

Missing Persons and Sequencing Laboratory Procedures

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1 Missing Persons and Sequencing (MPS) Laboratory and Technical Procedures Manual

This manual provides the laboratory guidelines to process samples for mitochondrial DNA (mtDNA) testing. It also describes the sample analysis, report writing, and reporting methods for mtDNA testing.

2 MPS Laboratory Guidelines

Due to the extremely sensitive nature of working with ~~mitochondrial DNA (mtDNA)~~, special precautions are needed to reduce the risk of possible contamination in the laboratory. These policies are in addition to common lab practices already in place in the CODIS section.

- Access to the CODIS Bone/Mitochondrial (Mito) lab should be restricted to authorized personnel who have work to conduct in the laboratory.
- An individual shall only enter the Bone/Mito lab if they are “clean.” An individual is to be considered “clean” if earlier in the same day they have not entered any laboratory space which contains **non-sealed** post-amplification product or materials. An individual will be considered “dirty” if they have entered a post-amplification laboratory space **with non-sealed amplified product** at any point during the day. Daily activities should be planned accordingly.
- The following items should be worn in the Bone/Mito DNA lab at all times: lab coat, shoe covers, sleeves, gloves, cap (hair pulled back and inside cap), mask, and eye protection. Lab coats and masks should be discarded and replaced periodically. Goggles may be periodically exposed to UV light and retained for use.
- Personnel should refrain from talking in the lab without a face mask on.
- When applicable, double gloving is strongly recommended. Gloves should be rinsed with 10% bleach and alcohol before beginning any procedure. Additional rinses should be conducted before entering any clean hood space or between handling of different samples as necessary.
- All bone processing (sanding, cutting, grinding) should be carried out behind the bone curtain to the extent possible.
- **Pink** cryo gloves should remain in the Mito lab at all times and be used when handling liquid nitrogen in the Mito lab. **Blue** cryo gloves should be used when filling the liquid nitrogen dewar and transporting the dewar.
- If possible, aprons and sleeves should be changed before moving from the bone cutting area to the extraction and amplification areas of the laboratory.
- All racks and other applicable plastics should be treated in the Ultraviolet Crosslinker before being used for extraction or amplification.
- If reagents are to be used in the hoods, the smallest volume possible should be transferred to labeled tubes for use within the hood. Once the work is complete the reagent aliquots should be discarded. If possible, UV the reagent before use (not be done with DNA, enzymes, etc.).

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- Tubes which are to be used for extraction and amplification should be placed in the Ultraviolet Crosslinker before use. Amplification plates should not be placed in the cross-linker (they will discolor if UV treated).
- Before and after working in a hood, the hood should be cleaned in the following order: DNAerase, 10% bleach, 70% Isopropyl alcohol, and treated with UV light for a minimum of 30 mins (if applicable).
 - **NOTE:** bleach should not be used to clean any surface with liquid waste containing PrepFiler Lysis Buffer or PrepFiler Magnetic Particles. These surfaces should be thoroughly wiped down with water, DNAerase, and alcohol before being wiped down with bleach.

3 Sample Processing

Bones, teeth, and reference samples are processed for entry into CODIS, for comparison to other unknown samples or reference samples, quality control purposes, or for research/training.

For note taking, the *MPS Sample Notes* form or any of the approved DNA notes pages may be used. For tracking samples throughout the MPS process, the most current version of the *MPS Lab Workbook* should be used. The workbook has the ability to directly import notes in the *MPS Sample Notes* format, create instrument import files, and house quantification results data. The *MPS Lab Workbook* should be used for each batch of samples processed in the laboratory. Directions for the use of the workbook can be found in the *MPS Lab Workbook User Guide*.

3.1 Bone - Sample Preparation

- 1) Clean the external surface of the bone:
 - a. Remove all flesh.
 - b. Soak the bone in 10% bleach for 1-2mins or wipe exterior of bone with 10% bleach.
 - c. Rinse with sterile distilled water and allow the bone to dry completely.
 - d. Using a Dremel ~~with an aluminum oxide grinding stone attached~~, sand the external surface of the bone.
 - e. Rinse with sterile distilled water, then with 100% ethanol, and then allow the bone to dry completely.
- 2) Using the autopsy saw ~~with vacuum attachment and the appropriate blade~~ or a Dremel ~~with a cutting disk~~, cut ~1/4inch square pieces from the bone.

NOTE: If using the autopsy saw vacuum while not attached to the autopsy saw, the opening must be guarded with wire/cloth mesh to prevent the loss of larger bone fragments into the vacuum. The mesh should be placed over the opening of the vacuum hose and then secured to the vacuum hose.
- 3) Place the bone cuttings in a labeled 50ml conical tube. Add enough 5% Tergazyme solution to cover the cuttings and allow to sit for 2 hours on a thermal mixer set to 23°C and 750rpm.

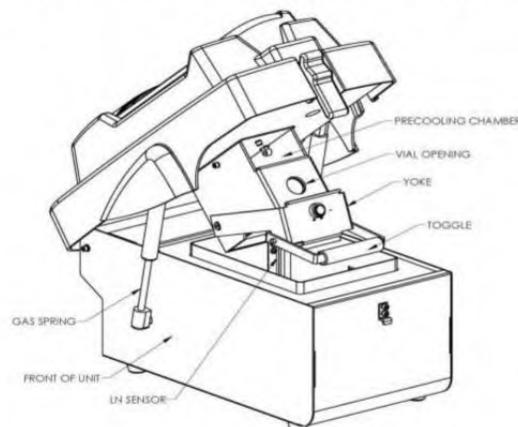
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- 4) After the incubation, place a paper towel over the drain of a clean sink **(to prevent potential sample loss)** and decant the Tergazyme solution. Add sterile distilled water to the tube and gently shake. Decant the water from the tube and repeat until the water runs clear. Alternatively, slits may be cut into the bottom of a weigh boat to allow the bone fragments to be easily rinsed off. Allow the bone cuttings to dry completely before continuing (overnight if **needed possible**).
- 5) Weigh the bone cuttings.

NOTE: If proceeding directly to the freezer/mill, tare the weight of the 50mL conical on the scale. Then after milling, the sample can be placed back into the conical and the weight can be measured.

- 6) To load a Freezer/Mill vial, first assemble it halfway by pushing a blunt end plug into a center cylinder. Add the bone cuttings and impactor to the vial, and close it with the flanged end plug. Shake the vial to make sure the impactor has ample room to move back and forth.

CAUTION: When handling Liquid Nitrogen (LN), chilled samples or vials, or touching parts of the Freezer/Mill that have been chilled by LN, always wear appropriate PPE.



- 7) Fill the 6775 Freezer/Mill with liquid nitrogen:
- Pour liquid nitrogen (LN) into the tub until it is approximately one third full (initially LN will boil off vigorously, but as the tub cools the boiling will subside)
 - Add more LN gradually, to avoid splashing and boiling over.
 - When the LN is up to the fill mark and boiling has subdued, push the lid down gently until the bottom of the latch can be engaged. Pause if splashing becomes excessive.
 - When the vapor stream **coming from the side port on the instrument** has subsided, lift the lid again and top off the liquid nitrogen in the tub, filling it to the mark on the inside wall of the tub.
 - Insert a loaded vial into the chamber.
 - One to three additional vials can also be put in the precooling chamber above the grinding chamber if necessary. **Vials should be labeled with the BCI case and item number if being precooled in the chamber together.**
 - Press the Freezer/Mill lid closed and then latch it closed.

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NOTE: A stream of condensed water vapor coming from the vent in the rear of the Freezer/Mill is normal.

- 8) Program the Freezer/Mill or select a saved protocol to run:
a. Program the Freezer/Mill as follows:

Bone Protocol	
Pre-Cool:	10 mins
Run Time:	2 mins
Cool Time:	2 mins
Cycles:	2
Rate:	10 CPS

- b. Selecting a Saved Protocol: Select "Saved Protocols" from the home screen, tap the protocol you wish to run, tap "Load"
- 9) Load the Sample Vial(s) into the Freezer/Mill
- Put on the pink cryogenic gloves when handling items which have come into contact with Liquid Nitrogen.
 - Unlatch the lid and as the lid is lifted the yolk should open as well.
 - Pick up a vial with a gloved hand and insert the vial into the chamber, blunt end first.
 - If necessary, additional vials can be precooled by inserting them into the precooling chamber in the coil housing.
 - Before closing the lid, be sure the Liquid Nitrogen level is sufficient.
 - Close the Lid.
 - Latch the lid shut.
- 10) Press "Run" to begin the grinding protocol.
- NOTE:** Be sure to check the Liquid Nitrogen level over the course of the run to ensure the level is sufficient. The instrument will pause and display "LN LEVEL LOW" if the liquid nitrogen in the tub is not at the correct operating level.
- 11) Once the grinding cycle is complete, unlatch the lid and let the gas cylinders raise the lid at its own speed. Do not forcefully lift the lid. If the yolk does not open by itself, open it by pulling the toggle to the right.
- 12) Remove the vial using the extractor tool or a gloved hand then do one of the following:
- Close the lid to conserve Liquid Nitrogen while another vial is being prepared,
 - Insert another vial and begin another grinding cycle, or
 - Leave the lid open and allow the Liquid Nitrogen to evaporate
- 13) Open the Vial: slip the open end of the extractor over the flanged end-plug, align the pegs in the end-plug with the slots in the Extractor, and turn the knob clockwise until the end-plug is drawn out. If the end-plug is drawn out within a millimeter or two of the end of the

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center cylinder, rock the extractor gently to dislodge the end plug. Always be careful that the vial does not fall and spill its contents.

- 14) Separate the impactor from the contents using a magnet, tongs, or pliers.
- 15) Place the bone dust in a 50mL conical or other tube, then weigh and record the amount of generated dust.
- 16) Clean the vial components immediately after processing:
 - a. scrub each vial component with a small brush and 10% bleach
 - b. rinse all parts with water and then scrub each vial component with a small toothbrush and 0.1% SDS
 - c. rinse all parts with water and place in 10% bleach
 - d. rinse all parts with water
 - e. rinse ONLY the metal end plugs and the metal impactor(s) with 200 proof ethanol and dry completely to prevent rusting. **DO NOT** rinse the plastic vial with ethanol as it can cause the vials to crack.
 - f. Expose all vial components to UV light for a minimum of 2hrs

3.2 Manual Extraction Procedures

Sample extraction should be performed using the PrepFiler BTA kit. The extraction may be performed either manually or on the AutoMate Express instrument.

NOTE: When extracting a sample, two tubes may be used for extraction with a minimum elution volume of 20µl per tube. This will ensure sufficient material for typing the sample with STRs on the CE instrument and mitochondrial typing on the sequencing instrument. Standards may be extracted in 100µl or more to prevent large dilutions being required prior to amplification. Elution Volumes may range from 20-200µl.

3.2.1 Bone – Sample Extraction with PrepFiler BTA Kit – Manual Procedure

WARNING: Contact with acids or bases (such as bleach) liberates toxic gases. DO NOT ADD acids, or bases (such as bleach) to any liquid wastes containing PrepFiler Lysis Buffer, PrepFiler BTA Lysis Buffer, or PrepFiler Magnetic Particles.

Preparation for Extraction:

- 1) If the kit is not being used for the first time proceed to step 2, otherwise:
 - a. Prepare one bottle each of Wash Buffer A and B (prepare the second bottle of each once the first bottles have been depleted).
 - i. Wash Buffer A: Fill the Wash Buffer A concentrate bottle to the shoulder with fresh 95% ethanol (93mL ethanol). Mark the bottle label to indicate the concentrate has been diluted.
 - ii. Wash Buffer B: Fill the Wash Buffer B concentrate bottle to the shoulder with fresh 95% ethanol (19.5mL ethanol). Mark the bottle label to indicate the concentrate has been diluted.

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- iii. Diluted Wash Buffer expires six months after prep or until their expiration date (whichever is sooner).
- 2) Prepare the following kit reagents:
 - a. Incubate Magnetic Particles at 37°C for 10 minutes then vortex for 1 min or until the particles are completely suspended.
 - b. If precipitate is present in the PrepFiler Lysis Buffer, heat at 37°C for 15 minutes then vortex.
 - c. Thaw 1.0M DTT (discard any remaining, do not re-freeze).
 - d. Turn on Thermal Shaker and set at 56°C.

Extraction:

- 1) Place approximately 0.05g of bone powder in 2.0mL screw-cap tube.
- 2) Create a Manipulation/Reagent Blank using an empty tube. ~~Label as "RB_DD-MM-YY_Initials" or "MB_DD-MM-YY_Initials"~~.
- 3) Prepare fresh lysis buffer mix as follows (add 3 to your number of samples to account for any pipetting losses which may occur):

<u>Lysis Buffer Solution Component</u>	<u>Volume Per Sample</u>
PrepFiler BTA Lysis Buffer	220µl
1.0M DTT	3µl
Proteinase K	7µl

- 4) Add 230µl of lysis buffer mix to each sample, then:
 - a. Cap the tube,
 - b. Vortex for 5 seconds,
 - c. Centrifuge briefly,
 - d. Gently flick the tube to re-suspend the substrate.
- 5) Place the tubes in the 56°C thermal shaker set to 1100rpm for 2 hours to overnight.
- 6) Allow the samples to equilibrate to room temperature and label new 1.5mL microcentrifuge tubes.
- 7) Centrifuge the samples at 10,000xg for 90 secs.
- 8) Transfer the clear lysate to a new labeled 1.5mL microcentrifuge tube (do not disturb the sediment).
- 9) Check to ensure the volume of the collected lysate is at least 180µl
 - a. If not, then add PrepFiler BTA Lysis Buffer to bring the volume to 180µl.
- 10) Add 300µl of PrepFiler Lysis Buffer to the tube, then vortex and centrifuge briefly.

NOTE: *May Stop Here. Supplemented Lysate is Stable For 24 Hours at Room Temperature. Do Not Chill the Lysate!*

- 11) Allow the sample to equilibrate to room temperature.
- 12) Vortex the PrepFiler Magnetic Particles for 5 secs then:
 - a. Invert the tube to be sure no visible pellet remains in the bottom of the tube
 - b. Centrifuge briefly
 - c. Vortex the magnetic particles every 5 mins until completed
- 13) Pipet 15 μ l of magnetic particles into each sample tube then:
 - a. Cap the tube
 - b. Vortex at low speed (~500-1,200rpm) for 10 secs
 - c. Centrifuge briefly
- 14) One sample at a time:
 - a. Add 300 μ l isopropanol (99.5% molecular biology grade) to the sample tube
 - b. Immediately cap
 - c. Vortex at low speed (~500-1,200rpm) for 5 secs
 - d. Centrifuge briefly
- 15) Once completed the above for all samples; place all tubes in shaker set to 23°C and mix at 700rpm for 10 mins.
- 16) Vortex at max speed for 10 secs and then centrifuge briefly
- 17) Place all tubes into the magnetic stand and wait 10 mins (or until the size of the pellet stops increasing)
- 18) While leaving the sample tube in the magnetic stand, carefully aspirate and discard all visible liquid phase (Be sure not to disturb the pellet)
- 19) Perform the following steps three times in a row:
 - a. Add the correct amount and type of wash buffer:
 - i. First Wash: 600 μ l of Wash Buffer A
 - ii. Second Wash: 300 μ l Wash Buffer A
 - iii. Third Wash: 300 μ l of Wash Buffer B
 - b. Cap the tubes and remove from the magnetic stand
 - c. Vortex for 15 secs then centrifuge briefly
 - d. Place the tubes on the magnetic stand for 1 minute
 - e. While still on the magnetic stand, remove all visible liquid phase (be sure not to disturb or aspirate the magnetic particle pellet)
- 20) Centrifuge the tubes briefly then place back on the magnetic stand for 30-60 secs.
- 21) Use a low volume pipettor to remove any residual liquid present
- 22) If residual liquid is still present, leave tubes on the magnetic stand, open the caps, and allow to air dry for 5 mins (air drying for more than 10 mins may reduce DNA yield).
- 23) Set the thermal shaker to 70°C.
- 24) Add 20 μ l-200 μ l of PrepFiler Elution Buffer to each tube and vortex at max speed until the pellet is re-suspended.
- 25) Place the tubes in a thermal shaker for 10 mins at 70°C and 900rpm.
- 26) Label new 1.5mL microcentrifuge collection tubes to be used for the final elution.
- 27) Vortex each tube at max speed until there is no visible magnetic particle pellet on the side of the tube and then centrifuge briefly.

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- 28) Place the tubes in the magnetic stand and wait for approximately 5 mins or until the size of the pellet stops increasing.
- 29) Carefully aspirate all visible liquid phase into the appropriate labeled 1.5mL microcentrifuge tube.

NOTE: Store the isolated DNA at 4°C for up to one week or at -20°C for prolonged storage.

3.2.2 Reference Sample Preparation and Extraction with PrepFiler BTA Kit – Manual Procedure

WARNING: Contact with acids or bases (such as bleach) liberates toxic gases. DO NOT ADD acids, or bases (such as bleach) to any liquid wastes containing PrepFiler Lysis Buffer, PrepFiler BTA Lysis Buffer, or PrepFiler Magnetic Particles.

Preparation for Extraction:

- 1) Depending on the sample age and substrate properties, determine the amount of sample to use. Example amounts to use are listed in the following table:

<u>Substrate</u>	<u>Amount</u>
Cotton Swab	Tip of swab
BODE Collector	Tip of Bode Collector
Blood Card	5x5mm Cutting

- 2) If the kit is not being used for the first time proceed to step 3, otherwise:
 - a. Prepare one bottle each of Wash Buffer A and B (prepare the second bottle of each once the first bottles have been depleted).
 - i. Wash Buffer A: Fill the Wash Buffer A concentrate bottle to the shoulder with fresh 95% ethanol (93mL ethanol). Mark the bottle label to indicate the concentrate has been diluted.
 - ii. Wash Buffer B: Fill the Wash Buffer B concentrate bottle to the shoulder with fresh 95% ethanol (19.5mL ethanol). Mark the bottle label to indicate the concentrate has been diluted.
 - iii. Diluted Wash Buffer expires six months after prep or until their expiration date (whichever is sooner).
- 3) Prepare the following kit reagents:
 - a. Incubate Magnetic Particles at 37°C for 10minutes then vortex for 1min or until the particles are completely suspended.
 - b. If precipitate is present in the PrepFiler Lysis Buffer, heat at 37°C for 15 minutes then vortex.
 - c. Thaw 1.0M DTT (discard any remaining DTT, do not re-freeze).
 - d. Turn on Thermal Shaker and set at 56°C.

Extraction:

- 1) Place sample in 1.5mL microcentrifuge tube

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- 2) Create a Manipulation/Reagent Blank using an empty tube. Label as "~~RB_DD-MM-YY_Initials~~" or "~~MB_DD-MM-YY_Initials~~".
- 3) Prepare fresh lysis buffer mix as follows (add 3 to your number of samples to account for any pipetting losses which may occur):

Lysis Buffer Solution Component	Volume Per Sample
PrepFiler BTA Lysis Buffer	220µl
1.0M DTT	3µl
Proteinase K	7µl

- 4) Add 230µl of lysis buffer mix to each sample, then:
 - a. Cap the tube,
 - b. Vortex for 5 seconds,
 - c. Centrifuge briefly,
 - d. Gently flick the tube to re-suspend the substrate.
- 5) Place the tubes in the 56°C thermal shaker set to 900rpm for 40 mins.
- 6) Allow the samples to equilibrate to room temperature and label new PrepFiler Spin Tubes with PrepFiler Filter Columns (insert PrepFiler Spin Column into a new PrepFiler Spin Tube).
- 7) Centrifuge the samples for 2 secs.
- 8) Transfer the sample tube contents into the appropriately labeled filter column/tube
 - a. Use a pipette to transfer the liquid contents
 - b. Use the pipette tip or tweezers to transfer the substrate
 - c. Centrifuge the sample tube and then use a pipette to transfer any residual lysate
- 9) Centrifuge the PrepFiler Spin Column/Tube for 90 secs at 8000rpm.
- 10) Remove and discard the filter column.
- 11) Transfer the clear lysate to the new 1.5mL tube.
- 12) Check to ensure the volume of the collected lysate is at least 180µl
 - a. If not, then add PrepFiler BTA Lysis Buffer to bring the volume to 180µl.
- 13) Add 300µl of PrepFiler Lysis Buffer to the tube, then vortex and centrifuge briefly.

***NOTE: May Stop Here. Supplemented Lysate is Stable For 24 Hours at Room Temperature.
Do Not Chill the Lysate!***

- 14) Allow the sample to equilibrate to room temperature.
- 15) Vortex the PrepFiler Magnetic Particles for 5 secs then:
 - a. Invert the tube to be sure no visible pellet remains in the bottom of the tube
 - b. Centrifuge briefly
 - c. Vortex the magnetic particles every 5 mins until completed
- 16) Pipet 15µl of magnetic particles into each sample tube then:
 - a. Cap the tube
 - b. Vortex at low speed (~500-1,200rpm) for 10 secs
 - c. Centrifuge briefly
- 17) One sample at a time:

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- a. Add 300µl isopropanol (99.5% molecular biology grade) to the sample tube
 - b. Immediately cap
 - c. Vortex at low speed (~500-1,200rpm) for 5 secs
 - d. Centrifuge briefly
- 18) Once completed the above for all samples; place all tubes in shaker set to 23°C and mix at 700rpm for 10 mins.
- 19) Vortex at max speed for 10 secs and then centrifuge briefly
- 20) Place all tubes into the magnetic stand and wait 10 mins (or until the size of the pellet stops increasing)
- 21) While leaving the sample tube in the magnetic stand, carefully aspirate and discard all visible liquid phase (Be sure not to disturb the pellet)
- 22) Perform the following steps three times in a row:
- a. Add the correct amount and type of wash buffer:
 - i. First Wash: 600µl of Wash Buffer A
 - ii. Second Wash: 300µl Wash Buffer A
 - iii. Third Wash: 300µl of Wash Buffer B
 - b. Cap the tubes and remove from the magnetic stand
 - c. Vortex for 15 secs then centrifuge briefly
 - d. Place the tubes on the magnetic stand for 1 minute
 - e. While still on the magnetic stand, remove all visible liquid phase (be sure not to disturb or aspirate the magnetic particle pellet)
- 23) Centrifuge the tubes briefly then place back on the magnetic stand for 30-60 secs.
- 24) Use a low volume pipettor to remove any residual liquid present
- 25) If residual liquid is still present, leave tubes on the magnetic stand, open the caps, and allow to air dry for 5 mins (air drying for more than 10 mins may reduce DNA yield).
- 26) Set the thermal shaker to 70°C.
- 27) Add 100µl-200µl of PrepFiler Elution Buffer to each tube and vortex at max speed until the pellet is re-suspended.
- 28) Place the tubes in a thermal shaker for 10 mins at 70°C and 900rpm.
- 29) Label new 1.5mL microcentrifuge collection tubes to be used for the final elution.
- 30) Vortex each tube at max speed until there is no visible magnetic particle pellet on the side of the tube and then centrifuge briefly.
- 31) Place the tubes in the magnetic stand and wait for approximately 5 mins or until the size of the pellet stops increasing.
- 32) Carefully aspirate all visible liquid phase into the appropriate labeled 1.5mL microcentrifuge tube.

NOTE: Store the isolated DNA at 4°C for up to one week or at -20°C for prolonged storage

3.3 Automated Extraction Procedures

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3.3.1 AutoMate Express Instrument

The AutoMate Express Forensic DNA Extraction System is a bench-top instrument which utilizes the PrepFiler Express and PrepFiler Express BTA chemistry.

To avoid significant problems such as data loss or run cancellation, always perform operations in the following order:

- 1) Before you insert or remove a protocol card, power OFF the instrument
- 2) Before you power ON the instrument:
 - a. Insert the protocol card
 - b. Close the instrument door
- 3) If you need to pause the instrument during an extraction run, press Stop before you open the instrument door
- 4) When you are *not* performing an extraction run or instrument test, you can open the instrument door with the power OFF or ON
- 5) Do not move instrument components such as the platform, magnets, and syringes while the instrument is powered on.

AutoMate Express Maintenance:

In order to maintain the instrument, preventative maintenance (PM) should be performed once per calendar year. In addition to yearly PMs, the below listed maintenance procedures should be performed while the instrument is in use:

Schedule	Procedure
As Needed (When in Use)	-Clean the Piercing Unit -Clean the Platform Surface -Clean the Tip and Tube Rack -Clean the Magnets -Maintain the D-Rings
Monthly (When in Use)	-Perform Axis and Temperature Test
Annually	-Replace the D-Rings -Planned Maintenance

-Clean the Piercing Unit

- 1) From the Main Menu, press *1* to display the Manual screen.
- 2) Press *3 (Clean)*, then press *1* to lower the piercing unit.
- 3) Wipe the tips with an alcohol wipe.
- 4) Press *ESC* to return the piercing unit to the original position.

-Clean the Instrument

- 1) Clean the clear door panel with a wet Kimwipe. Do not use ethanol to clean the panel.
- 2) Clean the cartridge rack, tip and tube rack, and magnets with mild detergent, and then rinse with deionized water. Allow the parts to dry before use.

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-Maintain the D-rings

- 1) Every two weeks, apply silicone grease to the nozzle D-rings on the syringe unit to maintain proper attachment of the tips to the nozzles and prevent leakage.
 - a. Place some silicone grease on a gloved finger
 - b. Apply the silicone grease on the surface of the D-rings attached to the nozzles. Do not put grease into the nozzles. If you accidentally put grease into a nozzle, remove the grease using a laboratory wipe or a dust free cloth.
 - c. Wipe off any excess grease on the edges of the nozzles using a laboratory wipe or a dust-free cloth. Excess grease interferes with the operation of the instrument.

-Replace the D-rings

- 1) Using the D-ring removal tool, press the D-ring from behind to create a loop in front. Grab the loop with pliers or a pipette tip, then stretch and remove the D-ring.
- 2) Using the D-ring installation tool, slip a new D-ring into the nozzle groove. Keep the flat inner surface of the D-ring in contact with the installation tool so that the D-ring does not roll. Ensure that the D-ring is placed properly on the nozzle to prevent leakage.

-Perform Axis Test

- 1) From the main menu, press 3 to enter the *Test* menu.
- 2) Press 1 from the *Tests* menu to display the *Axis test* screen.
- 3) Load 13 empty reagent cartridges into the cartridge rack and load the rack into the instrument.
- 4) Load the instrument tip and tube rack with the following:
 - a. Rows S: 13 sample tubes
 - b. Row T2: Load 13 tips inserted into tip holders
 - c. Row T1: Load 13 tips inserted into tip holders
 - d. Row E: Load 13 elution tubes (with the caps open)
- 5) Press *Start*.
- 6) If the test is passed, "ALL OK" will be displayed on the screen. If an error code is listed, check the *AutoMate Express Instrument User Guide* page 62 for more details.

-Perform Temperature Test

- 1) From the main menu, press 3 to enter the *Test* menu.
- 2) Press 2 from the *Tests* menu to display the *Temp test* screen.
- 3) Using the arrow key, change the *Set Temp* to 70°C.
- 4) Press *Start*.
- 5) The "Now Temp" value should reach the "Set Temp value" within a few minutes. When the Set value is reached, the alarm value will turn to 00.
- 6) If the test fails, contact technical support.

Setup the AutoMate Express Instrument

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- 1) Insert the protocol card and power on the instrument
 - a. Confirm the power is off.
 - b. Open the card slot and insert the card into the instrument
 - c. Power on the Instrument, wait for the “Main Menu” to be displayed, and then press *Start*.

3.3.2 Bone – Sample Extraction with PrepFiler BTA Kit – AutoMate Express Procedure

WARNING: Contact with acids or bases (such as bleach) liberates toxic gases. DO NOT ADD acids, or bases (such as bleach) to any liquid wastes containing PrepFiler Lysis Buffer, PrepFiler BTA Lysis Buffer, or PrepFiler Magnetic Particles.

Prepare Sample Lysate

- 1) Bring the thermal shaker temperature up to 56°C.
- 2) Place approximately 0.05g of bone powder into a new PrepFiler Bone and Tooth Lysate Tube
- 3) Create a Manipulation/Reagent Blank using an empty tube. Label as “~~RB_DD_MM_YY_Initials~~” or “~~MB_DD_MM_YY_Initials~~”.
- 4) Prepare fresh lysis buffer mix as follows (add 3 to your number of samples to account for any pipetting losses which may occur):

Lysis Buffer Solution Component	Volume Per Sample
PrepFiler BTA Lysis Buffer	220µl
1.0M DTT	3µl
Proteinase K	7µl

- 5) Add 230µl of lysis buffer mix to each sample, then:
 - a. Cap the tube,
 - b. Vortex for 5 seconds,
 - c. Centrifuge briefly,
 - d. Gently flick the tube to re-suspend the substrate.
- 6) Place the tubes in the 56°C thermal shaker set to 1,100rpm for at least 2 hours.
 - a. The sample can be incubated for up to 18 hours
- 7) Allow the samples to equilibrate to room temperature and label new PrepFiler Sample tubes.
- 8) Centrifuge the samples at 10,000xg for 90 secs.
- 9) Transfer the clear lysate to the new labeled PrepFiler Sample tube (do not disturb the sediment).
- 10) Check to ensure the volume of the collected lysate is at least 200µl
 - a. If not, then add PrepFiler BTA Lysis Buffer to bring the volume to 200µl.

Setup and Run the Automated DNA Extraction

- 1) Inspect the reagent cartridges. If precipitate forms in compartments 1 or 2 (lysis buffer and magnetic particle suspension), heat the cartridge in an incubator at 37°C for 30 minutes or

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until the precipitate is no longer visible. Heat only those cartridges that you plan to use that day.

- 2) Confirm that the power switch is in the off position.
- 3) Open the card slot and insert the protocol card with the arrow pointing toward the instrument and the label facing to the left.
- 4) Push the card completely into the card slot and then close the card slot.
- 5) Power on the instrument.
- 6) Open the instrument door, and then remove the tip and tube rack and the cartridge rack.
- 7) Remove as many cartridges from the kit as the number of samples to be extracted (up to 13 cartridges).
- 8) Shake and tap the reagent cartridges to resuspend the magnetic particles and to deposit any particles or liquid droplets underneath the foil seal into the compartments.
- 9) Load the reagent cartridges into the cartridge rack by sliding each reagent cartridge along the groove in the direction of the arrow until the reagent cartridge clicks into place. Ensure that the notches in the cartridge align with the notches in the cartridge rack.
- 10) Insert the loaded cartridge rack into the instrument.
- 11) Load the tip and tube rack in the following order:
 - Note:** If you are processing fewer than 13 samples, make sure to load the tips and tubes in the same positions as the reagent cartridges that are loaded in the cartridge rack.
 - a. Row S (fourth row): Load PrepFiler™ sample tubes containing the lysate.
 - i. **Note:** Before loading each sample tube in the rack, make sure that no salt precipitation is visible in the sample tube. Precipitate in sample tubes may cause instrument crash, tip clogging, or filter wetting. See PrepFiler Express™ and PrepFiler Express™ BTA Forensic DNA Extraction Kits User Guide for suggestions on preventing and/or dissolving precipitated salts.
 - ii. **Note:** Make sure that the LySep columns have been removed from the sample tubes.
 - b. Row T2 (third row): Load with AutoMate Express™ tips inserted into tip holders.
 - i. **Note:** One tip and tip holder set is required per sample.
 - c. Row T1 (second row): Leave empty.
 - d. Row E (first row): Load PrepFiler™ elution tubes, with the caps open and secured.
- 12) Insert the loaded tip and tube rack into the instrument with row E in the front, and then close the instrument door.
- 13) Press *Enter* then press 2 to select the PF Express BTA option for the PrepFiler Express BTA kit.
- 14) Select 20µl to 200µl depending on the sample type for the extraction volume. As a general rule, select lower volumes for samples and a higher volume for reference samples.
- 15) Press *Start*.
 - a. The screen will show the steps and the appropriate time remaining.
- 16) At the end of the run:
 - a. Press *Enter* to return to the main menu and then open the instrument door.
 - b. Remove the cartridge rack and tip and tube rack.
 - c. Remove and cap the elution tubes containing the purified DNA.

NOTE: Store the isolated DNA at 4°C for up to two weeks or at -20°C for prolonged storage.

- d. Properly dispose of the used reagent cartridges, tips, and tubes.
 - i. All PrepFiler Express waste should be bagged in a separate red or clear plastic bag and tied before being disposed of in the general trash. *Care should be taken to ensure the PrepFiler reagents do not come into contact with bleach as toxic gas will result.*
- e. Close the instrument door. After each run, clean the tip and tube rack as needed.

3.3.3 Reference Sample Preparation and Extraction with PrepFiler BTA Kit – AutoMate Express Procedure

WARNING: Contact with acids or bases (such as bleach) liberates toxic gases. DO NOT ADD acids, or bases (such as bleach) to any liquid wastes containing PrepFiler Lysis Buffer, PrepFiler BTA Lysis Buffer, or PrepFiler Magnetic Particles.

Prepare Sample Lysate

- 1) Bring the thermal shaker temperature up to 56°C.
- 2) Sample Input Amounts:

<u>Sample Type</u>	<u>Example Sample Input</u>
Liquid Samples (blood, saliva, etc.)	Up to 40µl
Blood on FTA paper or fabric	Up to 25-mm ² cutting or punch
Body fluids on fabric	Up to 25-mm ² cutting or punch
Body fluids on swabs	Up to one swab

- 3) Prepare fresh lysis buffer mix as follows (add 3 to your number of samples to account for any pipetting losses which may occur):

<u>Lysis Buffer Solution Component</u>	<u>Volume Per Sample</u>
PrepFiler BTA Lysis Buffer	220µl
1.0M DTT	3µl
Proteinase K	7µl

- 4) Insert a LySep Column into a hingeless PrepFiler sample tube, and then carefully transfer the sample into the LySep column.
- 5) Add 230µl of lysis buffer mix to each sample, and then tightly close the lid of the column/tube assembly.
- 6) Create a Manipulation/Reagent Blank using an empty tube. ~~Label as “RB_DD_MM_YY_Initials” or “MB_DD_MM_YY_Initials”.~~
- 7) Place the tubes in the 56°C thermal shaker set to 750rpm for 40 mins.
- 8) Allow the samples to equilibrate to room temperature.

- 9) Centrifuge the column/tube assembly for 2 mins at 10,000xg to transfer the lysate to the sample tube.
- 10) Check to ensure the volume of the collected lysate is at least 200µl
 - a. If not, then add PrepFiler BTA Lysis Buffer to bring the volume to 200µl.
- 11) To complete the removal of the substrate:
 - a. Carefully remove the LySep column from the sample tube. If there is clear lysate remaining in the LySep column, transfer the lysate to the sample tube.
 - b. Properly dispose of the LySep column.
 - c. If a pellet is visible in the sample tube, transfer the clear (no sediment) lysate to a new PrepFiler Sample Tube.

Setup and Run the Automated DNA Extraction

- 1) Inspect the reagent cartridges. If precipitate forms in compartments 1 or 2 (lysis buffer and magnetic particle suspension), heat the cartridge in an incubator at 37°C for 30 minutes or until the precipitate is no longer visible. Heat only those cartridges that you plan to use that day.
- 2) Confirm that the power switch is in the off position.
- 3) Open the card slot and insert the protocol card with the arrow pointing toward the instrument and the label facing to the left.
- 4) Push the card completely into the card slot and then close the card slot.
- 5) Power on the instrument.
- 6) Open the instrument door, and then remove the tip and tube rack and the cartridge rack.
- 7) Remove as many cartridges from the kit as the number of samples to be extracted (up to 13 cartridges).
- 8) Shake and tap the reagent cartridges to resuspend the magnetic particles and to deposit any particles or liquid droplets underneath the foil seal into the compartments.
- 9) Load the reagent cartridges into the cartridge rack by sliding each reagent cartridge along the groove in the direction of the arrow until the reagent cartridge clicks into place. Ensure that the notches in the cartridge align with the notches in the cartridge rack.
- 10) Insert the loaded cartridge rack into the instrument.
- 11) Load the tip and tube rack in the following order:
 - Note:** If you are processing fewer than 13 samples, make sure to load the tips and tubes in the same positions as the reagent cartridges that are loaded in the cartridge rack.
 - a. Row S (fourth row): Load PrepFiler™ sample tubes containing the lysate.
 - i. **Note:** Before loading each sample tube in the rack, make sure that no salt precipitation is visible in the sample tube. Precipitate in sample tubes may cause instrument crash, tip clogging, or filter wetting. See PrepFiler Express™ and PrepFiler Express™ BTA Forensic DNA Extraction Kits User Guide for suggestions on preventing and/or dissolving precipitated salts.
 - ii. **Note:** Make sure that the LySep columns have been removed from the sample tubes.
 - b. Row T2 (third row): Load with AutoMate Express™ tips inserted into tip holders.
 - i. **Note:** One tip and tip holder set is required per sample.

- c. Row T1 (second row): Leave empty.
 - d. Row E (first row): Load PrepFiler™ elution tubes, with the caps open and secured.
- 12) Insert the loaded tip and tube rack into the instrument with row E in the front, and then close the instrument door.
- 13) Press *Enter* then press 2 to select the PF Express BTA option for the PrepFiler Express BTA kit.
- 14) Select 20µl to 200µl depending on the sample type for the extraction volume. As a general rule, select lower volumes for samples and a higher volume for reference samples.
- 15) Press *Start*.
- a. The screen will show the steps and the appropriate time remaining.
- 16) At the end of the run:
- a. Press *Enter* to return to the main menu and then open the instrument door.
 - b. Remove the cartridge rack and tip and tube rack.
 - c. Remove and cap the elution tubes containing the purified DNA.

NOTE: Store the isolated DNA at 4oC for up to two weeks or at -20oC for prolonged storage.

- d. Properly dispose of the used reagent cartridges, tips, and tubes.
 - i. All PrepFiler Express waste should be bagged in a separate red or clear plastic bag and tied before being disposed of in the general trash. *Care should be taken to ensure the PrepFiler reagents do not come into contact with bleach as toxic gas will result.*
- e. Close the instrument door. After each run, clean the tip and tube rack as needed.

4 DNA Quantification

Applied Biosystems 7500 Real-Time PCR System

The Applied Biosystems 7500 Real-Time PCR System is a 96-well, five-color platform that uses fluorescence-based polymerase chain reaction reagents to provide quantitative detection of target nucleic acid sequences using real-time analysis. The standard curve method is used to determine the absolute target quantity in samples. With the standard curve method, the 7500 software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve; which is then used to interpolate the absolute quantity of target in the samples. In the MPS laboratory workflow, the 7500 is used to quantify DNA pre-amplification and for library quantification post-clean-up.

To maintain the instrument, preventative maintenance (PM) should be performed once per calendar year by an external vender. Additional routine maintenance should be conducted according to the below table. The 7500 software provides wizards for certain maintenance procedures. To launch a maintenance wizard: 1) open the HID Real-Time PCR Analysis Software, 2) click on the Instrument button in the top toolbar, 3) from the dropdown menu, select Instrument Maintenance Manager, 4) select the applicable maintenance task and then run the wizard by clicking "Start Calibration."

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Activity:	Frequency:	Actions to Take:
Background Assay	Monthly	7500 Wizard – “Background”
Disk Clean-Up	Monthly	Run Clean-Up and defragment the computer hard drive
Regions of Interest (R.O.I.)	Semi-Annually	7500 Wizard – “R.O.I.”
Optical Calibration	Semi-Annually	7500 Wizard – “Optical”
Pure Dye Calibration	Semi-Annually	7500 Wizard – “Dye”
Performance Check	Annually and After Service	Reference Section 10 of “LM-CODIS QA” for performance check parameters

4.1 Quantifiler Trio Kit

Quantifiler Trio Assay Background

Quantifiler Trio DNA quantification kit (Life Technologies) is designed to quantify the total amount of amplifiable human (and higher primate) DNA and human male DNA in a sample. The results from using the kit can aid in determining: 1) If sufficient human DNA or human male DNA is present to proceed with short tandem repeat (STR) analysis, 2) Degree of DNA degradation, and 3) How much sample to use in STR analysis applications.

The DNA quantification assay combines four 5’ nuclease assays. Each amplification well contains small human DNA, large human DNA, and human male DNA target-specific assays and an internal PCR control (IPC) assay. The target-specific assays consist of primers for amplifying the small human DNA (80 bases), large human DNA (214 bases), or human male DNA (75 bases) and one TaqMan (MGB or QSY quencher) probe labeled with VIC dye (small human DNA), ABY dye (large human DNA) or FAM dye (human male DNA) for detecting the amplified sequence. The IPC assay consists of IPC template DNA (a 130 base synthetic sequence not found in nature), primers for amplifying the IPC template DNA, and one TaqMan QSY quencher probe labeled with JUN dye for detecting the amplified IPC DNA. Successful amplification of the PCR control and the target DNA indicates that amplifiable DNA was detected. A scenario where the PCR control amplifies, but the sample does not, could indicate that sufficient amplifiable DNA is not present. In the absence of robust target DNA amplification, this assay can predict inhibition if the IPC also does not amplify or has a high C_T.

-Prepare Trio Master Mix and Set-up the Plate (to be performed in the Master Mix hood)

- 1) Required Materials:
 - a. Trio Primer Mix
 - b. Trio Reaction Mix
 - c. Disposable Tray or tube for preparing master mix
 - d. 96-well optical reaction plate
 - e. Extracted DNA samples
 - f. DNA quantification standards dilution series
 - g. Trio Dilution Buffer

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- h. Optical adhesive cover
- 2) Thaw the Trio Primer Mix, vortex 3-5 seconds, spin briefly.
 - 3) Gently vortex the PCR Reaction Mix, spin briefly.
 - 4) Use the chart to calculate the volume of the components needed to prepare the reactions. Also, the "Front End" tab of the MPS Workbook may be used for the calculations and printing a plate map.

Trio Master Mix Calculator		
Primer Mix	Number of samples + controls + 5	X 8µl
Reaction Mix	Number of samples + controls + 5	X 10µl

- 5) Mix components thoroughly and dispense 18µl the Quantifiler Trio PCR mix into each reaction well to be used on the QF plate.
- 6) Seal the reaction plate with the optical adhesive cover using a plate comb and ensuring the seal is secure all the way around the plate. Move plate to the DNA Hood for the addition of the standards and the sample DNA.

-Preparation of Standard Curve Samples (to be performed in the DNA hood)

- 1) After allowing for placement of the five recommended standards and the dilution buffer (blank) in duplicate, 84 samples fit on the plate.
- 2) Required Material
 - a. Low-bind microfuge tubes
 - b. Trio DNA Standard
 - c. Trio DNA Dilution Buffer
- 3) Refer to the chart for volumes:

Standard	ng/µl	Volumes of 100 ng/µl stock and dilution buffer	Dilution
1	50	10µl stock + 10µl dilution buffer	2x
2	5	10µl std 1 + 90µl dilution buffer	10x
3	0.5	10µl std 2 + 90µl dilution buffer	10x
4	0.05	10µl std 3 + 90µl dilution buffer	10x
5	0.005	10µl std 4 + 90µl dilution buffer	10x

- 4) Dispense the dilution buffer into the labeled tubes.
- 5) Vortex the DNA stock 3-5 seconds and quick spin before opening.
- 6) Prepare Standard 1, vortex and quick spin.
- 7) Using a new pipette tip, prepare Standard 2, vortex and quick spin.
- 8) Repeat the previous step until all standards are prepared.
- 9) Store prepared standards up to 1 week at 2-8°C in tubes.

-Preparation of Standard Curve Samples (to be performed in the DNA hood)

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- 7) Add 2µl of each standard, in duplicate, to columns 11 and 12. Load the lowest concentrated sample first and move in order of increasing DNA concentrations.
- 8) Add 2µl negative control (DNA Dilution Buffer) to the control wells.
- 9) Add 2µl of samples to the appropriate wells.
- 10) Seal the reaction plate with the optical adhesive cover using a plate comb and ensuring the seal is secure all the way around the plate. Remove the tabs on the seal. Or seal the reaction wells with optical flat top strip caps.
- 11) Centrifuge the plate at 3000 rpm for about 20 seconds.

-Procedure if no template has been created

- 1) Start the computer and turn on the Life Technologies 7500 instrument.
- 2) Open the HID Real Time PCR Analysis software.
- 3) Log into the software as "Guest".
- 4) In the Home screen, click the "Quantifiler Trio" icon.
- 5) In the Experiment Properties screen, enter a name for the experiment.
- 6) In the left navigational panel, click Setup > Plate Setup.
- 7) To define the samples, click Add New Sample in the Define Samples area on the right side, then type the name for the sample. Select the sample type (Unknown is the default for new samples). Save the sample and then repeat for each sample.
- 8) Click the Assign Targets and Samples tab. Targets are automatically assigned and the standard quantities are automatically specified.
- 9) Assign the samples to the plate wells.
- 10) Select wells.
- 11) In the Assign sample(s) to wells section to the left of the plate layout, locate the desired sample and select the checkbox in the Assign column next to the sample name. The target for each sample is set by default.
- 12) Repeat for all samples.
- 13) In the left navigational panel, click Setup > Run Method to view the parameters. The parameters are automatically specified.
- 14) Save the file in the experiments folder as an .eds file.

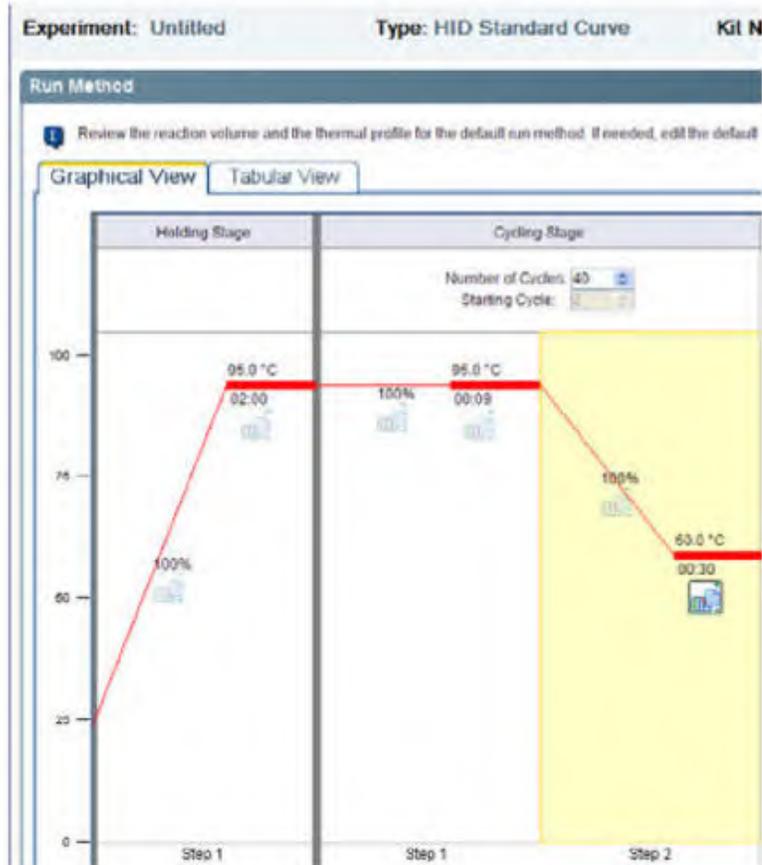
-Setting-up a previously prepared plate document on the 7500 Real-Time PCR Instrument

- 1) If the HID Real-Time Analysis Software is not already started, select the HID Real-Time PCR Analysis Software located on the computer desktop and turn on the 7500 instrument.
- 2) Log into the software as "Guest" and then click "Quantifiler Trio" icon.
- 3) Select File > Import, then select Browse to navigate to the appropriate Quantifiler Trio text file, then click Select. Click Start Import, then click Yes, and finally click Okay.
- 4) When all wells have been checked for the correct detectors and sample names, save the file.

-Preparing the Thermal Cycling Parameters

- 1) In the Experiment Menu, select Setup > Run Method.
- 2) Verify the sample volume is 20µl and the thermal profile is the following:

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-Running the Quantification Reactions

- 1) The computer and instrument are on and the software is open.
- 2) Press the tray door to open it.
- 3) Load the plate into the plate holder aligning so that well A1 is in the upper-left corner and the notched corner of the plate is in the upper-right corner.
- 4) Apply pressure to the right side of the tray at an angle to close the tray door.
- 5) Create an experiment for the run or use a template.
- 6) Click *Start Run* and it will prompt to save the experiment.
- 7) Before leaving the instrument unattended, verify that the run has begun by checking that the run time clock has begun to count down. During the run, you will see the amplification plot built.

-Reviewing and Analyzing the Run

- 1) Open the run file and select Analysis Settings in the upper-right corner. Click the CT Settings tab.
- 2) Verify the threshold is 0.1 and the baseline is set for automatic, then click Apply Analysis Settings.
- 3) Click Analyze.
- 4) In the left navigational panel, click Analysis > Standard Curve.
- 5) In the Target drop-down list, select All.

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- 6) Verify that slopes are between -3.0 and -3.6 (optimal = -3.3) and that the R2 value is >.98.
 - a. Delete points from the curve to achieve the proper slope.
 - b. Reanalyze if points are removed.
 - c. If values cannot be brought within range, consult with the TL. The results may still be useful but some troubleshooting is necessary.
- 7) The gap between the Small Autosomal, Large Autosomal and Male CT values may vary depending on the relative slopes of the targets and instrument performances.
- 8) Small amounts of background in the negative control or blanks do not invalidate results.
- 9) After the run has been analyzed and inspected for quality, save the file.

-Exporting Data

- 1) In the Experiment Menu, select Analysis. Click any Analysis screen, then click either View Plate Layout or View Well Table. Highlight the wells to export.
- 2) In the toolbar, click Export.
- 3) Under the Export Properties table, select Results as data to export, and then select it to be exported as One File. Select .xls as the File Type and specify an export location.
- 4) Click Start Export.
- 5) When export is complete, close the Export tool.

-Assessing the Quality of the Results

- 1) The closer the R2, slope and y-intercept values are to ideal, the more informative the quantitation results. If these values are off, consult with the TL. The results may still be useful but some troubleshooting is necessary.
- 2) The R2 value describes how well the data points of the standards fit the curve. An R2 = 1 is a perfect fit.
 - a. For best results, the R2 should be no less than 0.98. If it is less, outlier data points will be seen. Remove any data points that are far off the curve.
- 3) The slope describes how well the reaction is working. The theoretical maximum slope of -3.3 corresponds to a doubling of DNA at each cycle. In practice, the ideal slope should be -3.0 to -3.6.
 - a. A more negative slope (i.e. -3.7) may reflect a poorly performing reaction or bad data points in the curve.
 - b. Once bad data points have been removed, make no further adjustments on the slope.
 - i. Outlier data points to the right of the curve make the slope more negative.
 - ii. Those to the left of the curve make it more positive.
 - iii. Values in the middle of the range (those samples quanting between 0.5 and 2.0) will be least affected by variant slopes.
- 4) The y-intercept value is the cycle number when the 1.0 ng point of the standard curve crosses the threshold, and generally falls in the range of:
 - a. 25.5-28.5 for small autosomal
 - b. 23.5-26.5 for large autosomal
 - c. 25-28 for male

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- d. Small variation in the y-intercept may occur with different lots of standard curve DNA. An outlier Y-intercept may signal a problem with the standard curve that should be taken into consideration during interpretation.
 - e. A low Y-intercept may result in a falsely low quant and subsequent overloading of sample on e-gram. The cause may be a difference in a new batch of standard curve DNA or in measuring error in the first step of the standard curve dilution.
 - f. When the Y-intercept is different from the value normally obtained, consider increasing or decreasing the amount of DNA input into the amplification reaction.
- 5) The CT for the IPC generally falls in the in the range of 27-29 cycles.
- a. Where the sample DNA input is very high, the IPC CT will be slightly higher, reflecting competition for amplification reaction components.
 - b. A reaction where the IPC and the other targets are undetected has failed to amplify, most likely due to a soluble inhibitor in the casework DNA sample.
 - c. A reaction where the IPC is very low or undetected and the sample seems to have amplified adequately is partially inhibited. It may be helpful to perform a clean-up step.
 - d. A reaction where the IPC is within normal range and the sample is very low or undetected is an indication of the absence of any amplifiable human DNA.
- 6) When y-intercept, slope or line fit fall outside of ideal ranges, DNA quantity data is generally still sufficiently accurate to produce good typing results. Only in the most extreme circumstances would the benefits of repeating the Quantifiler test outweigh the additional time and sample resources required. This decision should be made in consultation with the TL.

-Assessing the Quantity of DNA Present in Samples

- 1) After determining the quality of the results, determine whether sufficient DNA is present to proceed. The quantification value from the small autosomal target should be used to determine amplification input amount.
- 2) The Trio Kit can detect DNA concentrations under 5 pg/μl. Significant variability in results below this level are due to stochastic effects.
- 3) The Degradation Index is a ratio of small/large target. As the value increases, a higher amount of degradation can be inferred. ~~Degradation ratios of 3 or above may prompt increasing the GlobalFiler amplification target value.~~ The Degradation Index can be affected by the degree of degradation of the large autosomal target DNA and the presence of PCR inhibitors.
- 4) The IPC CT flag is triggered for “undetermined” or very high values and may indicate inhibition.
- 5) The Quality Index is the concurrent consideration of both the degradation index and the IPCCT flag. ~~This may influence downstream sample clean-up or targeting decisions.~~
- 6) Potential Interpretations of Degradation and Quality Indexes:

<u>IPCCT flag?</u>	<u>Degradation Index</u>	<u>Interpretation</u>
No	<1	DNA not degraded or inhibited
	1-10	DNA slightly to moderately degraded

	>10 or blank	DNA is significantly degraded
Yes	>1 or blank	DNA is degraded and/or inhibited

5 DNA Amplification and Analysis Workflows

Depending on the sample type and testing **case-specific** requirements, there are several different workflow options for DNA amplification and analysis. ~~The following sections describe the laboratory procedures for each testing pipeline.~~ Depending on the sample to be tested and the available amount of starting material, considerations should be made for which workflow will be utilized. Limited sample may dictate only one workflow be performed (i.e. STRs), while samples with greater amounts of starting material may be tested in multiple workflows (i.e. STRs and mtDNA).

5.1 ~~GlobalFiler Kit~~

~~See the most current version of the "DNA Methods Manual" for information regarding conducting and analyzing data from the GlobalFiler Kit.~~

5.2 ~~Y-Filer Plus Kit~~

~~See the most current version of the "DNA Methods Manual" for information regarding conducting and analyzing data from the Y-Filer Plus Kit.~~

5.3 PowerSeq Nested CRM System, Promega PowerQuant MS, Library Dilutions, and MiSeq FGx Loading

PowerSeq Nested CRM System Background

The Promega PowerSeq CRM Nested System (Custom) generates 10 amplicons (size range of 144-237bp) covering the control region of the mitochondrial genome in one multiplex. Amplification of the target amplicons and incorporation of sequencing adapters happens in one PCR reaction. A series of bead clean-up steps, which remove unwanted product and leftover reaction components, yield libraries ready for quantification and sequencing.

NOTE: All samples shall be amplified in duplicate. A nuclear DNA input of 0.1ng-0.5ng ~~should~~ **may** be targeted for amplification.

-Amplification Protocol:

The following steps should be performed in the pre-amplification laboratory space:

- 1) Prepare the *Master Mix Hood* and *DNA Hood* by wiping down with DNAerase, 10% bleach, and 70% isopropyl alcohol. Then turn on the UV light in each hood for a minimum of ~~10~~ **minutes one cycle**.
- 2) Be sure to record all applicable lot numbers in the MPS workbook.
- 3) Vortex and centrifuge all tubes briefly.
- 4) Dilute DNA samples to the desired amount with TE or water.

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The following steps should be completed in the Master Mix Hood:

- 5) Begin in the *Master Mix Hood* by preparing the amplification master mix as follows:
 - a. PowerSeq 5X Master Mix = 5µl per reaction
 - b. PowerSeq 10X Primer Pair Mix = 2.5µl per reaction
 - c. Amp Grade Water = 2.5µl per reaction
 - d. Vortex for 5 secs and centrifuge briefly
 - e. NOTE: calculations can be performed in the “Amp and Index Guide - Promega” tab of the current version of the “MPS Workbook”.
- 6) Add 10µl of the amplification master mix to each well of the amplification PCR plate which will contain either a sample or a control.
- 7) Cover the plate with a plate seal and move the *DNA Hood* for the addition of the index sequences and samples.

The following steps should be conducted in the DNA Hood:

- 8) Add the Index Sequences to each well:
 - a. Add 2.5µl of the appropriate D7 Index to each well of the amplification plate.
 - b. Add 2.5µl of the appropriate D5 Index to each well of the amplification plate.
NOTE: The “Amp and Index Guide - Promega” tab of the “MPS Workbook” can be used to aid in Index selection and arrangement.
NOTE: Index D702 and D710 shall not be used on the same sequencing run. Validation data has shown low levels of cross-talk occurring when D702 and D710 are used together.
- 9) For the Amplification Negative:
 - a. add 10µl of water to the appropriate well, seal the well
- 10) Working in the *DNA Hood*, prepare the positive amplification control as follows:
 - a. Dilute the kit supplied 2800M to 0.05ng/µl by combining 398µl of TE or water with 2µl of stock 2800M.
 - b. Vortex and briefly centrifuge
 - c. Add 10µl of the diluted 2800M to the appropriate wells.
- 11) Add 10µl of each previously diluted (with TE or water) DNA sample to the appropriate wells.
- 12) Seal the plate with either an adhesive plate seal or strip caps and then briefly centrifuge.
- 13) Transport the plate to the **Post-amplification Laboratory** and place the plate in the thermocycler and run the following program (NOTE: be sure to set the ramp speeds to *MAX*):
 - 1 cycle of:
 - 96°C for 10 min
 - 30 cycles of:
 - 96°C for 5s
 - 60°C for 35s
 - 72°C for 5s
 - 1 cycle of:
 - 60°C for 2 min
 - Hold at 4°C indefinitely

14) Store the samples at -20°C or continue to the Purification Protocol.

-Purification Protocol

The following steps should be performed in the post-amplification laboratory space in a fume hood. All four bead purifications should be performed back-to-back without stopping or storing the samples between rounds.

Preparation of the Components

- 1) 50mM Tris-HCl (pH 8.0), 10mM CaCl₂
 - a. Make 100mM Tris-HCl (pH 8.0): 36mL Nuclease Free H₂O + 4mL 1M Tris-HCl (pH 8.0)
 - b. Make 20mM CaCl₂: 49mL Nuclease Free H₂O + 1mL 1M CaCl₂
 - c. Combine equal volumes of 100mM Tris-HCl (pH 8.0) and 20mM CaCl₂
- 2) Proteinase K
 - a. Reconstitute the included Proteinase K lyophilized powder with 500µl of 50mM Tris-HCl (pH 8.0), 10mM CaCl₂ to make a 20mg/mL of stock solution.
 - b. Prepare four aliquots of the 20mg/mL stock at 3.6µl per tube (the remaining amount can be left in the original tube). Store at -20°C until use.
 - c. To prepare the 360µg/mL ProK working solution: add 196.4µl of 50 mM Tris-HCl (pH 8.0), 10mM CaCl₂ to one of the previously made aliquots.
- 3) 80% Ethyl Alcohol
 - a. 10mL Nuclease Free H₂O + 40mL EtOH
- 4) 10mM Tris-HCl pH 8.5
 - a. 49.5 mL Nuclease Free H₂O + 0.5mL Tris-HCl pH 8.5

Initial Purification with ProK

- 1) Add 5µl of 360µg/mL Proteinase K working solution to each amplification reaction on the amp plate.
- 2) Completely mix the AMPure XP beads and add 30µl to each well of a midi plate (same number of wells as on the amplification plate).
- 3) Add the full volume of the Pro K and sample from the amp plate (~30µl) to the corresponding well of the midi plate. Pipette mix thoroughly and incubate at room temperature for 5 minutes.
- 4) Place the midi plate on the magnetic stand for 5 minutes at room temperature.
- 5) Remove and discard all of the supernatant and leave the plate on the magnetic stand.
- 6) Add 200µl of freshly prepared 80% ethanol and incubate for 30 seconds.
- 7) Remove and discard all of the supernatant.
- 8) Add 200µl of freshly prepared 80% ethanol and incubate for 30 seconds.
- 9) Remove and discard all of the supernatant.
- 10) While on the magnetic stand, allow the beads to air dry for 1-5 minutes.
- 11) For elution:
 - a. Remove the plate from the magnetic stand

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- b. Add 28µl of 10mMTris-HCl (pH 8.5)
- c. Pipette mix
Note: Ensure the beads have been fully resuspended. Extra pipetting or plate shaking may be required.
- d. Incubate for 2 minutes
- e. Place the plate on the magnetic stand until the samples are clear and a bead pellet has formed
- f. Remove 25µl of eluent and add to new midi plate

Additional Purification (the below steps must be repeated three times)

- 1) To the 25µl eluent obtained from the previous step, add 25µl of AMPure beads.
- 2) Pipette mix thoroughly and incubate at room temperature for 5 minutes.
- 3) Place the plate on a magnetic stand and incubate at room temperature for an additional 5 minutes.
- 4) Remove and discard the supernatant. Leave the plate on the magnetic stand.
- 5) Add 200µl of freshly prepared 80% ethanol and incubate for 30 seconds.
- 6) Remove and discard all of the supernatant.
- 7) Add 200µl of freshly prepared 80% ethanol and incubate for 30 seconds.
- 8) Remove and discard all of the supernatant.
- 9) While on the magnetic stand, allow the beads to air dry for 2-5 minutes.
- 10) For elution:
 - a. Remove the plate from the magnetic stand
 - b. Add 28µl of 10mMTris-HCl (pH 8.5)
 - c. Pipette mix
 - d. Incubate for 2 minutes
 - e. Place the plate on the magnetic stand until the samples are clear and a bead pellet has formed
 - f. Remove 25µl of eluent and add to new midi plate
- 11) Repeat steps 1-10 (second additional purification).
- 12) Repeat steps 1-10 again (third additional purification).
 - a. NOTE: Add the eluent to a new PCR plate instead of a midi plate.
- 13) The purified libraries may be stored at -20°C if needed.

Promega PowerQuant MS

-Promega PowerQuant MS Background

The PowerSeq™ Quant MS System determines the concentration of massively parallel sequencing (MPS) libraries compatible with Illumina MiSeq® platforms using qPCR. Data generated using the PowerSeq™ Quant MS System can be used to normalize the DNA concentration for MPS libraries to the desired concentration.

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The PowerSeq™ Quant MS system works by targeting the P5 and P7 Illumina adapter sequences for quantification of Illumina libraries. As a result, only DNA fragments with both adapters present will generate quantification values. The Master Mix includes BRYT Green® dsDNA-binding dye, passive reference dye, hot start polymerase, MgCl₂, dNTPs and a proprietary reaction buffer. The BRYT Green® dsDNA-binding dye is detected using the same filters and settings as the SYBR® Green I dye. The passive reference is a low level of CXR reference dye (identical to ROX™ dye). Data from the other dye channels are normalized to the signal from the passive reference. Serial dilutions of the included DNA standard are amplified in the same plate as the sample libraries, and the results are used to generate a standard curve. The standard curve is used to determine the DNA concentration of the sample libraries. The dynamic range of this assay is 20pM to 0.02pM. Sample libraries **must** **should** be diluted to fall in the dynamic range. If the concentration of a diluted library falls out of this range, the library **results may still be used and adjusted as needed**, ~~may be retested with a dilution that falls in the dynamic range of the assay.~~

Note: The 1X Dilution Buffer comes in a 150mL bottle and should be frozen until first use. Before first use, be sure to thaw the bottle in the refrigerator overnight to ensure it is ready for use.

-Master Mix Preparation

- 1) Prepare the master mix according to the following (per well):
 - a. 4µl, Amplification Grade Water
 - b. 10µl, 2X qPCR Master Mix
 - c. 2µl, 10X Primer Mix

NOTE: prepare enough for each sample/control/standard in triplicate with about 10% extra as overage. Master Mix calculations can be automatically performed in the “MPS Workbook – Quant Calculations” tab.

- 2) Add 16µl of the master mix to each well which will contain a sample/control/standard.
- 3) Add 4µl of library dilution buffer to each NTC well, then cap with optical strip caps.
- 4) Seal the plate with an optical seal while diluting the standards and preparing the samples.

-DNA Standards Dilution

- 1) Thaw the Quant MS DNA Standard (200pM).
- 2) Vortex and quick spin the DNA Standard.
- 3) Label four tubes as follows: 20pM, 2pM, 0.2pM, and 0.02pM.
- 4) Add 45µl of 1X Dilution Buffer to each of the four labeled tubes.
- 5) Add 5µl of the stock DNA standard to the 20pM tube, vortex, and quick spin.
 - a. Change gloves after handling the 200pM DNA Standard
- 6) Add 5µl of the 20pM dilution to the 2pM tube, vortex, and quick spin.
- 7) Add 5µl of the 2pM dilution to the 0.2pM tube, vortex, and quick spin.
- 8) Add 5µl of the 0.2pM dilution to the 0.02pM tube, vortex, and quick spin.

NOTE: DNA Standard dilutions can be stored for up to three months at 4°C.

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-Sample Dilutions

- 1) Dilute the sample libraries 1:100,000 in a Midi plate as follows:
 - a. For each sample, prepare three adjacent wells with the following amounts of 1X Dilution Buffer: 198µl, 198µl, and 180µl.
 - b. Mix 2µl of sample library with the first well containing 198µl of dilution buffer.
 - c. Mix 2µl of the above dilution with the second well containing 198µl of dilution buffer.
 - d. Mix 20µl of the above dilution with the third well containing 180µl of dilution buffer
 - i. This dilution will be used in the quantification reaction

-Reaction Setup

- 1) Remove the optical seal from the reaction plate prepared in Step #4 of the “Master Mix Preparation” section.
- 2) Add 4µl of the final library dilution (from 1.d. in the “Sample Dilutions” section) to the appropriate wells.
- 3) Add 4µl of each DNA standard to the appropriate wells (loaded in order from least concentrated DNA standard to most concentrated DNA standard).
- 4) Seal the plate with optical strip caps or and optical seal.
- 5) Briefly centrifuge the plate.
- 6) Place the plate on the 7500 and run the “PowerQuantMS” protocol.
 - a. Run settings should be as follows:
 - i. 95°C for 2 minutes
 - ii. 30 cycles of:
 - (1) 95°C for 15 seconds
 - (2) 60°C for 15 seconds
 - (3) 72°C for 45 seconds (with data collection)
 - b. Use the MPS Workbook export for plate record set-up:
 - i. Navigate to the “7500 Import File – PowerQuantMS” tab of the workbook.
 - ii. Click the grey *Generate 7500 Import .txt File* button.
 - iii. Select the range of data to be exported
 - iv. Save the .txt file on a thumb drive or in the run folder (and then to a thumb drive for transport to the DNA post-amplification laboratory and the 7500s).
 - v. Click *No*.
 - vi. Transport the newly created .txt file to the 7500s and import the plate setup.
 - (1) Launch the *HID Real-Time PCR Analysis Software v1.2*
 - (2) Logon as *Guest*
 - (3) Click on *Custom Assays*. Then click on *File, New Experiment*, and then *From Template* and select *Promega_PowerSeq Quant MS* from the list of saved template files
 - (4) Once the template has been loaded, click on *File*, then *Import*. Select *Browse* and navigate to the correct .txt file for import. Click *Start Import* then *Yes* when asked if you are sure you want to continue.

- (5) Check all of the imported information for accuracy.
 - (6) To save the experiment before the run begins, click *Save* then *Save As*. Name the file with the run name and then click *Save*.
 - (7) Click *Start Run*.
- 7) After the run is complete, remove and discard the plate and turn off the instrument.

-Data Analysis and Export

- 1) If the experiment to be analyzed is not already open and analyzed, in the *HID Real-Time PCR Analysis Software v1.2*, click *File » Open*.
 - a. Navigate to desired file, select the file, and click *Open*.
 - b. *Verify the threshold is set to 1.0 and the baseline is set for automatic, then click Apply Analysis Settings.*
 - c. Click *Analyze*
- 2) After analysis, results can be exported. Click *View Plate Layout » Highlight All Wells*.
- 3) Located on the top toolbar, click *Export*
 - a. Select data to export » *Results*
 - b. Select one file or separate files » *One File*
 - c. Ensure the correct file name
 - d. In the Custom Export tab check the data is exporting columns (A1, B1, etc.)
 - e. Click *Start Export*
 - f. This creates an excel data file
- 4) With all wells still highlighted, click *Print Report* located on the top toolbar.
- 5) Select *All Report Types*.
- 6) Click *Print* and chose to *save to CutePDF*. Ensure the correct run name is listed.
- 7) Add reports to the end of the file name.
- 8) Save file in appropriate folder and click *Save*.

-Data Assessment

- 1) Review the standard curve:
 - a. Manufacturer protocol lists slopes ranging from -3.30 to -3.70 to be the normal range. Validation data suggests slopes of -3.78 ± 0.30 to be within an acceptable range.
 - b. The R^2 value should be higher than 0.990.
 - c. The Y-intercept should be within a range of 16.42 ± 0.90 .
 - d. Standard curves falling outside of these listed ranges do not indicate a failed assay, but instead be analyzed with caution. Consult the TL for any concerns regarding the quality of the standard curve.
- 2) Determine Library Concentration:
 - a. To determine the original library concentration, multiply the average result of the sample replicates and then multiply by 100,000 to get a library concentration in pM. Dividing this result by 1,000 will give the library concentration in nM.
 - b. The MPS Workbook "Quant Calculations" tab may be used for these calculations by importing the 7500 results .xls file into the "Export from qPCR - Libraries" tab.

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-Library Dilutions

- 1) If needed, prepare the dilution buffer: 10mM Tris-HCl + 0.1% Tween 20 (pH 8.5)
 - a. 50µl of Tween20
 - b. 500µl of 1M Tris-HCl pH 8.5
 - c. 49,450µl of molecular biology grade water
- 2) Dilute all library samples down to 4nM.
 - a. The MPS Workbook “Quant Calculations” tab can be used to calculate the required dilution to achieve 4nM per sample.
 - b. Dilute sample libraries in 10mM Tris-HCl + 0.1% Tween 20 (pH 8.5).
 - c. Pipette mix or vortex and spin down all diluted libraries.
- 3) Pool all of the 4nM libraries by combining 5µl of each in a 0.5mL or 1.5mL tube labeled as “Pool” with the date, analysts initials, and run information.
 - a. If a library concentration does not reach 4nM, pool 5µl of the neat sample.
- 4) Vortex and spin the “Pool” tube.

The MiSeq FGx Instrument

MiSeq FGx Background

The MiSeq FGx is a sequencing instrument which utilizes sequencing by synthesis (SBS) technology. SBS chemistry uses a high-resolution camera to image the sequential incorporation of a fluorescently labeled dNTP, followed by cleavage of the terminator to allow incorporation of the next complementary base in an amplicon’s DNA sequence. This chemistry allows for all four reversible terminator-bound dNTPs (dATP, dGTP, dTTP, and dCTP) to be present during each sequencing cycle, natural competition minimizes incorporation bias, nearly eliminating errors and missed base calls associated with homopolymeric regions and repetitive DNA elements.

MiSeq FGx Maintenance:

In order to maintain the instrument, preventative maintenance (PM) should be performed once per calendar year. In addition to yearly PMs, regular instrument washes ensure the MiSeq FGx maintains optimal performance by flushing any remaining reagents from the fluidics lines and sippers, preventing salt accumulation and crystallization in the fluidics lines and sippers, and by preventing cross-contamination from run to run carryover. Instrument washes should be performed according to the following schedule when it is in a period of normal use:

Activity	Daily	Monthly	As Needed
Post-Run Wash	After every run		
Maintenance Wash		X	
Standby Wash			To prepare for idle (≥ 7 days unused)
Instrument Shutdown			X

NOTE: When the instrument is in *Standby Mode* a Standby Wash should be performed every 30 days.

-Post-Run Wash Procedure

A post-run wash should be performed after every sequencing run. Software prompts should be followed to load the wash components and perform the wash. The post-run wash takes approximately 30mins to complete.

- 1) Prepare fresh wash solution as follows:
 - a. Prepare a 10% Tween 20 solution as follows: Add 5mL 100% Tween 20 to 45mL nuclease-free water.
 - b. Prepare a 0.5% Tween 20 solution as follows: Add 25mL of 10% Tween 20 to 475mL nuclease-free water.
 - c. Invert several times to mix the 0.5% Tween 20 solution.
- 2) Prepare the hypochlorite wash solution as follows (if needed):
 - a. Prepare a 1:30 dilution of sodium hypochlorite as follows: Add 30µl of 6% sodium hypochlorite to 870µl of nuclease-free water.
 - b. Add 50µl of the 1:30 sodium hypochlorite dilution to 950µl of nuclease-free water in an Illumina MiSeq tube and pipette mix the solution.
- 3) Load the wash tray by adding 6ml of 0.5% Tween 20 wash solution to each reservoir of the wash tray.
 - a. If performing a post-run wash with sodium hypochlorite solution, do not fill position 17 with wash solution. Instead, insert the MiSeq tube containing .01% sodium hypochlorite solution into position 17.
 - b. If performing a post-run wash without sodium hypochlorite solution, fill position 17 with wash solution.
- 4) Add 350ml of the 0.5% Tween 20 wash solution to the 500ml *Wash Bottle*.
- 5) From the post-run wash screen select *Start Wash* and follow the on-screen prompts to load the wash cartridge and the wash bottle.
- 6) Remove the Waste Bottle, empty it, and return it to the instrument.
NOTE: *after a sequencing run, the waste bottle contains formamide and must be disposed of by emptying into an empty PR2 bottle from a previous run and placing this into the formamide bucket in the fume hood in the post-amp laboratory.*
- 7) After all bottles have been returned to the instrument, close the instrument door and select *Next*. The post-run wash will then start.
- 8) When the wash is complete, start a second post-run wash by repeating steps 1-7 from above.
NOTE: For this second wash omit the sodium hypochlorite solution and follow instructions under 3.b.
- 9) When the second wash is complete, leave the used flow cell, wash tray, and wash bottle containing the remaining wash solution on the instrument.

-Maintenance Wash Procedure

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While the MiSeq FGx is in a cycle of active use, a Maintenance Wash should be performed every 7 days to ensure optimal performance. The maintenance wash takes approximately 90mins to complete.

- 1) From the *Welcome* screen select *Perform Wash* then *Maintenance Wash*.
- 2) Prepare fresh wash solution as follows:
 - a. Prepare a 10% Tween 20 solution as follows: Add 5mL 100% Tween 20 to 45mL nuclease-free water.
 - b. Prepare a 0.5% Tween 20 solution as follows: Add 25mL of 10% Tween 20 to 475mL nuclease-free water.
 - c. Invert several times to mix the 0.5% Tween 20 solution.
- 3) Load the wash tray by adding 6ml of 0.5% Tween 20 wash solution to each reservoir of the wash tray.
- 4) Add 350ml of the 0.5% Tween 20 wash solution to the 500ml *Wash Bottle*.
- 5) Load the wash tray and wash bottle onto the instrument.
- 6) Select *Next* and the first wash begins.
- 7) After the first wash is complete, repeat above steps 2-6 to prepare and start the second wash.
- 8) After the second wash is complete, repeat above steps 2-6 to prepare and start the final wash.
- 9) When the final wash is complete, leave the used flow cell, wash tray, and wash bottle containing the remaining wash solution on the instrument. The sippers remain in the down position, which is normal. Leave the unused wash solution in the wash tray and wash bottle to prevent the sippers from drying out and air from entering the system.

-Standby Wash Procedure

If there are no plans to use the instrument for at least seven days, prepare the instrument to sit idle by performing a standby wash. A standby wash performs two consecutive washes that flush each position of any remaining reagents or salt accumulation. Each wash takes approximately 60 minutes. Allow approximately 2 hours to complete the standby wash.

When the standby wash is complete, the instrument is in standby mode and a message appears on the *Welcome* screen stating the status of the instrument. When the instrument is in standby mode, a maintenance wash must be performed before a sequencing run can be initiated. It is recommended to repeat the standby wash every 30 days that the instrument remains idle.

- 1) Make sure that a used flow cell is loaded on the instrument.
- 2) From the *Welcome* screen select *Perform Wash* and then select *Standby Wash*.
- 3) Prepare fresh wash solution as follows:
 - a. Prepare a 10% Tween 20 solution as follows: Add 5mL 100% Tween 20 to 45mL nuclease-free water.
 - b. Prepare a 0.5% Tween 20 solution as follows: Add 25mL of 10% Tween 20 to 475mL nuclease-free water.
 - c. Invert several times to mix the 0.5% Tween 20 solution.
- 4) Load the wash tray by adding 6ml of 0.5% Tween 20 wash solution to each reservoir of the wash tray.
- 5) Add 350ml of the 0.5% Tween 20 wash solution to the 500ml *Wash Bottle*.

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- 6) Load the wash tray and wash bottle onto the instrument.
- 7) Select *Next* and the first wash begins.
- 8) After the first wash is complete, repeat steps 3-7 to begin the second wash.
- 9) When the second wash is complete, leave the used flow cell, wash tray, and wash bottle containing the remaining wash solution on the instrument. The sippers remain in the down position, which is normal. Leave the unused wash solution in the wash tray and wash bottle to prevent the sippers from drying out and air from entering the system.

-Super Wash Procedure

Occasionally, the need will arise for a Super Wash to be conducted. To perform a Super Wash:

- 1) Perform three Post-Run washes (with sodium hypochlorite solution in position 17)
- 2) Perform another Post-Run wash
- 3) Perform a Maintenance Wash

-Shutting Down the MiSeq FGx

It is best to leave the instrument on at all times. However, if the instrument must be turned off, use the following procedure to shut down Windows and prepare the fluidics lines.

- 1) Perform a maintenance wash
- 2) Remove the waste bottle and discard the contents appropriately. Return the waste bottle to the reagent compartment. Close the reagent compartment door.
- 3) From the Manage Instrument screen, select *Shut Down*. This command shuts down the software.
- 4) Toggle the power switch to the *OFF* position.

Setting-Up a Sample Sheet on the MiSeq FGx:

The MiSeq FGx requires a sample sheet to be loaded before a sequencing run can begin. The sample sheet contains the run parameters, sample names, index assignments, and other run details.

- 1) On the MiSeq FGx, open the *Illumina Experiment Manager* software using the desktop shortcut.



- 2) To create a new sample sheet, click the *Create Sample Sheet* button.
 - a. If importing a sample setup sheet from the MPS Workbook, click *Edit Sample Sheet* and select the file to be imported. Verify all information is correct and then save the file to *D:/Illumina/MiSeq Control Software/Sample Sheets*.
- 3) Click the *MiSeq* button, and then click *Next*
- 4) Select *Other*, then *FASTQ Only*, then *Next*
- 5) The *Workflow Parameters* window will open.

Sample Sheet Wizard - Workflow Parameters

FASTQ Only Run Settings	FASTQ Only Workflow-Specific Settings
Reagent Cartridge Barcode* <input type="text"/>	<input type="checkbox"/> Custom Primer for Read 1
Sample Prep Kit <input type="text" value="TruSeq HT"/>	<input type="checkbox"/> Custom Primer for Index
Index Reads <input type="radio"/> 0 <input type="radio"/> 1 <input checked="" type="radio"/> 2	<input type="checkbox"/> Custom Primer for Read 2
Experiment Name <input type="text"/>	<input type="checkbox"/> Reverse Complement
Investigator Name <input type="text"/>	<input checked="" type="checkbox"/> Use Adapter Trimming
Description <input type="text"/>	<input checked="" type="checkbox"/> Use Adapter Trimming Read 2
Date <input type="text" value="5/ 1/2019"/>	
Read Type <input checked="" type="radio"/> Paired End <input type="radio"/> Single Read	
Cycles Read 1 <input type="text" value="301"/>	
Cycles Read 2 <input type="text" value="301"/>	
* - required field	

- 6) Fill out the *FASTQ Only Run Settings* as follows:

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- 11) Once all samples and required fields have been entered, the *Sample Sheet Status* indicator in the lower left-hand corner should read “Valid” and turn green. A red indicator reading “Invalid” means that required information has been omitted from the sample sheet, identical index pairs have been listed for more than one sample, or other possible mistakes are present. Review the Sample Sheet and make the required adjustments.
- 12) Click *Finish*.

Loading the MiSeq FGx:

-Prepare the MiSeq Reagents

- 1) Remove the sequencing cartridge from the freezer.
- 2) The HT1 buffer may be thawed at room temperature.
- 3) Place the sequencing cartridge in a Pyrex pan and fill with tap water until the water reaches the ‘Fill Line’ on the side of the cartridge.



- 4) Allow to thaw for at least 30 minutes.
- 5) Check to ensure all reagents are thawed before loading onto the MiSeq FGx.
- 6) Slowly invert the cartridge ten times to ensure mixing of all reagents, and then lightly tap on the bench top to remove any bubbles.
- 7) Place the reagent cartridge on ice or set aside at 2°C to 8°C (up to 6 hours) until ready to set up the run. For best results, proceed directly to loading the sample and setting up the run.
- 8) Prepare fresh 0.2N NaOH. If using 2N stock, dilute 100µl in 900µl of water. If using a different stock concentration, prepare a large enough dilution to counteract any small errors in pipetting.

-Preparing the Samples for Loading on the MiSeq FGx (*steps to be performed in the post-amplification hood*)

Denaturing the DNA

- 1) Label a 1.5mL tube “20pM Pool”.
- 2) Combine 5µl from the “Pool” with 5µl of 0.2N NaOH.
- 3) Quickly vortex and spin down.
- 4) Incubate for 5 minutes at room temperature
- 5) Immediately after the incubation, add 990µl of chilled HT1 buffer, vortex and spin
 - a. This dilution will yield a 20pM pool of the sample libraries.

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Preparing the PhiX

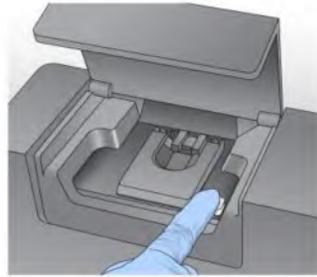
- 1) Combine the following, vortex and quick spin, then incubate the combined 10 μ l solution for 5 minutes at room temperature:
 - a. 2 μ l, 10nM PhiX Control
 - b. 3 μ l, 10mM Tris-HCl+0.1% Tween 20 (pH 8.5)
 - c. 5 μ l, 0.2N NaOH
- 2) Immediately after the incubation, add 990 μ l chilled HT1 buffer, vortex and spin
 - a. This dilution yields 20pM denatured PhiX which can be stored for up to two weeks at -200C.

Loading the Samples on the Cartridge

- 1) Label a 1.5mL tube as "Load" and combine the following to obtain a 600 μ l loading solution:
 - a. 225 μ l, HT1 buffer
 - b. 337.5 μ l, 20pM library pool
 - c. 37.5 μ l, 20pM PhiX
- 2) Vortex and quick spin.
- 3) Using a 1,000 μ l pipette tip, pierce the foil seal on the "Load" well of the MiSeq reagent cartridge.
- 4) Pipette 600 μ l from the "Load" tube into the loading well.
- 5) Load the reagent cartridge onto the MiSeq when prompted.

-Setting-Up a Run on the MiSeq FGx

- 1) From the welcome screen, select *Sequence*.
- 2) Then select *Research Use Only Run* for the PowerSeq Nested CRM kit.
- 3) The next screen contains a checkbox for using BaseSpace to save run data. Leave this box unchecked and click *Next*.
- 4) Follow the on-screen prompts to load the flow cell and reagents.
 - a. Cleaning the Flow Cell
 - i. Remove the flow cell from the container and rinse it with nuclease-free water to remove excess salt from both the glass and the plastic cartridge.
 - ii. Dry the flow cell off using a lint-free lens cleaning tissue and then wipe with an alcohol wipe being sure to not leave any streaks.
 - iii. Lightly massage the black gasket to ensure proper seal once loaded onto the MiSeq FGx
 - b. Loading the Flow Cell
 - i. Raise the flow cell compartment door, and then press the release button to the right of the flow cell latch.



- ii. Visually inspect the flow cell stage to make sure that it is free of lint. If lint or other debris is present, clean the flow cell stage using an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol. Carefully wipe the surface of the flow cell stage until it is clean and dry.
 - iii. Holding the flow cell by the edges, place the flow cell on the flow cell stage.
 - iv. Gently press down on the flow cell latch to close it over the flow cell.
 - v. Check the lower left corner of the screen to confirm that the RFID on the flow cell was read.
 - vi. Close the flow cell compartment door.
 - vii. Select *Next* on the Load Flow Cell screen. The Load Reagent Screen will open.
- c. Loading the Reagents and Reagent Cartridge
- i. Remove the PR2 bottle from the refrigerator and gently invert the bottle to mix, and then remove the lid.
 - ii. Open the reagent compartment door, raise the sipper handle until it locks into place, and then remove the wash bottle.
 - iii. Place the PR2 bottle in the wash bottle position and lower the sipper handle all the way down.



- iv. Check the lower left corner of the screen to confirm that the RFID on the PR2 bottle was read.
- v. Select *Next*.
- vi. Open the reagent chiller door and remove the wash tray present in the instrument.
- vii. Slide the reagent cartridge previously loaded with sample into the reagent chiller and then close the reagent chiller door.



- viii. Check the lower left corner of the screen to confirm that the RFID on the reagent cartridge was read.
- ix. Ensure the correct sample sheet has been selected. If not, navigate to the correct sample sheet and select it.
- x. Select *Next*.
- d. Review the run parameters and settings to ensure they are correct. Select *Next*.
- e. The system will then conduct a series of pre-run tests. Wait for all checks to pass (green checkmarks) then select *Start Run*. If any of the checks fail, refer to the “MiSeq FGx Instrument Reference Guide” for troubleshooting options.

6 **PowerSeq Nested CRM System** - Data Analysis and Reporting Workflows

6.1 **GlobalFiler Kit**

See the most current version of the “DNA Methods Manual” for information regarding data analysis for the GlobalFiler Kit.

6.2 **YFiler Plus Kit**

See the most current version of the “DNA Methods Manual” for information regarding data analysis for the Y Filer Plus Kit.

6.3 **PowerSeq Nested CRM System**

Analysis of Data Using GeneMarker HTS

GeneMarker HTS v1.2.2.1338 will be used for mtDNA analysis. The software provides rapid analysis of multiple samples using motif alignment technology that automates the recommendations of the DNA Commission of the International Society for Forensic Genetics: Revised and extended guidelines for mitochondrial DNA typing. This motif-based alignment provides recognition and proper assignment of motifs and INDELS consistent with phylogenetic and forensic considerations. As a result, very few manual edits of the alignments should be required.

The following steps outline the general procedure for analysis of mtDNA data generated in the BCI mitochondria laboratory. Reasonable deviations in the pathway may be made as long as the end product is the same.

Evaluating Post-Run Metrics on the MiSeq FGx

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The post-run screen displays numerous run metrics. The following metrics should be considered for quality and the suitability of the data for analysis:

- 1) *Q Score*: The Q-Score for all cycles on that run is displayed in the center of the post-run screen. Q scores are used to measure base calling accuracy. Low Q scores can lead to increase false-positive variant calls. The Q score default cutoff is set to $\geq Q30$. A Q score of 30 (Q30) is equivalent to the probability of an incorrect base call 1 in 1,000 times (i.e. the base call accuracy is 99.9%). In general, the default quality filter set at Q30 should be sufficient to provide good quality data.
- 2) *Cluster Density*: The cluster density metric is displayed in K/mm^2 and measures the number of clusters per square millimeter for the run. The acceptable range is 400-1650, with the validation runs ranging from 787 to 1067 (average of 905). A run with a cluster density falling outside of this range may still contain usable data and should be analyzed with caution.
- 3) *Clusters Passing Filter*: The clusters passing filter metric is displayed as a percentage and shows the percentage of clusters passing filter based on the Illumina chastity filter (which measures quality). A measure of 80% or higher is generally considered acceptable.
 - a. The chastity of a basecall is the ratio of the intensity of the greatest signal divided by the sum of the two greatest signals. If more than one base call has a chastity value of less than 0.6 in the first 25 cycles, then the reads do not pass the quality filter.
- 4) *Phasing and Pre-Phasing*: While not directly listed on the post-run screen, the phasing and pre-phasing metrics can be examined as another indicator of run quality. These values can be found in the Illumina Sequencing Analysis Viewer once the run file has been selected. Phasing is caused by a nucleotide blocker not being removed after signal detection. This results in no new nucleotide being added to the strand and an asynchronous reading for this strand. The affected strand will always be one nucleotide behind in the sequencing and pollute the light signal for that cluster. Pre-phasing is similar to phasing except that the affected strand is one cycle ahead of the others (due to the addition of a nucleotide with a defective terminator cap). Per the recommended guidelines from Illumina, the phasing percentage should be less than or equal to 0.250 while the pre-phasing percentage should be less than or equal to 0.150. Runs with phasing/pre-phasing values outside of these ranges may still provide useful data however.

Exporting Sample Files from the MiSeq FGx

- 1) After the run has completed the files must be moved for analysis.
- 2) Locate the latest run.
 - a. The list of runs on the instrument can be found here: Computer/Data (D:)/Illumina/MiSeqOutput/
 - b. Find the Flow Cell barcode ID for the current run and select the appropriate folder.
 - i. The file naming pattern places the Flow Cell ID at the end of the file name. The general structure is: run date_MiSeq FGx serial number_a four digit code_a string of zeros_Flow Cell ID.
- 3) Copy the run folder.

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- 4) On the MiSeq, open the MiSeqFGx (\\FORENSEQ) folder shortcut.
- 5) Paste the folder in the [\\FORENSEQ](#) folder.
- 6) Go to the MiSeq Server computer and open the MiSeq FGx ([\\FORENSEQ](#)) folder.
- 7) Copy the recently pasted run folder. Open the SBS Runs – Shortcut on the desktop and paste the folder into it.
- 8) Once copied over, the entire run folder will be backed up to the appropriate location for long term storage.
- 9) Once the run folder has been stored and verified to be complete, the original run folder may be deleted from the MiSeq FGx.
 - a. To delete a run folder from the MiSeq FGx, start on the home screen of the Illumina MiSeq FGx software.
 - b. Click the “Manage Files” button. Then navigate to the appropriate directory and select the folder to be deleted and click “Delete.” NOTE: before deleting a folder from the MiSeq FGx be sure it has been backed-up on either the server, and external drive, or the internal BCI network.

Trimming Fastq Files

- 1) Save all Fastq files to be trimmed in one folder.
- 2) Within the folder containing the Fastqs, create a new folder named “trim”.
- 3) Open Ubuntu (by clicking the  icon in the dock).
- 4) Change to the folder containing the Fastq files to be trimmed
 - a. For example, if the Fastq files are on the user’s desktop in a file named “Fastq Files” the file path would be as follows:
`- cd /mnt/c/Users/USER NAME/Desktop/FASTQ_files/`
 - b. Tip: cd= change directory. If you begin to type the file name and press *Tab* it should autofill the folder name.
- 5) Run Trimmomatic by entering the following text string:
`for j in *.gz; do java -jar /home/obci/Trimmomatic-0.38/trimmomatic-0.38.jar SE -phred33 $j trim/$j-trim90.fastq.gz MINLEN:90;done`
- 6) The trimmed Fastq files will be automatically placed into the “trim” folder and will have “trim” added to the file name.
- 7) Close Ubuntu.
- 8) Move the Fastq file folder containing the trimmed files to the appropriate batch folder.

Importing Samples into GeneMarker HTS

- 1) Create a new project folder to store the analysis on the GeneMarker HTS analysis computer.
- 2) Check the boxes to use the default reference and the default motif.
- 3) Set the “Alignment Options,” “Amplicon Settings,” and “Filter Options” to match settings shown in Appendix A.
 - a. The “Amplicon Settings” may be altered as needed.

- 4) To add the sample files, locate the .fastq or .fastq.gz files you wish to import. Each sample will have two .fastq/.fastq.gz files associated with it. One will be labeled as “R1” and the other as “R2.” Both files need to be imported into GeneMarker HTS.
- 5) Click “Add” and navigate to the .fastq/.fastq.gz. files and select all samples to be imported.
- 6) Click “OK” and wait for the analysis to complete. Files will continue to analyze until all samples have been processed. Samples are analyzed one at a time and completed samples can be reviewed while the remainder of the batch is processing.

Sample Analysis

- 1) Double click on a sample name to open the viewer window. The top of the viewer window shows a coverage map for the entire mitochondrial genome. Only the portion tested will be highlighted on the coverage map.
- 2) Below the coverage map is the reference sequence and the pile-up of the sample reads. This window may be scaled as needed. Right clicking on a base allows for edits if required.
- 3) Below the sample pile-up is the variant table. Clicking “Both” will show all variants above the 10% threshold. “Major” shows only variants above 50% with “Minor” showing variants between 10%-50%.
 - a. The Major/Minor distinction is one made by the software and cannot be modified.
- 4) In order for a variant to be called, it must meet the following criteria:
 - a. The variant percentage must be greater than or equal to 10%
 - b. The variant allele coverage must be greater than or equal to 200 reads
 - c. The total coverage at the position must be greater than or equal to 200 reads

Export of Variant Calls

- 1) On the F: drive, create a project folder for the current run. Then create a sub folder named “Major-Minor Reports.”
- 2) Navigate to the project folder created in step 1 of the “Importing Samples into GeneMarker HTS” section on the GeneMarker HTS analysis computer.
- 3) In the standard Windows search box, type “major” to show only the major reports in the project folder.
- 4) Click on the first sample, hit “ctrl+A” to select all, then hit “ctrl+C” to copy the files.
- 5) Navigate to the newly created project/run folder on the F: drive and paste these files into the “Major-Minor Reports” folder.
- 6) Repeat steps 3-5 but search for “minor” this time.
- 7) Once all the major and minor files are combined into the same folder, open the MPS workbook associated with the batch. On the “Sample Log” tab a button is available which uses a macro to combine the major and minor files into one. Once the files have been combined, save the file. The check box in the “Reports Combined” column can be used to keep track of which sample reports have been combined.

Motifs and Sequence Nomenclature

- 1) Motifs

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- a. Traditionally, alignment is focused on minimizing the number of mismatches between the reference and a sample read, or a “best fit” alignment; however, because forensic analysis has established conventions for the position of many common variants, this traditional approach to alignment is not always preferred when analyzing mtDNA. As a result, GeneMarker HTS provides option of using a motif: a list of variant calls within a specified region that are translated into an expected sequence.
 - b. When using a motif alignment, the alignments of reads that cross motif regions with expected sequences are adjusted to match the list of variants. Reads that start or end in motif regions are soft-clipped.
 - c. GeneMarker HTS comes supplied with a default motif file. The default motif file may be edited or a custom motif may be used. However, for routine casework the default motif file shall be used.
- 2) Sequence Nomenclature
- a. Nucleotide base positions are designated according to the standard one-letter code set by the International Union of Pure and Applied Chemistry (IUPAC) and are listed in the table below. Heteroplasmy is indicated by the use of IUPAC codes which designate more than one possible base.

- b. IUPAC Codes and base designations:

IUPAC Code	Base Designation
G	Guanine
A	Adenine
T	Thymine
C	Cytosine
N	G,A,T, or C
B	C,G, or T
D	A,G, or T
H	A,C, or T
K	G or T
M	A or C
R	A or G
S	G or C

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V	A,C, or G
W	A or T
Y	C or T

- c. Alignment and variant coding will be conducted in accordance with the SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis (2019). These rules are presented below and represent a blended approach of rule based and phylogenetic methods.

Rule 1 – Maintain known patterns of polymorphisms (a.k.a. known phylogenetic alignments). Most violations to known patterns of polymorphisms involve insertions and deletions. A phylogenetic alignment tool is available at empop.org.

Example: Maintain deletions at positions 249, 290 and/or 291 when present. See other examples in the SWGDAM mtDNA Nomenclature Examples document.

Rule 2 - Use nomenclature with the least number of differences unless it violates known patterns of polymorphisms.

Rule 3a – Homopolymeric C-Stretches in Hypervariable Region I (HVI): C stretches in HVI should be interpreted with a 16189C when the otherwise anchored T at position 16189 is not present. Length variation in the short A tract preceding 16184 should be noted as transversions.

Rule 3b - Homopolymeric C-Stretches in Hypervariable Region II (HVII): C stretches in HVII should be interpreted with a 310 C when the otherwise anchored T at position 310 is not present. C-stretches should be interpreted with a 311T when the anchored T at position 310 is followed by a second T.

Rule 4 – Maintain the AC Repeat Motif in the HVIII region from np 515-525.

Rule 5 – Prefer substitutions to insertions/deletions (indels).

Rule 6 – Prefer transitions to transversions unless this is in conflict with Rule 1.

Rule 7 – Place indels contiguously when possible.

Rule 8 – Place indels on the 3' end of the light strand.

Rule 9 – The 3107 nucleotide should not be reported in sample data. As 3107 in the rCRS is simply a placeholder intended to maintain historical nomenclature (*Andrews*

et al. 1999), differences from the rCRS (i.e., deletions) at this position are not biologically meaningful.

- d. Insertions are described by noting the site immediately prior to the insertion with respect to the light strand of the rCRS followed by a point and a '1' for the first inserted base, with sequential numbering for each inserted base thereafter (e.g. 315.1C). Insertions should not alter subsequent numbering of the sequence.
- e. Deletions are described by noting the deleted site followed by 'del'.
- f. Point Heteroplasmy occurs when more than one base above threshold is present at a given position and is: a) present in both sequencing directions, and b) above the level of background noise. Point heteroplasmy typically occurs at one or two positions within the mtDNA control region for a sample. The appropriate IUPAC code will be used to designate the site as being heteroplasmic.
- g. Length Heteroplasmy occurs in the homopolymeric C-stretch regions in HVI, HVII, and/or HVIII. This type of heteroplasmy occurs when a sample has at least two types with each one containing different totals of C nucleotides at a given C-stretch.
- h. Length heteroplasmy most often occurs when there is a substitution of a C for a T at position 16,189. The reference type in HVI is C5TC4. Sequences showing length heteroplasmy in HVI will be truncated to fit the format C5TC4 including the T to C change at 16,189. It will be noted if a sample has length heteroplasmy in HVI but the length heteroplasmy will not be used for comparison.
- i. Length variants in HVII are commonly observed in the number of C residues preceding a T residue at position 310. The reference type in HVII is C7TC5. When a 310C occurs, insertions relative to the rCRS at 303-315 do not need to be noted. However, it is often possible to determine unambiguously the dominant length variant in this region. The dominant type as called by GeneMarker HTS will be used for further comparison. Length heteroplasmy in HVII will be noted. Also, differences in sequences due to the absence of a common length variant is not exclusionary.

Guidelines for the Analysis of Controls

- 1) Positive Amplification Controls
 - a. The positive control, 2800M, is used to monitor the success of each step from amplification to sequencing. The positive control must be above all reporting thresholds across all amplicons and yield the following haplotype: 152C, 263G, 315.1C, 447C-477C, and 16519C.
 - b. If the positive control fails to amplify or produce the expected results, all samples associated with it fail, *and the extracts may be reamplified.*
- 2) Reagent Blank and Negative Amplification Control
 - a. The Reagent Blank (RB) or Manipulation Blank (MB) monitors for the presence of exogenous DNA from extraction sequencing. If a sample is re-extracted, a new RB is generated in the repeated extraction. The RB/MB must:

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- i. Be extracted concurrently and treated with the most sensitive conditions as the samples.
 - ii. Amplified using the same test kit, instrument model, sensitivity conditions as required by the sample(s) containing the least amount of DNA.
 - iii. Typed using the same instrument model and sequencing conditions as the least concentrated sample(s).
- b. The Negative Control (NC) monitors for the presence of exogenous DNA from the amplification procedure through the sequencing procedure.
- c. In the event that analyzable sequence **and called variants** (data above all applicable thresholds and aligned to the rCRS) are obtained from either the RB/MB or NC:
- i. An attempt will be made to determine the source of the ~~mtDNA~~ **analyzed sequence/variants**.
 - ii. The RB/MB or NC **analyzed sequence/variants** should be compared to the sample sequences to determine if any sample is the source. The samples and the RB/MB or NC sequence must differ at one or more base positions per amplicon to eliminate this possibility (length differences do not count).
 - iii. If the RB/MB or the NC base sequence (at a given position) is concordant with the associated sample sequence (at that position), the results of that that base (at the given position) are not used for comparison purposes. The sample sequence at that given position can only be used if one of the following is met:
 - (1) The RB/MB or NC base read coverage does not exceed the total read coverage threshold of 200 reads.
 - (2) The RB/MB or NC base read coverage exceeds the minimum read coverage threshold of 200 reads, but the coverage is less than the minimum variant frequency (10%) of the read coverage for the corresponding base of the associated sample.
 - (3) The RB/MB or NC base read coverage exceeds the minimum read coverage threshold of 200 reads and is greater than the minimum variant frequency (10%) of the read coverage for the corresponding base of the associated sample, but the sequence is not in concordance with the sample.

Sequence Interpretation, Reporting, and Comparison

- 1) The following criteria must be meant in order for a variant to be interpretable and reported:
 - a. The base pair position must have at least 200 total reads.
 - b. The variant basecall must have at least 200 reads.
 - c. The variant basecall must be at least 10% of the total reads at that base pair position.
- 2) When reporting sequence data for a sample:
 - a. A list of differences from the rCRS must be accompanied by the range of nucleotides of the region that was sequenced.
 - i. All possible alternate alignments will not be reported.
 - b. Samples are to be amplified in duplicate. Only variants which are consistent between the two duplicates for a sample will be used for comparison and reporting.

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- 3) When making sample comparisons, only regions with a common range will be evaluated.
- 4) The count of cytosine nucleotides in the HVI C-stretch will not be used for comparison purposes if a length heteroplasmy is present.
- 5) The number of cytosine nucleotides in samples with HVII length heteroplasmy will not be used for reporting purposes.
- 6) A sample which yields a mixture of DNA sequences ~~will be reported as inconclusive~~ **will not be used for comparison purposes.**
 - a. A sample may be a mixture if there are more than two heteroplasmic positions present.
- 7) The following conclusions can be made when comparing sequencing data from two samples:
 - a. **Exclusion** – when two samples being compared differ at two or more positions (**excluding length heteroplasmy**). These samples are excluded as coming from the same source.
 - b. **Inconclusive** – when two samples being compared differ at only one position.
 - i. Due to the possibility of undetected heteroplasmy, additional samples of known origin may be analyzed.
 - c. **Cannot Exclude** - when two samples being compared are consistent and demonstrate sequence concordance (having a common base at each position at which sequence data were obtained in the sample). These samples cannot be excluded as coming from the same source **or from having a maternal relationship.**
- 8) **The below chart may be used when making conclusions regarding comparisons between two samples involving sequence heteroplasmy:**

Criteria:	Conclusion:
Two identical heteroplasmic bases are present in both samples at the same position.	Concordant; this is not a difference.
One heteroplasmic base is present in one sample and a common base is present at the same position in the other sample.	Concordant; this is not a difference.
One heteroplasmic base is present in one sample and a different base is present at the same position in the other sample	This is a difference.

7 Frequency Calculations

7.1 ~~GlobalFiler Kit~~

~~See the most current version of the “DNA Methods Manual” for information regarding frequency calculations for the GlobalFiler Kit.~~

7.2 ~~Y-Filer Plus Kit~~

~~See the most current version of the “DNA Methods Manual” for information regarding frequency calculations for the Y-Filer Plus Kit.~~

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7.3 PowerSeq Nested CRM System

When sample comparisons are made and two samples cannot be excluded as coming from the same source, DNA statistical analysis will be performed using the CODIS Popstats database.

- 1) The most current version of the CODIS Popstats database is to be used to obtain a frequency estimate.
- 2) The data to be used will be limited to the sequence ranges in common for both samples being compared.
- 3) Only the samples in the database encompassing the same sequence ranges will be used in the search.
- 4) All sequence variants should be entered into the database search. However, length variants at positions 16193, 309, and 573 will be ignored during the search. As a result, any length variability at these positions will not affect the rarity of the database search.
- 5) When conducting a search, make sure the CODIS Popstats settings are as follows:

Database Information

Name: mtDNA_Population_Data

Description: Parson W and Dur A (2007) EMPOP-a forensic mtDNA database. FBI/Genetics 1(2), 88-92. Scientific Working Group on DNA Analysis Methods (SWGDM) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories, version 1.4

Confidence Interval (95%) Method

Clopper-Pearson One-Tailed

Normal Approximation Two-Tailed

Population Groups to Search

African Origin

Asian Origin

Caucasian Origin

Hispanic Origin

Native Origin

Length Heteroplasmy

309

573

16193

Maximum number of sequence differences for match: 1

Maximum number of sequence differences for display: 0

Minimum number of overlapping base pairs for search: 90

Minimum pop. group size for upper bound frequency estimate: 150

List the match pairs

Length heteroplasmy option

Ignore insertions at the specified length heteroplasmy sites

Consider insertions at the specified length heteroplasmy sites as individual differences

Searching the Database

- 1) Retrieve a Profile from the Database
 - a. From the main Analyst Workbench window, click the "Popstats" tab on the far left-hand side.
 - b. Enter the "Specimen ID" number and click *Retrieve*.
 - c. Click *Calculate*.
 - d. Print the report as a .pdf and save to the appropriate case folder.
- 2) Manually Enter a Profile to be Searched
 - a. The Ohio SDIS ORI (OHBCI0000) should be auto-populated in the *Agency ID* field. If not, add it.
 - b. Enter the sample name in the *Specimen ID* field.

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- c. Enter text into the *Comment* field if needed.
 - d. Under *Specimen Details* click the *mtDNA* header to activate the mtDNA data entry field.
 - e. At the bottom of the *Specimen Details* window, click the green "+" button to add a row for data entry.
 - f. Right click in the left most box of the newly added row to select which region data will be entered for.
 - i. HVI – 16,024-16,365
 - ii. HVII – 73-340
 - iii. Entire Control Region – 16,024-576

Note: may also enter the region positions manually.
 - g. Enter the variant in that region in the *Polymorphism* box.
 - h. Continue as above until all variants have been entered.
 - i. Click *Calculate*.
 - j. Print the report as a .pdf and save to the appropriate case folder.
- 3) **NOTE: While mtDNA sequence length variants for regions 302-310 (HVII) and 568-573 (HVIII) are entered, multiple C-stretch length variants at these positions are ignored during the database searches of concordant sequences containing this region. Therefore, will not add any additional rarity during searching. Also, the number of "C" nucleotides in samples with HVI length heteroplasmy is not considered for comparison purposes.**

Upper Bound Frequency Estimate

- 1) Frequency estimate calculation if the searched mtDNA sequence was observed at least once in the database:
 - a. The one-tailed Clopper and Pearson method for determining the upper bound frequency estimate of a mtDNA profile is used at a confidence level of 95% ($\alpha = 0.05$).
$$\sum_{k=0}^x \binom{n}{k} p_0^k (1 - p_0)^{n-k} = \alpha$$

Where: n = the number of profiles in the population database
x = the number of observations of the mtDNA profile in the database
k = 0, 1, 2, 3 ... x observations
p = the 95% confidence limit for the proportion of a population with the mtDNA profile of interest
 $\alpha = 0.05$ for a 95% confidence limit.

 - b. This equation requires a computer to calculate. This can be accomplished using the CODIS mtDNA Popstats program.
 - c. For example: A mtDNA sequence is observed once in the US Caucasian database containing 1,036 profiles. The upper bound frequency estimate using the Clopper-Pearson formula = 0.46%.
 - i. The above meaning: With 95% confidence, the maximum true frequency of the mtDNA profile in the US Caucasian population is 0.46%, or 1 in 217 individuals.

2) Frequency estimate calculation if the searched mtDNA sequence was not observed in the database:

a. In this case $x=0$ and the formula becomes

$$p_0 = 1 - \alpha^{1/n}$$

Where: α = the confidence coefficient (use 0.05 for a 95% confidence interval)

n = the number of individuals in the population

3) With the Clopper and Pearson method, an upper bound confidence interval of 95% is placed on the profile's frequency. In 95% of intervals constructed in this way, the true frequency is higher than this value; thus, with 95% confidence the actual haplotype frequency will be rarer than that provided by the estimate. The frequency estimates will be expressed as both a percentage and a frequency.

4) The CODIS Popstats program supplies frequency estimates for **numerous population groups** ~~the following population groups: African American, US Hispanic, US Caucasian, and US Asian.~~ These estimates are not intended to be used to draw any conclusions about the ethnicity of the contributors of the evidence.

EMPOP and/or HaploGrep Database Query

- 1) As a method to check the quality of the mtDNA results obtained, the mtDNA sequences may be searched in EMPOP or HaploGrep as needed.
- 2) These searches will not be used for reporting, but .pdf printouts of the results may be saved in the casefile for future reference.
- 3) A link to the EMPOP and HaploGrep websites can be found on the *Sample Log* tab of the MPS Workbook.

8 Report Wording

The following is a guideline intended to help achieve uniform content and style in technical reports. Departures from the statements below will at times be necessary to accommodate specific circumstances. Reporting that is highly accurate, grammatically correct, and easily understood by non-scientific readers shall be acceptable. For ease of reporting, report tables (similar to those in use by the DNA section) may be used for cases with large numbers of samples to be reported.

The body of the report may contain the following sections:

- Submitted Items
- Analytical Details and Results
- Conclusions and Statistical Information
- Remarks

8.1 Discrepancies

Any discrepancies between the description of submitted items and their actual contents should be noted in the Submitted Items section.

Reference Section 7.5 of the DNA Methods Manual for acceptable wording.

8.2 Analytical Details and Results

The Analytical Details and Results Section will precede the Conclusions and Statistical Information Section in the mtDNA report.

mtDNA Reports should contain the following analytical detail statement: Mitochondrial DNA (mtDNA) typing was performed using PCR with the PowerSeq™ CRM Nested kit for mtDNA sequence positions 16,013-16,569 and 1-592.

The following wording should be used for cases with mtDNA results:

- 1. mtDNA sequence(s) obtained:**
“mtDNA sequences were obtained from Items X and XX.”
- 2. Partial mtDNA sequence(s) obtained** (i.e. dropout of amplicons below threshold):
“mtDNA sequences were obtained from Item X (base positions 16XXX-16XXX and XX-XXX only) and Item XX.”
- 3. No mtDNA sequence(s) obtained:**
“Insufficient mtDNA was present for a DNA sequence to be obtained from Items X and XX.”
- 4. mtDNA sequence was obtained but was below set thresholds:**
“mtDNA sequence obtained from Item X was not of requisite quality for comparisons or for interpretations to be conducted.”
- 5. mtDNA sequence was obtained but was deemed uninterpretable due to contamination above the laboratory established threshold:**
“mtDNA sequence obtained from Item X was not of requisite quality for comparisons or for interpretations to be conducted.”
- 6. mtDNA sequence was obtained but results were inconsistent between duplicate amplifications:**
“A reliable mtDNA profile was unable to be obtained from Item X. Therefore, no conclusions can be reached regarding the source of Item X.”
- 7. mtDNA sequence was obtained but determined to be a mixture:**

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“The mtDNA sequence obtained from Item X indicates the presence of a mixture of mtDNA from more than one individual. mtDNA mixtures are not interpreted by the BCI laboratory; therefore, no comparisons can be performed using Item X.”

8. **mtDNA sequence was obtained from a relative of a missing person (only item in the report):**
“mtDNA sequence was obtained from *Jane Doe* (Item X), the *mother* of *Janie Doe*. ~~The mtDNA profile from *Jane Doe* (Item X) will be entered into the Relatives of Missing Persons Index of CODIS.~~”
9. **mtDNA sequence was obtained from a missing person of unknown origin (only item in the report):**
“A mtDNA sequence was obtained from Item X. ~~The mtDNA profile from Item X will be entered into the Unidentified Human Remains Index of CODIS.~~”
10. **mtDNA testing was started but was not completed or stopped before sequence data was obtained:**
“mtDNA testing was begun on Item X but will not be completed at this time.”
11. **No mtDNA testing performed on an item:**
“No mtDNA testing was performed on Item X.”

8.3 Conclusions and Statistical Information

The Conclusions and Statistical Information should follow the Analytical Details and Results section and contains any conclusions and statistical calculations made regarding the items examined.

Conclusions:

The following wording should be used as appropriate for stating the conclusions of mtDNA analysis.

1. **Exclusion:**
“The mtDNA sequences obtained from Item X and Item XX are different. As a result, (the source of Item X) is excluded as being the source of Item XX.”
2. **Exclusion with known sample from a maternal relative:**
“The mtDNA sequences obtained from the sample from the XXX (Item XX) and the known sample of *Jane Doe* are different. Mitochondrial DNA is known to be maternally inherited. As a result, the donor of the mtDNA from the XXX (Item XX) is excluded as a possible matrilineal relative of *Jane Doe*.”
3. **Inclusion:**
“The mtDNA sequences obtained from Item X and *Jane Doe* (Item XX) are concordant. As a result, *Jane Doe* cannot be excluded as being a possible source of Item X.”

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4. Inclusion with known sample from a maternal relative:

“The mtDNA sequences obtained from the sample from the XXX (Item XX) and the known sample of Jane Doe are concordant. Mitochondrial DNA is known to be maternally inherited. As a result, the donor of the mtDNA from the XXX (Item XX) cannot be excluded as a possible matrilineal relative of Jane Doe.”

5. Inclusion with partial sequence:

“The mtDNA sequences obtained from Item X and *Jane Doe* (Item XX) are concordant across the sequence range obtained in common to both samples. As a result, *Jane Doe* cannot be excluded as being a possible source of Item X.”

6. Inclusion with sequence heteroplasmy:

“The mtDNA sequences obtained from *Item X* and *Jane Doe* (Item XX) are the same, except at position XXXXX. At this position, the presence of a thymine (T) was observed in *Item X* and the presence of both a thymine (T) and a cytosine (C) was observed for *Jane Doe* (Item XX). As a result, the sequences are concordant and *Jane Doe* (Item XX) cannot be excluded as a possible source of Item X.”

7. Concordant Samples with Non- Concordant Reference:

“The mtDNA sequences obtained from Items X and XX are concordant. As a result, Items X and XX cannot be excluded as originating from a common source.” *(Include statement of exclusion as necessary if samples are not concordant with the submitted reference)*

8. Inconclusive:

“The mtDNA sequences obtained from Item X and *Jane Doe* (Item XX) are the same, except at position XXXXX. At this position, the presence of a thymine (T) was observed in Item X and the presence of a cytosine (C) was observed for *Jane Doe* (Item XX). As a result of this one base difference, no conclusion can be made as to whether or not *Jane Doe* is a possible source of Item X.”

Statistical Information:

The following reporting format should be used for results obtained from searches of pertinent Mitochondrial DNA Population Databases and the corresponding statistical calculations. Routine calculations should be reported for the three major U.S. databases (African American, U.S. Caucasian, and U.S. Hispanic) as well as any other population groups with one or more observations. The following wording and table should be used to report results from searches of the Mitochondrial DNA Population Database and the resultant statistical calculations:

“The mtDNA sequence obtained from Item X was searched against the CODIS mtDNA population database and has been observed in the three major U.S. population groups as follows:

Population Group	Number of Observations	Number of Profiles in the Population Group	Upper Bound Frequency Estimate	Approximate Occurrence in the Population
African American				
U.S. Caucasian				
U.S. Hispanic				

Notes: Search range included base positions XXX and XXX. Data from searches of additional population groups with no observations of the queried sequence is available upon request.”

If the database search returned results for additional population groups, the first sentence above should be edited accordingly:

“The mtDNA sequence obtained from Item X was searched against the CODIS mtDNA population database and has been observed in the three major U.S. population groups and others as follows:”

If the mtDNA sequence searched was less than the minimum range of HVI and HVII, the base positions stated in the “Notes” should be edited to state the base position range searched.

8.4 Remarks

Section 7.4 of the DNA Methods Manual may be referenced for additional wording as needed.

The following statements may be included in the Remarks section as appropriate:

1. **CODIS Upload Statement:**
“The DNA profile from Item XX has been entered into the CODIS database in accordance with state and national regulations. Regularly scheduled searches will be performed to assist in the identification of missing persons and unidentified human remains. If investigative information becomes available or a profile is removed from CODIS, your agency will be notified.”

2. **All cases with previously reported STR or Y-STR results:**
 ““This report supplements the original testing performed on Item(s) X issued on the [Section Name; i.e. “DNA” or “MPS”] report dated (insert date here).”
 “This report supplements the original testing performed on the database sample for (insert name).”

3. **All cases with mtDNA results obtained:**
 “All sequence data generated is contained within the control region of the human mitochondrial DNA.”

This document is uncontrolled if viewed outside the BCI document management system.

4. Samples are reported out as concordant:

“Concordant means that there is a base in common at every position.”

5. No mtDNA sequence was obtained:

“The control region of the human mtDNA was amplified but did not yield sufficient DNA for successful sequence analysis.”

6. Maternal references are being used:

“Mitochondrial DNA is maternally inherited. Therefore, individuals that share a maternal lineage will typically have the same mitochondrial DNA type.”

7. Contamination present which did not allow for comparisons to be made:

“No conclusions could be made for Item XX due to the detection of contamination above lab established thresholds.”

8. Request for submission of additional materials for testing due to incomplete sequence data:

“The National DNA Index System (NDIS) requires nucleotide positions 16,024-16,365 and 73-340 to be complete for uploading mitochondrial DNA sequences from unidentified human remains to NDIS. If uploading to NDIS is required, additional skeletal material should be submitted.”

9 Case Pocket Contents

MPS laboratory records are digital and are stored on a per batch or per case basis as needed. The records are stored on the F: drive. For each case, a PDF document should be uploaded to LIMS and should contain the following items as applicable:

1. MPS case-specific mtDNA Call Sheet
2. Sample Notes
3. Batch Workbook PDF File
4. Popstats Output
5. CODIS Specimen Details Report (Short)

As case circumstances dictate, additional documentation may be added as needed.

Appendix A – Laboratory Workflow Sheets

Promega Nested CRM Protocol Instructions (for Laboratory Use):

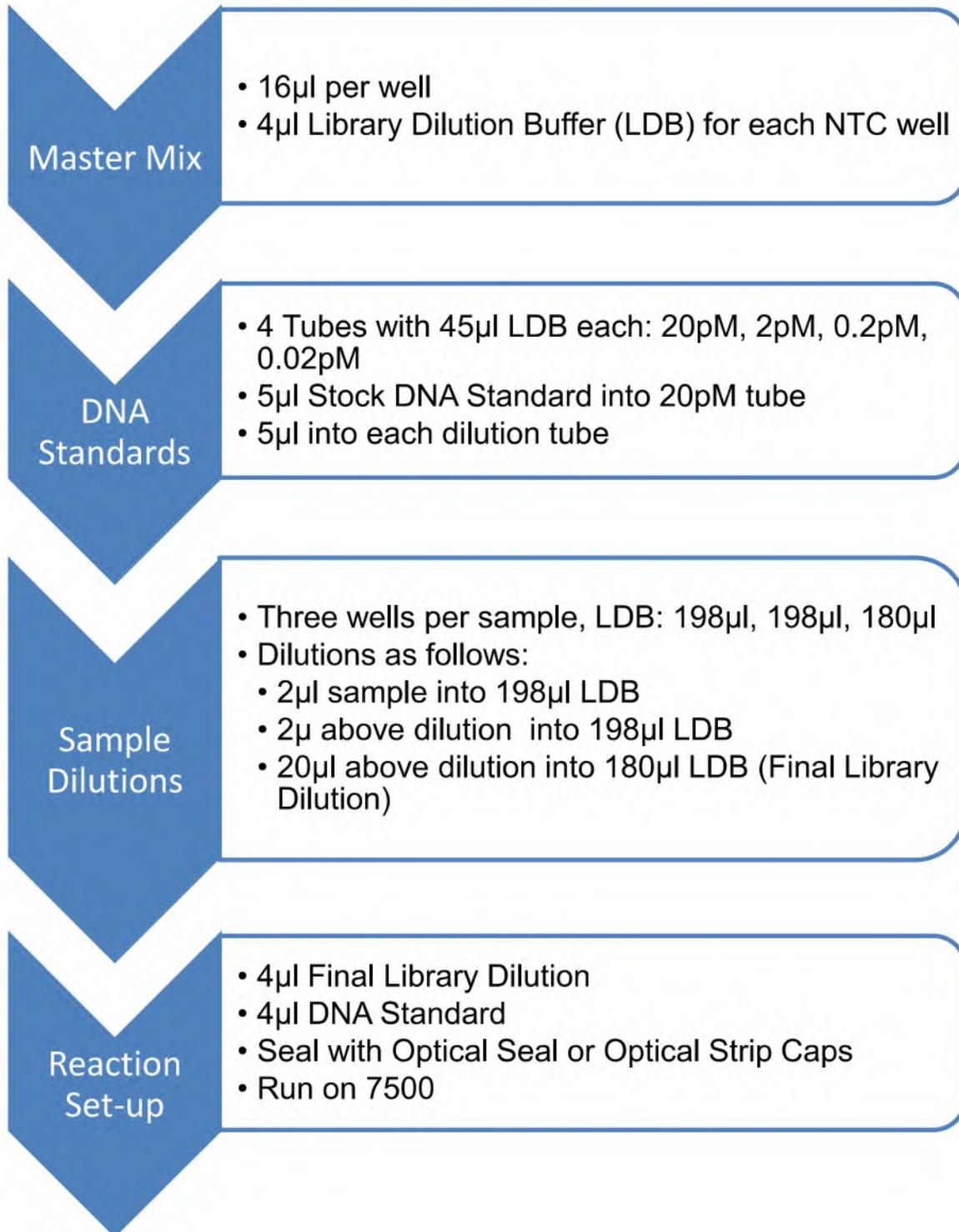
-Purification #1:



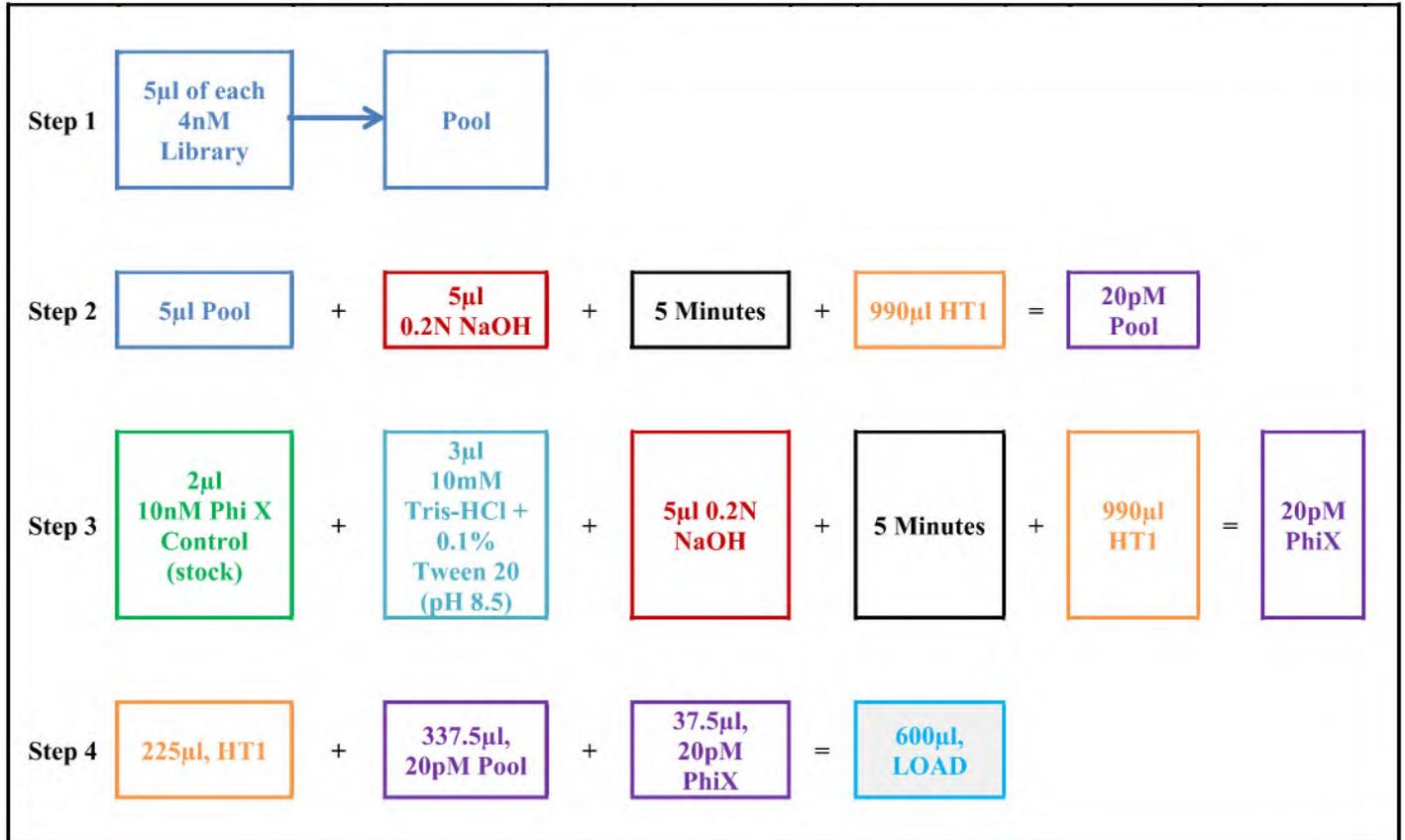
-Purification #2, #3, and #4:



PowerQuant MS Instructions (for Laboratory Use):



Library Dilution and MiSeq FGx Loading Instructions (for Laboratory Use):



Appendix B – GeneMarker HTS Software Settings

Use Default Motif

Create Motif Edit Motif

Alignment Options

Consensus
 Remove PCR Duplicates
 Keep Only Proper Pairs
 Motifs

Identity
 90

Soft Clipping at 3' Q ≤
 25

Sequencer:
 Illumina
 Ion Torrent
 Other

Amplicon Settings
 PHI Settings

OK Cancel

G Amplicon Settings

Amplicon Regions

	Chromosome	Start	End
1	chrM	16013	16126
2	chrM	16116	16226
3	chrM	16223	16408
4	chrM	16384	16486
5	chrM	16474	30
6	chrM	16555	152
7	chrM	136	257
8	chrM	246	364
9	chrM	342	436
10	chrM	429	592

Positions are inclusive (start through end)

Add Remove
 Load BED File Remove All

OK Cancel

G Filter Settings

Variant Filters Region Filters

Variant Percentage 10.0%

Variant Allele Coverage 200

Total Coverage 200

Allele Score Difference 10

Allele Balance Ratio

SNP 2.5

Indel 5.0

Save Load Default
 OK Cancel

G Filter Settings

Variant Filters Region Filters

Entire Reference Input Region

Chromosome	Start	End
chrM	16013	16569
chrM	1	592

Positions are inclusive (start through end)

Add Remove
 Load BED File Remove All

Save Load Default
 OK Cancel

Appendix C - Revised Cambridge Reference Sequence

16013 actattct
16021 ctgttcttc atggggaagc agatttgggt accaccaag tattgactca cccatcaaca
16081 accgctatgt atctgtaca ttactgccag ccacatgaa tattgtacgg taccataaat
16141 acttgaccac ctgtagtaca taaaaccca atccatca aaacccctc cccatgctta
16201 caagcaagta cagcaatcaa cctcaacta tcacacatca actgcaactc caagccacc
16261 cctcaccac taggatacca acaacctac ccaccctaa cagtacatag tacataaagc
16321 catttaccgt acatagcaca ttacagtcaa atcccttctc gtcccatgg atgaccccc
16381 tcagataggg gtccttgac caccatcctc cgtgaaatca atatccgca caagagtgtc
16441 actctctcg ctccgggcc ataacacttg ggggtagcta aagtgaactg tatccgacat
16501 ctggttcta cttcagggtc ataaagccta aatagccac acgttcccct taaataagac
16561 atcacgatg **16569**

1 gatcacaggc ctatcacct attaaccact cacgggagct ctccatgcat ttgtatttt
61 cgtctggggg gtatgcacgc gatagcattg cgagacgctg gagccggagc accctatgtc
121 gcagtatctg tcttgattc ctgcctcatc ctattatta tcgcacctac gttcaatatt
181 acaggcgaac atacttacta aagtgtgta attaattaat gctttagga cataataata
241 acaattgaat gtctgcacag ccActttcca cacagacatc ataacaaaaa attccacca
301 aacccccct CCCCgcttc tggccacagc acttaaacac atctctgcca aacccaaaa
361 acaagaacc ctaacaccag ctaaccaga tttcaaattt tatcttttgg cggtatgcac
421 ttttaacagt ccccccaa ctaacacatt atttcccct cccactcca tactactaat
481 ctcatcaata caacccccg ccatcctacc cagcacacac acaccgctgc taaccata
541 cccgaacca accaaacccc aaagacaccc cccacagttt atgtagctta c **592**

Appendix D – References

Andrews, R. M., Kubacka, I., Chinnery, P. F., Lightowlers, R. N., Turnbull, D. M., and Howell, N. Reanalysis and revision of the Cambridge Reference Sequence for human mitochondrial DNA, *Nature Genetics* (1999) 23:147.

GeneMarker HTS User Manual. GeneMarkerHTS-1.0-UG001, 2017.

MiSeq FGx Instrument Reference Guide. Part #15050524 Rev C., February 2015.

PrepFiler BTA Manual. 4463348 Rev C., January 2012.

PowerSeq CRM Nested System, Custom, Protocol. Application Note #AN322.

PowerSeq Quant MS System Technical Manual. TM511 – 3/18.

Quantifiler Trio DNA Quantification User Guide. 4485354, Revision G.

SWGDAM MtDNA Interpretation Guidelines – Approved 04/23/2019.

Appendix E – Abbreviations

The following abbreviations may be used in case file notes:

*Additional abbreviations listed in the DNA lab manual may also be used as needed.

~	about	IQ	DNA IQ extraction	prep	prepared
✓	checked	l	left	prev	previously
(-)	negative	lab	labeled	r	right
(+)	positive	lg	large	rec	received
∴	therefore	lt	light	s	suspect
?	unknown	ly	LySep Column/Tube		
amp	amplification	man	manila	ret	red evidence tape
attn	attention	mb	manipulation blank	rets	red evidence tape sealed
bet	blue evidence tape	me	manila envelope	RP	re-processing
bld	blood	med	medium	s	suspect
bp	back panel	mj	major	SEB	stain extraction buffer
		mn	minor		
bpb	brown paper bag	mtDNA	mitochondrial DNA	sec	second
brn	brown	nc	not collected	sm	small
CRM	PowerSeq CRM Nested System	nd	not detected		
ct	clear tape	ne	not examined	sme	small manila envelope
cts	clear tape sealed	neg	negative	std	standard
cont	containing	np	not performed	STR	short tandem repeat
ded	deduced	nr	none reported	sub	submitted
det	detective	nt	not tested	sw	swab
dh ₂ O	deionized/filtered	nv	non-victim	s/w	sealed with Quantifiler Trio
	water	obs	observed	TTP	tube to plate
dil	dilute	odh	Ohio dept of health	v	victim
DNSFC	data not suitable for comparison	oes	orange evidence sticker	vol	volume
env	envelope	ofc	officer	we	white envelope
er	evidence receiving	ONSE	organic non semen extraction		
et	evidence tape	orig	original		
exp	expires	os	oral swab		
ext	exterior	OSE	organic semen extraction (differential)		
Fp	front panel				
GF	Globalfiler	pb	paper bag		
gr	gram(s)	BTA	PrepFiler BTA kit		
Het	heterozygous	BTAE	PrepFiler Express BTA kit		
		pg	page		
Hom	homozygous	pkg	package		
hosp	hospital	plb	plastic bag		
ID	Identifiler	pos	positive		
int	interior	PQ	PowerSeq Quant MS System		