.3.2.5.1 Homozygous major paired with a heterozygous minor.

14.5.3.2.5.1.1 Enter all homozygote genotype combinations that have a major proportion paired with a heterozygous minor with a peak height ratio ≥ 23%.

14.5.3.2.5.1.1.1 If the genotype option's MR is < 2.2 also enter the minor genotype of this option to the table.

14.5.3.2.5.2 Heterozygous major paired with a homozygous minor.

14.5.3.2.5.2.1 Enter all heterozygote genotype combinations that have a major proportion paired with a homozygous minor where the major's peak height ratio is ≥ 23%.

14.5.3.2.5.2.1.1 If the genotype option's MR is < 2.2 also enter the minor genotype of this option to the table.

14.5.3.2.5.3 Heterozygous major paired with a heterozygous minor.

14.5.3.2.5.3.1 Enter all heterozygote genotype combinations that have a major proportion where the major and minor peak height ratios are ≥ 23%.

14.5.3.2.5.3.1.1 If the genotype option's MR is < 2.2 also enter the minor genotype of this option to the table.

14.5.3.2.6 Minor Allele Pair(s) – RMP Table (in addition to auto populated entry)

14.5.3.2.6.1 If two alleles are < STO, enter the remaining allele as "Allele,+". If this scenario is true, the remaining bullets in this section will be ignored.

14.5.3.2.6.2 Homozygous minor paired with a heterozygous major.

14.5.3.2.6.2.1 Enter all homozygote genotype combinations that have a minor proportion paired with a heterozygous major with a peak height ratio ≥ 23%. If the resulting homozygote genotype includes the allele in cell L60, it is already accounted for in the RMP and does not need to be added to the table.

14.5.3.2.6.2.1.1 If the genotype option's MR is < 2.2 also enter the major genotype of this option to the table. If the major genotype includes the allele in cell L60, it is already accounted for in the RMP and does not need to be added to the table.

14.5.3.2.6.3 Heterozygous minor paired with a homozygous major.

14.5.3.2.6.3.1 Enter all heterozygote genotype combinations that have a minor proportion paired with a homozygous major where the minor's peak height ratio is ≥ 23%. If the resulting heterozygote genotype(s) includes the allele in cell L60, it is already accounted for in the RMP and does not need to be added to the table.

14.5.3.2.6.3.1.1 If the genotype option's MR is < 2.2 also enter the major genotype of this option to the table. If the major genotype includes the allele in cell L60, it is already accounted for in the RMP and does not need to be added to the table.

14.5.3.2.6.4 Heterozygous minor paired with a heterozygous major.

14.5.3.2.6.4.1 Enter all heterozygote genotype combinations that have the minor proportion where the major and minor peak height ratios are ≥ 23%. If the resulting heterozygote genotype(s) includes the allele in cell L60, it is already accounted for in the RMP and does not need to be added to the table.

14.5.3.2.6.4.1.1 If a genotype option's MR is < 2.2 also enter the major genotype of this option to the table. If the major genotype includes the allele in cell L60, it is already accounted for in the RMP and does not need to be added to the table.

14.5.4 **Unrestricted Method** does not restrict genotypes utilizing PHRs, proportions or mixture ratios. Genotypes are determined qualitatively based on the alleles observed at each locus (i.e., all genotype combinations are considered possible) with consideration given to the STO. The unrestricted method results in the major and minor having identical genotypes (i.e., a mixture genotype), not a distinct major and minor genotype. The unrestricted method is allowed to be attempted on data containing a NOC of 2 or at least 2. The interpretation will be performed using the traditional deconvolution workbook. The workbook will clearly denote that the unrestricted method is being employed (e.g., noted on allele worksheet tab).

14.5.4.1 Are there any loci with complete dropout of information (excluding biological sex markers)?

14.5.4.1.1 Yes

14.5.4.1.1.1 If > 4 loci, the mixture will be deemed inconclusive due to limited/complex data. This must be documented within the case folder (e.g., documented on the relevant electropherogram).

14.5.4.1.1.2 If ≤ 4 loci, move to the next instruction.

14.5.4.1.1.2.1 We term this value (i.e., the number of loci with complete dropout) as **D.** This value (**D**) will be used in the next instruction.

14.5.4.1.2 No

14.5.4.1.2.1 Move to the next instruction.

14.5.4.2 Are there any one allele or two allele loci with all alleles < STO (do not consider biological sex markers)?

14.5.4.2.1 Yes

14.5.4.2.1.1 If > 4 loci, the mixture will be deemed inconclusive due to limited/complex data. This must be documented within the case folder (e.g., documented on the relevant electropherogram).

14.5.4.2.1.2 If ≤ 4 loci – we term this value (i.e., the number of one allele and two allele loci with all alleles < STO) as **B**.

14.5.4.2.1.3 Is the sum of **D** + **B** > 4 loci?

14.5.4.2.1.3.1 Yes – the mixture will be deemed inconclusive due to limited/complex data. This must be documented within the case folder (e.g., documented on the relevant electropherogram).

14.5.4.2.1.3.2 No – Move to the next instruction

14.5.4.2.2 No

14.5.4.2.2.1 Move to the next instruction.

14.5.4.3 Enter the following at each locus tab.

14.5.4.3.1 Allele RFU Assessment Table

14.5.4.3.1.1 Major – enter the allele with the largest RFU value to cell K10.

14.5.4.3.1.2 Minor – enter the allele with the smallest RFU value to cell M10. Check the "Check if no RMP for minor allele" box.

14.5.4.3.1.2.1 No minor entry at a 1 allele locus.

14.5.4.3.2 **Major Allele Pair(s) – RMP Table** – this table will be used to represent both the major and minor genotypes. Use the examples below to enter data to the table.

14.5.4.3.2.1 No entries will be made to the Minor Allele Pair(s) – RMP Table.

14.5.4.3.2.2 Example: 4 alleles observed (5, 7, 8, 9). Enter each of the following allele pair combinations. Note the "---" is used as a spacer from the bullet number to provide clarity to the example entry. The "---" will not be entered into the table.

14.5.4.3.2.2.1 --- 5,7

14.5.4.3.2.2.2 --- 5,8

14.5.4.3.2.2.3 --- 5,9

14.5.4.3.2.2.4 --- 7,8

14.5.4.3.2.2.5 --- 7,9

14.5.4.3.2.2.6 --- 8,9

14.5.4.3.2.3 Example: 3 alleles observed all ≥ STO (10, 11, 12). Enter each of the following combinations.

14.5.4.3.2.3.1 --- 10,10

14.5.4.3.2.3.2 --- 11,11

14.5.4.3.2.3.3 --- 12,12

14.5.4.3.2.3.4 --- 10,11

14.5.4.3.2.3.5 --- 10,12

14.5.4.3.2.3.6 --- 11,12

14.5.4.3.2.4 Example: 3 alleles observed with an allele* < STO (10*, 11, 12). Enter each of the following combinations.

14.5.4.3.2.4.1 --- 10,+

14.5.4.3.2.4.2 --- 11,11

14.5.4.3.2.4.3 --- 12,12

14.5.4.3.2.4.4 --- 11,12

14.5.4.3.2.5 Example: 3 alleles observed with two alleles* < STO (10*, 11*, 12). Enter each of the following combinations.

14.5.4.3.2.5.1 --- 10,+

14.5.4.3.2.5.2 --- 11+

14.5.4.3.2.5.3 --- 12,12

14.5.4.3.2.6 Example: 3 alleles observed with each allele* < STO (10*, 11*, 12*). Enter each of the following combinations.

14.5.4.3.2.6.1 --- 10,+

14.5.4.3.2.6.2 --- 11,+

14.5.4.3.2.6.3 --- 12,+

14.5.4.3.2.7 Example: 2 alleles observed all ≥ STO (15, 16). Enter each of the following combinations.

14.5.4.3.2.7.1 --- 15,15

14.5.4.3.2.7.2 --- 16,16

14.5.4.3.2.7.3 --- 15,16

14.5.4.3.2.8 Example: 2 alleles observed with one allele* < STO (15*, 16). Enter each of the following combinations.

14.5.4.3.2.8.1 --- 15,+

14.5.4.3.2.8.2 --- 16,+

14.5.4.3.2.9 Example: 2 alleles observed with each allele* < STO (15*, 16*). No entries to the major RMP table at this locus.

14.5.4.3.2.10 Example: 1 allele observed ≥ STO (20). Enter the following combinations.

14.5.4.3.2.10.1 --- 20,20

14.5.4.3.2.10.2 --- 20,+

14.5.4.3.2.11 Example: 1 allele* observed < STO (20*). No entries to the major RMP table at this locus.

14.5.5 Assessment of the major and minor. After deconvolution, the component averages must be assessed.

14.5.5.1 For results of the Unrestricted Method. If the Unrestricted Method was not performed continue to the next instruction.

14.5.5.1.1 Utilize the major component and minor component criteria below to assess the results of the unrestricted method. If either the major "component" or the minor "component" cannot be used, then the interpretation cannot be used. The workbook results will be marked as "Do not use: *Component(s)* below STO." Variations of this statement are acceptable if they convey the same meaning. The analyst may assess the case to determine if an Assumed Deconvolution is applicable.

14.5.5.2 Major Component – review the average per allele RFUs.

14.5.5.2.1 **If the number of contributors for the mixture is considered at least two**, all the major component dyes' average per allele RFUs must be ≥ the STOs. If one or more average per allele RFUs are < STO the interpretation cannot be used. The workbook results will be marked as "Do not use: major component below STO." Variations of this statement are acceptable if they convey the same meaning. The questioned profile may be suitable for the Major Deconvolution method. To make this determination, proceed to the Major Deconvolution method. The analyst may also assess the case to determine if an Assumed Deconvolution is applicable.

14.5.5.2.1.1 If the mixture's number of contributors is not considered at least two, please proceed forward with the instructions.

14.5.5.2.2 If the number of contributors for the mixture is considered two:

14.5.5.2.2.1 **If ≥ 1 average per allele RFU is > the STOs**, move forward to review the average per allele RFUs of the minor component.

14.5.5.2.2.2 **If all average per allele RFU are < the STOs**, the interpretation cannot be used. The questioned profile is too low level to deconvolute using the Traditional Deconvolution. The sample will be reported as at least 2 contributors. The Traditional Deconvolution results must be marked as "Do not use: major component below STO." Variations of this statement are acceptable if they convey the same meaning. The questioned profile may be suitable for the Major Deconvolution method. To make this determination, proceed to the Major Deconvolution method. The analyst may also assess the case to determine if an Assumed Deconvolution is applicable.

14.5.5.3 Minor Component – review the average per allele RFUs.

14.5.5.3.1 **If the number of contributors for the mixture is considered at least two**, all the minor component dyes' average per allele RFUs must be ≥ the STOs. If one or more average per allele RFUs are < STO the minor component cannot be used. The minor component is too low level to be certain it is comprised of alleles from only one individual. The interpreted major genotype may still be utilized for comparison. The Traditional Deconvolution results will be marked as "Minor component below STO. Minor genotype not used for comparisons." Variations of this statement are acceptable if they convey the same meaning. The analyst may also assess the case to determine if an Assumed Deconvolution is applicable.

14.5.5.3.1.1 If the mixture's number of contributors is not considered at least two, please proceed forward with the instructions.

14.5.5.3.2 If the number of contributors for the mixture is considered two:

14.5.5.3.2.1 **If ≥ 2 average per allele RFU are > the STOs**, the analyst may move forward with the interpretation.

14.5.5.3.3 **If < 2 average per allele RFU are < the STOs,** the interpretation for the minor component cannot be used. The minor component is too low level to be certain it is comprised of alleles from only one individual. The interpreted major genotype may still be utilized for comparison. The sample will be reported as at least 2 contributors. The Traditional Deconvolution results will be marked as "Minor component below STO. Minor genotype not used for comparisons." Variations of this statement are

acceptable if they convey the same meaning. The analyst may also assess the case to determine if an Assumed Deconvolution is applicable.

14.5.5.3.4 There are two **exceptions for the resolved method concerning differential fractions**. See the assumed deconvolution section for allowed assumptions using the E1 and/or E2 fractions that may assist with the results of the unrestricted method.

14.5.5.3.4.1 If all allele(s) in the minor component from a fraction (E1/E2) are included in the major component in the other fraction (E1/E2) that meets the criteria for use, the minor component can be used for comparison.

14.5.5.3.4.2 If all allele(s) in the minor component from a fraction (E1/E2) are included in the minor component in the other fraction (E1/E2) and one of the fractions meet the criteria for use, the minor component can be used for comparison.

14.5.5.3.4.3 If the differential exception is used, the NOC for each fraction will be X.

14.5.5.3.4.3.1 For example, if the analyst originally determines the E1 to be a NOC of 2 and the E2 to be a NOC of at least 2. Use of the differential exception in this scenario would result in both E1 and E2 being a NOC of 2, as the uncertainty in the E2 has been eliminated.

14.6 Sample Data: Assumed Deconvolution

14.6.1 When a contributor(s) can be reasonably expected to contribute their DNA to an evidence sample and the analyst determines this individual is included in the data an assumed deconvolution may be performed to separate the known individual(s) from the foreign alleles. A contributor is reasonably expected if at least one of the following apply:

14.6.1.1 The evidence sample is an intimate sample from the known individual. An evidence sample is deemed intimate in nature when it originates directly from the donor's body (e.g., fingernail clippings/swabbing, vaginal swabs, oral swabs, or a swabbing from any skin surface). An intimate sample is generally expected to yield DNA from the individual from whom the sample was collected.

14.6.1.2 The evidence profile was developed from clothing worn by the known individual. The clothing must be collected from the individual's body or identified by the individual as their clothing. Documented communication stating this must be maintained (e.g., MNPD 282 form, phone log, email, evidence label, etc.).

14.6.1.3 The sample was/is collected from an area, location, or item that the known individual is known to have occupied or touched (e.g., vehicle, residence, etc.). Documented communication stating this must be maintained (e.g., MNPD 282 form, phone log, email, evidence label, etc.).

14.6.1.4 The evidence profile originates from an alleged sexual assault case where a consensual partner(s) has been reported. Given the case scenario, the consensual partner may be considered a reasonably expected contributor.

14.6.2 An assumed deconvolution may be performed on evidence involving an alleged sexual assault using a single source profile obtained in one of the fractions. The single source profile obtained in either the E1 or E2 fraction can be used to help aid in mixture deconvolution of the corresponding fraction, if necessary, by assuming its presence. The profiles obtained from the two fractions originated from the same cutting, meaning there would be a reasonable expectation to obtain one individual's DNA in both fractions. Based on this expectation, any results from a single source fraction may be separated from a mixture result in the corresponding fraction to facilitate identification of the foreign individual's alleles.

14.6.3 The reasonably expected contributor's profile may be separated from the other mixture data to facilitate identification of the foreign alleles, a process termed "backing out". The remaining alleles can then be attributed to the foreign contributor using the instructions

in the procedure below. To "back out" the reasonably expected profile there must be called data at a minimum of 5 loci.

14.6.3.1 An assumed deconvolution of a mixture containing < 3 individuals will utilize the traditional deconvolution workbook, but meeting or not meeting the interpretation parameters is ignored. Save the traditional deconvolution file as "*Sample#_AssumedDecon_Initials*".

14.6.3.2 An assumed deconvolution of a mixture containing ≥ 3, but < 4 individuals can be performed when two contributors are reasonably expected. The deconvolution will be annotated on the electropherogram as instructed below.

14.6.4 **Assumed Deconvolution Method (< NOC 3):**

14.6.4.1 For mixtures containing < 3 individuals the analyst will use the traditional deconvolution workbook. The workbook will clearly denote that the assumed deconvolution method is being employed (e.g., noted on allele worksheet tab). The analyst must document what reference sample(s) or fraction(s) is being assumed. The assumption of the reasonably expected contributor's profile will be reflected in the report.

14.6.4.2 The analyst will assign the assumed individual's allele(s) to the assumed individual and the remaining allele(s) will be assigned to the foreign individual. Alleles belonging to the assumed individual will be wholly assigned to the assumed individual. Allele sharing between the assumed individual and the foreign individual will not be considered.

14.6.4.3 <u>Allele Entry – Assumed Individual</u>

14.6.4.3.1 The assumed individual's alleles will always (regardless of their proportion) be entered into the major cells of the allele RFU assessment table (K10 and K11). The stochastic threshold will be ignored when assigning the assumed individual's alleles.

14.6.4.3.2 Homozygous Assumed

14.6.4.3.2.1 If the assumed individual is homozygous and their allele is called, enter the allele into cells K10 and K11.

14.6.4.3.2.2 Major Allele Pair(s) RMP table – Entry will be auto populated. No additional entries will be made.

14.6.4.3.3 Heterozygous Assumed

14.6.4.3.3.1 If the assumed individual is heterozygous and their alleles are called, enter these alleles into cells K10 and K11.

14.6.4.3.3.2 Major Allele Pair(s) RMP table – Entry will be auto populated. No additional entries will be made.

14.6.4.3.4 Heterozygous Assumed with Dropout

14.6.4.3.4.1 If the assumed individual is heterozygous and only one of their alleles is called, enter the called allele into cell K10 only.

14.6.4.3.4.2 Major Allele Pair(s) RMP table – Enter the called allele to cell L52.

14.6.4.3.5 Complete Dropout of Assumed

14.6.4.3.5.1 If the assumed individual's alleles are not called, no entries to the allele RFU assessment table (K10 or K11) or the Major Allele Pair(s) RMP table will be made.

14.6.4.3.6 5 allele locus

14.6.4.3.6.1 If a locus contains 5 alleles, the locus tab will be blank. The analyst will use the justification box to describe the assumed individual's allele assignment. No assumed individual allele entries will be made to the K10 or K11 cells or the Major Allele Pair(s) RMP table. No foreign contributor determinations will be made at this locus.

14.6.4.3.7 Tri-Allele Assumed

14.6.4.3.7.1 At a locus containing a confirmed tri-allelic pattern the analyst will use the justification box to describe the assumed individual's allele assignment. No assumed individual allele entries will be made to the K10 or K11 cells or the Major Allele Pair(s) RMP table.

14.6.4.4 <u>Allele Entry – Foreign Individual</u>

14.6.4.4.1 The foreign individual's alleles will always (regardless of their proportion) be entered into the minor cells of the allele RFU assessment table (M10 and M11).

14.6.4.4.2 **If no foreign allele remains**, no entries will be made to the M10 or M11 cells or the Minor Allele Pair(s) RMP table.

14.6.4.4.3 **If a single foreign allele remains**, enter the allele into cell M10 only.

14.6.4.4.3.1 Minor Allele Pair(s) RMP table

14.6.4.4.3.1.1 If cell L60 has auto populated no additional entries will be made.

14.6.4.4.3.1.2 If cell L60 has not auto populated, enter the allele to cell L61.

14.6.4.4.4 **If two foreign alleles remain**, was a complete genotype for the assumed individual present at the locus (i.e., no allelic dropout of assumed individual)?

14.6.4.4.4.1 **If yes**, review the genotype combination that contains the assumed individual's genotype, do the two foreign alleles in this option have a PHR ≥ 23%? ***Note****: If this is a 5 allele locus that contains a confirmed tri-allelic pattern for the assumed individual the analyst must manually perform and document the PHR calculation of the two foreign alleles. The calculation must be documented in the case folder (e.g., document calculation on the relevant electropherogram*).

14.6.4.4.4.1.1 Yes

14.6.4.4.4.1.1.1 If this is a 5 allele locus containing a confirmed tri-allelic pattern for the assumed individual, enter the alleles into cells M10 and M11. For the Minor Allele Pair(s) RMP table the entry will be auto populated and no additional entries will be made.

14.6.4.4.4.1.1.2 If this is a 4 allele locus, enter the alleles into cells M10 and M11. For the Minor Allele Pair(s) RMP table the entry will be auto populated and no additional entries will be made.

14.6.4.4.4.1.1.3 If this is a 3 allele locus with at least one allele ≥ STO, enter the alleles into cells M10 and M11. For the Minor Allele Pair(s) RMP table the entry will be auto populated and no additional entries will be made.

14.6.4.4.4.1.1.4 If this is a 3 allele locus with all alleles < STO, enter the foreign allele with the smallest RFU value to cell M10. For the Minor Allele Pair(s) RMP table, enter one allele to cell L61 and the remaining allele to cell L62.

14.6.4.4.4.1.2 No

14.6.4.4.4.1.2.1 No foreign allele entries will be made to the M10 or M11 cells or the Minor Allele Pair(s) RMP table at this locus. The PHR suggests (note that Fusion can produce PHRs as low as 8.7% – see QMS WF 98598) the possibility of an additional contributor. Due to this locus, the data will be reported as "at least *X* contributors". For example, if the data was determined to contain 2 contributors, the data will

now be reported as at least 2 contributors. If this occurs more than once, the sub-bullet instruction will be implemented.

14.6.4.4.4.1.2.1.1 If the data was already considered to be at least 2 contributors due to this locus, the at least 2 NOC determination will be maintained. However, if this locus was not involved in the analyst's at least 2 contributors' determination, the NOC will move to 3. If the NOC moves to 3 the interpretation can be stopped and marked as, "Do not use: NOC moved to three contributors." Variations of this statement are acceptable if they convey the same meaning. The analyst may assess the data to determine if a Major Deconvolution can be performed.

14.6.4.4.4.2 **If no**:

14.6.4.4.4.2.1 Enter the foreign allele with the smallest RFU value to cell M10.

14.6.4.4.4.2.2 Minor Allele Pair(s) RMP table

14.6.4.4.4.2.2.1 If cell L60 has auto populated, enter the remaining allele to cell L61.

14.6.4.4.4.2.2.2 If cell L60 has not auto populated, enter one allele to cell L62 and the remaining allele to cell L62.

14.6.4.4.5 **If three foreign alleles remain**

14.6.4.4.5.1 No foreign allele entries will be made to the M10 or M11 cells or the Minor Allele Pair(s) RMP table at this locus. There is the possibility of an additional contributor. Due to this locus, the data will be reported as "at least *X* contributors". For example, if the data was determined to contain 2 contributors, the data will now be reported as at least 2 contributors.

14.6.4.4.5.1.1 If the data was already considered to be at least 2 contributors due to this locus, the at least 2 NOC determination will be maintained. However, if this locus was not involved in the analyst's at least 2 contributors' determination, the NOC will move to 3. If the NOC moves to 3 the interpretation can be stopped and marked as, "Do not use: NOC moved to three contributors." Variations of this statement are acceptable if they convey the same meaning. The analyst may assess the data to determine if a Major Deconvolution can be performed.

14.6.4.4.5.2 If three foreign alleles remain at more than one locus the profile must be interpreted as a NOC $X + 1$ (where X is the originally determined NOC). Given the new NOC determination the analyst must reevaluate the options for interpreting the question sample.

14.6.4.4.5.2.1 For example, if the original NOC determination was NOC 2, then the NOC will now be NOC 3. Interpretation options for NOC 3 may be utilized.

14.6.5 **Assumed Deconvolution Method (NOC 3 or at least 3):**

14.6.5.1 On the electropherogram, the analyst will annotate the two reasonably expected contributors whose presence is being assumed. This assumption will be reflected in the report.

14.6.5.2 **At each locus** the analyst will annotate the following:

14.6.5.2.1 The alleles assigned to assumed individual 1

14.6.5.2.1.1 Denote missing allele(s) with parenthesis (i.e., 12 (15)).

14.6.5.2.2 The alleles assigned to assumed individual 2

14.6.5.2.2.1 Denote missing allele(s) with parenthesis (i.e., 12 (15)).

14.6.5.2.3 Foreign alleles, if applicable.

14.6.5.2.3.1 **If no foreign allele(s) remains**, no foreign determination can be made.

14.6.5.2.3.2 **If one foreign allele remains** the allele will be treated as "Allele,+".

14.6.5.2.3.3 **If two foreign alleles remain**, the PHR (truncated to one decimal place) of the two alleles will be calculated and annotated at the locus.

14.6.5.2.3.3.1 If the PHR ≥ 23% the alleles will be treated as a heterozygous genotype.

14.6.5.2.3.3.2 If the PHR < 23% no foreign determinations will be made at this locus. The PHR suggests (note that Fusion can produce PHRs as low as 8.7% – see QMS WF 98598) the possibility of an additional contributor. Due to this locus, the data will be reported as "at least *X* contributors". For example, if the data was determined to contain 3 contributors, the data will now be reported as at least 3 contributors. If this occurs more than once, the sub-bullet instruction will be implemented.

14.6.5.2.3.3.2.1 If the data was already considered to be at least 3 contributors due to this locus, the at least 3 NOC determination will be maintained. However, if this locus was not involved in the analyst's at least 3 contributors' determination, the NOC will move to 4. If the NOC moves to 4 the interpretation can be stopped and annotated as, "Do not use: NOC moved to four contributors." Variations of this statement are acceptable if they convey the same meaning.

14.6.5.2.3.4 **If three foreign alleles remain**

14.6.5.2.3.4.1 No foreign determinations will be made at this locus. There is the possibility of an additional contributor. Due to this locus, the data will be reported as "at least *X*

contributors". For example, if the data was determined to contain 3 contributors, the data will now be reported as at least 3 contributors.

14.6.5.2.3.4.1.1 If the data was already considered to be at least 3 contributors due to this locus, the at least 3 NOC determination will be maintained. However, if this locus was not involved in the analyst's at least 3 contributors' determination, the NOC will move to 4. If the NOC moves to 4 the interpretation can be stopped and annotated as, "Do not use: NOC moved to four contributors." Variations of this statement are acceptable if they convey the same meaning.

14.6.5.2.3.4.2 If three foreign alleles remain at more than one locus the profile must be considered as a NOC ≥ 4. The interpretation can be stopped and annotated as, "Do not use: NOC moved to four contributors." Variations of this statement are acceptable if they convey the same meaning.

14.6.5.2.3.5. **If > three foreign alleles remain**

14.6.5.2.3.5.1 If > three foreign alleles remain at a locus the profile must be considered as a NOC ≥ 4. The interpretation can be stopped and annotated as, "Do not use: NOC moved to four contributors." Variations of this statement are acceptable if they convey the same meaning.

14.6.5.3 **At each dye channel** the analyst will annotate the following:

14.6.5.3.1 Foreign allele(s) RFU sum for the dye channel.

14.6.5.3.2 Foreign allele(s) RFU average per allele (rounded to one decimal place) for the dye channel.

14.6.5.3.3 An annotation stating the foreign allele RFU average is ≥ STO or < STO at the dye channel. The analyst will use this information to assess the foreign component as described in the next section of this procedure.

14.6.6 **Assessment of the foreign component.** Due to the assumption, when using the traditional deconvolution workbook, the STO results of the assumed individual's component averages (i.e., major column) will be ignored.

14.6.6.1 **If the number of contributors for the mixture is considered at least two or at least three**, all minor component dyes' average per allele RFUs must be ≥ the STOs. If one or more average per allele RFUs are < STO the interpretation of the foreign contributor cannot be used. The analyst may choose to use the assumed contributor's results (i.e., major). The workbook/electropherogram must clearly denote that the foreign

component results will not be used and provide justification (e.g., "Used for assumed profile only. Foreign component < STO and will not be used."). In this instance, the foreign component will be reported as inconclusive. If no results from the Assumed Deconvolution will be used, the analyst will mark the workbook/electropherogram as "Do not use: foreign component below STO." Variations of this statement are acceptable if they convey the same meaning. Lastly, the analyst may assess the data to determine if a Major Deconvolution can be performed.

14.6.6.1.1 If the mixture's number of contributors is not considered at least two or at least three, please proceed forward with the instructions.

14.6.6.2 If the number of contributors for the mixture is considered two or three:

14.6.6.2.1 **If ≥ 2 average per allele RFU are > the STOs**, the analyst may move forward with the interpretation.

14.6.6.2.2 **If < 2 average per allele RFU are < the STOs,** the interpretation for the foreign component cannot be used. The foreign component is too low level to be certain it is comprised of alleles from only one individual. If the NOC was determined to be X, the sample will be reported as at least X contributors. If the NOC was already determined to be at least X, this will be maintained.

14.6.6.2.2.1 For mixtures of < 3 individuals

14.6.6.2.2.1.1 The analyst may choose to use the assumed contributors results from the traditional deconvolution workbook. The workbook must clearly denote that the foreign component results will not be used and provide justification (e.g., "Used for assumed profile only. Foreign component < STO and will not be used."). The foreign component will be reported as inconclusive due to limited/complex data.

14.6.6.2.2.1.2 The analyst may choose to proceed to the Major Deconvolution method. The traditional deconvolution workbook will be marked as "Do not use: foreign component below STO." Variations of this statement are acceptable if they convey the same meaning.

14.6.6.2.2.2 For mixtures of ≥ 3, but < 4 individuals

14.6.6.2.2.2.1 The analyst will annotate the electropherogram with, "Do not use foreign component. Foreign component is below STO." Variations of this statement are acceptable if they convey the same meaning. The foreign component will be reported as inconclusive due to limited/complex data.

14.6.6.2.2.2.2 Where applicable, the analyst may choose to proceed to the Major Deconvolution method. The analyst will annotate the electropherogram with, "Do not use: foreign component below STO." Variations of this statement are acceptable if they convey the same meaning.

14.6.6.3 There are two exceptions for differential fractions (specific to mixtures of < 3 contributors).

14.6.6.3.1 If all allele(s) in the foreign minor component from a fraction (E1/E2) are included in the foreign major component in the other fraction (E1/E2), the foreign minor component can be used for interpretation.

14.6.6.3.2 If all allele(s) in the foreign minor component from a fraction (E1/E2) are included in the foreign minor component in the other fraction (E1/E2) and one of the fractions meet the criteria for use, the foreign minor component can be used for interpretation.

14.6.6.3.3 If the differential exception is used, the NOC for each fraction will be X.

14.6.6.3.3.1 For example, if the analyst originally determines the E1 to be a NOC of 2 and the E2 to be a NOC of at least 2. Use of the differential exception in this scenario would result in both E1 and E2 being a NOC of 2, as the uncertainty in the E2 has been eliminated.

14.7 Sample Data: Major Deconvolution: Use the steps below for directions on how to enter the discerned major component into the major deconvolution workbook. Save the major deconvolution file as "*Sample#_MajorDecon_Initials"*.

14.7.1 If the mixture contains a visually major contributor the major deconvolution workbook can be used to objectively support the interpretation of the major contributor and no determinations will be drawn from the minor alleles (i.e., minor(s) is inconclusive).

14.7.2 Within the Major Deconvolution Workbook follow the directions for automated entry of data (manual entry has not been validated).

14.7.3 Allele Entry:

14.7.3.1 Enter the proposed major contributor alleles into cells A13 and A14.

14.7.3.1.1 **If there is complete dropout** (i.e., no alleles at the locus) the analyst will type "INC" (lowercase/uppercase variations are accepted in formula) into cells A13 and A14. A justification will be provided for the inconclusive determination.

14.7.3.1.2 **The proposed major allele(s) must be ≥ STO. If the proposed major allele(s) are < STO**, the locus will be deemed inconclusive. The analyst will type "INC" (lowercase/uppercase variations are accepted in formula) into cells A13 and A14. A justification will be provided for the inconclusive determination.

14.7.3.1.3 **Homozygous proposals** will be entered as a genotype (e.g., 13,13).

14.7.3.1.3.1 If there is only one allele at a locus ≥ the AT, this allele is ≥ the STO, and this allele is thought to be contributed by the major (i.e., major is homozygous), the analyst will enter the allele as a genotype (e.g., 13,13) into the workbook as the proposed major contributor.

14.7.3.1.3.2 The RMP is calculated as the interpretation is performed. The 2p (i.e., "Allele,+") will not be used.

14.7.3.1.4 **Heterozygous proposals** will be entered as a genotype (e.g., 13,15).

14.7.3.1.4.1 If there are only two alleles ≥ the AT, these two alleles are ≥ the STO, and these alleles are thought to be contributed by the major (i.e., major is heterozygous), the analyst will enter the alleles into the workbook as the proposed major contributor.

14.7.3.1.4.2 The RMP is calculated as the interpretation is performed. The 2p (i.e., "Allele,+") will not be used.

14.7.3.1.4.3 **Amelogenin** is entered on the 'Allele Worksheet' tab of the major deconvolution workbook. See the Biological Sex Determining Markers section for further information on entry.

14.7.3.1.5 **DYS391** is not included in the major deconvolution method. No determinations will be made at this locus if more than one allele is present at DYS391.

14.7.3.2 **CAUTION**

14.7.3.2.1 **Degraded Data**

14.7.3.2.1.1 Analysts should use caution when entering major proposals for data with apparent degradation. Caution should especially be taken at two allele loci where one allele is > STO and the other is < STO *and* the PHR between these two alleles is ≥ 23% but ≤ 50%. If degradation is thought to interfere with the possible major profile the analyst may deem the locus as inconclusive. The analyst would type "INC" (lowercase/uppercase variations are accepted in formula) into cells A13 and A14. A justification will be provided for the inconclusive determination.

14.7.3.2.2 **Stochastic amplification**

14.7.3.2.2.1 If the sample's contributors are at/or around the STO (e.g., the traditional deconvolution resulted in a dye or multiple dyes below the STO for *both* major and minor component) caution should be taken at two allele loci where one allele is > STO and the other is < STO *and* the PHR between these two alleles is ≥ 23% but ≤ 50%. During the validation there was one such instance that resulted in the incorrect genotype determination at a locus. Due to the stochastic nature of this validation sample only 8 loci were determined. If this scenario occurs the analyst should attempt to reamplify the sample for a higher template, if possible. The analyst also has the discretion to deem this locus inconclusive. The analyst would type "INC" (lowercase/uppercase variations are accepted in formula) into cells A13 and A14. A justification will be provided for the inconclusive determination.

14.7.4 Locus Tab Review:

14.7.4.1 After allele entry, review the calculated major percent contribution (cell A18) and the PHRs within the 'List of Possible Genotypes' table.

14.7.4.2 **If the major percent contribution and PHRs meet the following thresholds**, then the major contributor for the locus is determined. If *any* of the following thresholds are not met, the locus will be deemed inconclusive.

14.7.4.2.1 The proposed major contributor alleles must have a percent contribution of ≥75% and;

14.7.4.2.2 The proposed major contributor alleles must have a PHR ≥60% and;

14.7.4.2.3 The heterozygous genotype combinations including an allele in the proposed major contributor must have a PHR ≤50%. *Note that the '>50%' column on the 'List of Possible Genotypes' table indicates when a PHR is ≥51%.*

14.7.4.2.3.1 If the proposed major contributor is heterozygous, the homozygous genotype combination of the proposed alleles will have a PHR of 100%. The homozygous genotype combinations will be ignored.

14.7.4.3 If a locus is deemed inconclusive, the analyst must type "INC" (lowercase/uppercase variations are accepted in formula) into cells A13 and A14. A justification must be provided for the inconclusive determination. When an analyst has deemed a locus inconclusive, the resulting major percent contribution does not constitute an interpretation of the major genotype at this locus. No RMP frequency will be calculated for the major genotype at the locus.

14.7.4.3.1 By typing "INC" into cells A13 and A14, the workbook will use the percent contribution for the heterozygous combination with the greatest percent contribution as the Major Percent Contribution if that combination has a PHR ≥ 50%. This is done to best assess the overall major percent contribution.

14.7.4.3.1.1 If the heterozygous combination with the greatest percent contribution has a PHR <50%, then the workbook will use the percent contribution of the most plausible homozygous major genotype (only the allele with the greatest RFUs). This is done to best assess the overall major percent contribution.

14.7.5 Results Assessment

14.7.5.1 The "Overall Contribution" tab of the Major Deconvolution Workbook lists each locus' major percent contribution. The overall major percent contribution is calculated by averaging the major percent contribution from each locus, except for loci containing a 0% or a 100% major contribution. This is done to prevent an artificial deflation or inflation of the overall major percent contribution. The workbook will do this automatically when the major percent contribution of a locus is 0% or 100%.

14.7.5.2 If the overall major percent contribution is ≥ 75% *and* ≥ 7 loci have been determined, the deconvolution results may be used for comparison to a reference sample.

14.7.5.2.1 When reviewing the profile, if the major component is at or around the STOs, the analyst has the discretion to call the questioned profile inconclusive even in instances with an overall major percent contribution ≥ 75% and ≥ 7 loci have been determined. If the analyst chooses to deem the questioned profile inconclusive the workbook results will be marked as do not use and a justification will be documented on the allele worksheet tab.

14.7.5.3 If the overall major percent contribution is < 75% *or* < 7 loci have been determined, the questioned profile must be deemed inconclusive. The workbook results will be marked as do not use and a justification will be documented on the allele worksheet tab.

14.8 Biological Sex Determining Markers

14.8.1 If a questioned profile appears to be from a biologically female individual, but there is a 'Y' allele at the Amelogenin locus or an allele at the DYS391 locus, this allele constitutes a second individual.

14.8.2 Amelogenin

14.8.2.1 The Amelogenin locus will not be deconvoluted using the deconvolution methods previously described. Amelogenin will be interpreted by the qualitative presence of each allele; even after subtracting a reasonably expected contributor's DNA profile, the full XX or XY can be attributed to the foreign contributor.

14.8.2.1.1 Single Source Data

14.8.2.1.1.1X only – can be determined to be X,X.

14.8.2.1.1.2X,Y – can be determined to be X,Y.

14.8.2.1.2 Mixture Data

14.8.2.1.2.1X only – the major can be determined to be X,X.

14.8.2.1.2.1.1 Before assigning X,X to the minor at Amelogenin, the analyst should determine if dropout of a Y allele is possible given the data. If dropout is considered a possibility, the minor will be undetermined at Amelogenin.

14.8.2.1.2.2X,Y – the major/minor can be determined to be only X,Y *OR* X,X and X,Y.

14.8.2.2 Amelogenin is not included in frequency calculations.

14.8.3 DYS391

14.8.3.1 DYS391 will undergo mixture interpretation as follows:

14.8.3.1.1 When performing the traditional deconvolution method:

14.8.3.1.1.1 If the PHR is ≤ 25%, the major and minor alleles can be determined.

14.8.3.1.1.2 If the PHR is >25%, the locus shall be deemed inconclusive.

14.8.3.1.2 When performing the assumed deconvolution method:

14.8.3.1.2.1 The analyst will pull out the assumed person's allele and the remaining allele will be assigned to the foreign individual.

14.8.3.1.3 When performing the major deconvolution method:

14.8.3.1.3.1 DYS391 is not included in the major deconvolution method. This locus will be deemed inconclusive if more than one peak is present.

14.8.3.2 DYS391 is not included in frequency calculations.

14.9 Comparison to Reference(s)

14.9.1 When comparing a reference profile to a questioned/evidence DNA profile the following terms will be used when drawing a conclusion.

14.9.1.1 ***Included*** – Statistical analysis may be performed to support an inclusion. Refer to the Statistical Analysis section.

14.9.1.1.1 ***Match:*** The question DNA profile is a complete profile and the DNA profile of the reference individual is the same at all loci tested.

14.9.1.1.2 ***Consistent with:*** The question DNA profile is a partial DNA profile where greater than 5 loci have been detected. The DNA profile of the reference individual is concordant with the DNA profile of the question sample at the loci detected.

14.9.1.2 ***Excluded:*** The reference profile will be deemed excluded as a contributor to the questioned profile if the DNA profile of the reference individual is different from the obtained/deduced question profile. Non-concordance at a single locus may not result in an overall exclusion when the difference may be explained scientifically (e.g., the possibility of drop-out, drop-in, stochastic amplification, degradation affecting PHR balance, etc.). In those instances, if the discrepancy can be scientifically explained/justified, the analyst can drop the locus in question from the frequency estimated and proceed with reporting the inclusion. The reasons for dropping the locus must be thoroughly, methodically, and explicitly explained and documented in the case folder.

14.9.1.3 ***Inconclusive comparison:*** An analyst may render a comparison inconclusive. This conclusion should be reserved for data that exhibits poor quality (i.e., degradation, inhibition, peak heights below stochastic threshold, peak height balance less than 50%,

etc.). An inconclusive comparison may occur when the non-concordance is observed in activity below stochastic threshold at no more than 2 loci. The non-concordance must be scientifically supported (e.g., elevated artifact, drop-in, drop-out, masked alleles in a deduction, degradation affecting PHR, stochastic amplification, etc.).

14.9.1.3.1 **Note**: For reports issued in the simplified table format, comparison conclusions will be reported by placing the name of the individual in the "Included" or "Excluded" columns.

14.10 Limitations

14.10.1 The deconvolution procedures described above are a model to interpret evidence data based on a validation data set created to imitate casework data. All models have limitations, and a validation data set cannot represent all iterations of casework data.

14.10.2 When a probative result is obtained for an evidence sample and partial profile is obtained for a reference sample, the reference sample will be re-typed, re-amplified, and/or re-extracted to produce a complete DNA profile (see Review of Casework Reference Sample Data in section 13) . To the extent possible, complete DNA typing results will be obtained for reference samples. If a partial and/or low-level profile from the reference standard must be used for comparison, locations where the analyst is confident that the known contributor is fully represented will be used for comparison. Locations exhibiting a single allele must be above stochastic threshold.

14.11 References

14.11.1 Internal Validation of Promega PowerPlex® Fusion using a Veriti Thermal Cycler and 3500 Series Genetic Analyzer (August 2014), Sorenson Forensics at MNPD-CL Forensic Biology Unit.

14.11.2 QMS Workflow 98598 – Fusion 5C Stochastic Threshold and Peak Height Ratio Reassessment Validation Study

14.11.3 QMS Workflow 109682 – Binary Mixture Interpretation Validation Study

14.11.4 Forensic Biology Quality Manual; Section 9.0 – Analytical Procedures

14.11.5 USACIL Advanced DNA Mixture Interpretation Workshop. May 5-7, 2014. Defense Forensic Science Center, Atlanta, GA

14.11.6 Scientific Working Group on DNA Analysis Methods. *Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories*. Approved 12 Jan. 2017, rev.

13 July 2021, SWGDAM, www.swgdam.org/publications/interpretation-guidelines-for-autosomal-str-typing-by-forensic-dna-testing-laboratories

14.11.7 National Research Council. The Evaluation of Forensic DNA Evidence (NRCII). Washington, D.C: National Academy, 1996.

15. Statistical Analysis

15.1 Scope

15.1.1 To describe the process of calculating the statistical frequency of evidentiary DNA profiles. Statistical analysis aids in the assessment (i.e., understanding the statistical weight) of an inclusion.

15.2 Equipment/Materials/Reagents

15.2.1 MNPD Statistical Workbook

15.2.2 Traditional Deconvolution excel file

15.2.3 Major Deconvolution excel file

15.3 Procedure

15.3.1 Random Match Probability (RMP)

15.3.1.1 The Random Match Probability (RMP) is a DNA profile rarity estimate. The RMP is the probability that the DNA of a randomly (unrelated) chosen person has the same profile as the DNA of an evidentiary sample. An RMP is the estimated frequency at which a particular profile would be expected to occur in a defined population group.

15.3.1.2 An RMP may be used when the number of contributors has been determined.

15.3.2 Formulae

15.3.2.1 Allele frequencies are as described in Hill et al. "Allele Frequency Values for Unrelated U.S. Population Samples (N=1036) at 29 Autosomal STR loci in Commercial STR Multiplex Kits." FSI: Genetics, 7 (2013) e82-e83. Further updates to this database are described in QMS WF 35074 – Forensic Biology DNA Stats Workbook Update Performance Check.

15.3.2.2 The genotype frequency associated with a particular pattern of alleles from a sample is based upon principles of Hardy-Weinberg equilibrium.

15.3.2.2.1 If a single source sample and/or a deconvoluted component under analysis demonstrates two different alleles (i.e., heterozygous), the genotypic frequency at a particular locus is determined by the equation 2pq, where p and q represent the frequencies of allele #1 and #2.

15.3.2.2.2 If a single source sample and/or a deconvoluted component under analysis consists of the same allele (i.e., homozygous), the genotypic frequency at a particular locus is

determined by the equation $p^2 + p(1-p)\theta$, where $\theta = 0.01$ and p represents the frequency of the allele.

15.3.2.3 If there are ≤ 5 occurrences of an allele in the database, the frequency of this allele will be the minimum allele frequency. The minimum allele frequency is calculated using 5/2N where N is the number of individuals sampled in the three separate ethnic groups (Caucasians, Hispanics, and African-Americans) from the database.

15.3.2.4 2p Rule (Allele,+)

15.3.2.4.1 For single alleles (i.e., Allele, +), the formula 2p will be applied, instead of $p^2 + p(1-p)\theta$, where $\theta = 0.01$ and p represents the frequency of the allele. This formula calculates for an "allele plus any" genotype combination in the event the sister allele has dropped out (e.g., allele < STO) or been filtered out as stutter. An assumption of contributors must be made for the 2p rule to be applied.

15.3.2.5 An RMP may be determined for a single source sample, deconvoluted components of a mixture, or mixtures using the product rule.

15.3.3 Modified Random Match Probability (mRMP)

15.3.3.1 At a locus, when there is one determined genotype, the RMP calculation is the probability for the genotype representing the possible contributor to a sample under the assumption of a defined number of contributors.

15.3.3.2 At a locus, when there is more than one determined genotype (i.e., allele pair) after mixture deconvolution, the RMP calculation (also referred to as a "modified RMP" or "mRMP") is the sum of the probabilities for all allele pairs representing the possible contributor(s) to a sample under the assumption of a defined number of contributors.

15.3.3.3 In addition to assumptions of the number of contributors, quantitative peak height information and mixture ratio/proportion assessments may be included in the interpretation and statistical support for the inclusion of a reference individual in an evidentiary profile. Calculations performed using interpretations incorporating this information are termed "restricted" (i.e., resolved and unresolved methods). When this quantitative peak height information is not included, the resultant calculation is termed "unrestricted" (i.e., unrestricted method).

15.3.3.3.1 The unrestricted method's RMP (i.e., unrestricted RMP) is utilized to represent the probability of randomly selecting an unrelated person from the population who could be a contributor to the DNA mixture.

15.3.3.4 At minimum, the loci and the assumptions used for statistical calculations must be documented in the case folder.

15.3.4 MNPD Statistical Workbook

15.3.4.1 Analysts can perform the statistical analysis using the validated Excel based program capable of calculating both standard RMP and mRMP utilizing the formulae described in the preceding sections of this technical procedure.

15.3.4.1.1 The statistical workbook is incorporated into the Traditional and Major Deconvolution excel files and calculates the RMP as the interpretation is performed. Interpretation determinations in these workbooks automate allele entries to the statistical workbook. No manual allele entries are necessary.

15.3.4.2 The statistical workbook also exists as a stand-alone version and may be used to calculate the RMP. Manual entry is required.

15.3.4.2.1 Open the MNPD Statistical Workbook, complete the case number, item number, and date fields at the top of the Entry page.

15.3.4.2.2 Enter the allele calls for the evidentiary sample into the corresponding boxes (Alleles 1 & 2) of the loci. If you are entering a homozygous genotype, you must enter the allele twice (Allele 1 & 2).

15.3.4.2.3 If multiple genotypic combinations are possible at a locus, utilize locations 1 through 6* for entry of these genotypes. The statistical workbook will calculate the genotypic frequency at each and sum them together to get a combined locus frequency to be applied using the product rule. If the sum of the frequencies is greater than 1, then a value of 1 will be given to the locus.

15.3.4.2.3.1 ***NOTE**: In the stand-alone workbook the maximum number of genotypes that can be entered per locus is six. In the Traditional Deconvolution excel file the maximum number of genotypes that can be entered per locus is seven. In the Major Deconvolution excel file the maximum number of genotypes that can be entered per locus is one.

15.3.4.2.4 If allelic dropout is possible (e.g., allele < STO), the statistical workbook will utilize the 2p calculation when the analyst enters the obligate allele in the first box location (Allele 1) and enters a + in the second box (Allele 2). After entry of the + symbol, press Enter or the Tab key to move to the next cell.

15.3.4.2.4.1 **NOTE**: Use the Tab key to progress after a + entry. If you use the mouse to click into another cell within the statistical workbook after entering a +, Excel will treat this as development of a formula. If this occurs, hit Esc to exit.

15.3.4.3 The bottom of the Entry page/Major RMP tab/Minor RMP tab will display the calculated profile frequencies for the Caucasian, African-American, and Hispanic populations from the referenced Hill et al database. The Entry page/Major RMP tab/Minor RMP tab should be converted to PDF for collection into the electronic casefile. The most common statistical frequency of the three populations will be selected for reporting in the final case report.

15.3.4.3.1 For the stand-alone version, once this page has been saved as a PDF, the Clear button at the top left corner of the Entry page can be clicked to clear out all of the entered data to prepare the statistical workbook for entry of new data (including case number, item number, and date).

15.4 Limitations

15.4.1 Statistical calculations for the results of each comparison in which a match or inclusion was made must be reported, except when the comparison is to a reasonably expected contributor.

15.4.2 Statistical calculations for more than one test may be reported together only if the results of those calculations are identical to each other.

15.5 References

15.5.1 Performance Check of a Statistical Calculations Workbook (December 2014), MNPD-CL Forensic Biology Unit.

15.5.2 ASCLD/LAB Board of Directors Clarification. Special Notice about Reporting DNA Test Results. 5/16/2014.

15.5.3 Hill et al. “Allele Frequency Values for Unrelated U.S. Population Samples (N=1036) at 29 Autosomal STR loci in Commercial STR Multiplex Kits.” FSI: Genetics, 7 (2013) e82-e83.

15.5.4 Scientific Working Group on DNA Analysis Methods (SWGDAM). "SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories." 14 Jan. 2010. Web. <swgdam.org>.

15.5.5 Scientific Working Group on DNA Analysis Methods (SWGDAM). "SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories." 13 July 2021. Web. <swgdam.org>.

15.5.6 Steffen et al. “Corrigendum to 'U.S. Population Data for 29 Autosomal STR Loci' [FSI; Genetics 7(2013)e82-e83].” FSI: Genetics, 31(2017) e36-e40.

15.5.7 QMS WF 35074 – Forensic Biology DNA Stats Workbook Update Performance Check

15.5.8 National Research Council. The Evaluation of Forensic DNA Evidence (NRCII). Washington, D.C: National Academy, 1996.

16. Forensic Biology Report Writing

16.1 Scope

16.1.1 To describe the process for reporting results from testing items of evidence

16.2 Equipment/Materials/Reagents

16.2.1 Completed Notes, Worksheets, and Interpreted DNA Data

16.2.2 LIMS software

16.3 Procedure

16.3.1 Access the LIMS software and open the case for which the Forensic Biology report will be drafted. Click on the REQUESTS tab. Right click on the request and select RELATED INDIVIDUALS. Highlight all suspects and victims listed on 282(s) in Available Persons. Click the double down arrow button to move the names to the Related Persons field and click OK.

16.3.2 For information in table format

16.3.2.1 Access the LIMS software and open the case for which the Forensic Biology report will be drafted. Click on the EVIDENCE tab. Right click on the parent item for entry of serological results and right click on the child item for the DNA result entry. Verify that the EVIDENCE TYPE has been selected and click on the … button to begin the entry for results.

16.3.2.1.1 Use the - to clear the box. Do not use this as a “not applied” or “not tested” option.

16.3.2.2 Screening Results

16.3.2.2.1 For serology results, do not enter results under the SEROLOGY heading, only enter under the SCREENING heading. Use the dropdown menu to select the results for the item.

16.3.2.2.2 Report serology results under the parent item, along with the metrics for that item. If no serology was performed for the case, leave boxes under SCREENING blank; do not enter N/A. This will ensure that the Serology Table does not show up on the report.

16.3.2.2.3 Table format for SCREENING:

Column Headings	**Detection of Possible Biological Stains:** *[Choose one of the following]*	**Blood:** *[Choose one of the following]*	**SEMEN:** *[Choose one of the following]*	**Forwarded for DNA:** *[Choose one of the following]*
SELECTIONS	Not tested	Not tested	Not tested	N/A
	Stain detected *(ALS positive – absorption or fluorescence)*	Identified *(HemaTrace positive)*	Not identified *(All tests performed negative)*	Yes *(this should only be used when stain/area referenced in previous columns is forwarded to DNA)*
	No stain detected *(ALS negative)*	Not identified *(all tests performed negative)*	Presumptive positive, confirmatory negative *(AP and/or p30 positive*, micro negative)*	No
	INC due to background interference	Presumptive positive, confirmatory negative *(KM positive, HemaTrace negative)*	Presumptive positive, not confirmed *(AP and/or p30 positive*, micro not performed)*	
		Presumptive positive, not confirmed *(KM positive, HemaTrace not performed)*	Sperm identified	
		Presumptive inconclusive, confirmatory negative	Presumptive positive, confirmatory inconclusive	
		Presumptive inconclusive, not confirmed	Presence of sperm inconclusive	

* If only one semen presumptive test is positive, still report as presumptive positive using the paragraph form.

16.3.2.3 DNA Results

16.3.2.3.1 Report DNA results under the child item. All boxes under DNA must have data. Do not leave any boxes empty (at minimum N/A can be entered).

16.3.2.3.2 Table format for DNA:

16.3.2.3.2.1**Note**: All boxes must have an entry (N/A, major, minor, etc.) if the item was tested for DNA. No boxes are to be left blank. If you need to clear results for an item, select - in all boxes for item.

Column Headings	**Y-Screen Results:** *[Choose one of the following]*	**STR Results:** *[Choose one of the following]*	**Contributors Assumed (#):** *[Choose one of the following]*	**Persons Reasonably Expected:** *[free type – must enter text (ie. name or N/A)]*	**Profiles for Comparison:** *[Choose one of the following]*	**Exclusions:** *[Free type – must enter text (i.e., name or N/A). If reporting major and/or minor, specify (i.e., Major: Name).]*	**Inclusions:** *[Free type – must enter text (i.e., name or N/A). If reporting major and/or minor, specify (i.e., Major: Name).]*	**Statistic:** *[Free type – must enter text (i.e., stat or N/A). If reporting major and/or minor, specify (i.e., Major: For XX loci, approximately 1 out of…." Or Major: For XX loci, rarer than 1 out of 100 trillion).]*	**Profiles Uploaded to CODIS:** *[Choose one of the following]*
SELECTIONS	Negative	Mixture	1		Foreign				Not applied *(eligible but not uploaded, another sample chosen)*
	No determinations	No determinations	1 with possible additional contributor		Major & minor				Not eligible

Column Headings	**Y-Screen Results:** *[Choose one of the following]*	**STR Results:** *[Choose one of the following]*	**Contributors Assumed (#):** *[Choose one of the following]*	**Persons Reasonably Expected:** *[free type – must enter text (ie. name or N/A)]*	**Profiles for Comparison:** *[Choose one of the following]*	**Exclusions:** *[Free type – must enter text (i.e., name or N/A). If reporting major and/or minor, specify (i.e., Major: Name).]*	**Inclusions:** *[Free type – must enter text (i.e., name or N/A). If reporting major and/or minor, specify (i.e., Major: Name).]*	**Statistic:** *[Free type – must enter text (i.e., stat or N/A). If reporting major and/or minor, specify (i.e., Major: For XX loci, approximately 1 out of…" Or Major: For XX loci, rarer than 1 out of 100 trillion).]*	**Profiles Uploaded to CODIS:** *[Choose one of the following]*
	Not applied	No result *(No data above AT)*	2		Major only, minor data too limited				One time search only
	Positive	Not tested	3		Mixed foreign				Yes
	Positive/NFT	Single source	4+		Mixture				Yes – major & minor
		Stop at quantitation	At least 2		N/A				Yes – major & partial minor
			At least 3		No foreign				Yes – major only
			N/A		None – Data too limited				Yes – partial

Column Headings	**Y-Screen Results:** *[Choose one of the following]*	**STR Results:** *[Choose one of the following]*	**Contributors Assumed (#):** *[Choose one of the following]*	**Persons Reasonably Expected:** *[free type – must enter text (ie. name or N/A)]*	**Profiles for Comparison:** *[Choose one of the following]*	**Exclusions:** *[Free type – must enter text (i.e., name or N/A). If reporting major and/or minor, specify (i.e., Major: Name).]*	**Inclusions:** *[Free type – must enter text (i.e., name or N/A). If reporting major and/or minor, specify (i.e., Major: Name).]*	**Statistic:** *[Free type – must enter text (i.e., stat or N/A). If reporting major and/or minor, specify (i.e., Major: For XX loci, approximately 1 out of…" Or Major: For XX loci, rarer than 1 out of 100 trillion).]*	**Profiles Uploaded to CODIS:** *[Choose one of the following]*
			No determinations		None – Data too complex				Yes – partial major & minor
					None – No interpretable data				Yes – partial major & partial minor
					Single source				Yes – partial major only
					Major only, minor data too complex				Yes – partial minor only

16.3.3 For information in paragraph format

16.3.3.1 Paragraph format will be used when the table cannot completely and/or accurately communicate results to the customer. Examples of when the paragraph format may be necessary include, but are not limited to, CODIS requests, Supplemental requests, and to report the submission or comparisons from reference standards. Often reports will include both table and paragraph information; however, the results should not be redundant.

16.3.3.2 Right click on the request and select EDIT FINDINGS. Right click on the request related to the results. Select ADD RESULT. Below are autotext statements and their codes. Other statements may be used in order to accurately report results and conclusions.

Circumstance	Autotext Code	Statement
When previous Forensic Biology Reports have been issued under the same CL #	SUPP	Refer to the official MNPD Forensic Biology Report(s) dated _____ for previous results.
When liquid blood is submitted, analyzed, and a portion of the blood is retained in dried form	LBT	A bloodstain card was prepared from a sample of Item(s)….
When liquid blood is submitted, not analyzed, and a portion of the blood is retained in dried form	LBNT	A bloodstain card was prepared from a sample of Item(s)….. No testing was performed on this item.
When hair is collected and repackaged	HAC	Possible hair was collected from Item… and labeled as_____.
When items are returned unopened	UNEXAM	Item(s)… is/are being returned unexamined.
When items are opened, but not chemical tested	UNTEST	No testing was performed on Item(s)…
When items are forwarded for DNA	DNA	Item(s)… is/are being forwarded for DNA analysis.

Samples used as a DNA Reference	REFDNA	A DNA profile/ DNA profiles was/were obtained for comparison from sample(s) of Item(s)…
(+) activity detected after assumptions	PACDNA	An indication/Indications of a possible additional contributor was/were present on the sample(s) of Item(s)…
When no further testing is performed on an item (i.e., unstained micro slides)	NFT	No further testing was performed on Item(s)…
When further testing is an option (not all items are fully tested)	FT	If further testing is desired, please contact the analyst for more information.
When DNA reference standards will potentially provide more information	STDS	If DNA standards in relation to the investigation are obtained, please contact the analyst for more information.
Various spellings of names*	SP	Spellings of names can differ among case submission paperwork, evidence packages, and/or paperwork submitted to the laboratory. This/these is/are the alternative name spelling(s) observed for the individual(s) listed above:
Additional testing to follow	FU	Additional testing will be performed on Items….. The results will be issued in a follow up report.
Samples were stopped after quantitation or Y-screen results indicated on report	Select the box in the Edit Request tab in the LIMS	Deoxyribonucleic Acid (DNA) was isolated and quantitated using the Promega Plexor HY Quantitation System
Evidence was outsourced to a vendor laboratory	OUT	Evidence was outsourced to [Bode Technology/DNA Labs International] on [date]. Biological screening and /or Y-screening and/or DNA analysis will be performed as applicable. You will be notified when casework is complete.
Additional testing will be forthcoming (i.e., outsourced case)	ADDTEST	Additional testing will be performed on the above listed evidence.

Additional testing was performed (i.e., outsourced case)	ADDTESTR	Additional testing was performed on the above listed evidence.
Contact the analyst for more information	CNCT	Please contact the undersigned analyst for more information.
DNA profile will be evaluated for CODIS upload (i.e., outsourced case)	CDSEVAL	The DNA profiles in this report will be evaluated for CODIS upload. If uploaded, notification will be made in a separate report.
Sample lost on instrumentation	LOST	Item [: Sample(s) was /were not recovered from the instrument [during extraction of these items]; therefore, no results were obtained. Item [ ] was resampled, re-extracted, and reported as Item { }.
Reference standard stopped at quant since all evidence stopped at quant	REFSTOP	A sample of this item was isolated and quantitated for DNA; however, no further processing was performed due to associated samples stopping at quantitation.
When a sample is re-extracted	RE-EXTRACT	A sample/Samples of this/these item(s) did not meet the Laboratory's Quality Control requirements (Item(s) XXXX [and XXXX]). A DNA profile/DNA profiles was/were obtained for comparison form the re-extraction of the sample(s) from this item/these items (Item(s) XXXX-RE [and XXXX-RE]).
Partial DNA profile obtained and sample re-extracted (primarily reference standards)	PARTREF	Item (): A partial DNA profile was obtained from Item () and will not be used for comparison. A DNA profile was obtained for comparisons from a re-extracted sample of this item (Item XX-RE).
Evidence received by the laboratory improperly sealed or unsealed	IMPROP	Evidence was received into the laboratory [unsealed/improperly sealed] per the MNPD-CL policy.

16.3.3.3 *Use of spelling statement

16.3.3.3.1 Examples of when the spelling statement is needed. The examples are not intended to be all encompassing.

16.3.3.3.1.1"Jr" is listed on one form, but not other forms.

16.3.3.3.1.2Middle name is on one form and no middle name is listed on other forms.

16.3.3.3.1.3Middle initial is different than middle initial on other forms.

16.3.3.3.1.4It appears names could be different people or if names are misspelled.

16.3.3.3.2 Examples of when a spelling statement is not needed. The examples are not intended to be all encompassing.

16.3.3.3.2.1One form has middle initial, and one form has middle name spelled out (and they are consistent)

16.3.3.3.2.2One form has middle initial, and the other form has no middle initial listed.

16.3.3.4 DNA Statements for Supplemental Request:

16.3.3.4.1 When a request includes DNA reference standards only to be compared to previously submitted evidence previously submitted, the results of the comparison will need to be reported in paragraph form since no evidence is associated with the request to allow for drop-down in the table.

Circumstance	Autotext Code	Statement
Single Source evidence profile identical to DNA Reference profile	SS	The (partial) (foreign/major/minor) DNA profile(s) from Item(s)… match(es)/is(are) consistent with the DNA profile obtained from the sample Item…
Unrestricted mixture	MIX+	_____ is included as a possible contributor to the (partial/foreign) mixed DNA profile(s) from Item(s)…
Calculated statistic below 100 trillion	STAT	For (# used in statistical analysis) loci, the frequency of occurrence of the (interpretable, mixed, major, minor) DNA profile obtained from the sample(s) of Item(s)… for unrelated individuals is approximately 1 out of _____.
Calculated statistic above 100 trillion	STATEX	STATEX: For (# used in statistical analysis) loci, the frequency of occurrence of the (interpretable, foreign, mixed, major, minor) DNA profile obtained from the sample(s) of Item(s)… for unrelated individuals is rarer than 1 out of 100 trillion.

DNA Reference profile excluded from the evidence data	XDNA	_____ is excluded as (the source of the foreign/major/minor DNA profile/ a possible contributor to the mixed DNA profile) obtained from the sample(s) of Item(s)…

16.3.3.5 Auto text statements for Supplement Serology reports in paragraph form

Circumstance	Autotext Code	Statement
When ALS testing is performed	ALS	A test/Tests for the detection of biological fluids was performed on the sample(s) of this/these Item(s) using the alternate light source (ALS). The result was….
When KM and HemaTrace or HemaTrace only is performed	KM/RSID	(A) Test(s) for the detection of blood was/were performed using Kastle-Meyer (KM) (and RSID) on the sample(s) of this/these Item(s). The results was/were (test) (result).
When KM only is performed	KM	A presumptive test gave/did not give chemical indications of blood on the sample(s) of this/these item(s).
When KM is inconclusive	KM?	A presumptive test for blood was inconclusive on the sample(s) of this/these Item(s) due to the results being outside the laboratory's validated parameters.
When AP only is performed	AP	A presumptive test/Presumptive tests gave/did not give chemical indications of semen on the sample(s) of this/these Item(s).
When AP and p30 or p30 only is performed	PRS	A Test(s) for the detection of seminal fluid was/were performed using (test(s)) on the sample(s) of this/these Item(s). The result(s) was/were (test) (result).
When AP and sperm search are performed		Tests for detection of seminal fluid and sperm were performed using an acid phosphatase test (AP) and microscopic examination on Item (X). The results were AP (result) and sperm (result).
When sperm search only is performed	Micro	A test/Tests for the detection of sperm was performed using microscopic

		examination o the sample(s) of this/these Item(s). The result was sperm (result).
When AP, p30, and sperm search are performed	TRS	Tests for the detection of seminal fluid and sperm were performed using (an acid phosphatase (AP) test, ABA card p30 test), and microscopic examination on the sample(s) of this/these Item(s). The results were AP (result), p30 (result), and sperm (result). Manually add the following to the end of the above statement: Acid phosphatase is an enzyme found in generally high levels in seminal fluid and in relatively lower levels in several other substances. The antigen p30 is a protein found in generally high levels in seminal fluid and in relatively lower levels in some other bodily fluids.

16.3.3.5.1 **Note**: Reports are not limited to the examples listed above. There might be circumstances to word the results differently; however, the results should not be redundant.

16.3.3.6 CODIS Statements:

16.3.3.6.1 If applicable, the following autotext statements related to CODIS upload shall be added to appropriate items following the same procedure described above. Additional statements may be used in order to accurately report any CODIS activity.

Circumstance	Autotext Code	Statement
Keyboard Search	CDSKB-	The (partial) (unknown/foreign/major/minor/mixed) DNA profile developed from the sample(s) of Item(s)…was/were searched in the CODIS database and no/a match was detected as of this report date.
Uploaded profiles	DBSEARCH	(An) STR profile(s) from the following item(s) has/have been entered into the Combined DNA Index System (CODIS) in accordance with local, state, and national regulations where regular searches will be performed: <item descriptions>. Notifications will be issued if there is a hit

		in the database or if a profile is removed from CODIS at any time in the future.
Removal due to elimination reference standard	CDSELIM	The (partial) (unknown/foreign/major/minor/mixed) DNA profile(s) from the sample(s) of Item(s)… has been removed from the CODIS database after comparison to (elimination reference standard).
Profile removed due to eligibility requirements	CDSREMOVE	The (partial) (unknown/foreign/major/minor/mixed) DNA profile(s) from the sample(s) of Item(s)… has been removed from the CODIS database. The profile does not meet the current CODIS eligibility requirements.
CODIS profile removed from unidentified person category after identification confirmed	CDSREMOVEID	The (partial) (unknown/foreign/major/minor/mixed) DNA profile from the sample of Item ...has been removed from the CODIS database after identification via previous CODIS match.
Case to Case Hit	CASEHIT	During a search of the CODIS database, a (high/moderate) stringency association was made between the (partial) (unknown/foreign/major/minor/mixed) DNA profile(s) obtained from the sample(s) of Item(s)… and (laboratory agency) Lab #_____ (agency, agency case #, officer). For additional information, please contact the undersigned analyst.
Evidence to DNA Reference Hit	REFHIT	During a search of the CODIS database, a (high/moderate) stringency association was made between the (partial) (unknown/foreign/major/minor/mixed) DNA profile(s) obtained from the sample(s) of Item(s)… and (laboratory agency) Lab #_____. The information being provided is an investigative lead only. In order to confirm this, a blood or buccal sample from (Name) (DOB) must be submitted for DNA testing.
With all Hit Reports	CDSRMS	Additional information may be found in RMS.

16.3.3.6.2 Click APPLY to assign these findings.

16.3.3.7 EDIT REQUEST Statements:

16.3.3.7.1 Additional information is necessary prior to submitting a report for technical review. Right click the request and select EDIT REQUEST. Click on the button that looks like … (beside "Complexity"). Complete all applicable data extension fields. The "Examination Start Date" shall reflect the date on which the laboratory process began (i.e., date of first inventory sheet or extraction sheet, if samples were previously cut and documented in screening report). For the "Disposition," one of the following two statements is commonly used:

Circumstance	Autotext Code	Statement
Disposition for Casework and CODIS Reports	DISP	The remainder of the evidence is being returned/forwarded/transferred to_____.
When a high stringency association from the DNA database is reported	HSTRING	High Stringency Association – Exact match between the compared profiles
When a moderate stringency association from the DNA database is reported	MODSTRING	Moderate Stringency Association - 1) Involves the comparison of mixture(s) or 2) the data present matches between the profiles being compared, but information may be missing in at least one of the profiles

16.3.3.8 In addition, check all applicable optional statements that apply to the corresponding report:

16.3.3.8.1 PCR was performed

16.3.3.8.1.1"…STR statement on final report"

16.3.3.8.2 Statistical calculations were performed

16.3.3.8.2.1"…DAT1 statement on final report"

16.3.3.8.3 Samples were stopped after quantitation

16.3.3.8.3.1"…Quant statement on final report"

16.3.3.8.4 Report is an amended report

16.3.3.8.4.1"…amended final report"

16.3.3.8.5 CODIS Associations are reported

16.3.3.8.5.1"…associations on final report"

16.3.3.8.6 Sample source could be characterized as trace

16.3.3.8.6.1"…Trace DNA Statement on Final Report"

16.3.3.9 If the report is amended, enter the original report date. Also, state what changes were made to the report in the "Additional Amend Statement – Optional" data extension field.

16.3.3.10 Other applicable metrics should be included under "Number of Tests Completed."

16.3.3.11 Once all applicable fields have been completed, click APPLY.

16.3.3.12 Right click on the request and select PRINT FINAL REPORT. Select the report to be printed to Screen.

16.3.3.13 Review the report to ensure fields which were auto-populated from the 282 form and the autotext statements are correct. If report is correct, close the screen containing the draft and right click on the request. Select SET MILESTONE then DRAFT COMPLETE. Select YES. In the Sign Report box, mark the circle next to SIGN, select ANALYST in the drop-down menu, and click in the field below. The analyst must scan or type individual barcode and type the PIN number. Click OK.

16.4 Limitations

16.4.1 A final report will not be released to a client until a successful technical and administrative review on the case has been completed.

16.5 References

16.5.1 MNPD-CL Forensic Biology Technical Procedures Manual

17. Case Review

17.1 Scope

17.1.1 To describe the process for performing the technical review on completed batch work and performing technical and administrative reviews on completed Forensic Biology casefiles.

17.2 Equipment/Materials/Reagents

17.2.1 Completed batch work

17.2.2 Forensic Biology Processing Batch Form and ReviewForm , if applicable

17.2.3 Completed electronic casefile

17.2.4 Forensic Biology Case Review Form or Forensic Biology Serology Review Form

17.2.5 LIMS and Adobe software

17.2.6 GeneMapper® ID-X Software v1.6

17.2.7 MNPD-CL network

17.2.8 QMS

17.3 Procedure

17.3.1 Completed batch work

17.3.1.1 Open the DNA Batch Workbook located on the MNPD-CL network.

17.3.1.2 Open the review form associated with the batch. The analyst review should be complete prior to submitting the batch of cases for technical review.

17.3.1.3 Elements listed in the Processing Batch Form and Review Form should be checked during the technical review of the batch. To notate suggestions, need for corrections, or requests for clarification in the DNA Batch Workbook or witness sheet(s), post comments on the PDF using the Comments.

17.3.1.4 Use of QMS, the MNPD-CL network, Plexor Software and GeneMapper ID-X should be utilized for the review.

17.3.1.5 The analyst will then be notified via Microsoft Planner and/or email that a batch review has been conducted. The analyst should ensure that the comments are not deleted as responses are made to the reviewer's comments.

17.3.1.6 Once corrected, the analyst will notify the reviewer that corrections are complete. Once all the elements of the batch review are verified, have the analyst add "Workbook Locked" with date and initials and lock the DNA Batch Workbook PDF. The reviewer will electronically sign the technical reviewer box of the Processing Batch Form and Review Form.

17.3.2 Completed electronic case file

17.3.2.1 Open the electronic case file located on the MNPD-CL network.

17.3.2.2 Open the review form associated with the case file. The analyst review should be complete prior to submitting the case file for technical review.

17.3.2.3 Elements listed in the technical review portion of the Forensic Biology Case Review Form, or the Forensic Biology Serology Review Form should be checked during the technical review. To notate suggestions, need for corrections, or requests for clarification in the case file, post comments on the PDFs using Adobe.

17.3.2.4 Review the case report and chain of custody in the LIMS software. If any comments or suggestions are needed for the case report, it must be printed from the LIMS to PDF on the MNPD-CL network.

17.3.2.5 If a discrepancy or needed correction is identified, the technical reviewer will select the option to 'Reject Findings' in the LIMS. Right click on the request to select Reject Findings. The correction and/or discrepancy by will be noted in the case folder, most often through use of a technical and/or administrative review form.

17.3.2.5.1 The 'Reject Findings' in the LIMS will be used when there is a technical discrepancy in the case file and/or report.

17.3.2.5.2 Additionally, the following information should be listed in the comment section: initials of individual performing the review, date of rejection, type of review (Tech or Admin review), and type of errors prompting the rejection (technical error, administrative error, or technical and administrative error).

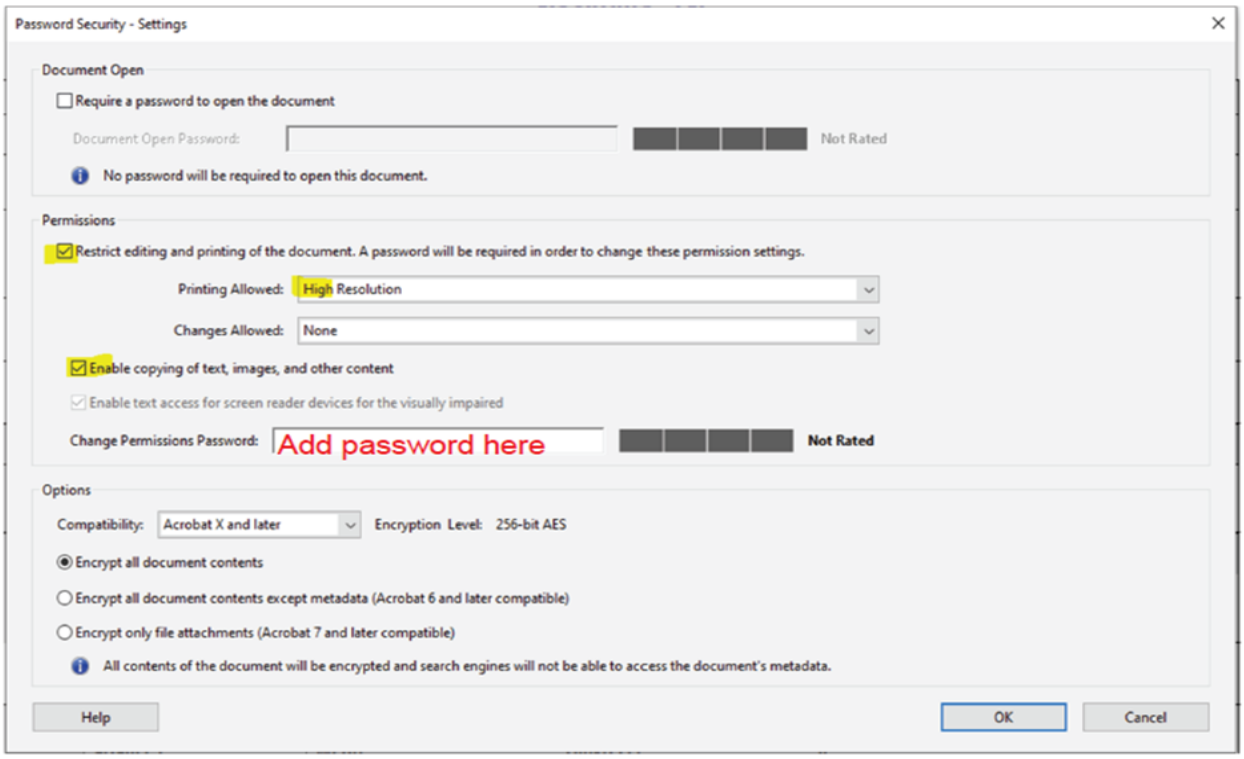
17.3.2.6 The analyst will also be notified via Microsoft Planner and/or email about any corrections needed or if the case is ready to prepare for administrative review. The analyst should ensure that the comments are not deleted as responses are made to the reviewer's comments.

17.3.2.7 Once corrected, the analyst will notify the reviewer that corrections are complete via Microsoft Planner and/or email. Once all the elements of the technical review are

verified, electronically sign the technical reviewer box of the Forensic Biology Case Review Form and set the Technical Review milestone in the LIMS.

17.3.2.7.1 To set the Technical Review milestone, open the main LIMS software and open the appropriate case file. Click on the Request tab and right click the appropriate request. Select Set Milestone, and Technical Review. Select Sign, scan the reviewer's personal barcode or type the user name in the barcode area and type in PIN.

17.3.2.8 The case file should now be prepared for administrative review by adding a coversheet and securing the case file. Secure the case file by clicking on the Protect tool in Adobe. Click on Advanced Options and then Encrypt with Password. Click Yes on the next dialog box. Fill in the settings as listed below.



17.3.2.9 The case file is now ready for an administrative review (alternatively, portions of the administrative review may be performed in conjunction with the technical review).

17.3.2.10 Elements listed in the review form administrative review section should be checked during the administrative review. Ensure at least one draft report is present in the case file folder.

17.3.2.11 If a discrepancy or needed correction is identified, the administrative reviewer will select the option to 'Reject Findings' in the LIMS. Right click on the request to select Reject

Findings. The correction and/or discrepancy by will be noted in the case folder, most often through use of a technical and/or administrative review form.

17.3.2.11.1 The 'Reject Findings' in the LIMS will be used when there is an administrative discrepancy in the report.

17.3.2.11.2 Additionally, the following information should be listed in the comment section: initials of individual performing the review, date of rejection, type of review (Tech or Admin review), and type of errors prompting the rejection (technical error, administrative error, or technical and administrative error).

17.3.2.12 The analyst will also be notified via Microsoft Planner and/or email about any corrections needed or if the case has been reviewed and released.

17.3.2.13 Once all the elements of the administrative review have been verified, electronically sign the administrative reviewer check box of the Forensic Biology Case Review Form. Finally, set the Administrative Review milestone in the LIMS.

17.3.2.13.1 To set the Administrative Review milestone, open the main LIMS software and open the appropriate case file.

17.3.2.13.1.1 Click on the Request tab and right click the appropriate request.

17.3.2.13.1.2 Select Set Milestone, and Administrative Review.

17.3.2.13.1.3 Select Sign, scan the reviewer's personal barcode or type the username in the barcode area and type in PIN.

17.3.2.13.1.4 Verify the analyst's signature has been added to the report in the LIMS by opening the released report.

17.3.2.14 Rename the case file folder to add the assignment that is listed on the Form 282 (ex. CL-21-003325 Casefile MDM - CID). Cut and paste this folder to the following location in the Completed folder depending on the month and year that the case was released: L:\Crime Lab Network Drive\Forensic Biology\Forensic Biology Unit\Electronic Casefiles\Completed.

17.3.2.14.1 The administrative reviewer will then zip the case file folder (Right click on folder -> Send to -> Compressed (zipped) folder). Move the zip file into the original folder.

17.3.2.14.2 Create a Sub Folder in the LIMS software under Case Attachments named DNA Request, Serology, or CODIS Request followed by the Request number (ex. DNA Request 0001). Upload the zip file into this Sub Folder after the case has been released by: right click sub folder -> select 'Add multiple attachments' -> drag and drop zip file in box -> select save. Leave the zip file in the original folder.

17.4 References

17.4.1 Forensic Biology Case Review Form

17.4.2 Forensic Serology Case Review Form

17.4.3 Processing Batch Form and Review Form

17.4.4 Interpretation Workbook Review Checklist

18. GenTegra-DNA Extract Preservation and Reconstitution

18.1 Scope

18.1.1 To describe the process of drying down DNA extracts for ambient temperature storage using GenTegra-DNA and a Savant SpeedVac.

18.1.2 To describe the process of reconstituting DNA extracts that have been dried down for ambient temperature storage using GenTegra-DNA.

18.2 Equipment/Materials/Reagents

18.2.1 Savant SpeedVac SPD1030 Integrated Vacuum Concentrator

18.2.2 GenTegra-DNA dry bulk tube

18.2.3 Glass condensation flask

18.2.4 CryoCool™ Heat Transfer Fluid

18.2.5 Autoclaved Ultrapure water

18.2.6 Centrifuge

18.2.7 Vortex

18.2.8 QIAgility (optional)

18.2.9 PCR Clean

18.2.10 Cover lock emergency release tool

18.2.11 Net

18.3 Procedure: Drying Extracts

18.3.1 Begin the SpeedVac SPD1030 start of day procedures before preparing any extracts for drying since the SpeedVac needs to be turned on for at least 45 minutes before running. The CryoCool™ in the refrigerated trap must be cold before the run begins.

18.3.2 SpeedVac SPD1030 Start of Day Procedures

18.3.2.1 Check all the hoses to ensure that they are secure. Remove the flask cap and white flask seal. Ensure there is no debris in the liquid in the refrigerated chamber. If any debris is present, remove it with a net. Obtain a clean glass condensation flask and ensure there are no cracks. Gently place the flask into the refrigerated chamber. As the flask is pressed into the chamber, the CryoCool™ level rises. Verify that the final CryoCool™ level is 10 to 15 mm below the rubber seal. If low, carefully pour more CryoCool™ into the chamber while holding down the flask. Immediately wipe clean any CryoCool™ that spills onto the rubber seal.

18.3.2.2 Fit the white insulating flask seal over the glass flask to secure the flask in the chamber. Its bevelled side faces upward to admit the Flask Cap.

18.3.2.3 Snap the black rubber flask cap over the mouth of the glass flask.

18.3.2.4 Turn the SpeedVac on.

18.3.2.4.1 Note: Do not switch OFF and ON within a short period of time as this could cause pressure to build up within the system and lock the system that could trigger the fuse. After switching OFF, allow sufficient time (minimum 15 minutes) for the pressure to stabilize before switching the system ON again.

18.3.2.5 Immediately verify by touch that the product is drawing air through the vent on the right side. If you cannot feel the air suction, switch the product OFF at once. Operating the product without a working fan, or with the air flow blocked, will damage the refrigeration system.

18.3.2.6 Clean the outside of the SpeedVac including the cover as well as the cover seal, chamber and rotor with a soft lint free cloth with PCR Clean, or similar, followed by autoclaved Ultrapure water.

18.3.3 Extract Drying

18.3.3.1 If the bulk tube of GenTegra-DNA has not been made into a solution yet, add 550 uL of autoclaved Ultrapure water to the tube and dissolve using gentle mixing for 5-10 minutes. This can be done using a slow vortex. A pipette may also be used so flow pressure can be applied directly to reagents that are on the bottom of the tube. **Avoid foaming.**

18.3.3.2 If already hydrated, check that the tube has not been open for more than three months. Mix by using a slow vortex or a pipette.

18.3.3.3 Obtain the extracts that will be dried down and transfer custody to yourself

18.3.3.4 Use the “Preservation Form – Drying Extracts” sheet for documentation. Name the file similar to “SP080724MDM” replacing with the appropriate date and analyst initials. “SP” refers to sample preservation. The sample names will need to be verified by a second person.

18.3.3.5 Navigate to the following location and make a folder with the same name as the file: G:\Instrument-DNA\Sample Preservation\Dry Down. Make sure to navigate to the appropriate year and month folder.

18.3.3.5.1 Within this folder, three documents should be saved:

18.3.3.5.1.1 Preservation Form – Drying Extracts

18.3.3.5.1.2 GenTegra-DNA Witness sheet

18.3.3.5.1.3 Review sheet (not needed at time of dry down)

18.3.3.6 Verify that each sample is at least 20µL. If not, add at least 10µL of autoclaved Ultrapure water. Document the sample name as well as the volume added in the Excel named "Sample Preservation Sample List" on SharePoint.

18.3.3.7 Add 5µl of GenTegra-DNA solution to each isolated DNA sample (can be done manually or with the QIAgility). Store the solution at 4°C when finished.

18.3.3.7.1 If adding GenTegra-DNA utilizing the QIAgility, see the instruction in the QIAgility Addition of GenTegra-DNA section.

18.3.3.8 Mix thoroughly and gently to disperse the GenTegra Matrix. This can be done by slowly pipetting or by tapping gently against your hand. It is crucial to avoid foaming at this step.

18.3.3.9 Quickly centrifuge to bring the matrix and sample to the bottom of the tube.

18.3.3.10 When at least 45 minutes have passed since turning on the SpeedVac, take the caps off the prepared extract tubes and place the extracts into the rotor. Ensure that the load is balanced. Use the control panel to set the parameters of the run to the following:

18.3.3.10.1 Run Time: 2 hours

18.3.3.10.2 Temperature: No

18.3.3.10.3 Vacuum Pressure: 5.1

18.3.3.10.4 Vacuum Type: Level

18.3.3.11 Press the Auto Run button to start the run. The cover locks and the lid locked indicator is illuminated.

18.3.3.11.1 Note: If the cover is not closed, the display will show "Lid" and the run will not start.

18.3.3.12 Once the run time expires, the machine will beep until the "Stop" button is pushed. ." Remove samples and ensure there is no liquid left in the tube. The sample can be dried again for a certain period (i.e., 10 minutes up to 2 hours) depending on how much liquid is left.

18.3.3.12.1 If removal of samples from the concentrator chamber during a power failure is required, insert cover lock emergency release tool into the vertical slot at the base of the front of the unit. Press the object gently into the slot until the lock releases. The cover can then be opened.

18.3.3.13 Return the caps to the extract tubes. The extracts can now be stored at room temperature.

18.3.3.14 When no more runs will be completed in the day, wipe the outside of the SpeedVac including the cover as well as the cover seal, rotor, and chamber with a soft lint free cloth with PCR Clean, or similar, followed by autoclaved Ultrapure water, turn the SpeedVac off, and remove the glass condensation flask using thermal protective gloves. Empty, defrost, and clean the flask by wiping off any condensation. Place the flask in a fume hood until thawed, then store under the counter. Fit the flask cap back into the seal to cover the opening of the refrigerated trap.

18.3.4 QIAgility Addition of Gen-Tegra DNA

18.3.4.1 QIAgility Template Preparation

18.3.4.1.1 Navigate to the file labeled “Sample Pres-QIA.txt” using the following path: L:\Crime Lab Network Drive\Forensic Biology\Forensic Biology Unit\Templates for Workbook

18.3.4.1.2 Make a copy of this file and navigate to G:\Instrument - DNA\QIAgility Import Files\Sample Preservation followed by the correct year and month. Paste the file and re-name it similar to “SP080724MDM” replacing with the appropriate date and analyst initials.

18.3.4.1.3 Copy the extract names to this file and save.

18.3.4.2 QIAgility Instructions

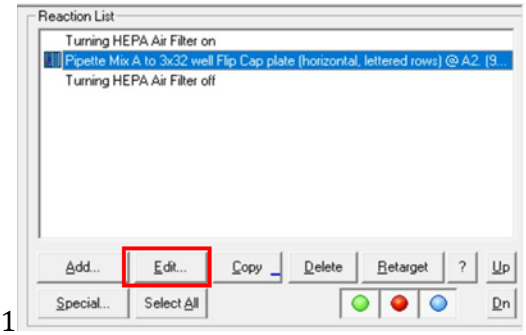
18.3.4.2.1 Log onto the computer, then turn on instrument using the switch on the back.

18.3.4.2.2 Launch QIAgility Software v4.18.1. The QIAgility instrument lid must be closed for the software and hardware to initialize upon start-up and for a run to proceed. Click Open on the dialog box.

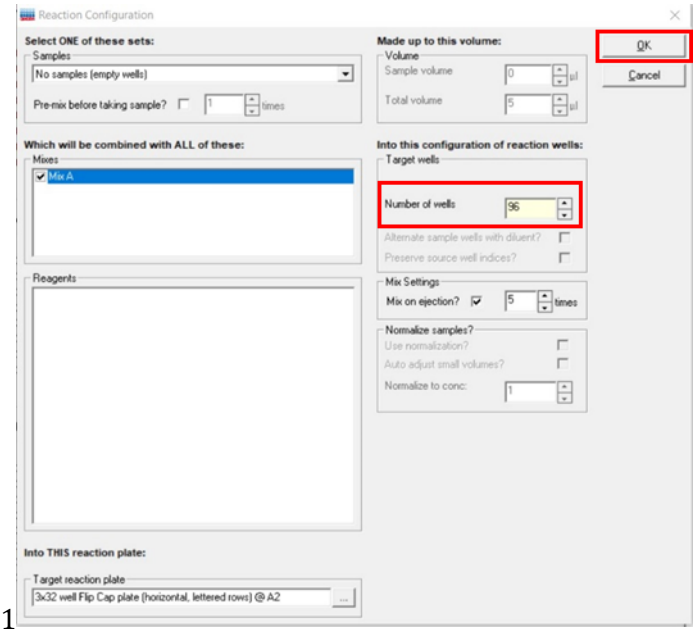
18.3.4.2.3 Open the sample preservation protocol (Sample Pres.QAS) using the following path after clicking “File” at the top left of the software screen and then “Open”: G:\Instrument - DNA\QIAgility- Prot.

18.3.4.2.4 Click on the A2 block.

18.3.4.2.5 Select “Pipette Mix A…” in the top right corner and hit the Edit button.

18.3.4.2.5.1 

18.3.4.2.6 In the following dialog box, change “Number of wells” from 96 to the number of extracts in your template file and hit OK.

18.3.4.2.6.1 

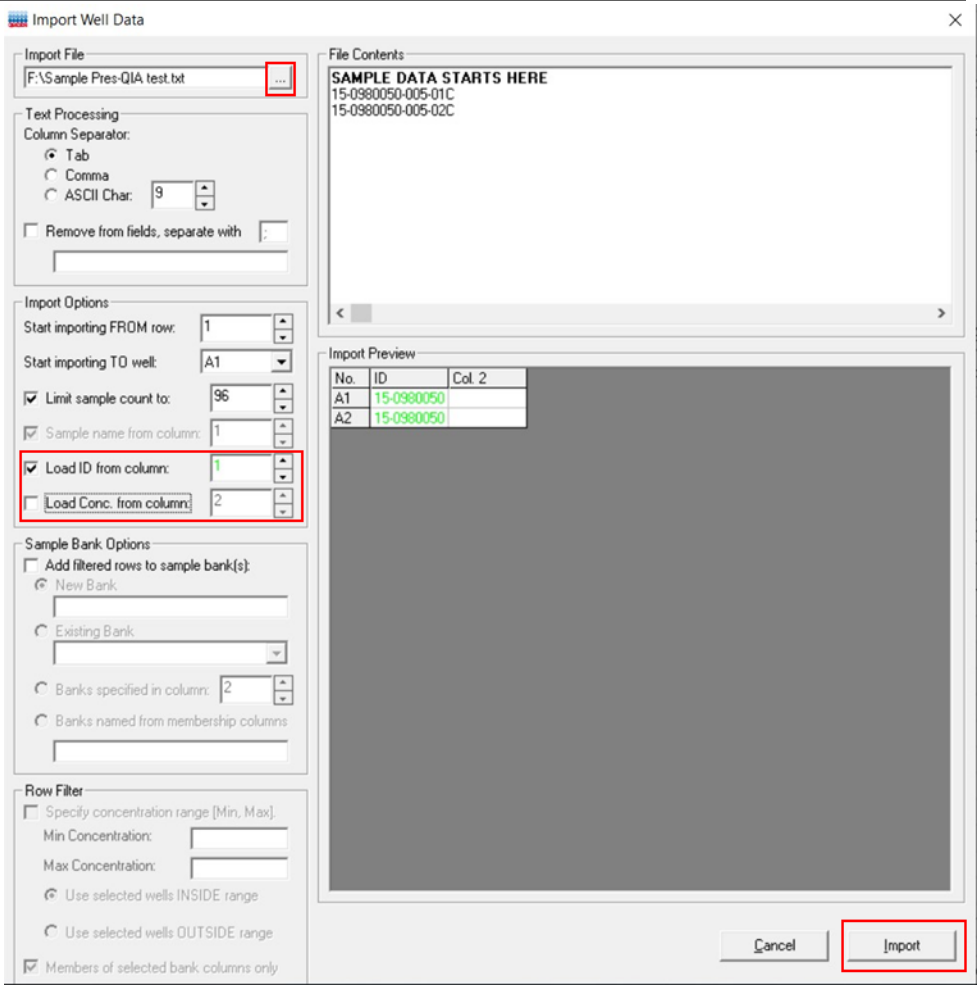
18.3.4.2.7 Select the Sample Block (A2) and click on the Import button.

18.3.4.2.8 In the following dialog box, navigate to your import file after clicking the three dots on the top left.

18.3.4.2.9 In the “Load ID from column:” check box, change the value to 1.

18.3.4.2.10 Uncheck the box labeled “Load Conc. from column:”

18.3.4.2.11 The dialog box should look like the one pictured below. Click Import and then Finish (Finish button appears after hitting Import).



18.3.4.2.11.1

18.3.4.2.12 Place the prepared GenTegra-DNA tube into position A on the Reagent Block (R1).

18.3.4.2.13 Load un-capped extract tubes into the 4 x 8 well sample racks in the Sample Block (A2) according to the placement indicated on the software.

18.3.4.2.14 Ensure that there are enough 50µL tips on the QIAgility worktable and present on the software. These tips appear as blue wells under the B1 section on the software. A white well indicates a tip is not present on the software. Ensure that the tip positions match between the worktable and the software.

18.3.4.2.15 To add or remove a tip from the software, right click on a well in B1 and select the appropriate action: Set selected tips to 'Available' or Set selected tips to 'Unavailable.' There is also the option to Set all tips on current plate to 'Available.'

18.3.4.2.16 Click the green start arrow on the toolbar.

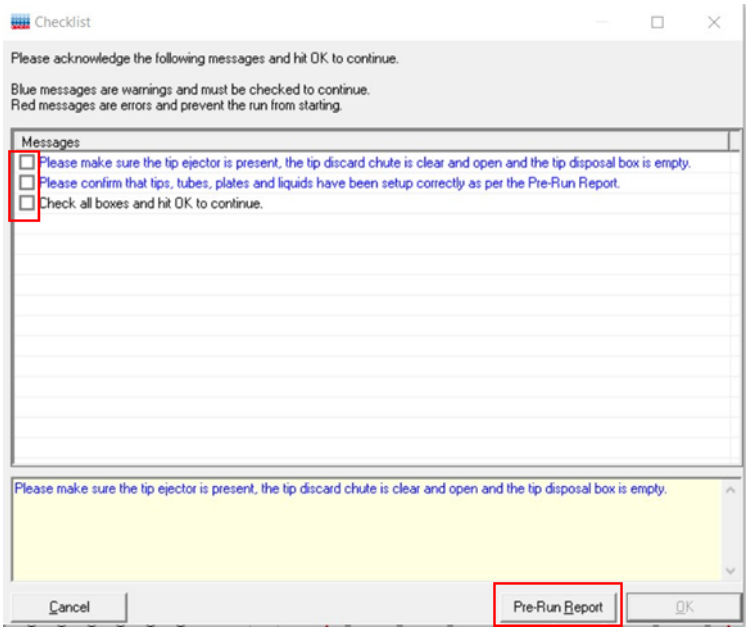
18.3.4.2.17 You will be prompted to save the QIAgility (.QAS) file for the run. Save it to the following location: G:\Instrument - DNA\Run Files\QIAgility\Hulk\Sample

Preservation and navigate to the appropriate year and month. Name it the same as your template file.

18.3.4.2.18 The Checklist dialog box will appear as pictured below. Click the Pre-Run Report button and print it to pdf. Save to the following location G:\Instrument - DNA\QIAgility Pre-Run Reports\Sample Preservation and navigate to the appropriate year and month. Name it the same as your template file.

18.3.4.2.19 Check the Pre-Run Report to verify the location and amount/volume of consumables and liquids that are required on the worktable for completion of the run file. The same lot of GenTegra-DNA can be combined in order to meet the volume requirement. Close the Pre-Run Report dialog box.

18.3.4.2.20 If no warnings or errors are listed, select the boxes, and click OK to start the run.

18.3.4.2.20.1 

18.3.4.2.21 NOTE: Care must be taken when opening the QIAgility lid during a run. It takes up to 10 seconds for the instrument to complete its current movement and for the pause to take effect.

18.3.4.2.22 Upon completion of the run, a Post-Run Report will appear. Print this to pdf and save it to the following location: G:\Instrument - DNA\QIAgility Post-Run Reports\Sample Preservation and navigate to the appropriate year and month. Name it the same as your template file. Close the Post-Run Report dialog box.

18.3.4.2.23 Place caps back onto extract tubes and pulse spin. The extract tubes are now ready to be dried down on the SpeedVac.

18.3.4.2.24 Refer back to Section 18.3.3.9 to continue with the drying down.

18.3.4.2.25 When all runs are completed for the day, re-cap the GenTegra-DNA tube and place into the refrigerator or discard into the biohazard trash if empty.

18.3.4.2.26 Perform the After Use maintenance procedures for the QIAgility.

18.3.4.2.27 Close the software. There will be a prompt asking if you want to save your changes. There is no need to save again since the QAS file was already saved at the beginning of the run.

18.3.4.2.28 A Quitting QIAgility dialog box will appear. Choose the option of "Move to Safety Position (recommended)."

18.3.4.2.29 Turn off the instrument and log out of the computer.

18.3.5 Limitations

18.3.5.1 The GenTegra-DNA solution should not be added to extracts greater than 245µL in volume.

18.3.5.2 The lower limit of GenTegra-DNA solution's protection abilities is 20µL. If the extract is <20µL, then additional diluent (autoclave Ultrapure water) must be added before adding the GenTegra-DNA solution to avoid loss of DNA.

18.3.5.3 The hydrated GenTegra-DNA tube should be used within 3 months. The dry form is good for three years.

18.3.6 Safety

18.3.6.1 Always use thermal protective gloves when removing the glass condensation flask at the end of the day. The cold temperature of the flask may cause pain and localized frostbite. Handle a full flask with care to avoid risk of injury.

18.3.6.2 The CryoCool™ is flammable and suspected of damaging fertility or the unborn child. Personal protective equipment should be worn at all times. It should be disposed of in the biohazard waste.

18.3.6.3 The SpeedVac lid can crush fingers. Do not reach between the device and lid when opening or closing the lid.

18.3.6.4 Personal protective equipment should be worn at all times when using GenTegra-DNA. Work should be conducted inside a safety enclosure when handling this reagent.

18.3.7 References

18.3.7.1 SpeedVac™ SPD1030/2030 Vacuum Concentrator Installation and Operation Manual (June 2018). ThermoFisher Scientific.

18.3.7.2 GenTegra-DNA dry BULK User Guide (August 2017). GenTegra.

18.3.7.3 Email Communication with ThermoFisher Scientific and GenTegra.

18.4 Procedure: Reconstituting Extracts

18.4.1 Obtain the samples that will be reconstituted and transfer custody to yourself.

18.4.2 Apply a volume of autoclaved Ultrapure water equivalent to the sample's volume prior to drying down. Find the original batch workbook to obtain this value, then check the Excel in SharePoint to see if any extra autoclaved water was added to the sample prior to drying down.

18.4.3 Use the "Preservation Form – Reconstitution" sheet for documentation. Name the file similar to "RSP080724MDM" replacing with the appropriate date and analyst initials. "RSP" refers to reconstituted sample preservation. The sample names will need to be verified by a second person.

18.4.4 Navigate to the following location and make a folder with the same name as the file: G:\Instrument - DNA\Sample Preservation\Reconstitution. Make sure to navigate to the appropriate year and month folder.

18.4.4.1 Within this folder, three documents should be saved:

18.4.4.1.1 Preservation Form – Reconstitution

18.4.4.1.2 GenTegra-DNA Witness sheet

18.4.4.1.3 Review sheet (not needed at time of reconstitution)

18.4.5 Incubate at room temperature (21–25°C) for 15 minutes.

18.4.6 Mix to solubilize the DNA by vortexing for 1 minute and then pulse spin.

18.4.7 The DNA is ready for use in downstream applications. It can be dried down again after use (see Drying Extract Procedure).

18.4.8 Limitations

18.4.8.1 This procedure may be repeated multiple times until a maximum of 75% of the original sample (and thus, GenTegra chemical matrix) is removed. It is important to keep record of the final sample volume before drying as this will be needed for reconstitution.

18.4.9 Safety

18.4.9.1 Personal protective equipment should be worn at all times when using GenTegra-DNA. Work should be conducted inside a safety enclosure when handling this reagent.

18.4.10 References

18.4.10.1 GenTegra-DNA dry BULK User Guide (August 2017). GenTegra.

18.4.10.2 Email communication from GenTegra.

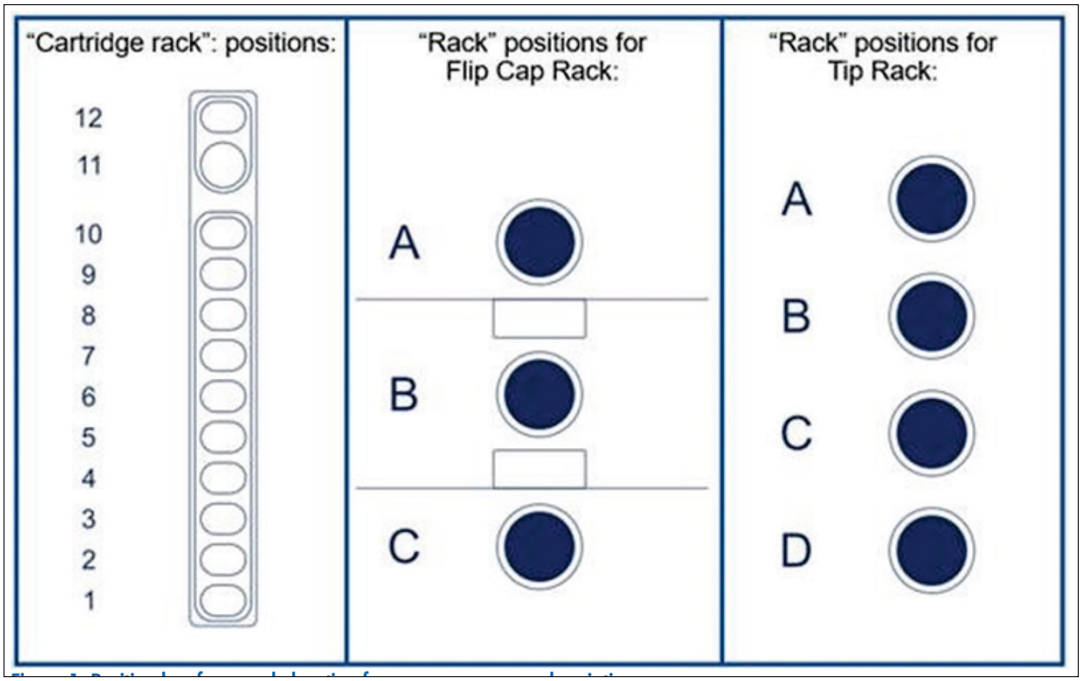
19. Appendix: EZ2 Sample Recovery Procedure

19.1 System Description

19.1.1 **Note: If the device was switched off unintentionally:** Start the device. The recovery screen should appear. If it is not possible to start the device, notify the FBU Supervisor(s) and DNA TL.

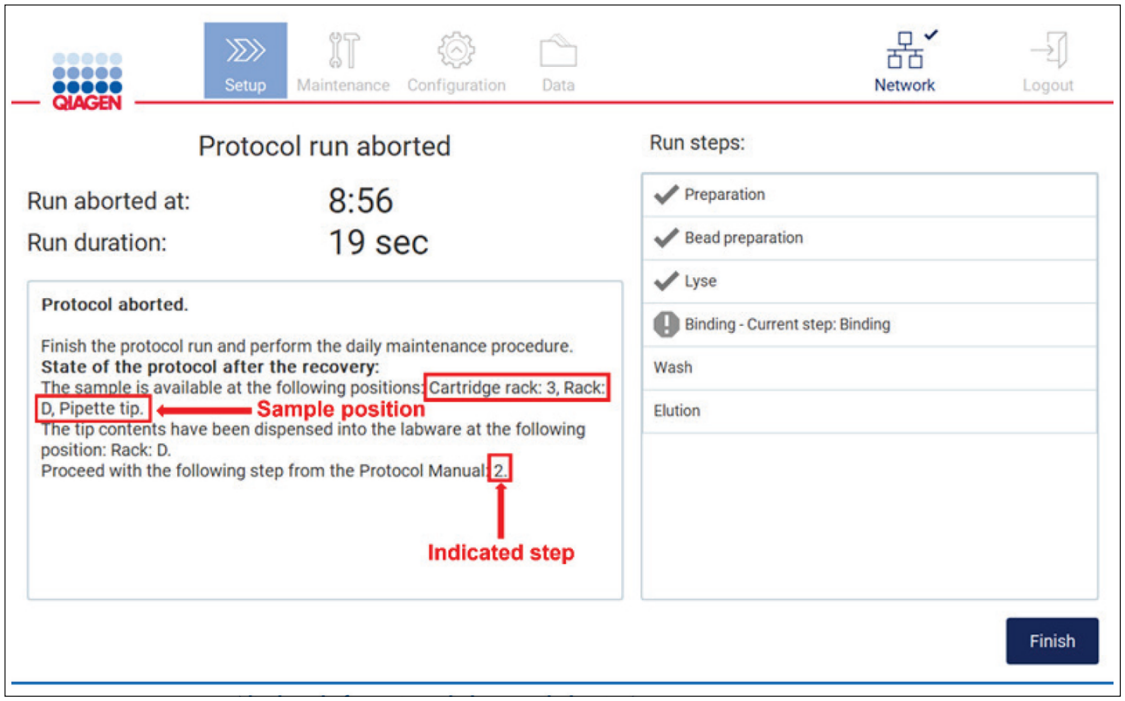
19.1.2 **Note: If the sample remains in the tip after the device switched off:** Place a tube under the tip and remove the tip from the pipette head. The liquid will now run out of the tip. If you have any problems with this step, notify the FBU Supervisor(s) and DNA TL.

19.1.3 Before proceeding, please review these general instructions.



19.1.3.1

19.1.3.1.1 This image represents the position key for the sample location from the recovery screen description (see next image).



19.1.3.2

19.1.3.2.1 This image represents the recovery screen (displayed after unintended protocol abortion).

19.1.3.3 Regardless of the respective recovery step, the following steps must be performed first:

19.1.3.3.1 The display message shows important information. Note the position of the sample and the step to be performed.

19.1.3.3.2 Open the door.

19.1.3.3.3 Remove and keep the sample containing tubes/cartridges.

19.1.3.3.3.1 Important: Label the sample tubes/cartridges and be careful not to mix up their order.

19.1.3.3.4 Proceed with the EZ2 screen's indicated recovery step using the following table.

EZ2 Screen's Indicated Step	Sample Recovery Procedure Step	Process Status
1	1	Sample untouched
2	2	Buffer MTL added to the sample (if applicable), beads may or may not have been added
3	3	During wash 1
4	3	During wash 2
5	3	During wash 3
6	4	During rinse step
7	5	During elution step

19.1.3.3.4.1

19.1.3.3.4.1.1 The analyst will document the reason the protocol run was aborted and the sample recovery procedure step being performed. This may be documented within the comments section of the extraction form.

19.2 Sample Recovery Procedure Steps

19.2.1.1 Step 1

19.2.1.1.1 Add new labware, by replacing the cartridges, tips and tip holders, and elution tubes. There may be magnetic beads in the elution tubes but since the sample was untouched, it can be discarded.

19.2.1.1.2 Restart the run.

19.2.1.2 Step 2

19.2.1.2.1 Recover the lysate and all beads, if already added. The screen will indicate the location of the sample at the time the run was aborted. The expected volume is approximately 900ul. Transfer 900uL from the sample location into a new clean 2 mL screw cap tube, making sure to recover as many beads as possible. Replace the cartridge, tips and tip holders, and original elution tubes.

19.2.1.2.2 Use as samples in the Large Volume protocol (old version, with no MTL from well 10). Note: Setup will be the same as the DNA Investigator Trace protocol.

19.2.1.3 Step 3

19.2.1.3.1 The instrument will display the location of the sample.

19.2.1.3.2 If no sample remains in the sample location skip this bullet and its sub-bullets and proceed to the next step. However, if sample is present in the sample location perform the following intermediary steps.

19.2.1.3.2.1 Allow a few minutes for the beads to settle. Carefully discard the supernatant (i.e., wash buffer) within the cartridge, making sure to avoid the beads, until ~500ul remains.

19.2.1.3.2.2 Re-suspend the beads by pipetting up and down.

19.2.1.3.3 Transfer the remaining beads (if present) from the cartridge into the elution tube. If the remaining beads have already been transferred to the elution tube (i.e., sample location is empty), proceed to the next step.

19.2.1.3.4 To get a bead pellet, centrifuge the elution tube at 6000x RCF for 1 minute.

19.2.1.3.5 Carefully discard wash buffer from the elution tube, until approximately 200ul are left.

19.2.1.3.6 Resuspend the beads within the elution tube by pipetting up and down and transfer the contents to a new 2mL sample tube. Replace the cartridges, tips and tip holders, and elution tubes.

19.2.1.3.7 Use sample in a Trace Protocol.

19.2.1.4 Step 4

19.2.1.4.1 Recover all beads and entire water used for the rinse by combining the sample from its well location and the elution tube (~1000ul total volume) into a tube. Resuspend the beads by pipetting up and down.

19.2.1.4.2 Split the ~1000ul collected during the previous step into two new 2 mL screw cap tubes (~500ul/tube). Label these two 2 mL tubes accordingly to keep track of samples and their fractions. Replace the cartridges, tips and tip holders, and elution tubes. If the starting sample count was 2, there should now be 4 spaces loaded onto the EZ2 (i.e., sample count doubles). If the starting sample count was > 12, a second EZ2 must be used and run concurrently (i.e., simultaneously or sequentially). If a second EZ2 is used, it must be documented within the comments section of the extraction form. Run these samples using the Large Volume Protocol. Note: Setup will be the same as the DNA Investigator Trace protocol.

19.2.1.4.3 When the run is completed, pool eluates of split samples after the run.

19.2.1.5 Step 5

19.2.1.5.1 Pipette the samples out of their location (if any present) into its respective elution tube. Place samples in a room temperature shaker for 5 minutes at 900 rpm.

19.2.1.5.2 Separate magnetic beads by centrifugation at 6000x RCF for 1 minute.

19.2.1.5.3 Carefully transfer the eluate to a new clean elution tube, being sure not to disturb the bead pellet.

20. Appendix: Exporting Data and Major Evaluation

20.1 Export data from GMID-X.

20.1.1 Within GMID-X, open the Display Plots of the sample of interest.

20.1.1.1 Ensure all artifacts have been removed from the data.

20.1.1.2 Change the plot settings to 'DNA Mix Export'.

20.1.1.3 In the toolbar select File > Export Table.

20.1.1.4 Name the file with, at minimum, the sample number and initials. Save as a tab-delimited text file (.txt) to a GM Export Files folder within the associated Batch Workbooks folder.

20.2 Open the Major Deconvolution workbook.

20.2.1 On the "Marker_Count" tab

20.2.1.1 Fill in the Scientist Initials, Case Number, Sample Name, and Date Performed cells.

20.2.1.2 Click the "Do The Magic" button.

20.2.1.2.1 Find and select the exported GMID-x file of interest. Click Open.

20.2.1.2.2 A pop-up window will alert the user that the import is complete. Click OK.

20.2.1.3 Click on the "Major Eval" button. After clicking the "Major Eval" button, the user is taken directly to the "Overall Contribution" tab.

20.2.1.3.1 Clicking the "Major Eval" button marks all loci as inconclusive. Marking a locus as inconclusive triggers the following:

20.2.1.3.1.1 The workbook will use the percent contribution for the heterozygous combination with the greatest percent contribution as the Major Percent Contribution if that combination has PHR ≥ 50%.

20.2.1.3.1.2 If the heterozygous combination with the greatest percent contribution has PHR < 50%, then the workbook will use the percent contribution of the most plausible homozygous major genotype (only the allele with the most RFUs).

20.2.1.3.2 This is performed to estimate the overall major percent contribution. Utilizing the estimated overall major percent contribution will direct the analyst to perform a resolved or unrestricted methods. The resulting major percent contribution does not constitute an interpretation of the major genotype.

20.2.2 Review the "Overall Contribution" tab.

20.2.2.1 If the overall major percent contribution ≥ 75.00% utilize the resolved method.

20.2.2.2 If the overall major percent contribution < 75.00% utilize the unrestricted method.

21. Appendix: Interpretation Parameters

21.1 The PHR, proportions and mixture ratio are used in conjunction to determine if a particular genotype combination is plausible. The Traditional Deconvolution workbook's locus tabs are conditionally formatted to highlight (yellow) genotype combinations if they meet the interpretation parameters.

21.2 PHRs of genotype combinations

21.2.1 Eliminate any PHR combinations that are <23%

21.2.2 Consider all combinations that contain PHR ≥ 23%

21.3 Proportions

21.3.1 A major/minor can be determined when the major proportion is ≥0.68 and the minor proportion is ≤0.32.

21.4 Mixture Ratio

21.4.1 The mixture ratio is dependent on the proportions and must be ≥2.2:1.

21.5 Allele Sharing: The traditional deconvolution workbook considers allele sharing using the following three rules:

21.5.1 **Rule 1:** Whenever possible, shared alleles are shared proportionately based on the unshared allele.

21.5.2 **Rule 2:** Whenever possible, peak height ratios are assumed to be 100%.

21.5.3 **Rule 3:** Minimum peak heights (ATs) are always maintained. This rule supersedes rules 1 and 2.

21.5.3.1 At three allele loci, rule 3 has been applied if the "Proportion PHR" within a genotypic combination is not equivalent.

21.5.3.2 At two allele loci, rule 3 has been applied if the "Shared Allele Portion" value does not match either of the allele RFU values *and* the PHR does not equal 100%.

21.5.3.3 Rule 3 does not apply to one allele loci.

22. Appendix: Traditional Deconvolution Workbook

22.1 Open the Traditional Deconvolution workbook. To enter information/data follow the automated entry directions on the "Marker_Count" tab (manual entry has not been validated). Save the major evaluation file as "*Sample#_MajorEval_Initials".* Save the traditional deconvolution file as "*Sample#_TradDecon_Initials*". Do not save over the workbook template.

22.1.1 **Important Note**: If a locus contains more than 4 alleles, its locus tab will be blank upon import. The workbook imports data to each locus tab that contains up to four alleles. Although data may not populate on the tab, the data is still imported to the workbook, located in a hidden tab, and it is from this hidden tab that the RFU values in the allele RFU assessment are pulled. Other than the confirmed tri-allele scenario for an assumed deconvolution, no determinations will be made at a locus containing more than four alleles when using the traditional deconvolution workbook. In this scenario, the analyst must denote it in the traditional deconvolution workbook and on the electropherogram (e.g., No determinations made at this locus due to 5 alleles).

22.2 Allele RFU Assessment Table

22.2.1 Note that an entry to the Allele RFU Assessment table does not necessarily represent an interpretation at the locus. In certain scenarios entries to this table will be for the sole purpose of estimating the RFU's of a component.

22.2.2 Display of cells K10, K11, M10 and M11.

22.2.2.1

Allele RFU Assessment				
	Major	Major RFU	Minor	Minor RFU
Allele 1	Cell K10		Cell M10	
Allele 2	Cell K11		Cell M11	
Total RFU		0		0

22.2.3 Entry example for single alleles for the major and minor.

22.2.3.1

Allele RFU Assessment				
	Major	Major RFU	Minor	Minor RFU
Allele 1	10		13	
Allele 2				
Total RFU		0		0

22.2.4 Entry example of identical alleles.

22.2.4.1

Allele RFU Assessment				
	Major	Major RFU	Minor	Minor RFU
Allele 1	10		12	
Allele 2	10		12	
Total RFU		0		0

22.2.5 Entry example of unique alleles

22.2.5.1

Allele RFU Assessment				
	Major	Major RFU	Minor	Minor RFU
Allele 1	10		13	
Allele 2	12		14	
Total RFU		0		0

22.2.6 When no determinations are made at a locus do not enter information into the allele RFU assessment table. Document using the justification cell. For example:

22.2.6.1

Allele RFU Assessment				
	Major	Major RFU	Minor	Minor RFU
Allele 1				
Allele 2				
Total RFU		0		0

Analytical Threshold	Stochastic Threshold	4 Allele Mixture Ratio
127	795	

RFUs Used in Average Calculation	
Major	0.0
Minor	0.0

Justification
No determinations were made at this locus.

22.2.7 The analyst, providing justification, may use the "Check if no RMP for minor allele" box for instances not explicitly stated in this document. Checking this box does as it describes if an entry has been made to M10 (i.e., the M10 entry will not be included in the RMP). If an entry has been made to M10 and M11, it will not work as described as it eliminates only the M10 entry from the RMP calculation. The analyst must thoroughly, methodically, and explicitly explain why the M10 entry is not being utilized in the RMP calculation. Stating simply "artifact interference" is not an acceptable justification.

22.2.7.1 Checked

Check if no RMP for minor allele.
☑

22.2.7.2 Unchecked

Check if no RMP for minor allele.
☐

22.2.8 When each allele is < STO at a 3, 2, or 1 allele locus, cell L60 will not auto populate with the entry made to cell M10.

22.2.9 When an allele is entered to cell K10 but not cell K11, cell L51 will not auto populate with the entry made to cell K10.

22.3 RMP Tables

22.3.1 Certain types of entries made to the Allele RFU Assessment table will auto populate to the RMP tables.

22.3.1.1 Cells L51 and M51 of the Major Allele Pair(s) – RMP table are locked to allow for auto population and do not allow manual entry.

22.3.1.2 Cells L60 and M60 of the Minor Allele Pair(s) – RMP table are locked to allow for auto population and do not allow manual entry.

22.3.1.3 Manual entries begin in cell L52 (major) and cell L61 (minor).

22.3.1.4

Major Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1	L51	M51
2	L52	
3		
4		
5		
6		
7		
Minor Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1	L60	M60
2	L61	
3		
4		
5		
6		
7		

22.3.2 Note that duplicate genotypes/allele pairs should not be entered to the RMP tables. For example, if a genotype (e.g., 15,16) meets the criteria for entry in two different genotype combinations (e.g., Major 15,16/Minor 11,11 and also Major 15,16/Minor 11,15) the genotype will only be entered once to the respective RMP table.

22.3.2.1 Allowed:

22.3.2.1.1

Major Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1		
2	15	16
3		
4		
5		
6		
7		

22.3.2.2 Not allowed:

22.3.2.2.1

Major Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1		
2	15	16
3	15	16
4		
5		
6		
7		

22.3.2.2.1.1 Entering in this way will cause the 15,16 to be counted twice in the RMP.

22.3.3 Example of both a homozygous and heterozygous entry.

Major Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1		
2	10	10
3	10	11
4		
5		
6		
7		
Minor Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1		
2	11	11
3	11	12
4		
5		
6		
7		

22.3.3.1

22.3.4 Example of an "Allele,+" entry. Leave the Allele 2 column blank.

Major Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1		
2	10	
3		
4		
5		
6		
7		
Minor Allele Pair(s) - RMP		
D2S441	Allele 1	Allele 2
1		
2	11	
3		
4		
5		
6		
7		

22.3.4.1

22.4 Component Averages Tab

22.4.1 The analyst must review the "Component Averages" tab. The RFUs of the major and minor component, at each locus, will automatically populate within the table.

22.4.2 If the major and/or minor RFU within a blue dye channel loci are ≥ STO (795 RFU) the cell will turn gray. If the RFU is < STO (795 RFU) the cell will turn yellow.

22.4.3 If the major and/or minor RFU within a green dye channel loci are ≥ STO (1290 RFU) the cell will turn gray. If the RFU is < STO (1290 RFU) the cell will turn yellow.

22.4.4 If the major and/or minor RFU within a yellow dye channel loci are ≥ STO (1255 RFU) the cell will turn gray. If the RFU is < STO (1255 RFU) the cell will turn yellow.

22.4.5 If the major and/or minor RFU within a red dye channel loci are ≥ STO (760 RFU) the cell will turn gray. If the RFU is < STO (760 RFU) the cell will turn yellow.

22.4.6 The average per allele RFUs of the major and minor component are calculated by totaling the component's RFUs and dividing by the total number of alleles for the component. The average per allele RFUs of each component are listed at the bottom of the table. The average per allele RFUs conditional highlighting follows the gray and yellow STO rules stated in the previous bullets.

22.5 Thresholds

Dye	Color	Analytical Threshold	Stochastic Threshold
Fluorescein	Blue	127	795
JOE	Green	131	1290
TMR-ET	Yellow	158	1255
CXR-ET	Red	153	760
WEN	Orange	100	

23. Equations for Traditional, Assumed, and Major Deconvolution methods

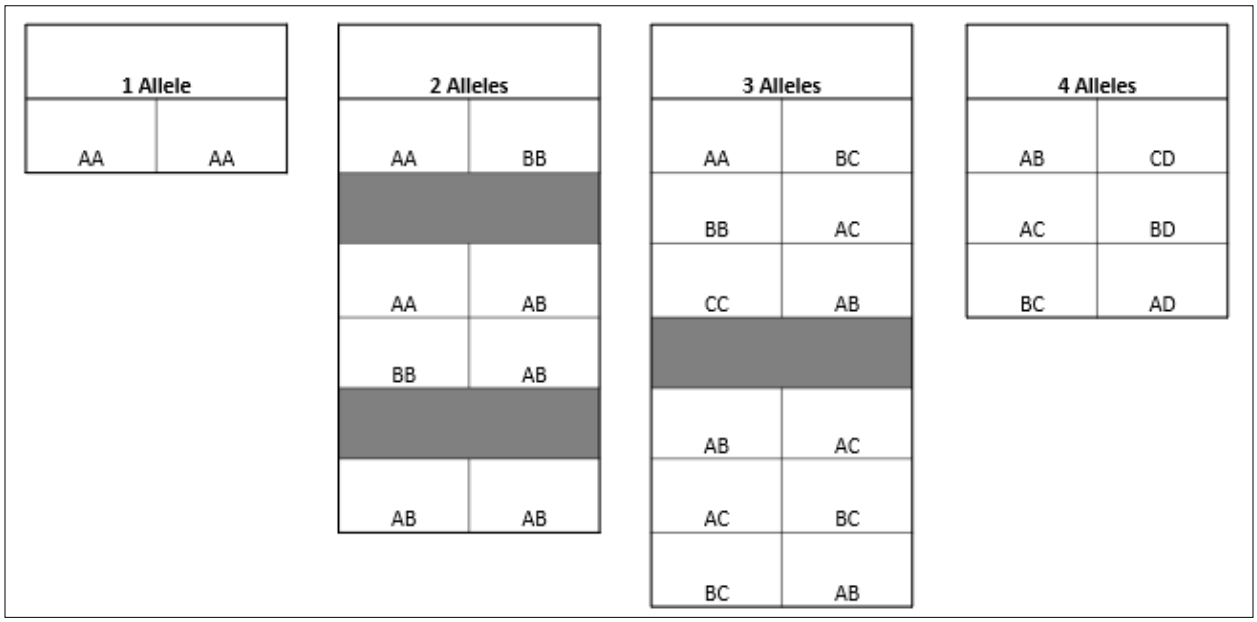
23.1 Peak Height Ratio

$$\left(\frac{PH2}{PH1}\right) * 100 = PHR$$

Where PH1 > PH2

23.2 Mixture Ratio and Proportions

Two Contributor Genotype Combinations



Mixture Ratio Calculation – general

$$\frac{Genotype\ Proprtion\ 1}{Genotype\ Proportion\ 2}:1$$

Where *Genotype Proportion* 2 < *Genotype Proportion* 1

Genotype Combinations – Three rules not applicable

- 1 allele – AA/AA
  - Unable to calculate both PHR or proportion of contributors

- 2 allele – AA/BB
  - Unable to calculate PHR
  - Proportion
    - Genotype 1 Proportion = $\frac{RFU\ of\ A}{RFU\ of\ A + RFU\ of\ B}$
    - Genotype 2 Proportion = $\frac{RFU\ of\ B}{RFU\ of\ A + RFU\ of\ B}$
- 2 allele – AB (major only)
  - Unable to calculate proportion
  - Calculate the PHR using the PHR equation
- 3 allele – AA/BC
  - Genotype 1 Proportion = $\frac{RFU\ of\ A}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C}$
  - Genotype 2 Proportion = $\frac{RFU\ of\ B + RFU\ of\ C}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C}$
- 3 allele – BB/AC
  - Genotype 1 Proportion = $\frac{RFU\ of\ B}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C}$
  - Genotype 2 Proportion = $\frac{RFU\ of\ A + RFU\ of\ C}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C}$
- 3 allele – CC/AB
  - Genotype 1 Proportion = $\frac{RFU\ of\ C}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C}$
  - Genotype 2 Proportion = $\frac{RFU\ of\ A + RFU\ of\ B}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C}$
- 4 allele – AB/CD
  - Genotype 1 Proportion = $\frac{RFU\ of\ A + RFU\ of\ B}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C + RFU\ of\ D}$
  - Genotype 2 Proportion = $\frac{RFU\ of\ C + RFU\ of\ D}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C + RFU\ of\ D}$
- 4 allele – AC/BD
  - Genotype 1 Proportion = $\frac{RFU\ of\ A + RFU\ of\ C}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C + RFU\ of\ D}$

- Genotype 2 Proportion = $\frac{RFU\ of\ B + RFU\ of\ D}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C + RFU\ of\ D}$

- 4 allele – BC/AD
  - Genotype 1 Proportion = $\frac{RFU\ of\ B + RFU\ of\ C}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C + RFU\ of\ D}$
  - Genotype 2 Proportion = $\frac{RFU\ of\ A + RFU\ of\ D}{RFU\ of\ A + RFU\ of\ B + RFU\ of\ C + RFU\ of\ D}$

Genotype Combinations – Three rules are applied

- **Rule 1 - Whenever possible, shared alleles are shared proportionately based on the unshared allele.**
  - Applicable to the following three allele genotype combinations:
    - AB/AC
    - AC/BC
    - BC/AB

1. Calculate the proportion of each unshared allele
   - Proportion of unshared allele 1 =

$$\frac{RFU\ of\ unshared\ allele\ 1}{RFU\ of\ unshared\ allele\ 1 + RFU\ of\ unshared\ allele\ 2}$$

   - Proportion of unshared allele 2 =

$$\frac{RFU\ of\ unshared\ allele\ 2}{RFU\ of\ unshared\ allele\ 1 + RFU\ of\ unshared\ allele\ 2}$$

2. Use the unshared allele proportion to calculate how to divide the shared allele.

   - Portion of shared peak paired with unshared allele 1 =

$$Proportion\ of\ unshared\ allele\ 1 * RFU\ of\ shared\ allele = X\ RFUs$$

   - Portion of shared peak paired with unshared allele 2 =

$$Proportion\ of\ unshared\ allele\ 2 * RFU\ of\ shared\ allele = X\ RFUs$$

3. Calculate PHR of the genotype combinations using the unshared alleles and the portion of shared peak.

   - Genotype 1

$$\frac{RFU\ of\ unshared\ allele\ 1}{RFU\ portion\ of\ shared\ peak\ paired\ with\ unshared\ allele\ 1} * 100$$

   - Genotype 2

$$\frac{RFU\ of\ unshared\ allele\ 2}{RFU\ portion\ of\ shared\ peak\ paired\ with\ unshared\ allele\ 2} * 100$$

   Where the numerator < denominator

4. Calculate Mixture Ratio

$$\frac{Proportion\ of\ unshared\ allele\ 1}{Proportion\ of\ unshared\ allele\ 2}:1$$

   Where *Proportion of unshared allele* 2 < *Proportion of unshared allele* 1

➢ **Rule 2 - Whenever possible, PHRs are assumed to be 100%.**

- Applicable to the following two allele genotype combinations – where the homozygote has the largest RFU value. If the homozygote has the smallest RFU value, proceed to section #2) of Rule 3's application to Rule 2.
  - AA/AB
  - BB/AB

1. Determine how much of the allele 1 (allele with the largest RFU value) goes with allele 2 (allele with the smallest RFU value).

   - Homozygote allele portion = $RFU\ of\ allele\ 1 - RFU\ of\ allele\ 2$

- When this results in a value < AT but >0 the Rule 3 section → Application to Rule 2 → #1 equations will be applied.
  - Heterozygote allele portion = $RFU\ of\ allele\ 2$

2. Heterozygote proportion

$$\frac{RFU\ of\ allele\ 2 + RFU\ of\ allele\ 2}{RFU\ of\ allele\ 1 + RFU\ of\ allele\ 2}$$

3. Homozygote proportion

$$\frac{RFU\ of\ allele\ 1 - RFU\ of\ allele\ 2}{RFU\ of\ allele\ 1 + RFU\ of\ allele\ 2}$$

4. Calculate PHR for heterozygote

$$\frac{RFU\ of\ allele\ 2}{RFU\ of\ allele\ 2} * 100$$

   - This should equal 100%.
   - No PHR can be calculated for the homozygote.

5. Calculate Mixture Ratio

$$\frac{Homozygote\ Proportion}{Heterozygote\ Proportion}:1$$

   - Where *Heterozygote Proportion < Homozygote Proportion*

➢ **Rule 3 – Minimum peak heights are always maintained.**

- Minimum peak heights /analytical thresholds (AT).
- Supersedes rules 1 and 2.

- <u>Application to Rule 1</u>
  - When calculating the portion of the shared peak that belongs to an unshared allele the portion is < AT
    - This affects the PHR.
  - When this occurs, calculate PHR as follows:

$$\frac{AT}{RFU\ of\ unshared\ allele} * 100$$

Where the numerator < denominator

- Application to Rule 2 – occurs in two instances:
  - #1) When the calculated homozygote allele portion results in a value <AT but >0.
    - Homozygote allele portion = $RFU\ of\ allele\ 1 - RFU\ of\ allele\ 2$

      Where allele 1 = allele with the largest RFU value

      Where allele 2 = allele with the smallest RFU value
    - If this results in a value < AT but >0,
      - Homozygote allele portion = AT
      - Heterozygote allele portion = $RFU\ of\ allele\ 1 - AT$
        - If RFU of allele 1 < 2*AT then the heterozygote allele portion will = AT.
    - Heterozygote proportion

$$\frac{RFU\ of\ allele\ 2 + (RFU\ of\ allele\ 1 - AT)}{RFU\ of\ allele\ 1 + RFU\ of\ allele\ 2}$$

    - Homozygote proportion

$$\frac{AT}{RFU\ of\ allele\ 1 + RFU\ of\ allele\ 2}$$

    - Calculate PHR for heterozygote

$$\frac{(RFU\ of\ allele\ 1 - AT)}{RFU\ of\ allele\ 2} * 100$$

      - Where the numerator < denominator

  - #2) When the homozygote genotype has the smallest RFU value resulting in a value <AT and <0.
    - Homozygote allele portion = $RFU\ of\ allele\ 2 - RFU\ of\ allele\ 1$

Where allele 1 = allele with the largest RFU value

Where allele 2 = allele with the smallest RFU value

- This result in a value < AT and <0, thus
  - Homozygote allele portion = AT
  - Heterozygote allele portion = $RFU\ of\ allele\ 2 - AT$
    - If RFU of allele 2 < 2*AT then the heterozygote allele portion will = AT.

- Heterozygote proportion

$$\frac{RFU\ of\ allele\ 1 + (RFU\ of\ allele\ 2 - AT)}{RFU\ of\ allele\ 1 + RFU\ of\ allele\ 2}$$

- Homozygote proportion

$$\frac{AT}{RFU\ of\ allele\ 1 + RFU\ of\ allele\ 2}$$

- Calculate PHR for heterozygote

$$\frac{(RFU\ of\ allele\ 2 - AT)}{RFU\ of\ allele\ 1} * 100$$

  - Where the numerator < denominator

➢ **Major Deconvolution percent contribution**

$$\frac{Total\ RFUs\ of\ Proposed\ Major}{Total\ Allelic\ RFUs\ at\ Locus} * 100$$