# 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.

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## 1. ForenSeq™ DNA Signature Prep Kit Sequencing Test Methods:

**1.1. Scope**: ForenSeq™ DNA Signature Prep Kit sequencing should be performed in cases involving reference standards or secondary reference standards where information beyond what is obtained with traditional STR analysis may assist in resolution of the case. 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 Prep 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. CODIS offender 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 which demonstrate mixed DNA profiles with autosomal STR analysis will 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 Prep Kit

1.5.5. MiSeq FGx® Reagent Kit

**1.6. Hazards/Safety:**

1.6.1. Two components of the ForenSeq™ DNA Signature Prep Kit, LNA1 and LNW1, have hazardous properties. **CAUTION:** Both reagents contain formamide and B-mercaptoethanol. Formamide is a suspected carcinogenic 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 Prep before or after autosomal STR analysis will be evaluated on a case-by-case basis.

1.8.1.2. DNA Signature Prep 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 Prep**

1.8.2.1. **Introduction**

1.8.2.1.1. The Verogen ForenSeq™ DNA Signature Prep 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 22 phenotype informative SNPs. Only Primer Set B is currently in use in the laboratory.

1.8.2.1.2. The ForenSeq™ DNA Signature Prep Kit locus-specific information is available at https://verogen.com/resources/product-documentation/ in the ForenSeq™ DNA Signature Prep Reference Guide VD2018005.

1.8.2.1.3. The ForenSeq™ DNA Signature Prep Kit reagents come in three boxes. Box 1 should be stored at -25°C to -15°C. After first use, the 2800M positive control should be stored at 2°C to 8°C. Box 2 should be stored at -25°C to -15°C. LNS2 should be stored at room temperature and LNW1 should be stored at 2°C to 8°C after opening of Box 2. Box 3 should be stored at 2°C to 8°C.

1.8.2.1.4. 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 Prep 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, balancing the number of samples in each column and row.

1.8.2.2.5.2. Assign i5 and i7 indexes to the sample setup, ensuring unique combinations are used. 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. For initial use, thaw one tube each of PCR1 reaction mix, DPMB primer mix, FEM enzyme, and 2800M positive control at room temperature. After first use, all reagents should be returned to -25°C to -15°C except the 2800M which should be stored at 2°C to 8°C.

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.

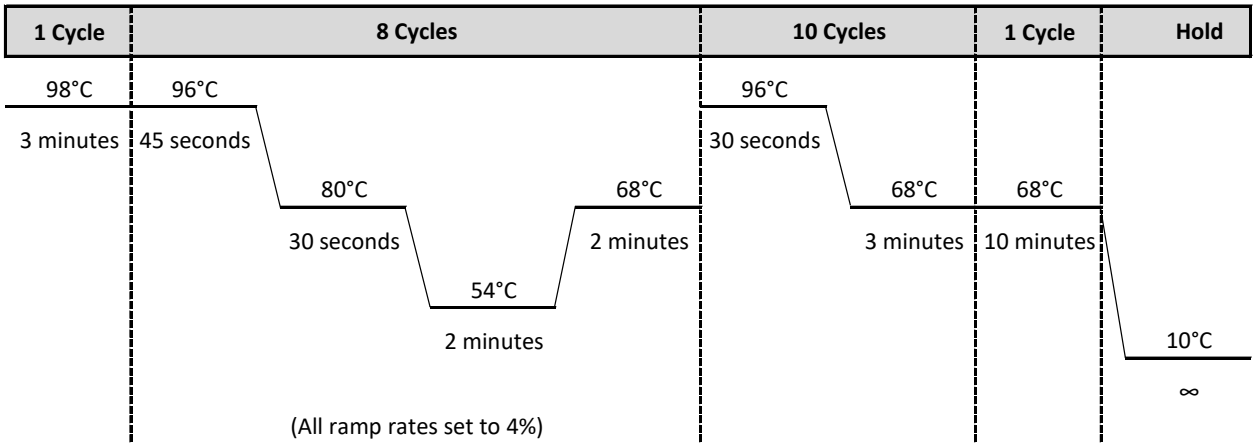
1.8.2.3.7. Add 10 µl master mix to each well of the FSP plate. Optionally, the master mix may be evenly distributed into each well of an eight-strip tube or column of a 96 well plate, then a multichannel pipette may be used to dispense.

1.8.2.3.8. Dilute 2 µl 2800M with 98 µl nuclease-free water in a 1.5 ml tube. Gently flick and centrifuge briefly. A new dilution shall be made for each run performed.

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

1.8.2.3.10. Seal the plate with a foil seal. Centrifuge plate briefly and place in a Veriti™ Thermal Cycler.

1.8.2.3.11. Select the **Sig Prep PCR1** protocol with the following amplification procedure. Approximately 3.5 hour cycling time.




1.8.2.3.12. 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.

1.8.2.4. **Enrich Targets (PCR2)**

1.8.2.4.1. Thaw index adapters and PCR2 reaction mix at room temperature for approximately 20 minutes. Only thaw index adapters that are being used.

1.8.2.4.2. Vortex the index adapters and centrifuge briefly.

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

1.8.2.4.4. Arrange i7 adapters in columns 1-12, or as many columns as are needed, of the ForenSeq Index Plate Fixture.

1.8.2.4.5. Arrange i5 adapters in rows A-H, or as many rows as are needed, of the ForenSeq Index Plate Fixture.

1.8.2.4.6. Place the FSP on ForenSeq Index Plate Fixture and remove the foil seal.

1.8.2.4.7. Add 4 µl i7 adapters to each column. Using a multichannel pipette is recommended. Replace the caps on the i7 tubes with new orange caps.

1.8.2.4.8. Add 4 µl i5 adapters to each row. Using a multichannel pipette is recommended. Replace the caps on the i5 tubes with new white caps.

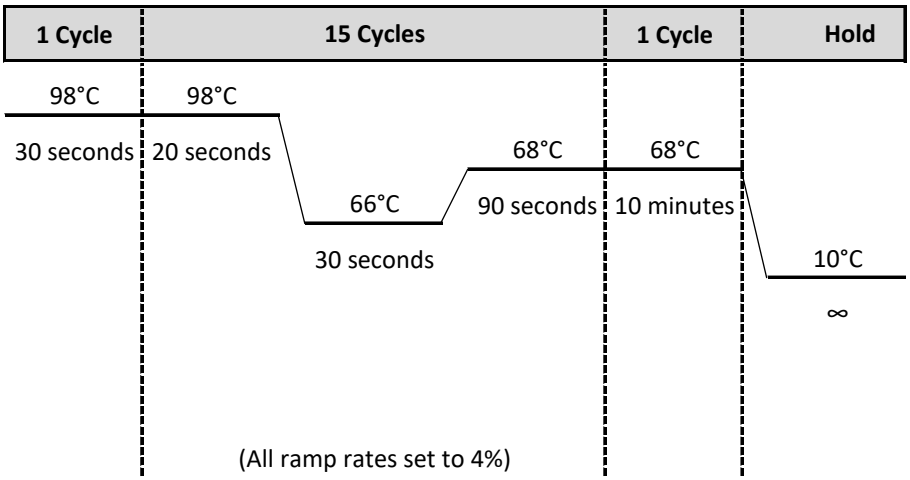
1.8.2.4.9. Vortex PCR2 reaction mix and centrifuge briefly.

1.8.2.4.10. Add 27 µl PCR2 reaction mix to each well. 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.

1.8.2.4.11. Seal the plate with a foil seal and centrifuge at 1000 x g (rcf) for 30 seconds.

1.8.2.4.12. Place plate in a Veriti™ Thermal Cycler.

1.8.2.4.13. Select the **Sig Prep PCR2** protocol with the following amplification procedure. Approximately 46 minute cycling time.




1.8.2.4.14. 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.

1.8.2.5. **Purify Libraries**

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

1.8.2.5.1.1. Vortex SPB before use and frequently during use to make sure that the beads are evenly distributed. Aspirate and dispense SPB slowly due to its viscosity.

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 SPB according to the number of libraries you are preparing.

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

1.8.2.5.4. Add 45 µl SPB 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 foil 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 (~2 minutes).

1.8.2.5.10. Remove the clear adhesive seal.

1.8.2.5.11. Remove and discard all supernatant from each well.

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

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

1.8.2.5.12.2. Incubate on the magnetic stand for 30 seconds.

1.8.2.5.12.3. Remove and discard all supernatant from each well.

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

1.8.2.5.14. Place the plate on the magnetic stand.

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

1.8.2.5.15.1. All EtOH must be removed from each well. Remaining EtOH can inhibit future steps.

1.8.2.5.16. Remove the plate from the magnetic stand.

1.8.2.5.17. Add 52.5 µl RSB to each well.

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

1.8.2.5.19. 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.

1.8.2.5.20. Incubate at room temperature for 2 minutes.

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

1.8.2.5.22. Remove the clear adhesive seal.

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

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

1.8.2.5.25. SAFE STOPPING POINT. The procedure may be continued immediately, or the plate can be sealed with a clear adhesive seal 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. Hold LNA1 up to light to make sure no crystals are present after vortexing.

1.8.2.6.4. Vortex LNB1 for at least 1 minute, stopping every 15 seconds to invert tube 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.

**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. Pour into a reagent reservoir.

1.8.2.6.9. Transfer 45 µl 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 plate 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 7.8.3.2.

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

1.8.2.6.15.1. Prepare 0.1N HP3 in a new 1.5 ml tube. Amounts account for overage.

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

1.8.2.6.15.1.2. Add 1.8 µl HP3 per sample.

1.8.2.6.15.1.3. Invert the tube several times to mix.

1.8.2.6.15.1.4. Set aside.

1.8.2.6.15.2. 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 the clear adhesive seal.

1.8.2.6.18. Remove and discard all supernatant from each well.

1.8.2.6.19. Remove the plate from the magnetic stand.

1.8.2.6.20. Wash two times with 45 µl LNW1 as follows:

1.8.2.6.20.1. Add 45 µl LNW1 to each well.

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

1.8.2.6.20.3. 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.

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

1.8.2.6.20.5. Remove the clear adhesive seal.

1.8.2.6.20.6. Remove and discard all supernatant from each well.

1.8.2.6.21. Remove the plate from the magnetic stand.

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

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

1.8.2.6.24. Remove the clear adhesive seal.

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

1.8.2.6.26. Remove the plate from the magnetic stand.

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

1.8.2.6.28. 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.

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

1.8.2.6.30. Remove the clear adhesive seal.

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

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

1.8.2.6.33. SAFE STOPPING POINT. The procedure may be continued immediately, or the plate can be sealed with a clear adhesive seal 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. Remove clear plastic seal.

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

1.8.2.7.5. The NLP plate should be sealed and stored at -25°C to -15°C for up to 30 days.

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

1.8.2.7.7. Vortex the PNL tube and centrifuge briefly.

1.8.2.7.8. 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. **Prepare the Human Sequencing Control**

1.8.2.8.1. Remove the HSC (Human Sequencing Control) and the HP3 (2N NaOH) from the ForenSeq™ DNA Signature Prep Kit and allow to thaw completely at room temperature.

1.8.2.8.2. Flick to mix the HSC and centrifuge briefly to pull contents to the bottom of the tube. Vortex the HP3 and centrifuge briefly.

1.8.2.8.3. Combine in a 1.5ml tube:

2 µl of HSC

2 µl of HP3

36 µl nuclease free water

1.8.2.8.4. Vortex and centrifuge briefly.

1.8.2.8.5. Incubate at room temperature for 5 minutes.

1.8.2.9. **Dilute and Denature Libraries**

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

1.8.2.9.2. If not previously thawed, remove the Hybridization Buffer (HT1) from the MiSeq FGx® Reagent Kit Box 1 (stored -25°C to -15°C) and thaw completely at room temperature.

1.8.2.9.3. Combine 591 µl of HT1 with 7 µl of the pooled normalized libraries (PNL). Pipette to mix.

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

1.8.2.9.5. Add 4 µl of the prepared HSC mixture. Pipette to mix.

1.8.2.9.6. Vortex and centrifuge briefly.

1.8.2.9.7. Denature at 96°C for 2 minutes in a pre-heated thermomixer. Invert several times to mix.

1.8.2.9.8. Place on ice or into a cold block for 5 minutes.

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 Verogen's Universal Analysis Software (UAS).

1.8.3.1.3. 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.

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 diluted denatured 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 Run**

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

1.8.3.3.2. Select **Create New Run** in the upper right corner.

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

1.8.3.3.4. The batch ID shall include the “S’, the year and the batch number. (ex. S2025-001).

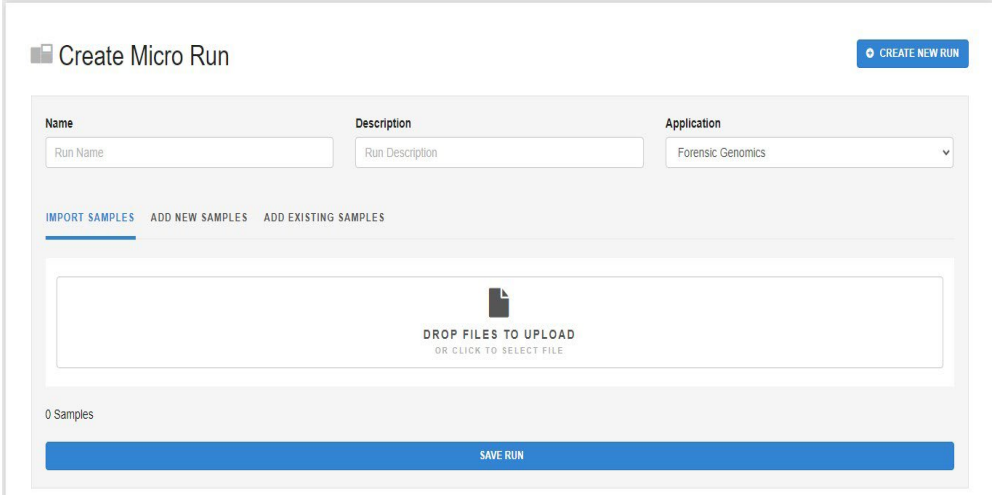
1.8.3.3.5. The run name shall be the batch ID and the date the run is started (ex. S2025-001_01Jan25).An optional description may be entered if desired.

1.8.3.3.6. Ensure that **Forensic Genomics** is selected in the Application box.

1.8.3.4. **Import or Enter Samples**

1.8.3.4.1. To import samples from the Sequencing Workbook (recommended) or other text file, select **Import Samples**.

1.8.3.4.2. Drag and drop or click to select the appropriate file for upload.

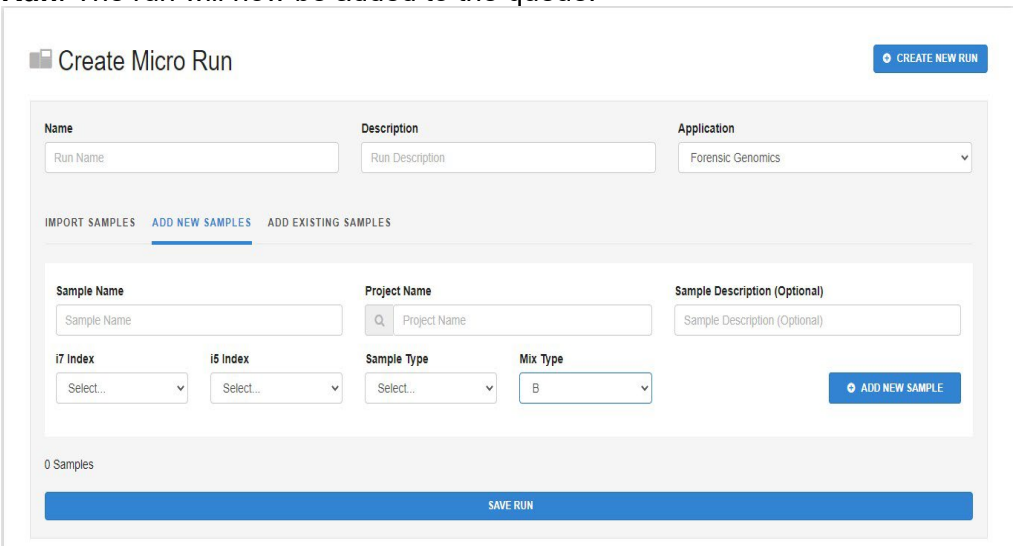



1.8.3.4.3. To manually enter sample details, select **Add New Samples** or **Add Existing Samples**.

1.8.3.4.3.1. Enter sample names, index combinations, and sample type into the appropriate fields.

1.8.3.4.3.2. Ensure Mix Type B is selected.

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




1.8.3.5. **Start the Sequencing Run**

1.8.3.5.1. On the Verogen MiSeq FGx®, ensure server 1.3 is selected and log in with a valid username and password.

1.8.3.5.2. Select **Forensic Genomics** as the run type.

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

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

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

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

1.8.3.5.5.2. 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.

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

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

1.8.3.6. **Evaluate the Sequencing Run**

1.8.3.6.1. 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 7.8.4.1.

1.8.4. **ForenSeq™ Universal Analysis Software v1.3 Data Analysis**

1.8.4.1. **ForenSeq™ Universal Analysis Software v1.3 Settings 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 internally determined and validated AT and IT are 1.5% and 4.5%, respectively, for all STR and SNP loci, with the exception of DYS389II at 5.0% and 15% and DYS448 and DYS635 both at 3.3% and 10%.

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 of 10 reads and IT of 30 reads.

1.8.4.2.6. Stutter is filtered by the UAS for back, forward, and double back stutter using internally determined and 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 square of the back stutter value is applied for filtering.

1.8.4.2.7. The ISP default analysis method has been set within the UAS incorporating the AT and IT thresholds and stutter percentages listed in Appendix 4.

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 0.1ng of target DNA shall have a stochastic of 200 reads. Samples between 0.1ng and less than 1ng shall have a stochastic of 100 reads. Samples with 1ng or greater target DNA require no stochastic application.

1.8.4.2.9. ST are not incorporated into the ISP default settings within the UAS. ST must be applied during interpretation either within the UAS (by toggling or untyping) or by using SeqLite.

1.8.4.3. **Evaluating Run Quality Metrics**

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

1.8.4.3.2. Analysis methods are then listed in the left column of the Project page. Some basic run information is also available in this left column including the number of samples in project and review of controls.

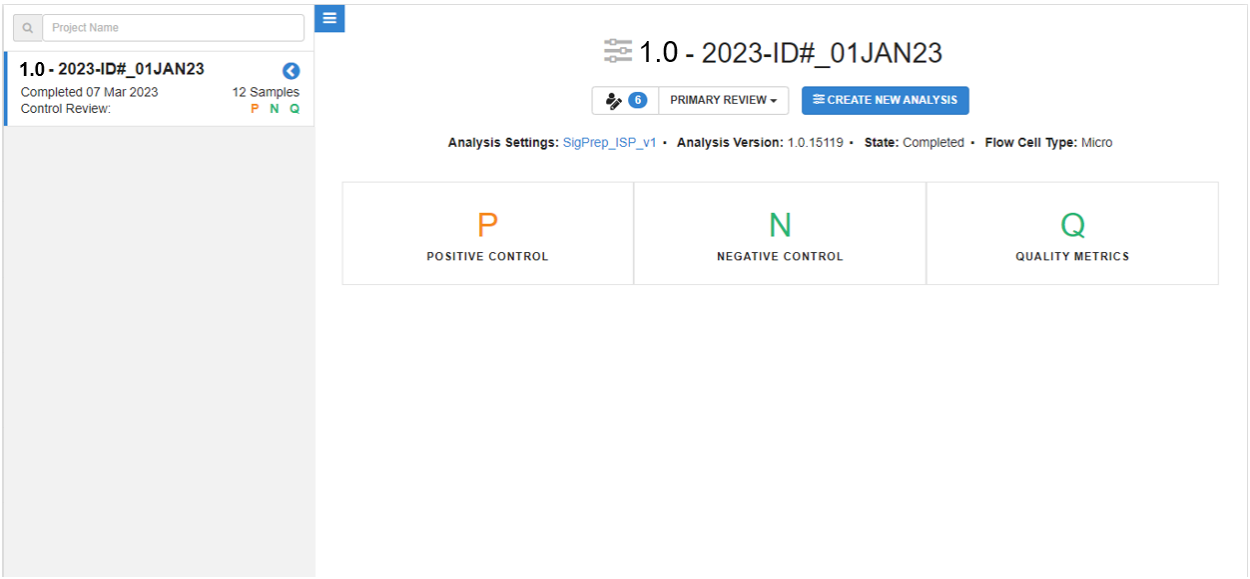
1.8.4.3.3. To access the results of the quality metrics and controls for a project, select the appropriate analysis method which will then open the Analysis Page. Note: projects analyzed with the ISP Default Analysis Settings will be listed as **1.0 – Batch ID**.

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

1.8.4.3.5. The preliminary results of the positive controls, negative controls and quality metrics are listed in a box in the middle of Analysis Page with color-coded run quality indicator icons. Each icon can be clicked to expand for additional details.

1.8.4.3.5.1. The Positive Control icon includes the results for the typing of the 2800M Control and the Human Sequencing Control (HSC).

1.8.4.3.5.2. The Negative Control icon includes the results for all reagent blanks and amplification negative controls.



1.8.4.3.6. 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. |

1.8.4.3.7. 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.

1.8.4.3.7.1. 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.

1.8.4.3.7.2. 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.

1.8.4.3.7.3. 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 IT at more than 5 loci shall be considered a failed control.

1.8.4.3.7.4. 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% |

1.8.4.3.8. Evaluate additional metrics, including Index CV and number of read counts across samples, under the Sample Representation tab, to further assess the quality of the run and samples.

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

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.

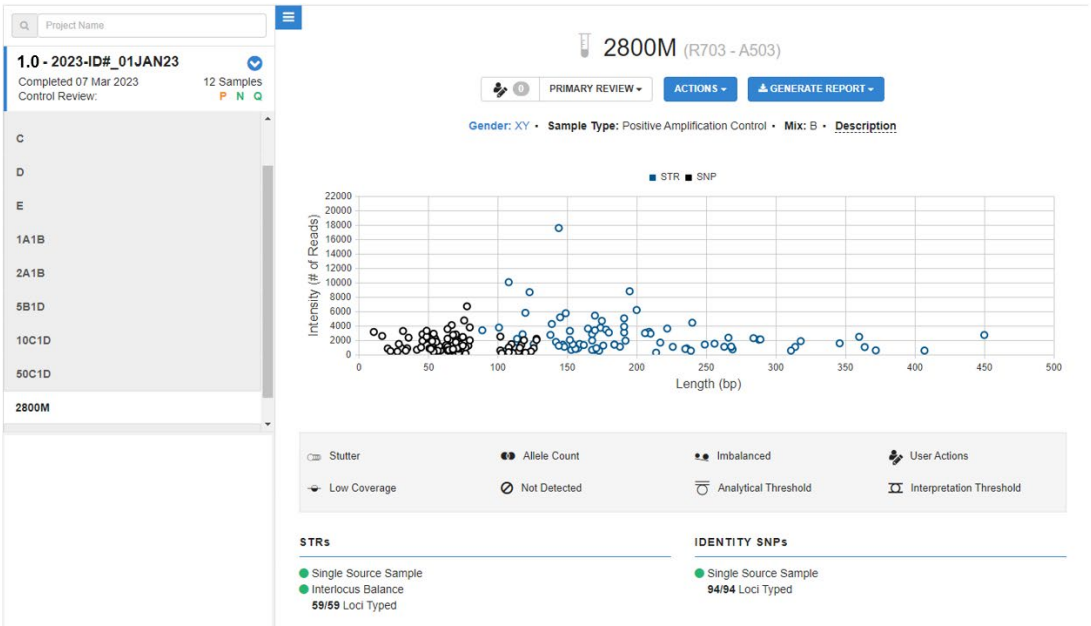
1.8.4.5. **General Sample Detail Information**

1.8.4.5.1. Navigate to sample results by selecting the blue arrow in the left column of the Project page to expand the sample list. Specific samples can then be opened for review in a Sample Details page.

1.8.4.5.2. Within each Sample Details page, results for STR and iiSNPs are viewable for interpretation. To access the piSNP and aiSNP results, navigate to Phenotype Estimation page as outlined in Section 6.12.

1.8.4.5.3. Sample and locus level quality indicators may be present which may aid in sample interpretation at the locus level in the Sample Details page.

1.8.4.5.4. Information about the sample includes a graph with read intensities of each typed STR and SNP locus, color-coded indicator used to identify single source samples, color-coded indicator highlighting interlocus balance performance, gender determination summary, and the typing results across the loci.




1.8.4.5.4.1. UAS utilizes sample results to indicate if a sample appears to be single source or may be a mixture.

1.8.4.5.4.1.1. If no or few locus level quality indicators are flagged, then the sample will have a green color-coded indicator for single source.

1.8.4.5.4.1.2. If greater than 5 STR loci and/or greater than 10 iiSNP loci yield locus level quality indicators, then an orange color-coded indicator will be displayed for single source sample indicating the possible presence of a mixture.

1.8.4.5.4.2. Interlocus balance of the STR loci is assessed internally within the UAS. 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.

1.8.4.5.5. Locus level quality indicators shall be evaluated and can aid in determination of single source contributor or mixture. Locus level quality indicators are listed in the image above.

## 1.8.5. Interpretation Guidelines for ForenSeq™ DNA Signature Prep Data Analysis

### 1.8.5.1. Sample Evaluation of Results

1.8.5.1.1. Each locus with quality indicator flags 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 or 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.

1.8.5.2.2.1. See also Interpretation Guide (Appendix 5).

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 threshold shall not be typed or 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.

1.8.5.2.4.2.3. If there are two typed alleles, with only one greater than the ST, the ACR should be evaluated in loci with an imbalance quality indicator.

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

1.8.5.2.4.2.3.2. 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.

1.8.5.2.4.2.3.3. 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.

1.8.5.2.4.2.4. If there are two typed alleles and an additional component, more than two typed alleles present at a locus, or one typed allele and multiple additional components, then the locus shall be considered inconclusive.

1.8.5.2.4.2.5. Gender determination may be used in assessing additional components in the X-STR and Y-STR loci. The presence of additional components outside of the gender expectations may warrant calling a locus inconclusive.

1.8.5.2.4.2.6. If a locus is inconclusive for genotype determination, then all alleles for that locus shall be toggled to untyped if the alleles are greater than stochastic.

1.8.5.2.4.2.7. Any homozygous alleles less than stochastic will be filtered from genotype determination using the SeqLite tool.

1.8.5.2.5. **SNP Loci Genotype Determinations**

1.8.5.2.5.1. Read counts below analytical threshold shall not be typed or interpreted.

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

1.8.5.2.5.2.1. 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.

1.8.5.2.5.2.1.1. **Exception:** If there are two untyped alleles (both below IT) in a piSNP or aiSNP locus, both may be typed to aid in phenotype and ancestry estimations.

1.8.5.2.5.2.2. 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.

1.8.5.2.5.2.2.1. The Allele Count Ratio (ACR) between the typed allele and the additional component shall be determined.

1.8.5.2.5.2.2.2. If the typed allele is above the ST for the sample and the ACR is between 0.10 to 0.20, then no additional typing shall be done. The genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

1.8.5.2.5.2.2.3. If the typed allele is above the ST for the sample and the ACR is <0.10, then no additional typing shall be done of the additional component. The genotype shall be considered as homozygous for the typed allele at the locus.

1.8.5.2.5.2.2.4. If the typed allele is not above the ST for the sample and the ACR is >0.20, then the additional component shall be typed resulting in a heterozygous genotype determination for the locus.

1.8.5.2.5.2.2.5. If the typed allele is not above the ST for the sample and the ACR is <0.20, then no additional typing shall be done. The genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

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

1.8.5.2.5.2.4. If a locus is inconclusive for genotype determination, then all alleles for that locus shall be toggled to untyped if the alleles are greater than ST.

1.8.5.2.5.2.5. Any homozygous alleles less than ST will be filtered from genotype determination 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.

1.8.5.3.2. 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) will be used to determine the major profile accordingly. Typed alleles are designated A-D in descending read count order. SNP loci in mixture samples will 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. Loci with 4 alleles (A-D)

1.8.5.3.4.4.1. 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.

1.8.5.3.4.5. Loci with 3 alleles (A-C)

1.8.5.3.4.5.1. 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.

1.8.5.3.4.5.2. 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.

1.8.5.3.4.5.3. 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.

1.8.5.3.4.6. Loci with 2 alleles (A and B)

1.8.5.3.4.6.1. 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.

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

1.8.5.3.4.7. Loci with 1 allele (A)

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

1.8.5.3.4.7.2. 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.

1.8.5.3.5. **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 mixture samples will 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. Loci with 5-6 alleles

1.8.5.3.5.4.1. 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. 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.

1.8.5.3.5.5. All remaining loci

1.8.5.3.5.5.1. 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 ≤0.20, then a heterozygous major profile (A, B) will be determined.

1.8.5.3.5.5.2. 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.

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

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

1.8.5.4. **General Interpretation Considerations for UAS**

1.8.5.4.1. If a locus is inconclusive for genotype determination for a single source profile, then all alleles for that locus shall be toggled to untyped if the alleles are greater than stochastic. The reason for the change shall be documented in the comment section.

1.8.5.4.2. Any homozygous alleles less than stochastic will be filtered from genotype determination using the SeqLite tool in a single source profile.

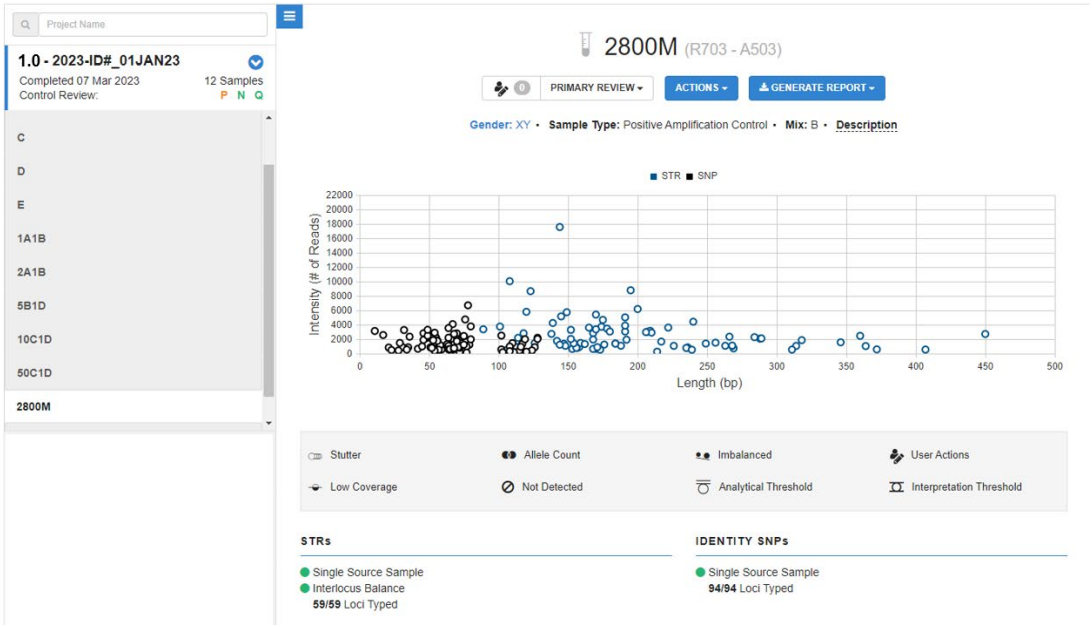
1.8.5.4.3. Only alleles determined as the major profile of a mixture shall be typed for a mixture sample. The reason for any changes to the remaining portion of the profile shall be documented in the comments section.

### 1.8.5.5. Exporting Sample Details

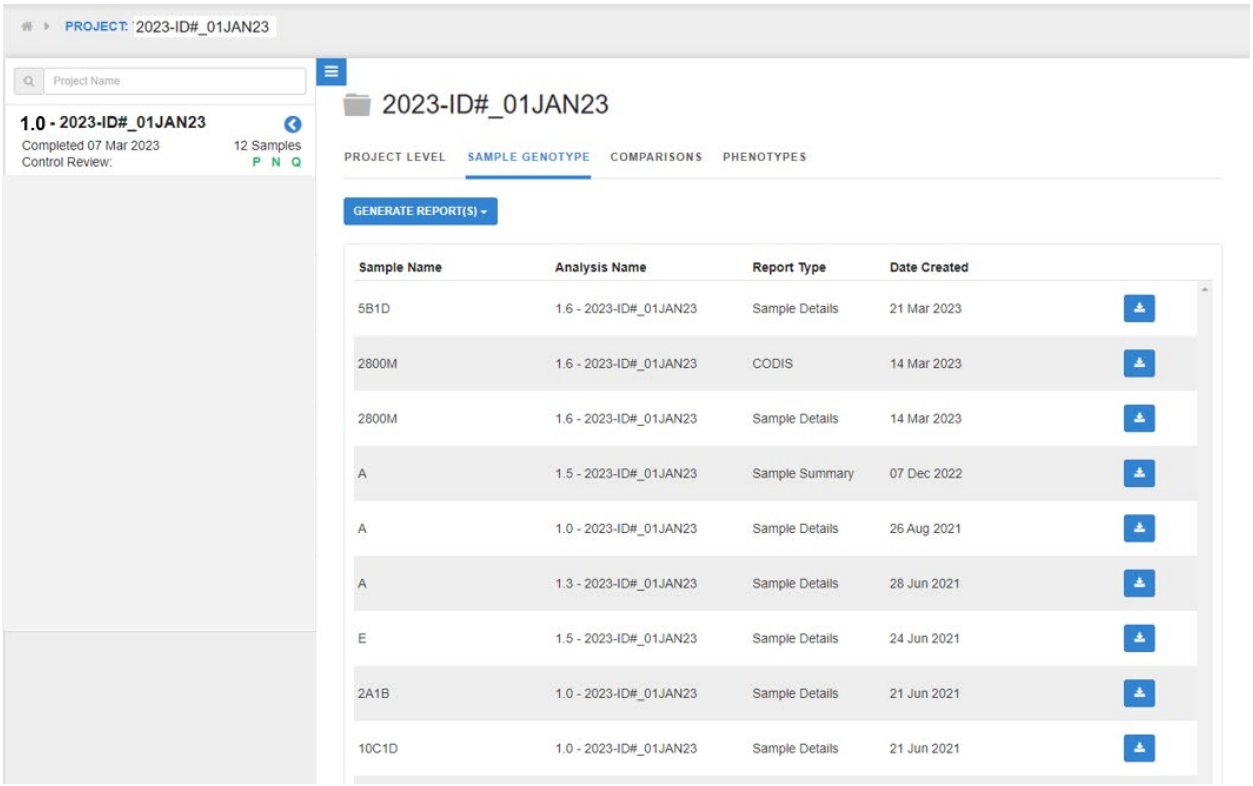
1.8.5.5.1. After all typing and genotype determinations, a Sample Details Report shall be generated from the UAS.

1.8.5.5.2. Sample Details Reports can be generated in two different ways.

1.8.5.5.2.1. At the top of a sample details page select **Generate Report** to create for individual samples.




1.8.5.5.2.2. To populate reports for more than one sample, go the Project page, navigate to the Sample Genotype tab, and select **Generate Report(s)**. Select **Sample Details** and then multiple samples can be toggled on for generation of Sample Details Reports.




1.8.5.5.3. Sample Details Reports shall be exported from the UAS to be utilized for SeqLite.

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

1.8.5.6. **SeqLite**

1.8.5.6.1. Sample Details report shall be imported into the Seqlite tool, an Excel program used to apply ST, document final allele calls for a sample, and generate additional interpretation files.

1.8.5.6.2. The appropriate concentration-based ST shall be selected within Seqlite.

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

1.8.5.6.4. 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 Prep project/run shall be recorded on the appropriate batch review worksheet. The technical review of all data for the DNA Signature Prep 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 Prep Batch data electronic records shall be retained in the "zSigPrep" 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 Prep 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 Prep 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 autosomal STR 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 appropriate NIST 2017 population data using the racial information provided by the contributing agency. If racial information is unknown or unobtainable, calculations shall be performed using the Combined 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 nomenclature 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.

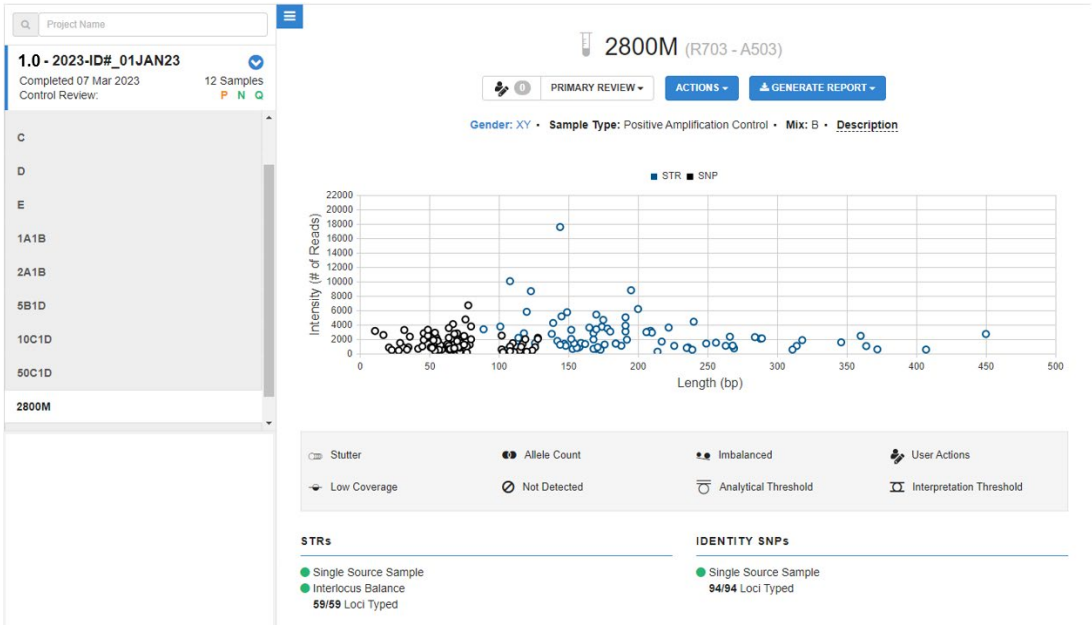
1.12.2.11.2.1. If the 1/LR is less than 2, it shall be rounded up to 2.0 (limited support for exclusion).

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

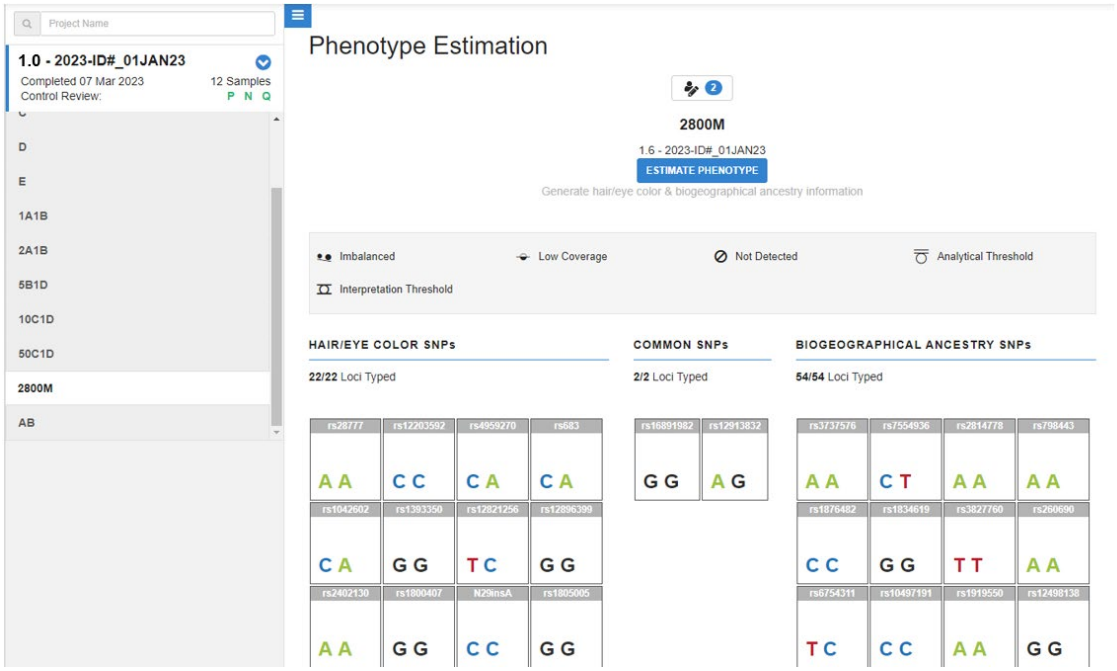
1.13.1. At the analyst's discretion, the piSNPs and aiSNPs may be utilized for phenotype and ancestry estimations in order to provide investigative information for missing person and unidentified remains cases.

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 Sample Details page, click the **Actions** drop-down button and select **Phenotype Estimation**.




1.13.4. A table of piSNP and aiSNP results will be displayed. Data for the piSNPs and aiSNPs shall be interpreted using the Interpretations Guidelines outlined in Section 6.8.5 before generating a phenotype estimation report. Select **Estimate Phenotype** to generate phenotype estimation from the SNP information.



1.13.5. 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.

1.13.6. 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.

**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, Inc. *ForenSeq™ DNA Signature Prep Reference Guide*. Document# VD2018005, Rev. D (or current version). San Diego, CA, 2022. https://verogen.com/resources/product-documentation/?_products=forenseq-dna-signature-prep-kit

1.14.3. Verogen, Inc. *ForenSeq™Universal Analysis Software v1 Guide*. Document# VD2018007, Rev. A (or current version). San Diego, CA, 2018. https://verogen.com/resources/product-documentation/?_products=forenseq-universal-analysis-software-v1-3

1.14.4. *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.

## 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. 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 samples shall be run on a single standard flow cell.

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:

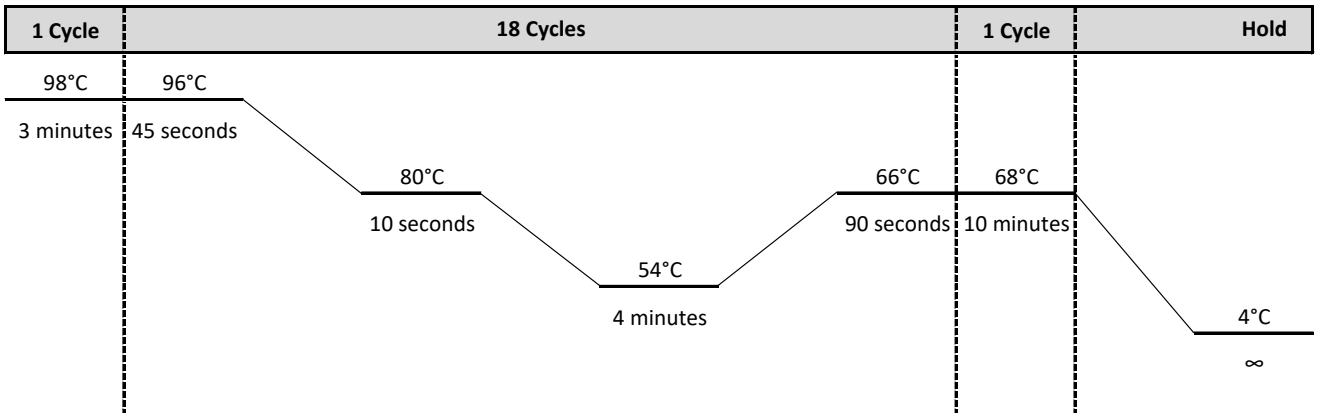
2.8.2.3.10.1. In the Control DNA Dilution 1, combine 2 µl of 10 ng/µl NA24385 with 48 µl of nuclease-free water in order to prepare 50 µl of 400 pg/µl NA24385. Gently pipette to mix and centrifuge briefly. A new dilution shall be made for each run performed.

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 100 x g for 30 seconds.

2.8.2.3.13. Place on the 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 that day, prepare 1.5 ml per sample.

2.8.2.4.3.2. If the Purify Libraries step will not be completed that day, prepare 1.0 ml per sample.

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 100 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 100 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 100 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 100 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 100 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.

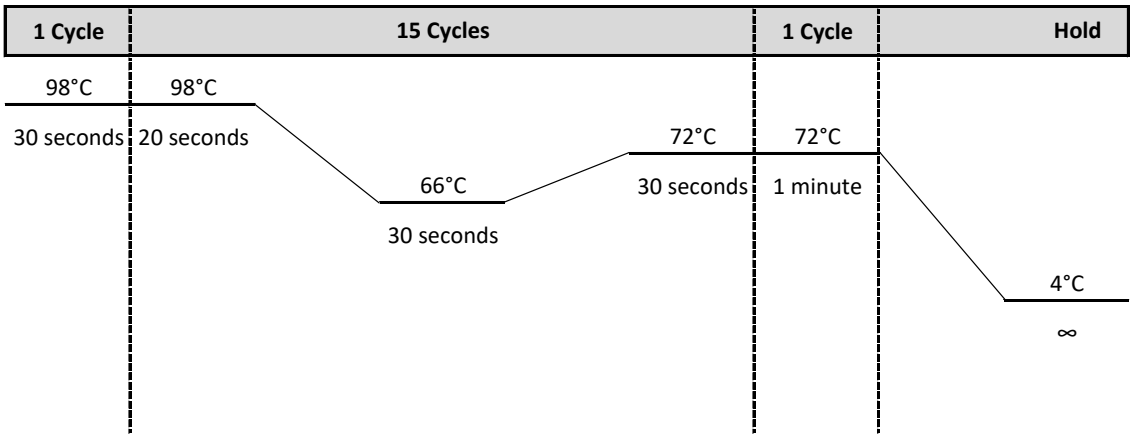
2.8.2.5.5. Briefly centrifuge kPCR2 and then pipette to mix.

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 100 x g for 30 seconds.

2.8.2.5.9. Place on the 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 5.8.3.2.1.

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.

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 100 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 100 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.

2.8.2.7. **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.

2.8.2.7.7. 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 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 Verogen's 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.

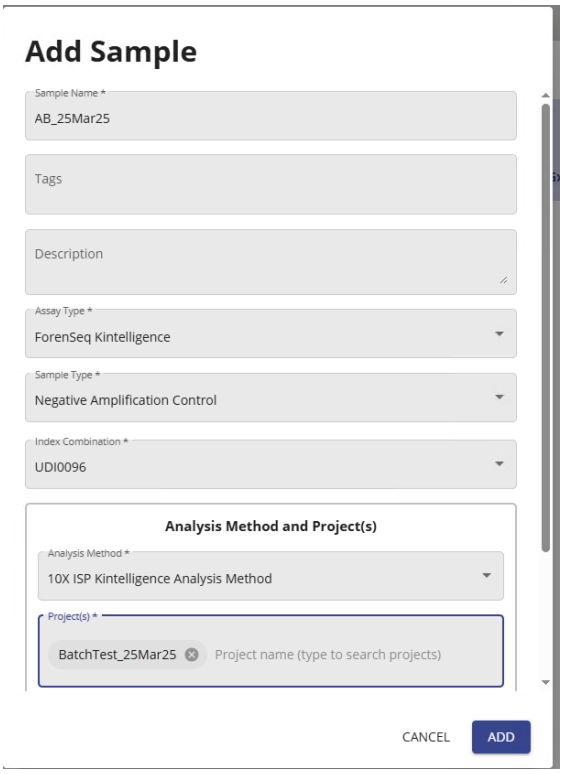
2.8.3.3.4.10. Select the appropriate Index Combination.

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 Verogen 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 6.**

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

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 $\geq$ 50% autosomal SNP heterozygosity and $\geq$ 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.

2.8.4.6.1.2. 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**

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 analyst's case 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 each casework sample shall be stored in the analyst's case folder on the DNA Data Drive.

2.9.5.1. Within the Project tab, click on the casework 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 casework sample.

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 an investigative memo for missing person and unidentified remains cases.

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. *ForenSeq™ 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.

# 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.

## 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 Verogen 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 Verogen 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 Verogen 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 Verogen ForenSeq™ Kintelligence Kit.

## SEQUENCING STATEMENTS FOR ACCREDITATION PROCESS

The Indiana State Police Forensic Services Division approved DNA Sequencing with the ForenSeq™ DNA Signature Prep Kit, Primer Set B for casework use on July 1, 2023. The Laboratory is currently in the process of adding this type of analysis to its ISO/IEC 17025 Forensic Testing Scope of Accreditation by ANSI National Accreditation Board (ANAB).

The Indiana State Police Forensic Services Division approved DNA Sequencing with the ForenSeq™ DNA Kintelligence Kit for casework use on April 21, 2025. The Laboratory is currently in the process of adding this type of analysis to its ISO/IEC 17025 Forensic Testing Scope of Accreditation by ANSI National Accreditation Board (ANAB).

# 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 Prep Kit, Primer Set B, 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
# Verogen's Universal Analysis Software v1.3

## 1 Setting Up User Profile

1.1 Open the UAS.

1.2 Select "Create One" next to the section that states "Need an account?"

1.3 Enter the new user's ISP email address and create password.

1.4 Click the blue bar with a check mark.

1.5 The new user must be approved by an administrator.

1.5.1 Log into an administrator account.

1.5.2 Select the administrator's email address at the top of the screen.

1.5.3 Select "Maintenance" from the menu.

1.5.4 Select "User Management".

1.5.5 Approve the new user.

1.5.6 Users can also be changed from standard to administrator as well as enabled or disabled on this screen.

## 2 Creating an Analysis Method

2.1 Log into an administrator account.

2.2 Select the administrator's email address at the top of the screen.

2.3 Select "Maintenance" from the menu.

2.4 Select "Analysis Values".

2.4.1 The ISP default analysis method is "SigPrep_ISP_v1"

2.4.1.1 Analytical threshold is 1.5% for most loci, 5% for DYS389II, and 3.3% for DYS448 and DYS635.

2.4.1.2 Interpretation threshold is 4.5% for most loci, 15% for DYS389II, and 10% for DYS448 and DYS635.

2.4.1.3 See Appendix 4 for stutter value table.

2.5 Select "New Template".

2.6 Enter a name for the new template and select "Save".

2.7 Ensure the correct primer mix (Mix A or Mix B) is selected.

2.8 Click the check boxes by the loci the need to be edited.

2.9 Edit the necessary information (Analytical Threshold, Interpretation Threshold, or Stutter Filter).

2.10 To edit the Intralocus Balance flag percentage, select the "Edit" button in the gray banner containing that information.

2.11 Edit the appropriate Intralocus Balance value and select "Save".

## 3 Enable Sample History

3.1 Log into an administrator account.

3.2 Select the administrator's email address at the top of the screen.

3.3 Select "Maintenance" from the menu.

3.4 Select "Define Content"

3.5 Sample History is at the top of the page.

3.6 Ensure "Enable Sample History" is toggled on (to the right).

## 4 Data Management

4.1 Log into an administrator account.

4.2 Select the administrator's email address at the top of the screen.

4.3 Select "Maintenance" from the menu.

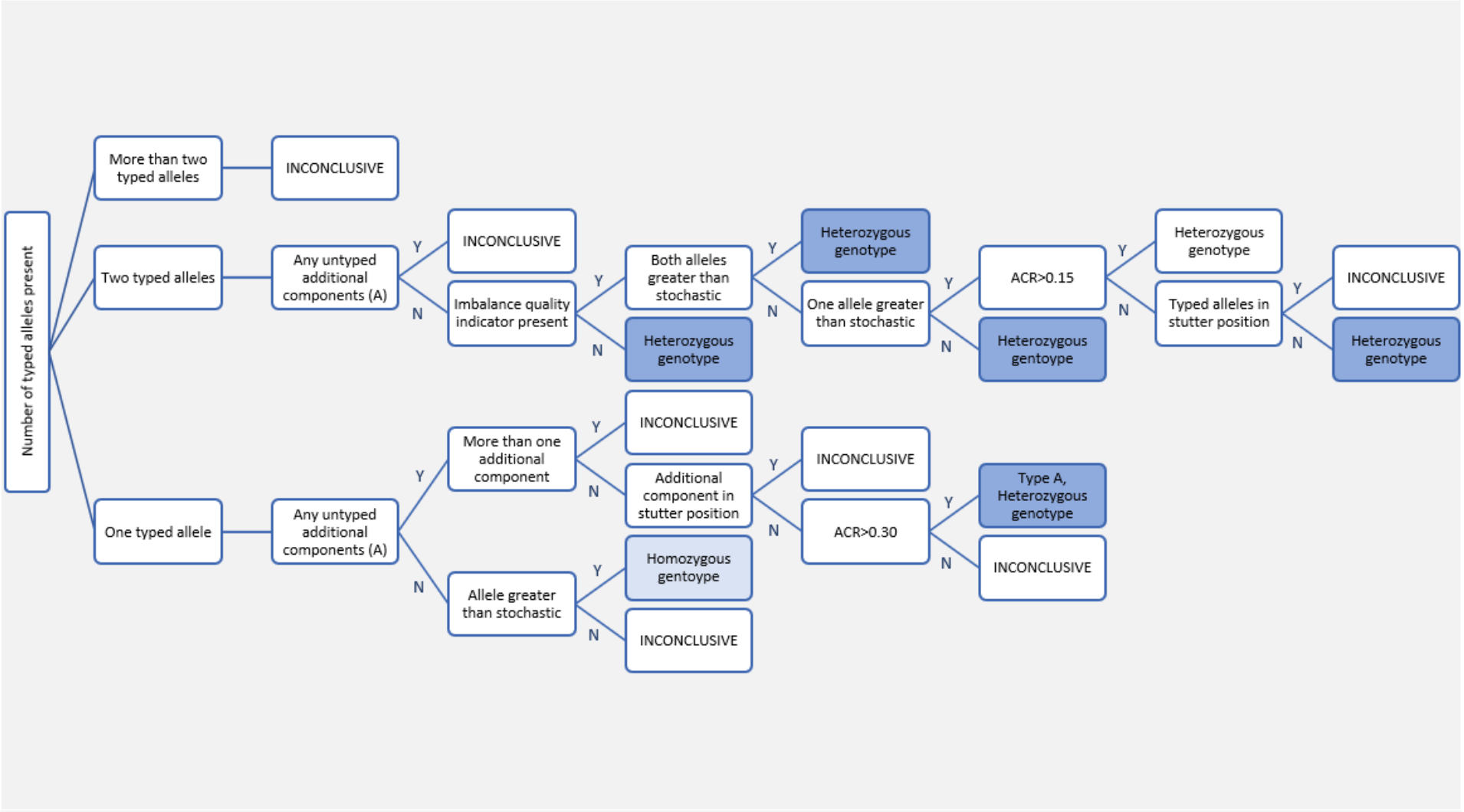
4.4 Select "Export Data".

# INDIANA STATE POLICE
# FORENSIC BIOLOGY SECTION
# TEST METHODS
# APPENDIX 4
# ForenSeq™ DNA Signature Prep Stutter Filters

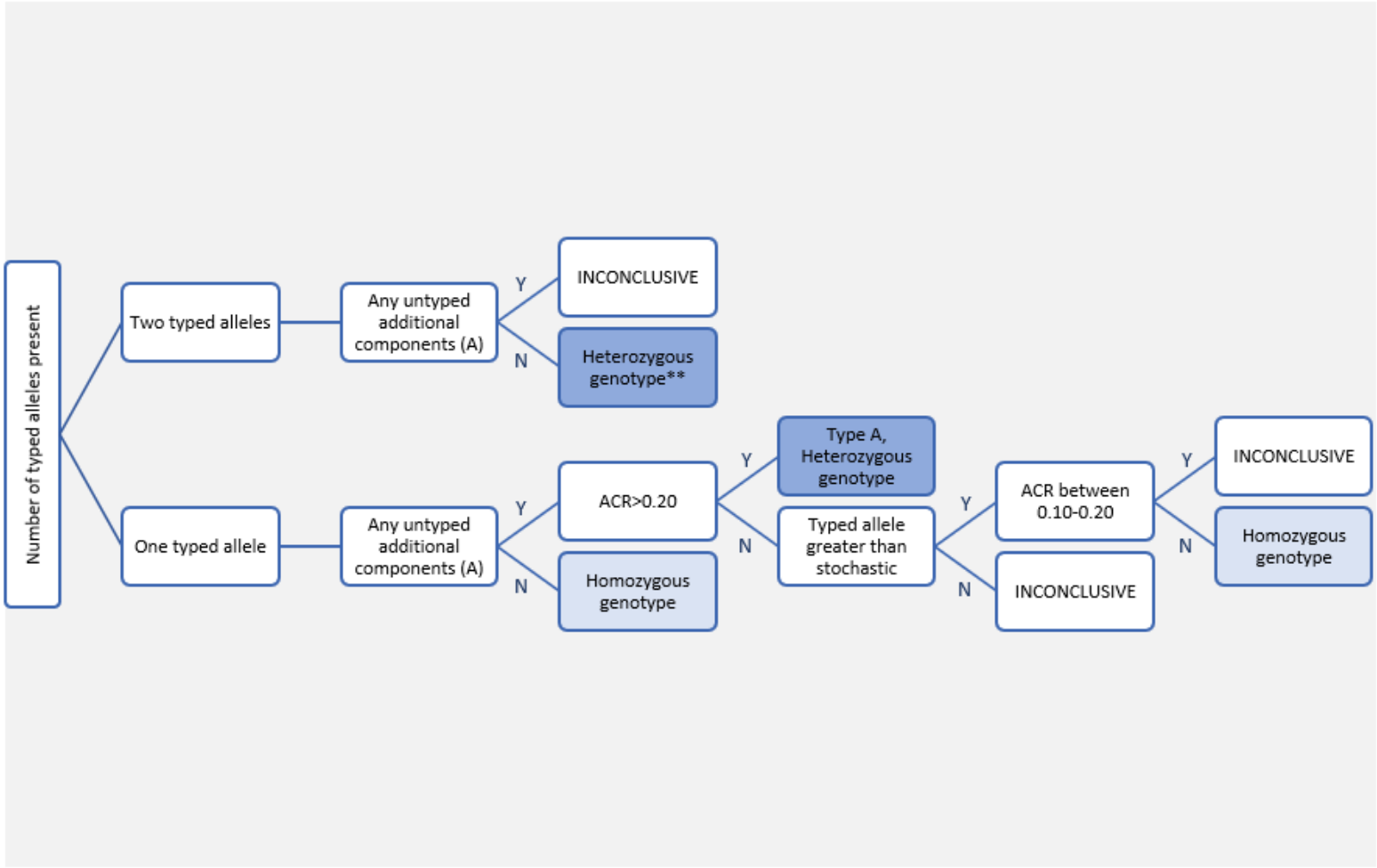
Stutter filters utilized by the Universal Analysis Software have been internally determined and validated through methods outlined in the Verogen MiSeq FGx® and ForenSeq™ DNA Signature Prep Kit Primer Set B validation. The back stutter filter values listed in the table below are the mean + 3SD of the average observed stutter for each locus rounded to the nearest whole percent. The forward and double back stutter are listed as the square of the corresponding back stutter.

| | Back Stutter | Forward & Double Back Stutter | | | Back Stutter | Forward & Double Back Stutter |
|---|---|---|---|---|---|---|
| D1S1656 | 23.00% | 5.29% | | DXS7132 | 21.00% | 4.41% |
| TPOX | 7.00% | 0.49% | | DXS10074 | 12.00% | 1.44% |
| D2S441 | 5.00% | 0.25% | | DXS10103 | 17.00% | 2.89% |
| D2S1338 | 23.00% | 5.29% | | HPRTB | 6.00% | 0.36% |
| D3S1358 | 13.00% | 1.69% | | DXS7423 | 7.00% | 0.49% |
| D4S2408 | 6.00% | 0.36% | | DYS505 | 10.00% | 1.00% |
| FGA | 25.00% | 6.25% | | DYS570 | 20.00% | 4.00% |
| D5S818 | 10.00% | 1.00% | | DYS576 | 14.00% | 1.96% |
| CSF1PO | 7.00% | 0.49% | | DYS522 | 9.00% | 0.81% |
| D6S1043 | 15.00% | 2.25% | | DYS481 | 44.00% | 19.36% |
| D7S820 | 11.00% | 1.21% | | DYS19 | 13.00% | 1.69% |
| D8S1179 | 23.00% | 5.29% | | DYS391 | 15.00% | 2.25% |
| D9S1122 | 14.00% | 1.96% | | DYS635 | 10.00% | 1.00% |
| D10S1248 | 17.00% | 2.89% | | DYS437 | 11.00% | 1.21% |
| TH01 | 11.00% | 1.21% | | DYS439 | 8.00% | 0.64% |
| vWA | 21.00% | 4.41% | | DYS389I | 17.00% | 2.89% |
| D12S391 | 30.00% | 9.00% | | DYS389II | 19.00% | 3.61% |
| D13S317 | 7.00% | 0.49% | | DYS438 | 3.00% | 0.09% |
| PentaE | 9.00% | 0.81% | | DYS612 | 40.00% | 16.00% |
| D16S539 | 20.00% | 4.00% | | DYS390 | 12.00% | 1.44% |
| D17S1301 | 20.00% | 4.00% | | DYS643 | 7.00% | 0.49% |
| D18S51 | 25.00% | 6.25% | | DYS533 | 9.00% | 0.81% |
| D19S433 | 13.00% | 1.69% | | Y-GATA-H4 | 15.00% | 2.25% |
| D20S482 | 17.00% | 2.89% | | DYS385a-b | 24.00% | 5.76% |
| D21S11 | 12.00% | 1.44% | | DYS460 | 12.00% | 1.44% |
| PentaD | 5.00% | 0.25% | | DYS549 | 7.00% | 0.49% |
| D22S1045 | 15.00% | 2.25% | | DYS392 | 23.00% | 5.29% |
| DXS10135 | 20.00% | 4.00% | | DYS448 | 4.00% | 0.16% |
| DXS8378 | 8.00% | 0.64% | | DYF387S1 | 20.00% | 4.00% |

# APPENDIX 5
# ForenSeq™ DNA Signature Prep Interpretation Guide (STRs)



# ForenSeq™ DNA Signature Prep Interpretation Guide (SNPs)




**If ACR is <0.10, the locus may be called inconclusive at the analyst's discretion.

# APPENDIX 6
# Verogen's Universal Analysis Software v2.0+

## 5 Setting Up User Profile

5.1 Open the UAS log-in screen.

5.2 Go to Register As A New User.

5.3 Enter email and password and select Register.

5.4 An administrator will log in.

5.5 Go to Settings, User Management.

5.6 The administrator will enable the person who registered.

## 6 Creating an Analysis Method

6.1 Log into an administrator account.

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

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

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

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

## 7 Enable Sample History

7.1 Log into an administrator account.

7.2 Go to Settings, then System Settings.

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

## 8 Data Management

8.1 Log into an administrator account.

8.2 Go to Settings, then Data Management.

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

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

8.5 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.

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