FORENSIC BIOLOGY SECTION

SEQUENCING TEST METHODS

FOREWORD

The analysts of the Forensic Biology Section shall have a minimum of a baccalaureate or an advanced degree in a natural science or a closely related field. Analysts shall have successfully completed college course work covering the subject areas of genetics, biochemistry, molecular biology, and statistics. All analysts undergo a formalized training program covering forensic techniques and instrumentation. Successful completion of the Training Program is required before analysis of evidence is performed. Additionally, all analysts participate in external proficiency testing from an accredited vendor.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
TABLE OF CONTENTS

1. **ForenSeq® DNA Signature Plus Methods**
    a. DNA Signature Plus Procedure
    b. MiSeq FGx® Procedure
    c. Data Analysis
    d. Single Source Interpretation Guidelines
    e. Mixture Interpretation Guidelines
    f. Phenotyping and Ancestry Estimations

2. **ForenSeq® Kintelligence Methods**
    a. Kintelligence Procedure
    b. MiSeq FGx® Procedure
    c. Data Analysis
    d. Database Upload
    e. Phenotyping and Ancestry Estimations

APPENDICES:

1. Definitions Appendix 1
2. Sequencing Report Wording Guidelines Appendix 2
3. DNA Signature Plus Stutter Filters Appendix 3
4. Universal Analysis Software v2.0+ Settings Appendix 4

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

1. ForenSeq® DNA Signature Plus Kit Sequencing Test Methods:

1.1. Scope: ForenSeq® DNA Signature Plus Kit sequencing should be performed in cases where information beyond what is obtained with traditional STR analysis may assist in resolution of the case. This analysis is targeted for single source samples or mixtures with a predominant contributor. DNA analysis performed prior to sequencing shall be in accordance with the Indiana State Police Forensic Services Division Forensic Biology Casework Test Methods.

1.2. Precautions/Limitations: Samples that may be suitable for sequencing with the DNA Signature Plus Kit should be evaluated by a member of the sequencing team to determine the most appropriate sample(s) to analyze in a case. The following should be considered:

1.2.1. Cases that have not provided meaningful or conclusive autosomal results should be considered for analysis.

1.2.2. Cases with previously determined unidentified human remains or missing person profiles may be considered for analysis.

1.2.3. Unknown evidentiary profiles, DNA standards, and CODIS reference profiles shall not have ancestry and phenotyping modeling reports run. This application shall only be used for human remains or missing person cases.

1.2.4. Due to the limitations of the technology, items demonstrating autosomal STR results with indistinguishable or complex mixtures shall not be considered for sequencing. Exceptions require Technical Leader approval.

1.2.5. For optimal results, samples from various cases/analysts may be pooled to achieve desired flow cell load.

1.3. Related Information:

1.3.1. Worksheet Manual

1.4. Instruments:

1.4.1. Verogen MiSeq FGx® Sequencing System – A DNA sequencing platform that delivers multiplex results of an array of STR and SNP loci from a prepared library while also providing interpretation functions using the fully integrated Universal Software Analysis (UAS) feature.

1.5. Reagents/Materials:

1.5.1. Ethanol

1.5.2. Nuclease Free Water (NFH_2O)

1.5.3. Amp Grade Water

1.5.4. ForenSeq® DNA Signature Plus Kit

1.5.5. MiSeq FGx® Reagent Kit

1.5.6. MiSeq FGx® Reagent Micro Kit

1.6. Hazards/Safety:

1.6.1. Two components of the ForenSeq® DNA Signature Plus Kit, LNA1 and LNW1, have hazardous properties. **CAUTION:** Both reagents contain formamide and B-mercaptoethanol. Formamide is a suspected carcinogen and a reproductive toxicant. B-mercaptoethanol is a toxin and irritant that can cause nervous system and organ damage. Therefore, universal precautions shall be used when working with these samples. In addition, appropriate waste receptacles shall be utilized when disposing of these reagents after use.

1.6.2. The cartridge of the MiSeq FGx® Reagent Kit contains formamide in reservoir 8. After a sequencing run using this cartridge, the contents of reservoir 8 shall be removed using universal precautions inside a fume hood and properly disposed.

1.6.3. In addition, the waste bottle within the MiSeq instrument contains potentially hazardous chemicals after a sequencing run. The contents shall be disposed of in the appropriate waste receptacle using universal precautions.

1.7. Reference Materials/Controls/Calibration Checks:

1.7.1. An assessment of the run quality metrics shall serve as a check of the accuracy and specificity of test results in addition to the testing of controls.

1.8. Procedures/Instructions:

1.8.1. **See the Forensic Biology Casework Test Methods for extraction and quantification procedures**

1.8.1.1. Performing DNA sequencing with DNA Signature Plus before or after autosomal STR analysis will be evaluated on a case-by-case basis.

1.8.1.2. DNA Signature Plus analysis may be performed on extracts previously used for autosomal STR testing or may require re-extraction of a sample.

1.8.1.3. Quantification results from previous autosomal STR testing may be used for amplification of the sample.

1.8.2. **ForenSeq® DNA Signature Plus**

1.8.2.1. **Introduction**

1.8.2.1.1. The Qiagen ForenSeq® DNA Signature Plus Kit is a sequencing multiplex containing two primer sets that amplifies Short Tandem Repeats (STRs) and Single Nucleotide Polymorphisms (SNPs). Primer Set A amplifies 27 autosomal STRs, 7 X-STRs, 24 Y-STRs, and 94 identity informative SNPs. Primer Set B amplifies the same markers as Primer Set A plus 56 ancestry informative SNPs and 24 phenotype informative SNPs, with 2 ancestry informative SNPs also being phenotype informative SNPs. Only Primer Set B is currently in use in the laboratory.

1.8.2.1.2. The ForenSeq® DNA Signature Plus Kit locus-specific information is available at https://www.qiagen.com/us/resources/kithandbookandprotocol/hb-3613-001-hb-forenseq-dna-sig-plus-1024-ww in the ForenSeq® DNA Signature Plus Kit Handbook, October 2024.

1.8.2.1.3. The preparation of the Amplify and Tag Targets step (PCR1) shall be performed in the PCR amplification set-up area. All further steps shall be performed in a post-PCR area.

1.8.2.2. **Sample Preparation**

1.8.2.2.1. The ideal target concentration is 1ng, however samples may be amplified in a range from 0.05-2ng. The Normalization Dilution Worksheet in the Sequencing Workbook is recommended for this purpose.

1.8.2.2.2. An attempt should be made to maximize samples on a flow cell. For Primer Set B, the capacity of a standard flow cell is 32 samples, and the capacity of a micro flow cell is 12 samples.

1.8.2.2.3. No fewer than 8 samples containing DNA shall be run on a flow cell. If fewer than 8 samples are available, additional positive control samples or standards may be added to the run to reach a minimum of 8 samples.

1.8.2.2.4. The maximum number of samples per flow cell may be exceeded if the overage is comprised of negative controls.

1.8.2.2.5. A ForenSeq® DNA Signature Plus Sequencing Record worksheet shall be used to track library prep. It is recommended to use the Sequencing Workbook for this purpose.

1.8.2.2.5.1. Add samples to sequencing record and assign unique dual index (UDI) adaptors to the sample setup, ensuring each sample is assigned a different UDI. Libraries may be re-run as necessary as long as the index assignments don't overlap with those for the new libraries.

1.8.2.3. **Amplify and Tag Targets (PCR1)**

1.8.2.3.1. Thaw PCR1 reaction mix for 30 minutes at room temperature while letting DPMB and NA24385 positive control stand at room temperature for 30 minutes. Remove FEM from frozen storage immediately before use and return to frozen storage immediately after use.

1.8.2.3.2. Label a 96-well plate FSP (for ForenSeq Sample Plate) to use for amplification. Crosslink the plate.

1.8.2.3.3. Determine the number of samples to be amplified including controls. Add approximately 10% extra reagent for overage to compensate for the loss that occurs during reagent transfer. Calculate the required amount of each component of the PCR master mix. Multiply the volume (µl) per sample by the total number of reactions to obtain the final volume (µl).

Components of Master Mix/sample:
4.7 µl PCR1
0.3 µl FEM
5.0 µl DPMB

1.8.2.3.4. Do not vortex the FEM enzyme. Pipette to mix.

1.8.2.3.5. Vortex PCR1 and DPMB tubes and centrifuge briefly.

1.8.2.3.6. Add the calculated volume of each component to a 1.5 ml tube. Pipette to mix and centrifuge briefly.

1.8.2.3.7. Add 10 µl master mix to each well of the FSP plate. Optionally, the master mix may be evenly distributed into each well of an eight-strip tube or column of a 96 well plate, then a multichannel pipette may be used to dispense.

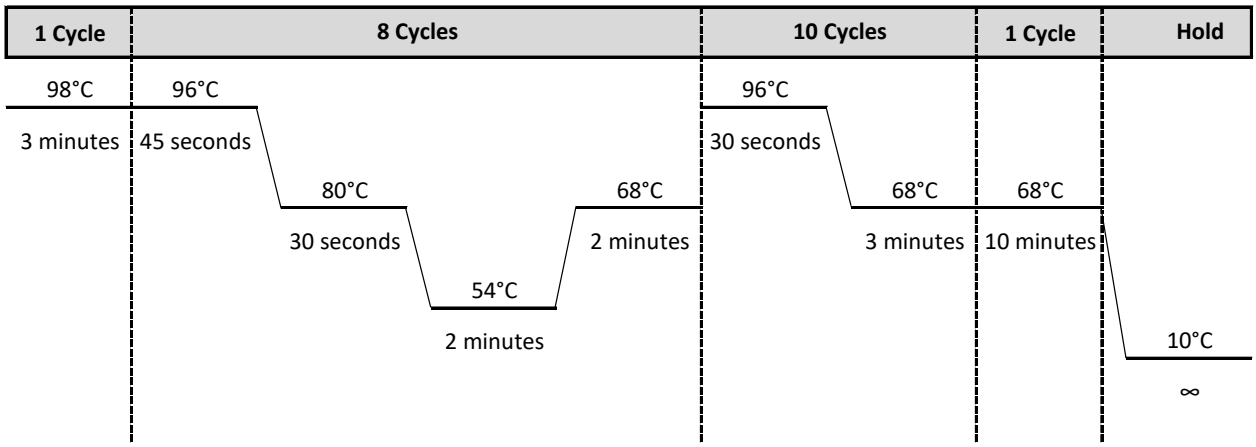
1.8.2.3.8. Invert NA24385 positive control at least three times to mix and then centrifuge briefly.

1.8.2.3.9. Dilute 2 µl NA24385 with 98 µl nuclease-free water in a 1.5 ml tube. Gently invert at least three times and centrifuge briefly. A new dilution shall be made for each run performed.

1.8.2.3.10. Add 5 µl sample, positive amplification control, or negative amplification control to the appropriate well in the FSP plate. Pipette to mix.

1.8.2.3.11. Seal the plate with a Microseal ‘A’ film. Centrifuge plate briefly and place in a VeritiPro™ Thermal Cycler.

1.8.2.3.12. Select the **Sig Plus PCR1** protocol with the following amplification procedure with a lid temperature of 105°C and a ramp mode of 0.2°C per second. Approximately 4.5 hour cycling time.



1.8.2.3.13. SAFE STOPPING POINT. Remove samples after the amplification process is complete and proceed immediately or the plate may be stored at 2°C to 8°C for up to two days. If storing at 2°C to 8°C before proceeding to PCR2, then seal plate with Microseal ‘B’ film. The plate may also be left on the thermal cycler overnight.

1.8.2.4. **Enrich Targets (PCR2)**

1.8.2.4.1. Thaw UDI plate and PCR2 reaction mix at room temperature for approximately 20 minutes.

1.8.2.4.2. Briefly mix UDI plate at 1800 rpm on plate shaker and centrifuge briefly.

1.8.2.4.3. Centrifuge the FSP at 1000 x g (rcf) for 30 seconds.

1.8.2.4.4. Pierce the foil cover of UDI plate to transfer 8 µl UDI adaptor to each sample well in the FSP plate. Seal used wells of UDI plate when finished.

1.8.2.4.5. Invert PCR2 reaction mix, centrifuge briefly, and then pipette to mix.

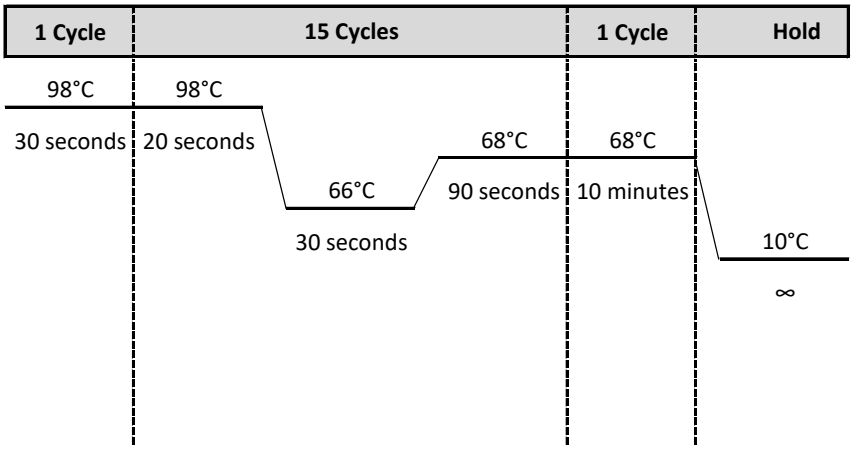
1.8.2.4.6. Add 27 µl PCR2 reaction mix to each well. Pipette to mix.

1.8.2.4.6.1. If more than 8 samples are being processed, PCR2 reaction mix may be evenly distributed into each well of an eight-strip tube or column of a 96 well plate, then a multichannel pipette may be used to dispense.

1.8.2.4.7. Seal the plate with a Microseal ‘A’ film seal and centrifuge at 1000 x g (rcf) for 30 seconds.

1.8.2.4.8. Place plate in a VeritiPro™ Thermal Cycler.

1.8.2.4.9. Select the **Sig Plus PCR2** protocol with the following amplification procedure with a lid temperature of 100°C. Approximately 46 minute cycling time.



1.8.2.4.10. SAFE STOPPING POINT. Remove samples after the amplification process is completed and proceed immediately. Alternatively, the plate may be stored at 2°C to 8°C for up to seven days or left on the thermal cycler overnight. If storing at 2°C to 8°C before proceeding to next step, then seal plate with Microseal 'B' film.

1.8.2.5. **Purify Libraries**

1.8.2.5.1. Let RSB and SPB2 stand for approximately 30 minutes to bring to room temperature.

1.8.2.5.1.1. Vortex SPB2 for at least 1 minute, inverting to mix before use and frequently during use to make sure that the beads are evenly distributed. Aspirate and dispense SPB2 slowly due to its viscosity.

1.8.2.5.1.2. Vortex RSB and invert to mix before use.

1.8.2.5.2. Label a midi plate PBP (for Purification Bead Plate) and a PCR plate as PLP (for Purified Library Plate).

1.8.2.5.3. Prepare SPB2 according to the number of libraries you are preparing.

Number of libraries	Procedure
<16	Add 50 µl SPB2 x number of libraries to a 1.5 ml tube.
16-96	Add [50 µl SPB2 x (number of libraries/8)] + 5 µl SPB2 to each well of a column of a new midi plate or a reagent reservoir.
>96	Add (50 µl SPB2 x number of libraries) + 200 µl SPB2 to a reagent reservoir.

1.8.2.5.4. Add 45 µl SPB2 to each well of the PBP plate according to the sequencing record.

1.8.2.5.5. Centrifuge the FSP plate at 1000 x g (rcf) for 30 seconds and remove the seal.

1.8.2.5.6. Transfer 45 µl sample from each well of the FSP to the corresponding well of the PBP. Discard the FSP plate.

1.8.2.5.7. Seal the PBP plate with a plastic adhesive seal and shake at 1800 rpm for 2 minutes.

1.8.2.5.8. Incubate at room temperature for 5 minutes.

1.8.2.5.9. Place the plate on a magnetic stand and wait until the liquid is clear (~5 minutes).

1.8.2.5.10. Remove and discard all supernatant from each well.

1.8.2.5.11. On the magnetic stand, wash two times as follows:

1.8.2.5.11.1. Add 200 µl freshly prepared 80% EtOH to each well.

1.8.2.5.11.2. Incubate on the magnetic stand for 30 seconds.

1.8.2.5.11.3. Remove and discard all supernatant from each well.

1.8.2.5.12. Centrifuge at 1000 x g (rcf) for 30 seconds.

1.8.2.5.13. Place the plate on the magnetic stand.

1.8.2.5.14. Remove residual EtOH from each well. A 20 µl pipette is recommended.

1.8.2.5.15. Remove the plate from the magnetic stand.

1.8.2.5.16. Add 52.5 µl RSB to each well.

1.8.2.5.17. Seal the plate with a plastic adhesive seal and shake at 1800 rpm for 2 minutes.

1.8.2.5.18. Make sure after shaking that beads are in solution. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 2 minutes.

1.8.2.5.19. Incubate at room temperature for 2 minutes.

1.8.2.5.20. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).

1.8.2.5.21. Transfer 50 µl from each well of the PBP to the corresponding well of the PLP plate.

1.8.2.5.22. Seal the plate with a plastic adhesive seal and centrifuge at 1000 x g (rcf) for 30 seconds.

1.8.2.5.23. SAFE STOPPING POINT. The procedure may be continued immediately, or the plate can be sealed with a Microseal ‘B’ film and stored at -25°C to -15°C for up to a year.

1.8.2.6. **Normalize Libraries**

1.8.2.6.1. This set of reagents contains formamide. LNA1 and LNW1 contain β-mercaptoethanol. These reagents, tips, and waste from these steps shall be disposed of in the appropriate hazardous waste container.

1.8.2.6.2. Thaw HP3 and LNA1 at room temperature for at least 30 minutes. Let LNB1 and LNW1 stand for approximately 30 minutes to bring to room temperature.

1.8.2.6.3. Vortex LNA1 with intermittent inversion.

1.8.2.6.4. Vortex LNB1 for at least 1 minute, stopping every 15 seconds to invert tube at least 5 times. Pipette to mix until the bead pellet at the bottom is resuspended.

1.8.2.6.5. Label a midi plate NWP (for Normalization Working Plate) and a PCR plate NLP (for Normalization Library Plate).

1.8.2.6.6. Create a master mix of LNA1 and LNB1. The amounts listed account for overage. Multiply each volume by the number of samples.

Components of Master Mix/sample:
46.8 µl LNA1
8.5 µl LNB1

1.8.2.6.7. Vortex, then invert the tube several times to mix.

1.8.2.6.8. Transfer into a reagent reservoir.

1.8.2.6.9. Add 45 µl of the LNA1/LNB1 Master Mix to each well of the NWP that will contain a library.

1.8.2.6.10. To clear any beads that may have aspirated, place the PLP on the magnetic stand and wait until the liquid is clear (~2 minutes).

1.8.2.6.11. Remove the clear adhesive seal from the PLP plate.

1.8.2.6.12. Transfer 20 µl from each well of the PLP plate to the corresponding well of the NWP plate.

1.8.2.6.13. Seal the NWP with a clear adhesive seal and shake at 1800 rpm for 30 minutes.

1.8.2.6.14. If sequencing immediately after library prep, begin thawing the MiSeq FGx® Reagent Kit Box 1 (stored -25°C to -15°C) as described in 1.8.3.2.1.

1.8.2.6.15. While the plate is shaking, perform the following:

1.8.2.6.15.1. Prepare 0.1N HP3. The amounts listed account for overage. Multiply each volume by the number of samples.

Components of 0.1N HP3
33.3 µl nuclease-free water
1.8 µl HP3

1.8.2.6.15.2. Invert the tube several times to mix and set aside.

1.8.2.6.15.2.1. Add 33.3 µl nuclease-free water per sample.

1.8.2.6.15.2.2. Add 1.8 µl HP3 per sample.

1.8.2.6.15.2.3. Invert the tube several times to mix.

1.8.2.6.15.2.4. Set aside.

1.8.2.6.15.3. Add 30 µl LNS2 to each well of the NLP plate that will contain library.

1.8.2.6.16. Immediately after the NWP has finished shaking, place the NWP on the magnetic stand and wait until the liquid is clear (~2 minutes).

1.8.2.6.17. Remove and discard all supernatant from each well.

1.8.2.6.18. Remove the plate from the magnetic stand.

1.8.2.6.19. Wash two times as follows:

1.8.2.6.19.1. Add 45 µl LNW1 to each well.

1.8.2.6.19.2. Seal with a clear adhesive seal and shake at 1800 rpm for 5 minutes.

1.8.2.6.19.3. Make sure after shaking that beads are in solution. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 5 minutes.

1.8.2.6.19.4. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

1.8.2.6.19.5. Remove and discard all supernatant from each well.

1.8.2.6.20. Remove the plate from the magnetic stand.

1.8.2.6.21. Seal with clear plastic adhesive seal and centrifuge at 1000 x g (rcf) for 30 seconds.

1.8.2.6.22. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

1.8.2.6.23. Remove residual supernatant from each well. A 20 µl pipette is recommended.

1.8.2.6.24. Remove the plate from the magnetic stand.

1.8.2.6.25. Add 32 µl freshly prepared 0.1 N HP3 to each well.

1.8.2.6.26. Seal the plate with a clear plastic adhesive seal and shake at 1800 rpm for 5 minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 5 minutes.

1.8.2.6.27. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

1.8.2.6.28. Transfer 30 µl from each well of the NWP to the corresponding well of the NLP plate. Pipette to mix.

1.8.2.6.29. Seal with clear plastic adhesive seal and centrifuge at 1000 x g (rcf) for 30 seconds.

1.8.2.6.30. SAFE STOPPING POINT. The procedure may be continued immediately, or the plate can be sealed with a Microseal ‘B’ film and stored at -25°C to -15°C for up to 30 days.

1.8.2.7. **Pool Libraries**

1.8.2.7.1. Determine which libraries to pool for sequencing.

1.8.2.7.2. Label a 1.5 ml tube PNL (for Pooled Normalized Libraries).

1.8.2.7.3. Transfer 5 µl of each library from NLP plate to an eight-tube strip or column of a 96 well plate.

1.8.2.7.4. The NLP plate should be sealed with a Microseal ‘B’ film and stored at -25°C to -15°C for up to 30 days.

1.8.2.7.5. Transfer the contents of each well to the PNL tube.

1.8.2.7.6. Vortex the PNL tube and centrifuge briefly.

1.8.2.7.7. SAFE STOPPING POINT. The procedure may be continued immediately, or the tube can be stored at -25°C to -15°C for up to 30 days.

1.8.2.8. **Denature and Dilute Libraries**

1.8.2.8.1. Preheat thermomixer to 96°C.

1.8.2.8.2. If not previously thawed, remove the Hybridization Buffer (HT1) from the MiSeq FGx® Reagent Kit Box 1 and thaw completely at room temperature.

1.8.2.8.3. Thaw HSC and HP3 for at least 30 minutes at room temperature.

1.8.2.8.4. Invert to mix the HSC and centrifuge briefly. Vortex the HP3 and centrifuge briefly.

1.8.2.8.5. Label a 1.5 ml tube Denatured HSC.

1.8.2.8.6. Combine in Denatured HSC tube:

2 µl of HSC

2 µl of HP3

36 µl nuclease free water

1.8.2.8.7. Pipette gently to mix and centrifuge briefly.

1.8.2.8.8. Incubate at room temperature for 5 minutes.

1.8.2.8.9. Label a 1.5 ml tube DNL for denatured normalized libraries.

1.8.2.8.10. Add 591 µl of HT1 to the DNL tube.

1.8.2.8.11. Place the PNL tube in the preheated 96°C thermomixer and incubate for 2 minutes.

1.8.2.8.12. Transfer 7 µl library from the PNL tube to the DNL tube immediately. Pipette to mix.

1.8.2.8.13. Add 4 µl of the Denatured HSC to the DNL tube. Pipette to mix.

1.8.2.8.14. Vortex and centrifuge briefly.

1.8.2.8.15. Immediately transfer entire DNL to the reagent cartridge as described in 1.8.3.2.4.

1.8.2.8.16. The PNL can be stored at -25°C to -15°C for up to 30 days.

1.8.3. Verogen MiSeq FGx® Sequencing System

1.8.3.1. Introduction

1.8.3.1.1. The Verogen MiSeq FGx® Sequencing System is a sequencing instrument that performs sequencing by synthesis, a type of targeted DNA sequencing. The system uses instrument-specific reagents and flow cells to measure fluorescence signals of labeled nucleotides. The system also includes internal imaging hardware and data analysis software.

1.8.3.1.2. The data produced by the Verogen MiSeq FGx® Sequencing System is analyzed with internal software that performs base calling, demultiplexes, aligns data to targeted regions and reports STR and SNP calls. Analyst data analysis occurs in the Universal Analysis Software (UAS).

1.8.3.2. Reagent Cartridge Preparation

1.8.3.2.1. Thaw the MiSeq FGx® Reagent Kit (Box 1). The MiSeq FGx® Reagent Kit (Box 1) contains the reagent cartridge and the hybridization buffer (HT1). Thaw the HT1 at room temperature. Thaw the reagent cartridge in a room temperature water bath for approximately 60-90 minutes. Do not submerge the cartridge

above the maximum water line. Alternatively, thaw the reagent cartridge overnight in the refrigerator.

1.8.3.2.1.1. The thawed reagent cartridge may be stored on ice for up to 6 hours if necessary. The MiSeq FGx® Reagent Kit may not be thawed and refrozen.

1.8.3.2.2. Once thawed, invert the cartridge at least ten times to mix. Ensure all reagent reservoirs are completely thawed and there are no precipitates. Remove bubbles and excess water by firmly tapping the cartridge on the benchtop. Dry the bottom of the cartridge with a lab wipe.

1.8.3.2.3. In a fume hood, pierce the foil of the highlighted sample position on the reagent cartridge, using a clean pipette tip.

1.8.3.2.4. Load the entire volume of the denatured normalized libraries (DNL) into the highlighted position on the reagent cartridge, taking care to avoid bubbles.

1.8.3.2.5. Tap the cartridge to ensure contents move to the bottom of the well.

1.8.3.3. **Create a Project and Run**

1.8.3.3.1. Log in to the ForenSeq® Universal Analysis Software.

1.8.3.3.2. Select Create Project at top of Home screen.

1.8.3.3.2.1. Enter project name. The project name shall be the batch number and the date the first run is started (ex. S2026-001_01Jan26).

1.8.3.3.2.1.1. The batch number shall include the “S’ and the year and the number for the sequencing run in the following format: S2026-001

1.8.3.3.2.2. Save project.

1.8.3.3.3. Select **Create Run** at top of Home screen.

1.8.3.3.3.1. Enter run name. The run name shall be the batch number and the date the run is started (ex. S2026-001_01Jan26) to allow for multiple sequencing runs of samples in a batch.

1.8.3.3.3.2. Select the appropriate type of flow cell for the run (micro or standard).

1.8.3.3.3.3. Save run.

1.8.3.3.4. Select appropriate run. Add samples to a run.

1.8.3.3.5. Select **Import Samples** to import samples from the Sequencing Workbook (recommended) or other text file,.

1.8.3.3.6. Select Add Sample to manually enter sample details.

1.8.3.3.6.1. Enter sample names, assay type, sample type, UDI index combination, analysis method, and project name into the appropriate fields.

1.8.3.3.6.2. Select **Add**. Repeat for all samples in the run, then select **Save Run**. The run will now be added to the queue.

1.8.3.4. **Start the Sequencing Run**

1.8.3.4.1. On the Verogen MiSeq FGx®, ensure server 2.0 is selected and log in with a valid username and password.

1.8.3.4.2. Select **Sequencing** as the run type.

1.8.3.4.3. Select the run previously created in the Universal Analysis Software.

1.8.3.4.4. Remove the flow cell and the PR2 from the MiSeq FGx® Reagent Kit Box 2.

1.8.3.4.5. Load the flow cell, PR2, waste bottle and previously prepared reagent cartridge as prompted by the software.

1.8.3.4.5.1. The flow cell must be thoroughly rinsed with water to remove excess salts. Dry the flow cell, taking care not to damage the gasket.

1.8.3.4.5.2. Use an alcohol wipe or a lab wipe moistened with ethanol to clean the flow cell glass. Do not use alcohol on the flow cell gasket. Ensure flow cell glass is clean and dry and that the gasket is properly seated before loading.

1.8.3.4.6. The instrument will perform a pre-run check, verifying all the RFID tags for the consumables. When complete, select **Start Run**.

1.8.3.4.7. If a RFID tag cannot be read, it can be entered manually using the keyboard. If necessary, contact Qiagen technical support for assistance.

1.8.3.5. **Evaluate the Sequencing Run**

1.8.3.5.1. When the run is complete, select **Next**. Ensure the sequencing run completed successfully. The sequencing run status may be viewed on the sequencer in real time or evaluated in the Universal Analysis Software after the run completes. Run Quality Metrics should be evaluated as described in 1.8.4.3.

1.8.4. **ForenSeq® Universal Analysis Software v2.0 Data Analysis**

1.8.4.1. **ForenSeq® Universal Analysis Software v2.0Settings are detailed in Appendix 3.**

1.8.4.2. **Introduction**

1.8.4.2.1. Run information generated by the MiSeq FGx® Sequencing System is stored on the ForenSeq server. Upon run completion, files are transferred to the Universal Analysis Software (UAS) which initiates the analysis of data using the default analysis settings.

1.8.4.2.2. UAS performs analysis processes including demultiplexing, sequence alignment, allele counting, genotype calling, stutter determination, noise filtering, and quality assessment.

1.8.4.2.3. Analytical thresholds (AT) and interpretation thresholds (IT) are percent-based values of the total read count at each locus.

1.8.4.2.4. The UAS default and internally validated AT and IT settings are both 1.5% for all STR and SNP loci, with the exception of DYS389II both at 4.5% and DYS481 and DYS681 both at 2%.

1.8.4.2.5. For instances of low read coverage, a minimum read count of 650 reads is used to establish the AT and IT which correlates to an AT and IT of 10 reads.

1.8.4.2.6. Stutter is filtered by the UAS for back, forward, and double back stutter using default and internally validated back (n-3/n-4/n-5/n-6) stutter parameters. For forward (n+3/n+4/n+5/n+6) and double back (n-6/n-8/n-10/n-12) stutter, the default stutter parameters are used.

1.8.4.2.7. The default analysis method, “SigPlus_ISP_v1”, has been set within the UAS incorporating the AT and IT thresholds and stutter percentages listed in Appendix 3.

1.8.4.2.8. Stochastic thresholds (ST) have been internally determined and validated. For all STR loci, a stochastic threshold of 450 reads shall be applied. A concentration based stochastic threshold shall be applied to SNP loci. Samples with less than or equal to 0.1ng of target DNA shall have a stochastic threshold of 200 reads. Samples greater than 0.1ng shall have a stochastic threshold of 100 reads.

1.8.4.2.9. Stochastic thresholds are not incorporated into the ISP default settings within the UAS. ST must be applied during interpretation and documented using SeqLite.

1.8.4.3. **Evaluating Run Quality Metrics**

1.8.4.3.1. To open the analysis of a project (plate) or run, select the Project or Run name.

1.8.4.3.2. Before evaluating the quality metrics and controls, ensure that the analysis state is listed as completed.

1.8.4.3.3. Navigate to the **Metrics** tab.

1.8.4.3.4. Quality Indicator Results for Controls and Quality Metrics

Color Coded Indicator	Positive Control	Human Sequencing Control	Negative Control	Quality Metrics
Green	All STR and SNP loci are typed and concordant with 2800M DNA Control.	Concordant genotype determined with sufficient coverage for the sample.	All designated reagent blanks or negative controls have no typed STR or SNP alleles.	All metrics are in recommended ranges.
Orange	At least 1 locus is untyped or disconcordant with 2800M DNA Control. Total number of reads for sample may be less than 85,000 reads.	Discordant genotype determined or overall intensity not met.	At least 1 STR or SNP has demostrated typed results.	At least 1 metric is not within recommended ranges.

1.8.4.3.5. Controls or quality metrics highlighted in green meet or pass recommended parameters for the run or sample. If highlighted in orange, further evaluation of the run quality metrics or sample is required, but the run or sample data may still be suitable for interpretation. If run quality metrics are flagged as orange, the Technical Leader shall be notified.

1.8.4.3.6. On the **Sample Quality** tab, ensure that Run Quality Metrics meet the following recommended target ranges.

Run Quality Metric	Recommended Ranges
Cluster Density	400 - 1650 k/mm^2
Cluster Passing Filter	≥80%
Phasing	≤0.25%
Pre-phasing	≤0.15%

1.8.4.3.7. Verify the Read and Index Quality Metrics all indicated a green check mark.

1.8.4.3.8. Assess the results of the Human Sequencing Control (HSC), an internal sequencing control, for either pass or fail results. Consult with TL if a failed result is obtained.

1.8.4.3.9. Evaluate the Sample Representation values to further assess the quality of the run and samples.

1.8.4.3.9.1. Samples demonstrating less than 85,000 reads are considered to have low coverage and should be interpreted with caution.

1.8.4.3.10. On the **Positive / Negative Controls** tab, ensure the positive amplification control (PC) and negative controls meet required parameters.

1.8.4.3.10.1. The positive control information includes the number of loci typed for the NA24385 control and the pass filter reads value or sample read count.

1.8.4.3.10.2. Incomplete coverage or dropout may occur in the typing of a positive control and shall not be considered a failed result. A positive control is considered a failed control if incorrect genotypes are determined for the sample.

1.8.4.3.10.3. Negative controls should have no typed results as no template DNA should be present in the samples. A negative control which demonstrates read counts above AT/IT at more than 5 loci shall be considered a failed control.

1.8.4.4. **Interpreting, Comparing and Reporting DNA Results Associated with Failed Controls and Contamination Events**

1.8.4.4.1. The Technical Leader shall be notified if a positive or negative control fails and/or if sample contamination occurs. Sample contamination is defined as additional peaks present in an unknown or reference sample that can be conclusively identified to not have originated in the sample (e.g., case to case contamination or a mixture in a reference sample).

1.8.4.4.2. Assessment of the integrity of the associated DNA results shall be done in consultation with the Technical Leader. This shall include the possible cause and effect of the failed control or contamination. Additionally, an assessment of the risk associated with moving forward with interpretation shall be documented, as well as the risks associated with retesting (e.g., unnecessary consumption of evidence).

1.8.4.4.2.1. If retesting is performed, the reasons for doing so shall be documented.

1.8.4.4.2.2. If results are determined to be suitable for interpretation without retesting, the appropriate interpretations, comparisons, and statistical results and a description of the failed control or contamination event shall be reported per the Report Writing Guidelines.

1.8.4.4.2.3. If results are determined to be not suitable for interpretation and retesting cannot be performed, the results shall be reported as not suitable for comparison per the Report Writing Guidelines.

1.8.4.4.3. Documentation in the case record shall include at a minimum: the forensic sample, reference or control test results that failed or was contaminated, the likely or known cause of the failed control or contamination, the likely or known

source of the contamination, the impact on the integrity of the DNA results, and the determination if the test result is suitable or unsuitable for interpretation.

1.8.4.5. General Sample Detail Information

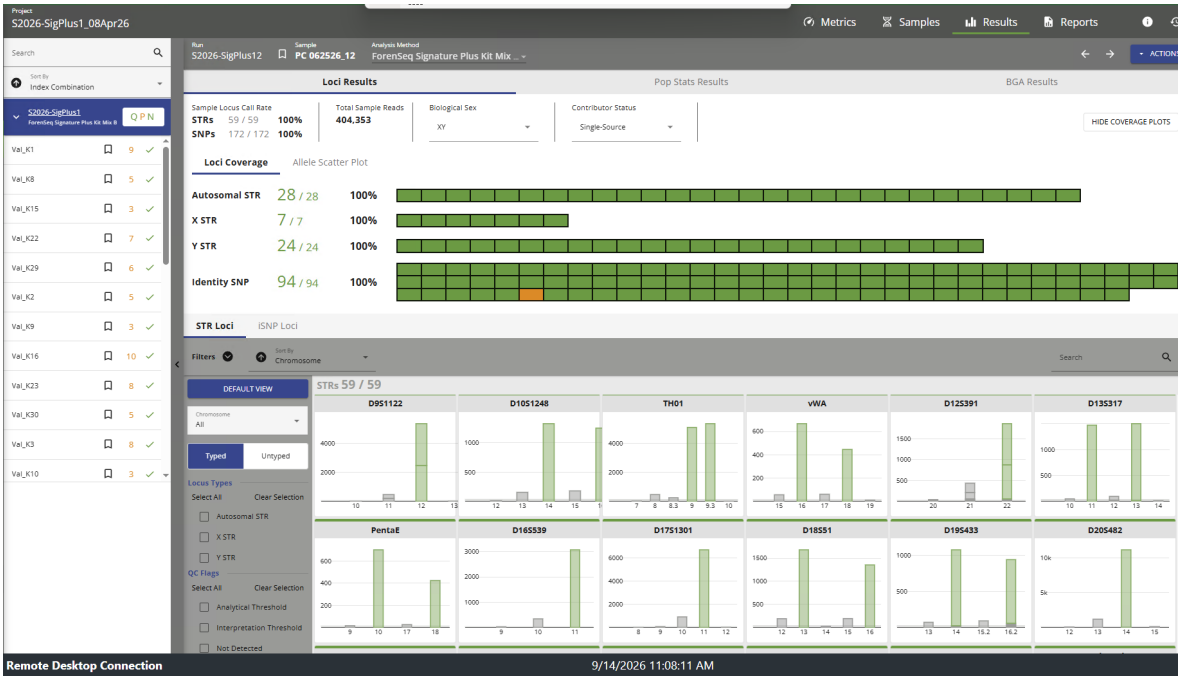
1.8.4.5.1. Within the Project, navigate to sample results by selecting the **Results** tab.

1.8.4.5.2. Evaluate the Biological Sex and Contributor Status.

1.8.4.5.3. Results for STR and iiSNPs are viewable for interpretation on the **Loci Results** tab. To access the piSNP and aiSNP results, navigate to **BGA Results** tab.

1.8.4.5.4. Information about the sample includes a Loci Coverage bar graph with color coded quality flags and an Allele Scatter Plot with read intensities of each typed STR and iiSNP locus, Biological Sex and Contributor Status, Sample Locus Call Rate, Total Sample Reads and the typing results across the loci. Several filters are present to allow for ease in interpretation of a specific type of marker or quality flags.

1.8.4.5.5. Sample and locus level quality indicators (highlighted in orange) may be present which may aid in sample interpretation at the locus level.



1.8.4.5.6. Interlocus balance of the STR loci is assessed internally within the UAS with a 60% threshold. A green indicator shows that the balance is within the recommended range while an orange indicator shows that the balance falls outside of optimal range.

1.8.4.5.7. Locus level quality indicators shall be evaluated and can aid in determination of single source contributor or mixture.

1.8.4.5.8. Locus level quality indicators are listed in the image below from the Universal Analysis Software for ForenSeq® Signature Plus Module User Guide, October 2024.

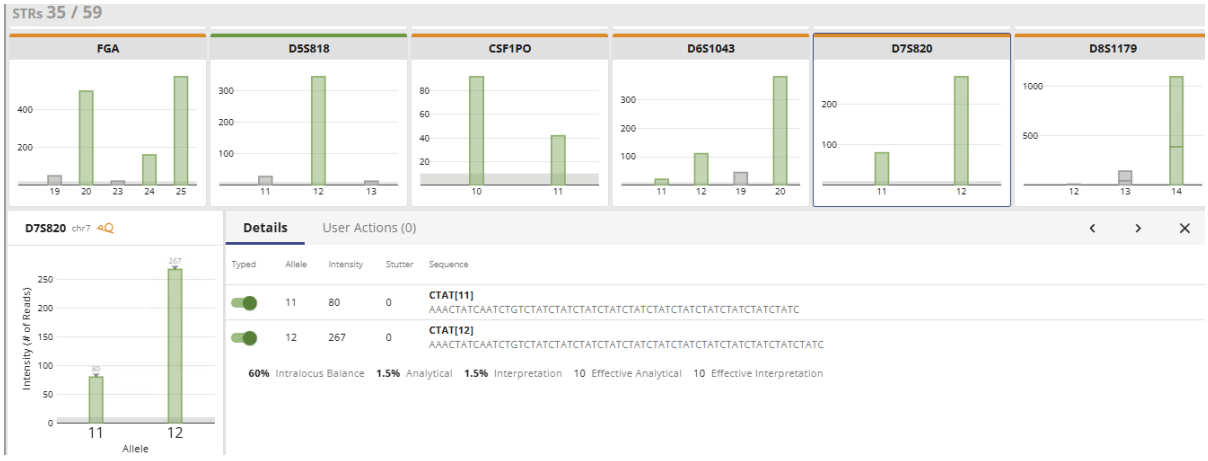
Symbol	Name	Description
	Analytical Threshold	The locus has an expected allele signal below the AT and no alleles greater than or equal to the IT.
	Interpretation Threshold	The locus has an expected allele signal greater than or equal to the AT, but below the IT.
	Not Detected	The software did not detect a signal for the locus.
	User Modified	A user typed or untyped an allele at the locus.
	Imbalanced	The locus is not homozygous, not hemizygous, and falls below the intralocus balance setting.
	Allele Count	The locus has more typed alleles than expected for a single-source sample of the indicated biological sex.
	Unexpected Allele*	The locus has an unexpected allele (non-reference or alternate) that is greater than or equal to the AT.
	Detected	An unexpected locus was typed (e.g., an NTC with typed loci, or an XX (Female) sample types Y SNPs above the threshold).
	Stutter†	At least one sequence exceeds the stutter filter percentage at a stutter position of a possible parent allele.

* SNPs only.
† STRs only.

1.8.5. Interpretation Guidelines for ForenSeq® DNA Signature Plus Data Analysis

1.8.5.1. Sample Evaluation of Results

1.8.5.1.1. Each locus with quality indicator flags (orange) shall be reviewed.



1.8.5.1.2. It shall be at the analyst's discretion, based on experience and training, to determine if read counts are true alleles, stutter, noise, or artifacts. If read counts are determined to not be true alleles, then the analyst shall untype the read counts and indicate a reason in the comment field.

1.8.5.1.2.1. **Stutter:** Read counts that fall in a stutter position that exceed the stutter filters applied by the UAS software may be removed by the analyst. The

sequence of the possible stutter shall be reviewed to further assess the association to the allele before removal.

1.8.5.1.2.2. **Sequence-Based Noise:** Read counts that exceed the filter range for noise may be removed by the analyst. The sequence of the possible noise shall be reviewed to further assess the association to the allele before removal. Sequence-Based Noise that differs by more than 1 bp can also be present and removed by the analyst.

1.8.5.1.2.3. **Artifacts:** A documented artifact has been observed at STR locus D7S820 with the addition of a single T bp at the end of the sequence resulting in +.1 base pair allele call. This artifact typically occurs at a much lower intensity than the primary allele read count. An artifact has also been observed at the piSNP locus rs201326893_Y152OCH which shows as read counts for the allele T (an unexpected genotype allele at this locus) typically under IT for the locus. However, this artifact may show as a typed allele in a low read count sample.

1.8.5.1.3. Single source, mixture, and gender determination

1.8.5.1.3.1. The overall profile, including results of all STR and SNP loci, shall be evaluated to determine if a sample is single source or a mixture.

1.8.5.1.3.2. Untyped read counts under AT /IT may indicate the presence of an additional contributor.

1.8.5.1.3.3. If there are no typed genotype results indicating the presence of a mixture, then the number of contributors does not need to be increased due to the presence of untyped read counts. Analyst's discretion may be used to determine if the untyped read counts interfere with the ability to interpret the typed component.

1.8.5.1.3.4. Generally, a single source profile should contain no more than two alleles at each autosomal STR or SNP locus. For gender specific markers, profiles from female individuals should contain no more than two alleles for the X-STR loci and no alleles for the Y-STR loci. Profiles from male individuals should contain only one allele for each X-STR and Y-STR locus, with the exception of DYS385a-b and DYF387S1 which may have up to two alleles.

1.8.5.1.3.4.1. Three allele patterns in autosomal STR loci as well as unexpected two allele patterns in the Y-STR loci have been observed and may not necessarily indicate the presence of a mixture. A sample with only one autosomal STR locus demonstrating this pattern could be an indication of a tri-allelic locus. A sample with only one Y-STR locus demonstrating this pattern could be an indication of a duplication event.

1.8.5.1.3.4.2. Other patterns outside of the general expectations for a single source profile may be observed. Consultation with the Technical Leader is required to determine the necessary action for the locus.

1.8.5.1.3.4.3. Biological anomalies such as tri-alleles or duplications should be confirmed through repeating the amplification and library preparation. Alternatively, amplification with PowerPlex® Fusion 6C or PowerPlex® Y23, for applicable loci, may be performed.

1.8.5.1.3.5. Analysis algorithms within UAS based on allele counts are used for preliminary gender determination for a sample. However, gender determination shall be established by the analyst and then gender information can be used to aid in interpretation and/or typing of gender specific markers.

1.8.5.1.3.6. Profiles with typed alleles in excess of the expectations of a single source profile or of tri-allelic or duplication patterns shall be defined as mixtures. Additionally, a profile shall be defined as a mixture if the only additional alleles present are typed in the gender specific markers.

1.8.5.1.3.7. Determination of the number of contributors in a mixture shall be done at the analyst's discretion, based on experience and training, and should take into account number of alleles present as well as allele count ratios and possible genotype pairings.

1.8.5.2. **Single Source Profile Interpretation**

1.8.5.2.1. Due to stochastic effects, low coverage, and intralocus imbalance issues, careful evaluation of untyped additional components shall be performed using the established Analytical/Interpretation and Stochastic Thresholds.

1.8.5.2.2. The following guidelines shall be used to aid in genotype determinations for read counts below thresholds if present.

1.8.5.2.3. A profile shall be evaluated for stutter and/or artifacts prior to the interpretation of untyped additional components.

1.8.5.2.4. **STR Loci Genotype Determinations**

1.8.5.2.4.1. Read counts below analytical and interpretation threshold shall not be interpreted.

1.8.5.2.4.2. Read counts below interpretation threshold may be used in genotype determinations in some instances.

1.8.5.2.4.2.1. If no typed alleles are present, then read counts below IT shall not be used for interpretation. Therefore, there shall not be any toggling/typing of read counts below IT.

1.8.5.2.4.2.2. If there is one typed allele, then additional untyped read counts (i.e., an additional component) below IT shall be further analyzed to determine if typing is warranted. Read counts below IT that become typed (toggled) can be used for interpretation.

1.8.5.2.4.2.2.1. If the additional component is in a stutter position and not previously identified as stutter, then no additional typing shall be done, and the genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

1.8.5.2.4.2.2.2. If the additional component is not in stutter position, then the Allele Count Ratio (ACR) between the typed allele and the additional component shall be determined.

1.8.5.2.4.2.2.2.1. ACR shall be calculated by dividing the read counts of the additional component by the read counts of the typed allele.

1.8.5.2.4.2.2.2.2. If the ACR is >0.30, the additional component shall be typed resulting in a heterozygous genotype determination for the locus.

1.8.5.2.4.2.2.2.3. If the ACR is not greater than 0.30, then no additional typing shall be done, and the genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

1.8.5.2.4.3. If there are two typed alleles, with only one greater than the stochastic threshold and with an imbalance quality indicator, the ACR should be evaluated.

1.8.5.2.4.3.1. If the ACR is ≥0.15 between the two typed alleles, then the heterozygous genotype shall be called.

1.8.5.2.4.3.2. If the ACR is <0.15 between the two typed alleles, then the heterozygous genotype shall be called if the two alleles are not in a stutter position of each other.

1.8.5.2.4.3.3. If the ACR is <0.15 between the two typed alleles, then the genotype shall be considered inconclusive due to ambiguity for the sister allele determination if the alleles are in a stutter position of each other.

1.8.5.2.4.4. Gender determination may be used in assessing the X-STR and Y-STR loci.

1.8.5.2.4.5. Any homozygous alleles less than stochastic threshold will be indicated with brackets using the SeqLite tool.

1.8.5.2.5. **SNP Loci Genotype Determinations**

1.8.5.2.5.1. Read counts below analytical and interpretation threshold shall not be interpreted.

1.8.5.2.5.1.1. If there are two typed alleles with an ACR of <0.10, then at the analyst's discretion the locus may be called inconclusive.

1.8.5.2.5.1.2. Any homozygous alleles less than the stochastic threshold will be indicated with brackets using the SeqLite tool.

1.8.5.3. **Mixture Profile Interpretation**

1.8.5.3.1. Mixture profiles may be suitable for interpretation. Additional sampling, extraction, or amplification should be considered before interpreting a mixture result.

1.8.5.3.1.1. Complex and/or indistinguishable mixtures are not suitable for interpretation. Mixtures with ≤ 85% mixture contribution (~5:1 mixture ratio) from the primary contributor with Fusion 6C/STRmix shall not be interpreted. Mixtures demonstrating the presence of more than three individuals shall not be interpreted.

1.8.5.3.2. A profile shall be evaluated for stutter and/or artifacts prior to the genotype determinations and no untyped additional components shall be typed for interpretation.

1.8.5.3.3. The following guidelines shall be used to determine the genotype of the major profile of the mixture. The minor profile shall not be utilized for interpretation.

1.8.5.3.3.1. A major profile shall be determined at 12 or more autosomal STR loci in order for the profile to be suitable for interpretations.

1.8.5.3.3.2. If a major profile is unable to be determined based off the guidelines, then the mixture will be deemed to be an indistinguishable mixture, and no conclusions shall be drawn.

1.8.5.3.4. **Two-Person Mixture Genotype Determinations**

1.8.5.3.4.1. An assessment of the STR loci (autosomal, X, and Y) shall be performed to determine the gender of the contributors to the mixture, if possible.

1.8.5.3.4.2. The allele count ratios of STR loci (autosomal, X, and Y) and iiSNP loci will be used to determine the major profile. Typed alleles are designated A-D in descending read count order. aiSNP and piSNPs in a two-person mixture sample shall not be interpreted or utilized for phenotype or ancestry estimations.

1.8.5.3.4.3. Allele count ratios shall be calculated by dividing the read counts of an allele (B) by the read counts of another allele (A).

1.8.5.3.4.4. **STR Loci**

1.8.5.3.4.4.1.Loci with 4 alleles (A-D)

1.8.5.3.4.4.1.1. If the allele count ratio of the two highest alleles (A and B) is ≥ 0.60 and the allele count ratio of the lowest high allele (B) and the highest low allele (C) is ≤ 0.30, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.4.4.2. Loci with 3 alleles (A-C)

1.8.5.3.4.4.2.1. If the allele count ratio of the two highest alleles (A and B) is ≥ 0.60 and the allele count ratio of the lowest high allele (B) and the lowest allele (C) is ≤ 0.30, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.4.4.2.2. If the allele count ratio of the two highest alleles (A and B) is ≤ 0.30 and the allele count ratio of the lowest high allele (B) and the lowest allele (C) ≥ 0.50, then a homozygous major profile (A) will be determined.

1.8.5.3.4.4.2.3. If the allele count ratio of the two highest alleles (A and B) is ≤ 0.15, then a homozygous major profile (A) will be determined.

1.8.5.3.4.4.3.Loci with 2 alleles (A and B)

1.8.5.3.4.4.3.1. If the allele count ratio of the two alleles (A and B) is ≥ 0.60, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.4.4.3.2. If the allele count ratio of the two alleles (A and B) is ≤ 0.20, then a homozygous major profile (A) will be determined.

1.8.5.3.4.4.4. Loci with 1 allele (A)

1.8.5.3.4.4.4.1. If the allele read count is greater than the stochastic threshold, then a homozygous major profile (A) will be determined.

1.8.5.3.4.4.4.2. A major profile (A) may be determined when the allele is below stochastic, only for X and Y STR loci, if the major profile is

deemed to be from a male individual. **Exception**: one allele below stochastic at DYS385a-b or DYF387S1 may not be used for interpretation.

1.8.5.3.4.5. **iiSNP Loci**

1.8.5.3.4.5.1.Loci with 2 alleles (A and B)

1.8.5.3.4.5.1.1. If the allele count ratio of the two alleles (A and B) is ≥ 0.70, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.4.5.1.2. If the allele count ratio of the two alleles (A and B) is ≤ 0.20, then a homozygous major profile (A) will be determined.

1.8.5.3.4.5.2.Loci with 1 allele (A)

1.8.5.3.4.5.2.1. If the allele read count is greater than the stochastic threshold, then a homozygous major profile (A) will be determined.

1.8.5.3.5. **Three-Person Mixture Genotype Determinations**

1.8.5.3.5.1. An assessment of the STR loci (autosomal, X, and Y) shall be performed to determine the gender of the contributors to the mixture, if possible.

1.8.5.3.5.2. The allele count ratios of STR loci (autosomal, X, and Y) will be used to determine the major profile accordingly. Typed alleles are designated A-F is descending read counts order. SNP loci in a three-person mixture sample shall not be interpreted or utilized for phenotype or ancestry estimations.

1.8.5.3.5.3. Allele count ratios shall be calculated by dividing the read counts of an allele (B) by the read counts of another allele (A).

1.8.5.3.5.4. **STR Loci**

1.8.5.3.5.4.1. Loci with 5-6 alleles

1.8.5.3.5.4.1.1. If the allele count ratio of the two highest alleles (A and B) is ≥0.60 and the allele count ratio of the lowest high allele (B) and the highest low allele (C) is ≤0.30, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.5.4.1.2. If the allele count ratio of the two highest alleles (A and B) is ≤0.30, then a homozygous major profile (A) will be determined.

1.8.5.3.5.4.2. All remaining loci

1.8.5.3.5.4.2.1. If the allele count ratio of the two highest alleles (A and B) is ≥0.60 and the allele count ratio of the lowest high allele (B) and the highest low allele (C) is ≤0.30, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.5.4.2.2. If the allele count ratio of the two highest alleles (A and B) is ≤0.20, then a homozygous major profile (A) will be determined.

1.8.5.3.5.4.2.3. If only 2 alleles are typed and the allele count ratio is ≥0.60, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.5.4.3. If only 1 allele is typed and the allele is greater than the stochastic threshold, then a homozygous major profile (A) will be determined.

1.8.5.4. **Exporting Sample Details**

1.8.5.4.1. After genotype determinations have been completed, a Sample Report shall be generated from the UAS and exported for use in SeqLite.

1.8.5.4.2. For each sample, in the **Actions** drop-down menu, select and generate appropriate reports.

1.8.5.4.3. Additionally, a Project Report shall be generated to document the performance of positive and negative controls associated with the sample.

1.8.5.4.4. A Phenotype & Ancestry Report should be generated for missing persons and unidentified human remains cases.

1.8.5.4.5. The Sample Reports and Project Reports shall be saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

1.8.5.5. **SeqLite**

1.8.5.5.1. Sample Report shall be imported into the SeqLite tool, an Excel program used to apply stochastic threshold, document final allele calls for a sample, document a major profile for mixtures, and generate additional interpretation files.

1.8.5.5.2. The appropriate concentration-based stochastic threshold shall be selected within SeqLite.

1.8.5.5.3. For CODIS eligible samples, a CMF file may be created and saved in the appropriate CMF server file for upload.

1.8.5.5.4. Additionally, a.txt file can be created to be used for statistical calculations within DBLR. The .txt file shall be saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

1.9. Records

1.9.1. The appropriate worksheets as contained in the Sequencing Workbook or the equivalent working document shall be used to record all procedures.

1.9.2. All data sheets, notes, and other information generated from the laboratory examination shall be retained in the case record.

1.9.3. The batch technical review of a DNA Signature Plus project/run shall be recorded on the appropriate batch review worksheet. The technical review of all data for the DNA Signature Plus analysis shall be recorded on the appropriate technical review worksheet.

1.9.4. Sequencing run data shall be exported using the data management tool within the Universal Analysis Software. The export file shall include all projects, samples and reports generated from the run and shall be retained in the appropriate batch folder on the network drive. All files stored on the server shall be routinely backed up to ensure proper security of data.

1.9.4.1. Do not edit the compressed export file generated by the Universal Analysis Software.

1.9.5. Any case data not retained in the batch folder shall be retained in the case record and/or in a case folder on the network drive. All files stored on the server shall be routinely backed up to ensure proper security of data.

1.9.6. Sequencing run data may be purged from the Universal Analysis Software as necessary to maintain database space.

1.9.7. DNA Signature Plus Batch data electronic records shall be retained in the “zSigPlus” folder on the IRL DNA Data Drive and not duplicated per individual case.

1.10. Interpretations of Results

1.10.1. See Section 1.8.5 for DNA Signature Plus interpretation guidelines and Forensic Biology Casework Test Methods Section 3 for Relationship Comparison guidelines.

1.10.2. If it has been determined that a sample is unsuitable for interpretation, an explanation as to why the sample is uninterpretable should be given. Examples include:

1.10.2.1. No results: Insufficient read counts were detected.

1.10.2.2. Inconclusive: Read counts were detected; however, due to limited data, poor quality data, or an indistinguishable mixture, no conclusions were drawn.

1.10.3. Test results from an unknown sample that are suitable for comparison to a reference sample may have the following conclusions reported:

1.10.3.1. Included: The DBLR™ calculated Likelihood Ratio favors inclusion.

1.10.3.2. Excluded: The profile obtained from the unknown had discrepant genotypes as compared to the profile of the reference sample (i.e., was not the same) and can be visually excluded or the DBLR™ calculated Likelihood Ratio favors exclusion.

1.10.3.3. Uninformative: The DBLR™ calculated Likelihood Ratio falls in the uninformative range.

1.10.4. If conclusive results are obtained from a sample, appropriate CODIS eligible profiles shall be entered into CODIS for searching. The analyst and technical reviewer shall evaluate CODIS eligibility before submitting profiles for entry.

1.11. Report Writing for DNA Signature Plus Analysis

1.11.1. Rules for Forensic Relationship Comparisons are listed in Forensic Biology Casework Test Methods Section 3.

1.11.1.1. Possible mutations at X-STR and Y-STR loci shall be considered. The analyst shall consult with the Technical Leader to determine an appropriate course of action.

1.11.2. See the Forensic Biology Casework Test Methods Appendix 7 for formatting, general rules, specific reporting examples, and the Relationship Comparison Report Appendix.

1.11.3. See Appendix 2 for sequencing specific report wording.

1.12. Direct Comparisons Statistics using DBLR™

1.12.1. Statistics shall only be performed with single source samples or major profiles determined from mixtures.

1.12.2. Y-STR, X-STR, aiSNP and piSNP loci shall only be used for interpretation purposes and not for statistical evaluations.

1.12.2.1. Direct comparison statistics shall be calculated using the Kinship module of DBLR™.

1.12.2.1.1. In the H1 screen, construct the pedigree by leaving a single individual.

1.12.2.1.1.1. The symbol used is not required to reflect the sex of the corresponding sample.

1.12.2.1.2. Add H2 by clicking the “**+**” symbol next to H1.

1.12.2.1.2.1. Add the unknown, unrelated individual by choosing either the male or female symbol from the toolbar, as appropriate.

1.12.2.2. Choose **Numeric Kinship** in the bottom, right corner.

1.12.2.3. Add the profiles to the box marked **Load Samples**. This may be done by dragging and dropping the appropriate files, or by selecting **Browse** and navigating the file tree.

1.12.2.3.1. DBLR™ does not have an option to omit a locus; therefore, loci indicating potential drop-out or in which a major profile could not be determined shall be omitted using the SeqLite tool.

1.12.2.4. Under **Configuration**, add the appropriate Analysis Name. At a minimum, the name shall include the case number, followed by an underscore and the DBLR run number, increasing sequentially (e.g., 23L1234_DBLR1, 23L1234_DBLR2). If additional characters are added to the "Analysis Name" designation, those characters shall be added after the specified naming convention.

1.12.2.5. Under **Parameters**, choose the appropriate Allele Frequencies file. Verify that "Model Fst" is checked and set to 0.01. Linkage can be left on as a default as it will not be applied by the program for this comparison.

1.12.2.5.1. Direct comparisons shall be performed with the Combined NIST 2017 population data.

1.12.2.6. Select **Confirm Inputs** once all profiles are loaded and settings are verified.

1.12.2.7. Link the unknown and reference both to the node in H1. In H2, link the unknown to one node and the reference to the second node. This may be accomplished by dragging the sample name to the appropriate position in the pedigree, or by clicking **Add Relationship** and selecting the person in the pedigree and its corresponding sample from the drop-down menus.

1.12.2.7.1. Verify that the correct Contributor Position is selected if a STRmix deconvolution is used as a sample input.

1.12.2.7.2. When F_{ST} is enabled, a warning message will show that there are unexplained contributor positions until all loaded samples are linked to the pedigree. This must be resolved before a likelihood ratio can be calculated.

1.12.2.8. Click **Compute Likelihood Ratios** and review the data.

1.12.2.9. Save a copy of the Kinship Reports in the casefile and the run folder on the DNA server (See Forensic Biology Casework Test Methods Appendix 9 for naming requirements).

1.12.2.9.1. The report will list linked loci together whenever Linkage is enabled. However, the model is only applied when needed based on the pedigree and sample profiles.

1.12.2.10. If the analyst cannot clearly exclude an individual, DBLR™ may be used to assist in the comparison. If DBLR™ is used to determine an exclusion, the appropriate exclusion statement shall be included in the report for that individual.

1.12.2.10.1. LR values less than 1 favor the defense proposition (H2). LR values less than 1 will be evaluated for the verbal equivalent as 1/LR.

1.12.2.10.1.1. 1/LR values between 2 and 99 will be reported as limited support for exclusion and will include the hypotheses and calculated 1/LR value in the report as stated in the report wording guidelines.

1.12.2.10.1.2.1/LR values above 99 shall use the general exclusion statement as stated in the report wording guidelines.

1.12.2.10.1.3.An LR value of zero indicates no scientific support for the prosecution proposition (H1) and shall use the general exclusion statement as stated in the report wording guidelines.

1.12.2.11. DBLR™ LR values shall be rounded to two significant figures for reporting.

1.12.2.11.1. When the LR is in the support of H1, it shall be rounded down to two significant figures.

1.12.2.11.1.1. When the LR in support of H1 is less than 2, it shall be rounded down to 1.0 (uninformative).

1.12.2.11.2. When the LR is in support of H2, the 1/LR value shall be rounded up to two significant figures.

1.12.2.11.2.1. If the 1/LR is less than 2, it shall be rounded up to 2.0 (limited support for exclusion).

1.13. Investigative Information on Phenotype and Ancestry Estimations Using DNA Signature Plus

1.13.1. At the analyst's discretion, the piSNPs and aiSNPs may be utilized for phenotype and ancestry estimations in order to provide investigative information for missing person and unidentified remains cases.

1.13.2. A full piSNP profile shall be developed for phenotype estimations to be performed. At least 27 complete aiSNP loci are required in order to utilize an ancestry estimation.

1.13.3. From the Results or Samples tabs, click the **Actions** drop-down button and select **Phenotype & Ancestry Estimation Report**.

1.13.4. Hair and eye color phenotype percentages and the ancestry result as generated from the estimation shall be issued to the investigating agency via a memo as shown in Appendix 2.

1.13.5. The Phenotype & Ancestry Report shall be exported and saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

1.14. References:

1.14.1. Verogen, Inc. *MiSeq FGx® Sequencing System Reference Guide*. Document# VD2018006, Rev. F (or current version). San Diego, CA, 2021. https://verogen.com/resources/product-documentation/?_products=miseq-fgx-system

1.14.2. *Verogen MiSeq FGx® and ForenSeq™ DNA Signature Prep Kit Primer Set B Validation for Missing Person and Unidentified Human Remains Applications*. Indiana State Police Forensic Services Division, 2023. Indianapolis, IN.

1.14.3. QIAGEN. *ForenSeq® DNA Signature Plus Kit Handbook*. Document No. HB-3613-001, October 2024. https://www.qiagen.com/us/resources/kithandbookandprotocol/hb-3613-001-hb-forenseq-dna-sig-plus-1024-ww

1.14.4. QIAGEN. *Universal Analysis Software for ForenSeq® Signature Plus Module User Guide*. Document No. HB-3616-001, October 2024. https://www.qiagen.com/us/resources/usermanual/hb-3616-001-ug-uas-dna-sigplus-1024-ww

1.14.5. *Verogen ForenSeq® DNA Signature Plus Kit Primer Set B with VeritiPro™ Validation (Including PowerQuant®)*. Indiana State Police Forensic Services Division, 2026. Indianapolis, IN.

2. ForenSeq® Kintelligence Kit Sequencing Test Methods:

2.1. Scope: Sequencing utilizing the Kintelligence Kit shall only be performed for unsolved, violent, felony crimes against persons or unidentified human remains cases. DNA analysis performed prior to sequencing shall be in accordance with the test methods outlined in the DNA Methods section of the Forensic Biology Casework Test Methods document.

2.2. Precautions/Limitations: Samples that may be suitable for sequencing should be evaluated by a member of the sequencing team to determine the most appropriate sample(s) to analyze in a case. The following should be considered:

2.2.1. Unsolved, violent, felony cases with profiles uploaded to CODIS may be considered for analysis.

2.2.2. Cases with critical public safety implications may be considered for analysis.

2.2.3. Unidentified human remains cases may be considered for analysis.

2.2.4. Due to the limitations of the technology, items which demonstrate mixed DNA profiles with autosomal STR analysis shall not be considered for sequencing. Exceptions require Technical Leader approval.

2.2.5. For optimal results, samples from various cases/analysts may be pooled to achieve desired flow cell load.

2.3. Related Information:

2.3.1. Worksheet Manual

2.4. Instruments:

2.4.1. Verogen MiSeq FGx® Sequencing System – Sequencing platform that delivers SNP results from a prepared library while also providing interpretation functions using the fully integrated Universal Software Analysis (UAS) feature.

2.4.2. Quantus™ Fluorometer – Fluorescence based quantification system used for the detection of dsDNA.

2.5. Reagents/Materials:

2.5.1. Ethanol

2.5.2. Amp Grade Water

2.5.3. ForenSeq® Kintelligence Kit

2.5.4. Promega® QuantiFluor® ONE Kit

2.5.5. MiSeq FGx® Reagent Kit

2.6. Hazards/Safety:

2.6.1. The cartridge of the MiSeq FGx® Reagent Kit contains formamide in reservoir 8. After a sequencing run using this cartridge, the contents of reservoir 8 shall be removed using universal precautions inside a fume hood and properly disposed.

2.6.2. In addition, the waste bottle within the MiSeq instrument contains potentially hazardous chemicals after a sequencing run. The contents shall be disposed of in the appropriate waste receptacle using universal precautions.

2.7. Reference Materials/Controls/Calibration Checks:

2.7.1. An assessment of the run quality metrics shall serve as a check of the accuracy and specificity of test results in addition to the testing of controls.

2.8. Procedures/Instructions:

2.8.1. **See Forensic Biology Casework Test Methods, DNA Test Methods Section 2.8 for extraction and quantification procedures**

2.8.1.1. Autosomal STR analysis shall be performed on the sample prior to sequencing with the Kintelligence kit.

2.8.1.2. Sequencing analysis may be performed on extracts previously used for autosomal STR testing or may require re-extraction of a sample.

2.8.1.3. Quantification results from previous autosomal STR testing may be used for amplification of the sample.

2.8.2. **ForenSeq® Kintelligence**

2.8.2.1. **Introduction**

2.8.2.1.1. The ForenSeq® Kintelligence Kit is a single SNP multiplex which targets 10,230 SNP markers. 106 X-SNPs, 85 Y-SNPs, 56 Ancestry SNPs, 24 Phenotype SNPs, 94 Identity SNPs and 9,867 Kinship SNPs are genotyped and analyzed by the Universal Analysis software in the MiSeq instrument.

2.8.2.1.2. The ForenSeq® Kintelligence Kit reagents come in four boxes. Boxes 1 and 4 should be stored at 2°C to 8°C. Boxes 2 and 3 should be stored at -25°C to -15°C.

2.8.2.1.3. The preparation of the Amplify and Tag Targets step (PCR1) shall be performed in the PCR amplification set-up area. All further steps shall be performed in a post-PCR area.

2.8.2.2. **Sample Preparation**

2.8.2.2.1. The ideal target concentration is 1ng, however samples may be amplified in a range from 0.125-1ng. The Normalization Dilution Worksheet in the Sequencing Workbook is recommended for this purpose.

2.8.2.2.2. Care should be taken to minimize the amount of non-DNA containing samples, such as negative controls, or low DNA quantity samples present on each flow cell. Three to four samples should be run on a single standard flow cell.

2.8.2.2.2.1. For flow cells containing three samples, it is recommended there be no more than one negative control.

2.8.2.2.2.2. For flow cells containing four samples, it is recommended that at most two negative controls and at most three samples containing DNA are included. If poor performance as demonstrated by a low read intensity is observed for a sample, it is recommended the prepared library be rerun on a flow cell containing a total of three multiplexed libraries.

2.8.2.2.3. A ForenSeq® Kintelligence Sequencing Record worksheet shall be used to track library prep. It is recommended to use the Sequencing Workbook for this purpose.

2.8.2.2.3.1. Add samples to sequencing record, balancing the sample type in each flow cell column.

2.8.2.2.3.2. Assign UDI adapters to the sample setup, ensuring unique combinations are used. Libraries may be re-run as necessary as long as the adapter assignments don't overlap with those for the new libraries.

2.8.2.3. **Amplify and Tag Targets (PCR1)**

2.8.2.3.1. Thaw kPCR1 and FEM at room temperature. Let NA24385 and KPM stand for 30 minutes to bring to room temperature.

2.8.2.3.2. Label a 96-well semi-skirted PCR plate as KSP (for Kintelligence Sample Plate), a 1.5 mL tube as Master Mix, a 1.5 mL tube as Control DNA Dilution 1, and a 1.5 mL tube as Control DNA Dilution 2. Crosslink the plate and tubes.

2.8.2.3.3. Dilute 1 ng gDNA to 40 pg/µl using nuclease-free water. Gently pipette to mix.

2.8.2.3.4. Determine the number of samples to be amplified including controls. Add approximately 10% extra reagent for overage to compensate for the loss that occurs during reagent transfer. Calculate the required amount of each component of the PCR master mix. Multiply the volume (µl) per sample by the total number of reactions to obtain the final volume (µl).

- **Components of Master Mix/sample:**
  - 18.5 µl kPCR1
  - 5.0 µl KPM
  - 1.5 µl FEM

2.8.2.3.5. Do not vortex the FEM enzyme. Flick tube to mix. Centrifuge briefly.

2.8.2.3.6. Invert NA24385 three times or flick to mix. Centrifuge briefly.

2.8.2.3.7. Vortex kPCR1 and KPM to mix and centrifuge briefly.

2.8.2.3.8. Add the calculated volume of each component to the 1.5 ml Master Mix tube. Pipette to mix and centrifuge briefly.

2.8.2.3.9. Add 25 µl master mix to each well of the KSP plate.

2.8.2.3.10. Dilute 10 ng/µl NA24385 stock as below. Alternatively, the NA24385 may be quantified with Quantifiler® Trio and diluted using the new quantitation values.

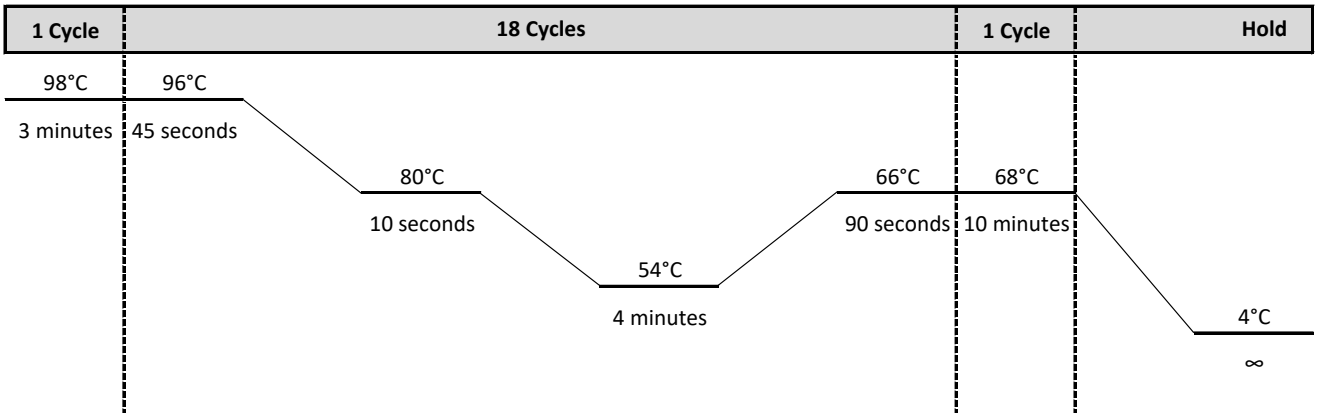
2.8.2.3.10.1. In the Control DNA Dilution 1, combine 2 µl of 10 ng/µl NA24385 with 48 µl of nuclease-free water in order to prepare 50 µl of 400 pg/µl NA24385. Gently pipette to mix and centrifuge briefly. A new dilution shall be made for each run performed.

2.8.2.3.10.2. In the Control DNA Dilution 2, combine 10 µl of 400 pg/µl NA24385 with 90 µl of nuclease-free water in order to prepare 100 µl of 40 pg/µl NA24385. Gently pipette to mix and centrifuge briefly. A new dilution shall be made for each run performed.

2.8.2.3.11. Add 25 µl sample, positive amplification control, or negative amplification control to the appropriate well in the KSP plate. Pipette to mix.

2.8.2.3.12. Seal the plate with a Microseal 'A' film. Centrifuge plate at 1000 x g for 30 seconds.

2.8.2.3.13. Place on the VeritiPro™ thermal cycler. Select the **kPCR1** protocol with the following amplification procedure with a lid temperature of 100°C and a ramp mode of 0.2°C per second. Cycling time is approximately 4.5 hours.



2.8.2.3.14. SAFE STOPPING POINT. Remove samples after the amplification process is completed and proceed immediately or the plate may be stored at 2°C to 8°C for up to two days. The plate may also be left on the thermal cycler overnight.

2.8.2.4. **Purify Targets**

2.8.2.4.1. Let ProK, RSB, and SPB2 stand for 30 minutes to bring to room temperature.

2.8.2.4.2. Label a Midi plate as Purification Bead Plate 1 and a 96-well semi-skirted PCR plate as Purified Targets Plate.

2.8.2.4.3. Prepare the appropriate volume of fresh 80% EtOH from absolute ethanol.

2.8.2.4.3.1. If the Purify Libraries step will be completed the same day 80% EtOH can be prepared for both purification steps at one time.

2.8.2.4.3.1.1. If the Purify Libraries step will be completed that day and one preparation of EtOH is desired, prepare 1.5 ml per sample. Additional sample overage may be added to ensure sufficient volume for pipetting.

2.8.2.4.3.1.2. If 80% EtOH is prepared only for the Purify Targets step, prepare 1.0 ml per sample. Additional sample overage may be added to ensure sufficient volume for pipetting.

2.8.2.4.4. Invert ProK to mix and centrifuge.

2.8.2.4.5. Vortex and invert RSB to mix.

2.8.2.4.6. Prepare the SPB2 tube for use. For first time use, add 7.5 µl ProK to the SPB2 tube. Select checkbox on SPB2 label to indicate addition. For subsequent use, ensure checkbox on SPB2 label is selected.

2.8.2.4.7. Vortex the ProK/SPB2 tube for ≥1 minute and invert several times to mix.

2.8.2.4.8. Immediately dispense 75 µl ProK/SPB2 to each well of the Purification Bead Plate 1.

2.8.2.4.9. Briefly centrifuge KSP.

2.8.2.4.10. Pipette to mix and then transfer 45 µl reaction from each well of the KSP to the corresponding well of the Purification Bead Plate 1.

2.8.2.4.11. Discard the KSP.

2.8.2.4.12. Seal the Purification Bead Plate 1 and shake at 1800 rpm for 2 minutes.

2.8.2.4.13. Incubate at room temperature for 10 minutes.

2.8.2.4.14. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

2.8.2.4.15. Centrifuge at 1000 x g for 30 seconds.

2.8.2.4.16. Place on the magnetic stand and wait until the liquid is clear (~1 min).

2.8.2.4.17. **Critical step!** Remove and discard **all** supernatant.

2.8.2.4.18. Keep on the magnetic stand and wash as follows:

2.8.2.4.18.1. Add 200 µl fresh 80% EtOH to each well. Avoid disturbing the bead pellet.

2.8.2.4.18.2. Incubate for 30 seconds.

2.8.2.4.18.3. Remove and discard all supernatant.

2.8.2.4.19. Wash a **second** time.

2.8.2.4.20. Seal and centrifuge at 1000 x g for 30 seconds.

2.8.2.4.21. Place on the magnetic stand and wait until the liquid is clear (~1 minute).

2.8.2.4.22. **Critical step!** Remove **all** residual EtOH from each well.

2.8.2.4.23. Remove from the magnetic stand.

2.8.2.4.24. Add 30 µl RSB to the bottom of each sample well.

2.8.2.4.25. Seal and shake at 1800 rpm for 2 minutes.

2.8.2.4.26. **Critical step!** If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.

2.8.2.4.27. Incubate at room temperature for 2 minutes.

2.8.2.4.28. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

2.8.2.4.29. Transfer 28 uL supernatant from each well of the Purification Bead Plate 1 to a fresh well in the **same plate**. Some bead carryover is normal.

2.8.2.4.30. Add 45 µl ProK/SPB2 to each sample well.

2.8.2.4.31. Seal and shake at 1800 rpm for 2 minutes.

2.8.2.4.32. Incubate at room temperature for 5 minutes.

2.8.2.4.33. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

2.8.2.4.34. Centrifuge at 1000 x g for 30 seconds.

2.8.2.4.35. Place on the magnetic stand and wait until the liquid is clear (~1 minute).

2.8.2.4.36. **Critical step!** Remove and discard **all** supernatant.

2.8.2.4.37. Keep on the magnetic stand and wash as follows:

2.8.2.4.37.1. Add 200 µl fresh 80% EtOH to each well. Avoid disturbing the bead pellet.

2.8.2.4.37.2. Incubate for 30 seconds.

2.8.2.4.37.3. Remove and discard all supernatant.

2.8.2.4.38. Wash a **second** time.

2.8.2.4.39. Seal and centrifuge at 1000 x g for 30 seconds.

2.8.2.4.40. Place on the magnetic stand and wait until the liquid is clear (~1 minute).

2.8.2.4.41. **Critical step!** Remove **all** residual EtOH from each well.

2.8.2.4.42. Remove from the magnetic stand.

2.8.2.4.43. Add 27 µl RSB to the bottom of each well.

2.8.2.4.44. Seal and shake at 1800 rpm for 2 minutes.

2.8.2.4.45. **Critical step!** If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.

2.8.2.4.46. Incubate at room temperature for 2 minutes.

2.8.2.4.47. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

2.8.2.4.48. Transfer 25 µl supernatant from each well of the Purification Bead Plate 1 to the corresponding well of the Purified Targets Plate.

2.8.2.4.49. SAFE STOPPING POINT. Proceed immediately to the next step or the plate may be sealed and stored at -25°C to -15°C overnight.

2.8.2.5. **Enrich Targets (PCR2)**

2.8.2.5.1. Let kPCR2 and the appropriate UDI adapters stand for 30 minutes to bring to room temperature.

2.8.2.5.2. Vortex kPCR2 and the UDI adapters to mix and centrifuge briefly.

2.8.2.5.3. Seal and centrifuge the Purified Targets Plate at 1000 x g for 30 seconds.

2.8.2.5.4. Add 5 µl UDI adapter to each sample well. The total volume of the well should be 30 µl.

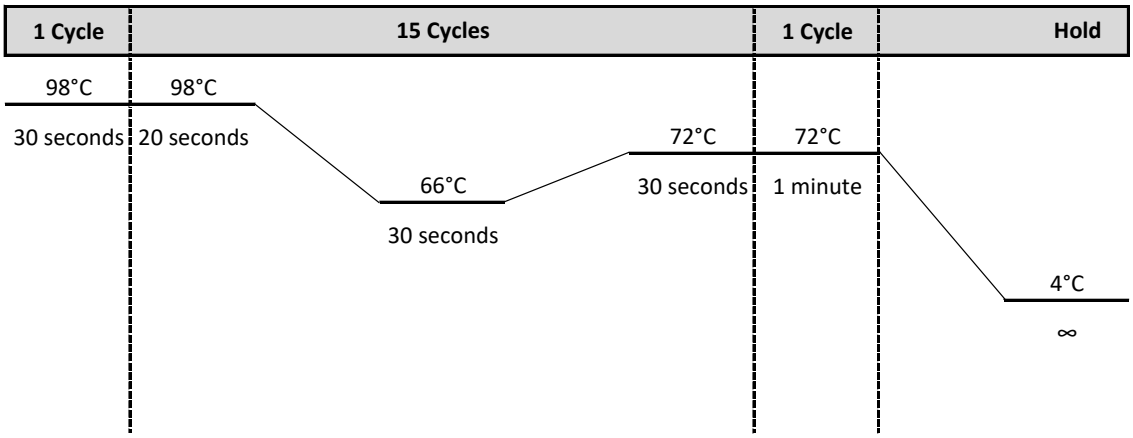
2.8.2.5.5. Briefly centrifuge kPCR2 and then pipette to mix.

2.8.2.5.6. Add 20 µl kPCR2 to each well, avoiding excess carry-over of viscous kPCR2 to the sample wells.

2.8.2.5.7. Pipette to mix in each well.

2.8.2.5.8. Seal and centrifuge at 1000 x g for 30 seconds.

2.8.2.5.9. Place on the VeritiPro™ thermal cycler. Select the **kPCR2** protocol with the following amplification procedure with a lid temperature of 100°C. Cycling time is approximately 30 minutes.



2.8.2.5.10. SAFE STOPPING POINT. Remove samples after the amplification process is completed and proceed immediately or the plate may be stored at 2°C to 8°C for up to seven days. The plate may also be left on the thermal cycler overnight.

2.8.2.6. **Purify Libraries**

2.8.2.6.1. If beginning a sequencing run that day, begin thawing the MiSeq FGx® Reagent Kit (Box 1). See 2.8.3.2.1.

2.8.2.6.2. Let ProK, RSB, and SPB2 stand for 30 minutes to bring to room temperature.

2.8.2.6.3. Label a Midi plate as Purification Bead Plate 2 and a 96-well PCR plate as Purified Library Plate.

2.8.2.6.4. If 80% EtOH was not prepared within the day, prepare 0.5 mL fresh 80% EtOH per sample. Additional sample overage may be added to ensure sufficient volume for pipetting.

2.8.2.6.5. Invert ProK to mix and centrifuge.

2.8.2.6.6. Vortex and invert RSB to mix.

2.8.2.6.7. Prepare the SPB2 tube for use. For first time use, add 7.5 µl ProK to the SPB2 tube. Select checkbox on SPB2 label to indicate addition. For subsequent use, ensure checkbox on SPB2 label is selected.

2.8.2.6.8. Vortex the ProK/SPB2 tube for ≥1 minute and invert several times to mix.

2.8.2.6.9. Briefly centrifuge Purified Targets Plate.

2.8.2.6.10. Immediately dispense 45 µl ProK/SPB2 to each well of the Purification Bead Plate 2.

2.8.2.6.11. Transfer 45 µl reaction from each well of the Purified Targets Plate to the corresponding well of the Purification Bead Plate 2.

2.8.2.6.12. Seal the Purification Bead Plate 2 and shake at 1800 rpm for 2 minutes.

2.8.2.6.13. Incubate at room temperature for 5 minutes.

2.8.2.6.14. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

2.8.2.6.15. **Critical step!** Remove and discard **all** supernatant.

2.8.2.6.16. Keep on the magnetic stand and wash as follows:

2.8.2.6.16.1. Add 200 µl fresh 80% EtOH to each well. Avoid disturbing the bead pellet.

2.8.2.6.16.2. Incubate for 30 seconds.

2.8.2.6.16.3. Remove and discard all supernatant.

2.8.2.6.17. Wash a **second** time.

2.8.2.6.18. Seal and centrifuge at 1000 x g for 30 seconds.

2.8.2.6.19. Place on the magnetic stand and wait until the liquid is clear (~1 minute).

2.8.2.6.20. **Critical step!** Remove **all** residual EtOH from each well.

2.8.2.6.21. Remove from the magnetic stand.

2.8.2.6.22. Add 52.5 µl RSB to the bottom of each sample well.

2.8.2.6.23. Seal and shake at 1800 rpm for 2 minutes.

2.8.2.6.24. **Critical step!** If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.

2.8.2.6.25. Incubate at room temperature for 2 minutes.

2.8.2.6.26. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

2.8.2.6.27. Transfer 50 µl supernatant from each well of the Purification Bead Plate 2 to the corresponding well of the Purified Library Plate.

2.8.2.6.28. Seal and centrifuge at 1000 x g for 30 seconds.

2.8.2.6.29. SAFE STOPPING POINT. Proceed immediately to the next step or seal the plate and store at -25°C to -15°C. The plate shall be sealed and stored at -25°C to -15°C for up to 1 year.

2.8.2.6.30. If sequencing results indicate the potential excess primer dimers, the Purify Libraries step may be repeated to attempt to optimize results.

2.8.2.7. **Normalize Libraries**

2.8.2.7.1. Let RSB stand for 30 minutes to bring to room temperature.

2.8.2.7.2. Label either a 1.5 ml tube or a 96-well PCR plate as Normalized Library.

2.8.2.7.3. Vortex RSB and invert to mix.

2.8.2.7.4. Place the Purified Library Plate on the magnetic stand.

2.8.2.7.5. Quantify the libraries using the Promega® QuantiFluor® ONE fluorometric method.

2.8.2.7.5.1. Warm QuantiFluor® ONE dsDNA Dye and QuantiFluor® ONE Lambda DNA standard (400 µg/ml) to room temperature before use. Protect tubes from light throughout process.

2.8.2.7.5.2. The Quantus™ Fluorometer shall be calibrated each day quantification is performed using a blank sample and a 400 ng standard sample.

2.8.2.7.5.3. Prepare the blank sample by adding 200 µl QuantiFluor® ONE dsDNA Dye to an empty 0.5 ml PCR tube.

2.8.2.7.5.4. Prepare the 400 ng standard sample by adding 2 µl QuantiFluor® ONE Lambda DNA standard (400 µg/ml) to 400 µl QuantiFluor® ONE dsDNA Dye in an empty 0.5 ml PCR tube. Vortex well.

2.8.2.7.5.5. Prepare unknown samples by adding 2 µl unknown samples to 200 µl QuantiFluor® ONE dsDNA Dye to empty 0.5 ml PCR tubes. Vortex well or pipette to mix. Take care not to introduce bubbles.

2.8.2.7.5.6. Incubate samples for 5 minutes at room temperature.

2.8.2.7.5.7. Select the ONE DNA protocol on the Quantus™ Fluorometer.

2.8.2.7.5.8. If needed, calibrate the Quantus™ Fluorometer by reading the blank and standard. Save calibration.

2.8.2.7.5.9. Enter the volume of the unknown samples added for quantitation (2 µl) and desired concentration units.

2.8.2.7.5.10. Measure fluorescence. The number displayed represents the concentration of the original sample.

2.8.2.7.6. If the concentration of the library is > 0.75 ng/µl, prepare RSB to dilute each library to 0.75 ng/µl as follows:

2.8.2.7.6.1. Use the formula $C_1V1/C_2=V_2$ to calculate the value for V_2, where:

- C_1 is the library quantification result
- V_1 is 8 µl undiluted library
- C_2 is 0.75 ng/µl
- V_2 is the final volume of diluted library

2.8.2.7.6.2. Calculate the requisite volume of RSB (V_2 – 8 µl).

2.8.2.7.6.3. Add the calculated volume of RSB to the corresponding well of the Normalized Library Plate or tube. Use the tube when the library is ≥15 ng/µl.

2.8.2.7.6.4. Libraries ≤ 0.75 ng/µl are used at the existing concentration and do not require diluting.

2.8.2.7.6.5. Negative controls > 0.75 ng/µl shall also be normalized as stated above due to the potential existence of excessive primer dimers and their effect on flow cell sequencing with multiplexed samples.

2.8.2.7.7. Transfer 8 µl of each purified library from the Purified Library Plate to the corresponding well of the Normalized Library Plate or tube. This results in a Normalized Library Plate or tube containing 0.75 ng/µl libraries.

2.8.2.7.8. SAFE STOPPING POINT. Proceed immediately to the next step, or seal the plate or cap the tube and store at -25°C to -15°C. The Normalized Library Plate or tube shall be sealed or capped and stored at -25°C to -15°C for up to 30 days.

2.8.2.8. **Pool Libraries**

2.8.2.8.1. Select 3-4 libraries to pool for sequencing.

2.8.2.8.2. Label a 1.5 ml tube Pooled Libraries.

2.8.2.8.3. Transfer 5 µl of each library from the Normalized Library Plate or tube to the Pooled Libraries tube.

2.8.2.8.4. Cap and vortex the Pooled Libraries tube. Centrifuge briefly.

2.8.2.8.5. SAFE STOPPING POINT. The procedure may be continued immediately. The Pooled Libraries tube shall be stored at -25°C to -15°C for up to 6 months.

2.8.2.9. **Dilute and Denature Libraries**

2.8.2.9.1. Thaw HP3 and HT1 at room temperature.

2.8.2.9.2. Let HSC stand for 30 minutes to bring to room temperature.

2.8.2.9.3. Label four 1.5 mL tubes as 12 pM Denatured Library, 20 pM Denatured Library, Denatured HSC, and 0.2 N NaOH.

2.8.2.9.4. Vortex HP3 and centrifuge briefly.

2.8.2.9.5. Vortex HT1 to mix.

2.8.2.9.6. Invert HSC to mix and centrifuge.

2.8.2.9.7. In the 0.2 N NaOH tube, combine 90 µl nuclease-free water with 10 µl HP3 to prepare 0.1 ml 0.2 N NaOH.

2.8.2.9.8. Invert the tube several times to mix. Use within **12 hours**. Freshly diluted NaOH is essential for denaturation.

2.8.2.9.9. In the Denatured HSC tube, combine 2 µl HSC with 2 µl 0.2 N NaOH to prepare denatured HSC. Pipette to mix, cap, and centrifuge briefly. Do NOT vortex HSC with 0.2 N NaOH.

2.8.2.9.10. Incubate at room temperature for 5 minutes.

2.8.2.9.11. Add 36 µl HT1 to the Denatured HSC tube. Pipette to mix. Denatured HSC can be stored at room temperature for up to 1 day.

2.8.2.9.12. In the 20 pM Denatured Library tube, combine 5 µl 0.75 ng/µl library pool with 5 µl 0.2 N NaOH. Pipette to mix, cap, and centrifuge briefly.

2.8.2.9.13. Incubate at room temperature for 5 minutes.

2.8.2.9.14. Add 990 µl HT1 to the 20 pM Denatured Library tube to prepare 1 ml 20 pM denatured library. The 20 pM denatured library shall be stored at -25°C to -15°C for up to 3 weeks.

2.8.2.9.15. In the 12 pM Denatured Library tube, combine 360 µl 20 pM library pool, 238 µl HT1, and 2 µl denatured HSC in order to dilute the 20 pM library to 12 pM. Pipette to mix, cap, and centrifuge briefly.

2.8.2.9.16. Immediately transfer the entire volume of the 12 pM Denatured Library tube to the reagent cartridge.

2.8.3. **Verogen MiSeq FGx® Sequencing System**

2.8.3.1. **Introduction**

2.8.3.1.1. The Verogen MiSeq FGx® Sequencing System is a sequencing instrument that performs sequencing by synthesis, a type of targeted DNA sequencing. The system uses instrument-specific reagents and flow cells to measure fluorescence

signals of labeled nucleotides. The system also includes internal imaging hardware and data analysis software.

2.8.3.1.2. The data produced by the Verogen MiSeq FGx® Sequencing System is analyzed with an internal software that performs base calling, demultiplexes, aligns data to targeted regions and reports SNP calls. Analyst data analysis occurs in Universal Analysis Software (UAS).

2.8.3.1.3. The MiSeq FGx® Reagent Kit materials come in two boxes. Box 1 should be stored at -25°C to -15°C. Box 2 should be stored at 2°C to 8°C.

2.8.3.2. **Reagent Cartridge Preparation**

2.8.3.2.1. Thaw the MiSeq FGx® Reagent Kit (Box 1). The MiSeq FGx® Reagent Kit (Box 1) contains the reagent cartridge and the hybridization buffer (HT1). Thaw the HT1 at room temperature. Thaw the reagent cartridge in a room temperature water bath for approximately 60-90 minutes. Do not submerge the cartridge above the maximum water line. Alternatively, thaw the reagent cartridge overnight in the refrigerator.

2.8.3.2.1.1. The thawed reagent cartridge may be stored on ice for up to 6 hours if necessary. The MiSeq FGx® Reagent Kit may not be thawed and refrozen.

2.8.3.2.2. Once thawed, invert the cartridge at least ten times to mix. Ensure all reagent reservoirs are completely thawed and there are no precipitates. Remove bubbles and excess water by firmly tapping the cartridge on the benchtop. Dry the bottom of the cartridge with a lab wipe.

2.8.3.2.3. In a fume hood, pierce the foil of the highlighted sample position on the reagent cartridge, using a clean pipette tip.

2.8.3.2.4. Load the entire volume of the 12 pM Denatured Library tube into the highlighted position on the reagent cartridge, taking care to avoid bubbles.

2.8.3.2.5. Tap the cartridge to ensure contents move to the bottom of the well.

2.8.3.3. **Create a Project and Run**

2.8.3.3.1. Each amplification batch shall have an associated project. The project shall contain one to four associated sequencing runs, each on a flow cell.

2.8.3.3.2. Log in to the ForenSeq® Universal Analysis Software.

2.8.3.3.3. Create project.

2.8.3.3.3.1. Select **Create Project** on homepage.

2.8.3.3.3.2. Enter project name. The project name shall be the batch number and the date the first run is started (ex. K2025-001_18Mar25).

2.8.3.3.3.3. Save project.

2.8.3.3.4. Create run.

2.8.3.3.4.1. Select **Create Run** on homepage.

2.8.3.3.4.2. Enter run name. The run name shall include the batch number of the project, flow cell number, and date of flow cell run (ex. K2025-001_FC1_18Mar25).

2.8.3.3.4.3. Choose **Standard** flow cell type.

2.8.3.3.4.4. Save run.

2.8.3.3.4.5. In upper right corner, select **Samples**.

2.8.3.3.4.6. Select Add **Sample**.

2.8.3.3.4.7. Add Sample Name (ex. case number and item).

2.8.3.3.4.8. Select ForenSeq Kintelligence for Assay Type.

2.8.3.3.4.9. Select the appropriate Sample Type.

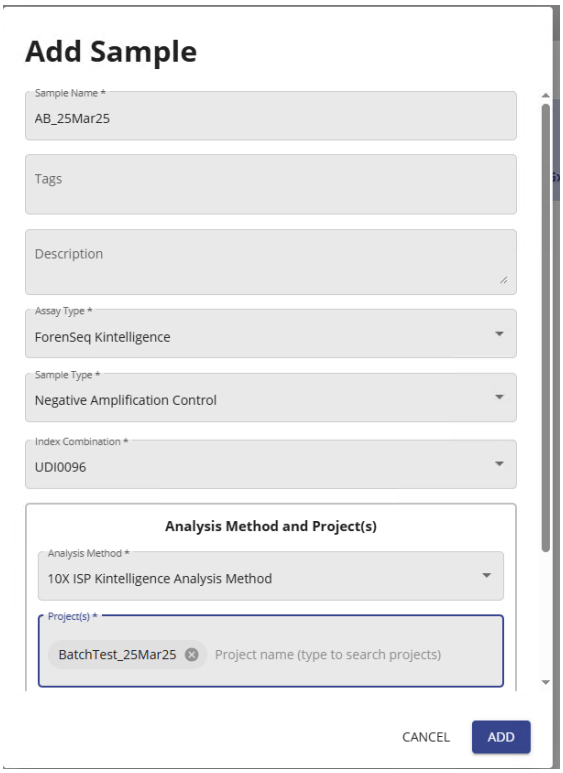
2.8.3.3.4.10. Select the appropriate Index Combination.

2.8.3.3.4.11. Type in associated project.

2.8.3.3.4.12. Select 10X ISP Kintelligence Analysis Method.

2.8.3.3.4.13. Select **Add**.

2.8.3.3.4.14. Repeat for all samples.



2.8.3.4. **Start the Sequencing Run**

2.8.3.5.1 On the Verogen MiSeq FGx®, ensure server 2.0+ is selected and log in with a valid username and password.

2.8.3.5.2 Select **Sequencing.**

2.8.3.5.3 Select the run previously created in the Universal Analysis Software.

2.8.3.5.4 Remove the flow cell and the PR2 from the MiSeq FGx® Reagent Kit Box 2 (store at 2°C to 8°C.)

2.8.3.5.5 Load the flow cell, PR2, waste bottle and previously prepared reagent cartridge as prompted by the software.

2.8.3.5.5.1 The flow cell must be thoroughly rinsed with water to remove excess salts. Dry the flow cell, taking care not to damage the gasket.

2.8.3.5.5.2 Use an alcohol wipe or a lab wipe moistened with ethanol to clean the flow cell glass. Do not use alcohol on the flow cell gasket. Ensure flow cell glass is clean and dry and that the gasket is properly seated before loading.

2.8.3.5.6 The instrument will perform a pre-run check, verifying all the RFID tags for the consumables. When complete, select **Start Run**.

2.8.3.5.7 If an RFID tag cannot be read, it can be entered manually using the keyboard. If necessary, contact Qiagen technical support for assistance.

2.8.3.5 **Evaluate the Sequencing Run**

2.8.3.5.1 When the run is complete, select **Next**. Ensure the sequencing run completed successfully. The sequencing run status may be viewed on the sequencer in real time or evaluated in the Universal Analysis Software after the run completes. Run Quality Metrics should be evaluated as described in 2.8.4.3.

2.8.4 ForenSeq® Universal Analysis Software v2.0+ Data Analysis

2.8.4.1. **ForenSeq® Universal Analysis Software v2.0+ Settings are detailed in Appendix 4.**

2.8.4.2. **Introduction**

2.8.4.2.1. Run information generated by the MiSeq FGx® Sequencing System is stored on the ForenSeq server. Upon run completion, files are transferred to the Universal Analysis Software (UAS) which initiates the analysis of data using the default analysis settings.

2.8.4.2.2. UAS performs analysis processes including demultiplexing, sequence alignment, allele counting, genotype calling, noise filtering, and quality assessment.

2.8.4.2.3. Analytical thresholds (AT) and interpretation thresholds (IT) are percent-based values of the total read count at each locus.

2.8.4.2.3.1. AT and IT are both set to 1.5% in the default analysis method, “10X ISP Kintelligence Analysis Method”.

2.8.4.2.3.2. AT and IT are both set to 3.0% in the optional analysis method, “20X ISP Kintelligence Analysis Method”. This method may be utilized for high template or high read count samples.

2.8.4.3. **Evaluating Run Quality Metrics and Controls**

2.8.4.3.1. To open the analysis of a project (plate), select the project name.

2.8.4.3.2. For each run, select the **QPN**.

2.8.4.3.3. Quality Indicator Results for Controls and Quality Metrics

2.8.4.3.4. Controls or quality metrics highlighted in green pass recommended parameters for samples. If highlighted in orange, further evaluation or interpretation of the sample is required.

Color Coded Indicator	Positive Control	Human Sequencing Control	Negative Control	Quality Metrics
Green	All loci have calls and concordant with NA24385 DNA Control.	The control meets or exceeds the UAS criteria for intensity and concordance.	All designated reagent blanks or negative controls have no typed called alleles.	All metrics are in recommended ranges.
Orange	At least 1 locus is not called or has a disconcordant call to the NA24385 DNA Control.	The control does not meet at least one UAS criteria for intensity and/or concordance.	At least one locus has demostrated an allele call.	At least 1 metric is not within recommended ranges.

2.8.4.3.5. On the Sample Quality tab, ensure that Quality Metrics meet the following guidelines under the Run Metrics tab.

Run Quality Metric	Recommended Ranges
Cluster Density	400 - 1650 k/mm^2
Cluster Passing Filter	≥80%
Phasing	≤0.25%
Pre-phasing	≤0.15%

2.8.4.3.6. Verify the **Read and Index Quality Metrics** all indicate a green check mark. If run quality metrics are flagged as orange, the Technical Leader shall be consulted.

2.8.4.3.7. The Human Sequencing Control is an internal sequencing control that presents either pass or fail results. The actual sequencing result is not viewable in the UAS; however, any discordant results will be listed. Consult with the Technical Leader if a failed result is obtained.

2.8.4.3.8. Evaluate the **Sample Representation** values to further assess the quality of the run and samples.

2.8.4.3.8.1. Samples demonstrating less than 10,000,000 read counts are considered to have low coverage and should be interpreted with caution.

2.8.4.3.9. On the **Positive/Negative Controls** tab

2.8.4.3.9.1. The **Positive Control** includes the results for the typing of the NA24385 Control.

2.8.4.3.9.2. Incomplete coverage or dropout/dropin may occur in the typing of a positive control and shall not be considered a failed result. The Technical

Leader shall be consulted if there is a discordance rate > 5% or 511 loci. A discordance rate >20% or 2,046 loci is considered a failed positive control.

2.8.4.3.9.3. A negative control passes when the call rate is less than the following amounts:

2.8.4.3.9.3.1.10X Analysis method – < 2%

2.8.4.3.9.3.2.20X Analysis method – < 1%

2.8.4.4. **Interpreting, Comparing and Reporting DNA Results Associated with Failed Controls and Contamination Events**

2.8.4.4.1. The Technical Leader shall be notified if a positive or negative control fails and/or if sample contamination occurs. Sample contamination is defined as additional peaks present in an unknown or reference sample that can be conclusively identified to not have originated in the sample (e.g., case to case contamination or a mixture in a reference sample).

2.8.4.4.2. Assessment of the integrity of the associated DNA results shall be done in consultation with the Technical Leader. This shall include the possible cause and effect of the failed control or contamination. Additionally, an assessment of the risk associated with moving forward with interpretation shall be documented, as well as the risks associated with retesting (e.g., unnecessary consumption of evidence).

2.8.4.4.2.1. If retesting is performed, the reasons for doing so shall be documented.

2.8.4.4.2.2. If results are determined to be suitable for interpretation without retesting, the appropriate interpretations, comparisons, and statistical results and a description of the failed control or contamination event shall be reported per the Report Writing Guidelines.

2.8.4.4.2.3. If results are determined to be not suitable for interpretation and retesting cannot be performed, the results shall be reported as not suitable for comparison per the Report Writing Guidelines.

2.8.4.4.3. Documentation in the case record shall include at a minimum: the forensic sample, reference or control test results that failed or was contaminated, the likely or known cause of the failed control or contamination, the likely or known source of the contamination, the impact on the integrity of the DNA results, and the determination if the test result is suitable or unsuitable for interpretation.

2.8.4.5. **General Sample Detail Information**

2.8.4.5.1. For each sample, evaluate the **Biological Sex** and **Contributor Status**.

2.8.4.5.1.1. The UAS determines the Biological Sex as male if there are $\geq$ 10 Y-SNPs typed. If there are no Y-SNPs typed and the call rate is $\geq$ 50%, the Biological Sex is determined as female.

2.8.4.5.1.2. The UAS determines the contributor status is a mixture if any of the following conditions are met:

2.8.4.5.1.2.1. The sample has $\geq$ 10 Y-SNPs with 2 alleles.

2.8.4.5.1.2.2. The sample has $\geq$ 10 Y-SNPs typed and $\geq$ 10 X-SNPs with 2 alleles.

2.8.4.5.1.2.3. The sample has no Y-SNP loci typed but has ≥ 50% autosomal SNP heterozygosity and ≥ 40% X-SNP heterozygosity.

2.8.4.5.1.3. If inconclusive results are indicated for either category, further evaluation shall be performed to determine suitability for further analysis. Limited allele/noise determinations may be made by the analyst with changes recorded in the history of the sample report by the UAS.

2.8.4.5.1.4. No further analysis shall be performed if the contributor status indicates a mixture and/or the biological sex determination does not match the STR results.

2.8.4.6. **Exporting Reports**

2.8.4.6.1. For each sample, in the **ACTIONS** drop-down menu, select and generate appropriate reports.

2.8.4.6.1.1. The Sample Report documenting all SNP calls and edits shall be generated for all samples and controls.

2.8.4.6.1.2. The Project Report shall be generated for the analysis method used for interpretation.

2.8.4.6.1.3. The Kintelligence GEDmatch™ PRO Report and the Kintelligence Phenotyping and Ancestry Report shall be generated for all samples with more than approximately 6,000 SNPs.

2.8.4.7. **Uploading to Databases**

2.8.4.7.1. The Project analyst should upload the Kintelligence results into the appropriate database(s).

2.8.4.7.2. **GEDmatch™ PRO**

2.8.4.7.2.1. Log in to GEDmatch™ PRO and select **UPLOAD DNA**

2.8.4.7.2.2. Select **Upload A ForenSeq® Kintelligence Kit**

2.8.4.7.2.3. Enter the case number and item number in the **Case Name** field (ex. 25I-00123-001A). No spaces or underscores are allowed in the file name. The **Alias** field can be left blank.

2.8.4.7.2.4. Select the biological sex of the sample

2.8.4.7.2.5. Leave the **Mitochondrial haplogroup** and **Y haplogroup** fields blank.

2.8.4.7.2.6. For **Source of Profile** select **other**, then enter “Indiana State Police Laboratory”.

2.8.4.7.2.7. For **Law Enforcement Agency**, enter “Indiana State Police Laboratory”.

2.8.4.7.2.8. For **Agency Contact, phone and email address**, enter the analyst’s name, phone number and email.

2.8.4.7.2.9. Select the appropriate category for the sample type.

2.8.4.7.2.10. Select the correct sample file GEDmatch™ PRO report for upload.

2.8.4.7.2.11. Select **Upload.**

2.8.4.7.2.12. After the kit has completed processing, return to the **GEDmatch PRO™ Dashboard.**

2.8.4.7.2.13. Select **Create New Project.**

2.8.4.7.2.14. Title the project with the case number. Select **Create Project**.

2.8.4.7.2.15. Select **Add Your Kit to the Project**, enter kit number or kit case name, and select **Add Kit**.

2.8.4.7.2.16. Under **Project Members**, select **Invite a Member**.

2.8.4.7.2.17. Enter the email addresses for all members of the FIGG team and select **Project Admin** for project permission.

2.8.4.7.2.18. Alternatively, the data upload can be started from the **GEDmatch PRO™ Dashboard** screen by selecting **Upload New Kit+** and following the steps listed above.

2.8.4.7.3. **Family Tree DNA (utilize steps below or instructions provided by Othram)**

2.8.4.7.3.1. Make a copy of the GEDmatch™PRO report file folder containing any samples for upload. Rename the folder “Family Tree DNA Upload Files”.

2.8.4.7.3.2. Simplify the Kintelligence GEDmatch™ PRO report file name in the copied folder for the sample so that it contains only the case number and item number with no spaces or underscores. (ex. 25I-00123-001A.txt)

2.8.4.7.3.3. The sample data is uploaded to Family Tree DNA through the sFTP website. https://sft.genebygene.com/WebInterface/login.html#/

2.8.4.7.3.4. Select **Add Files** and navigate to the renamed sample file and select the file for upload.

2.8.4.7.3.5. Email the appropriate paperwork to solve@othram.com. Retain copies of the paperwork in the casefile.

2.8.4.7.3.6. Othram will return a match list.

2.9. Records

2.9.1. The appropriate worksheets as contained in the Sequencing Workbook or the equivalent working document shall be used to record all procedures.

2.9.2. All data sheets, notes, and other information generated from the laboratory examination shall be retained in the case record.

2.9.3. All generated UAS reports shall be retained in the project folder within the Kintelligence folder on the DNA Data Drive.

2.9.4. The technical review of all Kintelligence data shall be recorded on the appropriate technical review worksheet.

2.9.5. Kintelligence sequencing fastq files for all project samples and controls shall be stored in the project folder within the Kintelligence folder on the DNA Data Drive.

2.9.5.1. Within the Project tab, click on the sample and navigate to the “i” information icon in the upper right corner of the screen.

2.9.5.2. Select the icon to view the Analysis Folder Path.

2.9.5.3. On the D: drive of the server computer, navigate to the appropriate data folder using the Analysis Folder Path.

2.9.5.4. Copy and save the fastq files for the sample. The file name shall be renamed to add the case number and sample name.

2.9.6. Sequencing run data may be archived and deleted from the Universal Analysis Software as necessary to maintain database space.

2.10. Interpretations of Results

2.10.1. If it has been determined that a sample is unsuitable for upload, an explanation as to why the sample is uninterpretable should be given. Examples include:

2.10.1.1. Insufficient read counts were detected.

2.10.1.2. The sample demonstrated the presence of a mixture.

2.11. Report Writing for Kintelligence Analysis

2.11.1. Formatting and general rules for report writing are listed in Forensic Biology Casework Test Methods.

2.11.2. See Appendix 2 for specific reporting examples.

2.12. Investigative Information on Phenotype and Ancestry Estimations Using Kintelligence

2.12.1. The Kintelligence Phenotype and Ancestry estimations shall be provided to the FIGG genealogist for use as investigative information during the FIGG process.

2.12.2. At the analyst's discretion, the piSNPs and aiSNPs may be utilized for phenotype and ancestry estimations in order to provide a separate investigative memo for missing person and unidentified remains cases.

2.12.2.1. If phenotype and ancestry estimations have previously been reported, an additional investigative memo should not be issued unless the estimations have changed due to improved sequencing results.

2.12.3. Hair and eye color phenotype percentages and the ancestry result as generated from the estimation shall be issued to the investigating agency via a memo as shown in Appendix 2.

2.12.4. The Phenotype Report shall be exported and saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

2.13. References:

2.13.1. Verogen, Inc. *MiSeq FGx® Sequencing System Reference Guide*. Document# VD2018006, Rev. F (or current version). San Diego, CA, 2021. https://verogen.com/resources/product-documentation/?_products=miseq-fgx-system

2.13.2. Verogen, Inc. *ForenSeq Kintelligence Kit Reference Guide*. Document# VD2020053, Rev. C (or current version). San Diego, CA, 2023. https://verogen.com/resources/product-documentation/

2.13.3. Verogen, Inc. *Universal Analysis Software Kintelligence Module Version 2 Reference Guide*. Document# VD20022002, Rev. A (or current version). San Diego, CA, 2022. https://verogen.com/resources/product-documentation/

2.13.4. Verogen, Inc. *SNP Typing in Universal Analysis Software and Kinship Estimation with GEDmatch PRO Technical Note.* San Diego, CA, 2021. https://verogen.com/resources/product-documentation/

2.13.5. *Validation of Verogen ForenSeq™ Kintelligence on the Illumina MiSeq FGx®*. Indiana State Police Forensic Service Division, 2025. Indianapolis, IN.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 1
DEFINITIONS

Allele Count Ratio – A ratio of read counts of two alleles, determined by dividing the lower read count allele by the higher read count allele.

Cluster – A distinct spot on a flow cell made up of ~1000 copies of a library containing one amplicon.

DNA Library – A DNA sample that is ready for sequencing.

Flow Cell – A MiSeq FGx® component where the libraries are amplified and sequenced.

Index Adapters – A DNA sequence added to a sample for sample multiplexing and flow cell binding.

Isoallele – An allele that has the same number of repeats as another allele, but a different sequence.

Sequencing by Synthesis – A sequencing process that establishes the DNA sequence as the strand is being synthesized.

Single Nucleotide Polymorphism (SNP) – A single-base sequence variation at a particular point in the genome.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 2
REPORT WORDING

Report wording general rules and formatting shall follow those listed in the Forensic Biology Casework Test Method Appendix 7. This report wording appendix applies to results not covered by the Forensic Biology Casework Test Method.

DNA SIGNATURE PLUS RESULTS

SINGLE UNKNOWN PROFILE

The DNA profile [from ITEMDESCRIPTION] was interpreted as originating from a single male/female/individual [(Unknown Male/Female/Individual #)].[1/2/3]

UNKNOWN MIXTURE

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two/three individuals. A major male/female profile was determined [(Unknown Male/Female/Individual #)]. The remaining portion of the profile was determined to be unsuitable for comparison.[1/2/3]

MIXTURE WITH LR

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two/three individuals. PERSON was considered as a contributor to the major profile. The remaining portion of the profile was determined to be unsuitable for comparison.[1/2/3]

► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON and N-1 unknown individual[s] than if it originated from N unknown, unrelated individuals.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON is **included**.

ANCESTRY AND PHENOTYPING RESULTS TO FOLLOW IN MEMO

piSNP and aiSNP STATEMENT

Analysis of phenotype informative and ancestry informative SNPs has been completed and will be provided separately.

KINTELLIGENCE RESULTS

SUITABLE FOR UPLOAD

A SNP DNA profile was developed using the ForenSeq® Kintelligence Kit and was suitable for upload into law enforcement accessible Forensic Investigative Genetic Genealogy database(s). Forensic Investigative Genetic Genealogy information will be provided in a separate investigative memo.

SNPs DETECTED BELOW 6,000 THRESHOLD

The SNP DNA profile developed using the ForenSeq® Kintelligence Kit demonstrated limited data and is not suitable for upload into law enforcement accessible Forensic Investigative Genetic Genealogy database(s).

MIXTURE

The SNP DNA profile developed using the ForenSeq® Kintelligence Kit was a mixture and is not suitable for upload into law enforcement accessible Forensic Investigative Genetic Genealogy database(s).

NO PROFILE DEVELOPED

A SNP DNA profile was unable to be developed using the ForenSeq® Kintelligence Kit.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

MEMO FOR ANCESTRY AND PHENOTYPE ESTIMATIONS:

Investigator Name
Agency
Address
City, IN Zip

Date
Agency Case Number:
ISP Forensic Services Division Case Number:

This notice is to inform you of potential investigative information. Analysis of phenotype (pi) and ancestry informative (ai) SNP markers has been performed utilizing the [ForenSeq® DNA Signature Plus Kit, Primer Set B/ForenSeq® Kintelligence Kit], to aid in the identification of the unidentified human remains for the above referenced case. This information is for investigative purposes only.

The genetic results are based only on the data developed at the time of reporting and do not supersede any additional genetic testing that may be performed. Conclusions based on genetic prediction models may not account for age-related changes, human factors that affect physical appearance (i.e., dying, bleaching, highlighting), and/or differences in subjective descriptions. Therefore, genetic modeling of phenotypic traits may not correspond with the physical characteristics of an individual. These results should be utilized with caution.

The following results supplement DNA information for item XXX described in the ISP issued Certificate of Analysis Request XXX. The following phenotype estimations were yielded from *sample description*.

Hair Color			
Brown	Red	Black	Blond

Eye Color		
Brown	Intermediate	Blue

The genetic analysis suggests that the unidentified human remains are consistent with an individual of *European/East Asian/African/Ad Mixed American* ancestry. [This individual may possess *Brown/Blue/Intermediate* eye color and *Brown/Red/Black/Blond* natural hair color. (>80%)/A range of phenotype results was observed; therefore, specific hair and eye color characteristics could not be determined/Additional descriptions with supervisor approval.]

Phenotypic hair and eye color traits are an estimate and should not be used as the sole basis for an inclusion or exclusion of an individual. To confirm an identification, submit appropriate family reference DNA standards. Please contact the reporting analyst for further discussion regarding the information provided in this report.

Analyst
Biology Section
Indiana State Police Forensic Services Division
Analyst work phone number
Analyst work email

Year/Lab/Memo#

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

APPENDIX 3
ForenSeq® DNA Signature Plus Stutter Filters

Stutter filters utilized by the Universal Analysis Software are the UAS default values and were internally validated through methods outlined in the Verogen MiSeq FGx® and ForenSeq® DNA Signature Plus Kit Primer Set B with VeritiPro™ validation.

	Back Stutter	Forward Stutter	Double Back Stutter		Back Stutter	Forward Stutter	Double Back Stutter
D1S1656	23.60%	4.80%	4.50%	DXS7132	20.20%	4.50%	2.10%
TPOX	7.30%	0.50%	0.50%	DXS10074	12.40%	1.90%	4.30%
D2S441	5.50%	1.60%	0.30%	DXS10103	19.60%	3.80%	3.80%
D2S1338	26.50%	3.30%	3.20%	HPRTB	8.30%	2.80%	2.50%
D3S1358	17.20%	2.20%	1.50%	DXS7423	8.40%	0.80%	0.60%
D4S2408	7.10%	1.70%	1.70%	DYS505	9.90%	2.00%	1.20%
FGA	26.90%	3.70%	4.70%	DYS570	19.20%	3.70%	2.00%
D5S818	11.40%	13.00%	1.30%	DYS576	16.80%	2.70%	1.80%
CSF1PO	9.30%	13.30%	0.90%	DYS522	9.70%	0.90%	0.90%
D6S1043	15.20%	6.20%	2.00%	DYS481	50.20%	6.30%	13.60%
D7S820	10.30%	2.30%	1.10%	DYS19	15.50%	2.40%	2.40%
D8S1179	24.90%	3.90%	4.00%	DYS391	14.90%	4.00%	1.90%
D9S1122	14.60%	1.60%	1.30%	DYS635	10.30%	3.20%	3.00%
D10S1248	17.60%	3.30%	2.30%	DYS437	13.10%	2.10%	1.60%
TH01	11.90%	1.00%	0.80%	DYS439	8.60%	2.40%	0.70%
vWA	18.70%	5.60%	3.30%	DYS389I	16.10%	1.50%	1.80%
D12S391	32.10%	3.80%	4.10%	DYS389II	18.50%	18.60%	6.60%
D13S317	7.30%	3.50%	0.50%	DYS438	3.40%	1.70%	0.10%
PentaE	12.10%	9.10%	1.50%	DYS612	39.10%	10.40%	9.20%
D16S539	18.50%	3.10%	2.30%	DYS390	12.70%	2.00%	1.30%
D17S1301	20.40%	2.30%	2.50%	DYS643	7.00%	1.30%	0.50%
D18S51	22.90%	10.60%	3.70%	DYS533	9.30%	1.90%	2.60%
D19S433	13.30%	2.10%	1.30%	Y-GATA-H4	18%	6.10%	3.20%
D20S482	15.90%	2.80%	1.20%	DYS385a-b	25.40%	6.10%	4.40%
D21S11	13.30%	3.60%	18.30%	DYS460	11.60%	1.30%	1.30%
PentaD	5.60%	5.30%	0.30%	DYS549	7.10%	2.20%	4.40%
D22S1045	19.80%	4.00%	1.70%	DYS392	24.30%	0.60%	3.40%
DXS10135	20.60%	4.00%	2.50%	DYS448	2.00%	0%	0%
DXS8378	8.70%	1.50%	1.00%	DYF387S1	26.00%	4.40%	5.80%

APPENDIX 4
Verogen's Universal Analysis Software v2.0+

1 Setting Up User Profile

1.1 Open the UAS log-in screen.

1.2 Go to Register As A New User.

1.3 Enter email and password and select Register.

1.4 An administrator will log in.

1.5 Go to Settings, User Management.

1.6 The administrator will enable the person who registered.

2 Creating an Analysis Method

2.1 Log into an administrator account.

2.2 Go to Settings, then Analysis Methods, then Create Analysis Method.

2.3 Under Options select "Copy Analysis Method", select the default method, give it a new name (ex. 10X ISP Kintelligence Analysis Method).

2.4 If needed, adjust the analytical threshold, interpretation threshold and/or Intralocus Balance.

2.5 To select a method as the default method, select the star icon in the left panel.

3 Enable Sample History

3.1 Log into an administrator account.

3.2 Go to Settings, then System Settings.

3.3 Enable "Run, Project, and Sample History" to record user actions.

4 Data Management

4.1 Log into an administrator account.

4.2 Go to Settings, then Data Management.

4.3 Select "Archive" , then the appropriate project folder to be archived.

4.4 Select "Next". Archive FASTQs should already be selected. To archive only FASTQ files for the project, Select "Next" to review and confirm.

4.5 To archive and delete project for data management, Select "Complete archive with all run files" and "Delete data after archive complete" and then Select "Next" to review and confirm.

4.6 Archived data may be re-imported (if needed) by selecting Import in Data Management.