ForenSeq DNA Signature Prep Reference Guide

VEROGEN PROPRIETARY
Document # VD2018005 Rev. C
August 2020

For Research, Forensic, or Paternity Use Only– not for any clinical or therapeutic use in humans or animals.

This product includes 2800M Control DNA manufactured by Promega Corporation for distribution by Verogen, Inc.

Promega

Notice to Purchaser: Limited License

The purchase price of the Product includes a limited, non-transferable license under U.S. and foreign patents owned by BIO-RAD Laboratories, Inc., to use this Product. No other license under these patents is conveyed expressly or by implication to the purchaser by the purchase of this Product.

The Product is sold under license from Affibody AB, Sweden.

Phusion DNA Polymerase is manufactured by Thermo Fisher Scientific. Phusion is a trademark or registered trademark of Thermo Fisher Scientific, or its subsidiaries.

Chinese Patent No. ZL201580018499.1
Russian Patent No. RU2708337C2
United States Patent No. US10422002B2

Revision History

Document #	Date	Description of Change
VD2018005 Rev. C	August 2020	Reverted the HT1 volume to 591 µl.
VD2018005 Rev. B	July 2020	Updated volumes added to DNL tube to 589 µl HT1 and 4 µl denatured HSC. Added Chinese, Russian, and US patent numbers to the legal notice.
VD2018005 Rev. A	June 2018	Updated document number to Verogen document number. Updated technical support information with Verogen contacts. Updated the location of SDS information on the Verogen website. Added information for the ForenSeq DNA Signature Prep Kit, 96 reactions, catalog # TG-450-1002. Added cap insert colors to kit components. Added maximum plexity by flow cell type for DNA Primer Mix A and DNA Primer Mix B. Updated protocol steps for Amplify and Tag DNA procedure, and separated instructions by input type. Added additional thermal cycler recommendations. Updated information for autosomal STRs, Y haplotype markers, and X haplotype markers to be consistent with hg38 human genome build and polymorphism distributions in STRBase. Added information about interpreting loci D22S1045 and DYS392.

Document #	Date	Description of Change
15049528 v01	September 2015	Updated introduction to indicate that DPMA contains primer pairs for 58 STRs and 94 identity-informative SNPs Updated this document to current format for library prep documentation. Revised instructions to be more succinct Changed reference from PCR Product to library following amplification Removed reference to obsolete Experienced User Card and added reference to new protocol guide and checklist Removed kit box and tube part numbers Removed pipettes from *Consumables* as they are standard lab items Removed thermal cycler from pre-PCR *Equipment* Corrected 2800M Control Alleles in the *Loci* tables for the following. • rs279844, DYS612, Y-GATA-H4, DXS10103 • rs1805007, rs1294331, rs1413212, rs993934 • rs1355366, rs2399332, rs1979255, rs2046361 • rs251934, rs338882, rs1336071, rs214955 • rs727811, rs763869, rs1463729, rs3780962 • rs735155, rs2111980, rs2920816, rs1335873 • rs1886510, rs354439, rs722290, rs1821380 • rs1382387, rs729172, rs1024116, rs1736442 • rs719366, rs1800407, rs2814778, rs3737576 • rs1876482, rs3827760, rs1229984, rs3811801 • rs870347, rs1871534, rs2196051, rs3814134 • rs1079597, rs1572018, rs1800414, rs2593595 • rs4411548, rs2042762, rs3916235, rs310644 Removed loci rs7520386 from Identity Informative SNPs *Loci* table Corrected amplicon lengths for DXS8378 in the X Haplotype Markers *Loci* table Changed *Loci* table headers from Target Start to Amplicon Start Position and Target End to Amplicon End Position and defined positions Incorporated alternate procedures to prepare FTA Card into *Amplify and Tag Targets* procedures Added 2800M as a positive template control to prepare FTA Card

15049528 Rev. D	February 2015	• Updated introduction to indicate that DNA Primer Mix A supports 7 X haplotype markers
		• Removed 1000 µl pipettes and tips from *Consumables and Equipment*
		• In the *Loci* tables:
		  • Moved SNPs rs16891982 and rs12913832 from phenotypic-informative SNP to ancestry-informative SNP and indicated that they are used for both predictions.
		  • Corrected the vWA minimum and maximum amplicon length

Document #	Date	Description of Change

15049528 Rev. C	January 2015	• Updated *Loci* tables:
		  • Revised autosomal STR, Y haplotype marker, and X haplotype marker amplicon lengths
		  • Removed X haplotype marker DXS10148
		  • Changed right column heading of identity, phenotypic, and ancestry-informative SNPs to 2800M Control Alleles
		• Added number of reactions supported to *Kit Contents*
		• Changed catalog numbers for kit, guide, and experienced user card
		• Changed MiSeq FGx Reagent Kit name and catalog number

15049528 Rev. B	September 2014	• Modified the reagent volumes in the *Amplify and Tag Targets* and *Prepare FTA Card* master mix tables to actual reagent volumes without overage • Corrected locus D5S818 2800M Control alleles to 12,12 • Updated *Additional Resources* to remove updated support page url and remove web navigation instructions and written urls • Separated seal and shake as separate substeps • Updated SDS link to support.illumina.com/sds.html
15049528 Rev. A	August 2014	Initial release.

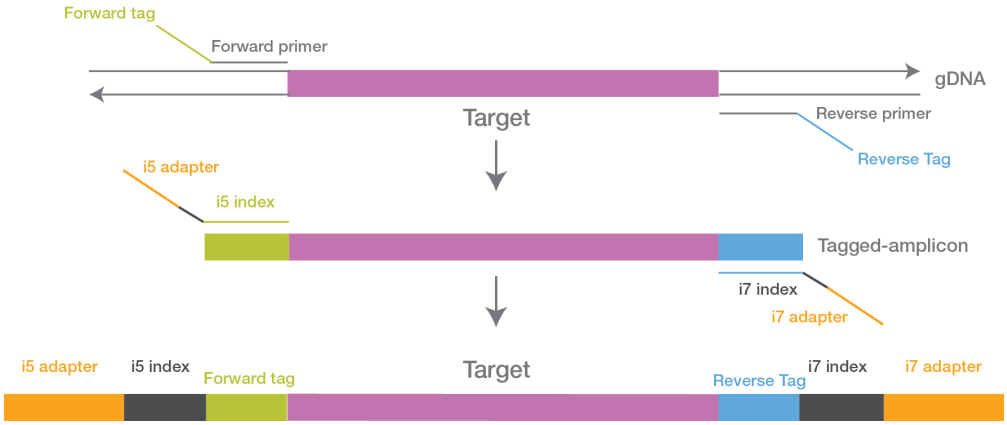
Table of Contents

Introduction

This protocol explains how to prepare DNA libraries using the reagents provided in the Verogen ForenSeq™ DNA Signature Prep Kit to genotype database or casework reference samples in a single sequencing run.

A primer mix containing a pair of tagged oligos for each target sequence is mixed with the DNA sample. PCR cycles link the tags to copies of each target to form DNA templates consisting of the regions of interest flanked by universal primer sequences. The tags are used to attach indexed adapters, which are then amplified using PCR, purified, pooled into a single tube, and then sequenced. The index sequences allow the sequencing system to separate and isolate the data generated from each sample.

Figure 1 ForenSeq DNA Signature Prep Overview



Targeted primer mixes enable analysis of autosomal, Y- and X-chromosome Short Tandem Repeat (STR) targets, identity-informative SNPs, with the option to include ancestry-informative and phenotypic-informative SNPs depending on which primer mix is used. The ForenSeq DNA Signature Prep enables analysis of these markers on gDNA ranging from high-quality single source to difficult samples. This process is done within a single reaction with integrated indexing to support sequencing of up to 96 database (DNA Primer Mix A) or 32 casework (DNA Primer Mix B) samples per standard flow cell run. ForenSeq DNA Signature Prep applies the long paired-end read capability and high data quality of your Illumina sequencing system.

The ForenSeq DNA Signature Prep Kit offers:

- Multiplexing—Amplify STR and SNP amplicons in a single reaction, and sequence up to 96 samples in a single sequencing run.
- Two different primer mixes:
  - DNA Primer Mix A—Contains primer pairs for 58 STRs (including 27 autosomal STRs and 7 X and 24 Y haplotype markers) and 94 identity-informative SNPs.
  - DNA Primer Mix B—Contains all markers in DNA Primer Mix A, plus primer pairs for 56 ancestry-informative SNPs and 22 phenotypic-informative SNPs (2 ancestry-informative SNPs are also used for phenotype prediction).
- Library generation—Allows for simultaneous preparation of up to 96 samples to generate libraries of PCR products within a single plate. Each library is a collection of amplified DNA fragments from a single sample.

DNA Input Recommendations

It is important to quantify the input DNA and assess the DNA quality before beginning the ForenSeq DNA Signature Prep protocol. Follow these DNA input recommendations:

- 1 ng of human genomic DNA (gDNA) input is recommended.
- Use a fluorometric based method for quantification, such as qPCR.
- The ForenSeq DNA Signature Prep Kit is compatible with lysates from buccal swabs and FTA card stains as DNA input.
  - If using crude lysates, 2 µl input material is required per sample. See *Equipment* on page 30 for recommended lysis buffers.
  - If using FTA paper, a 1.2 mm FTA card punch per sample is required.

Protocol Introduction

- Processing fewer than eight samples at the same time, including positive and negative controls, can cause problems with pipetting accuracy due to the small volumes used when preparing the master mix.
- For experimental and sequencing planning, refer to the supported maximum sequencing plexity on the MiSeq FGx as described in Table 1.

Table 1 Maximum Sequencing Plexity by Flow Cell Type

Primer Mix	Standard Flow Cell	Micro Flow Cell
DNA Primer Mix A	96	36
DNA Primer Mix B	32	12

- Create a sample sheet to record the positions of each sample and index adapter. For more information, see the *ForenSeq Universal Analysis Software User Guide (document # VD2018007)*.
- Follow the protocol in the order shown using the specified volumes and incubation parameters.
- Confirm kit contents and make sure that you have the required equipment and consumables. For more information, see *Supporting Kit Information* on page 26.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.
- Set up PCR-1 (copy and tag) in a pre-PCR environment.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is effective for thermal cycling and easy to cut when using fewer than 96 wells.

Mixing

- Always centrifuge plates and tubes briefly after mixing.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

Library Prep Workflow

Figure 2 ForenSeq DNA Signature Prep Workflow

Amplify and Tag Targets

This process amplifies and tags the gDNA using a ForenSeq oligonucleotide primer mix with regions specific to DNA sequences upstream and downstream of STRs and SNPs.

This protocol requires Control DNA 2800M and a negative PCR amplification control (nuclease-free water) in each experiment. If these controls are not included, troubleshooting support is limited.

NOTE
Processing fewer than eight samples at the same time, including positive and negative controls, can affect pipetting accuracy due to the small volumes used when preparing the master mix.

Consumables

- 2800M (Control DNA 2800M)
- One of the following:
  - DPMA (DNA Primer Mix A)
  - DPMB (DNA Primer Mix B)
- FEM (Enzyme Mix)
- PCR1 (PCR1 Reaction Mix)
- 1.5 ml microcentrifuge tubes (2)
- 96-well 0.3 ml PCR plate, skirted or semiskirted
- Human gDNA:
  - Purified DNA (1 ng per sample)
  - Crude lysate (2 µl per sample)
  - FTA card (1.2 mm punch per sample)
- [For FTA card] 1X TBE buffer (100 µl per FTA card punch)
- Microseal 'A' film
- Microseal 'B' adhesive seal
- Nuclease-free water
- [Optional] RNase/DNase-free 8-tube strip and caps

NOTE
Use Microseal 'A' when sealing the plate before placing on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

About Reagents

- For information on the loci detected with DPMA and DPMB, see *Loci* on page 32.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
DPMA or DPMB	-25°C to -15°C	Thaw at room temperature.
FEM	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
PCR1	-25°C to -15°C	Thaw at room temperature.
1X TBE buffer	-25°C to -15°C	Thaw at room temperature.
2800M	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2. Create a sample sheet to record the positions of each sample and index adapter.
3. Save the following PCR1 program on the thermal cycler in the post-amplification area.

CAUTION
Failure to use the thermal ramping mode for your thermal cycler can have an adverse effect on results. See ramping modes for selected *Thermal Cyclers* on page 31.

- Choose the preheat lid option and set to 100°C
- 98°C for 3 minutes
- 8 cycles of:
  - 96°C for 45 seconds
  - 80°C for 30 seconds
  - 54°C for 2 minutes, with specified ramping mode
  - 68°C for 2 minutes, with specified ramping mode
- 10 cycles of:
  - 96°C for 30 seconds
  - 68°C for 3 minutes, with specified ramping mode
- 68°C for 10 minutes
- Hold at 10°C

NOTE
The PCR1 program takes approximately 3.5 hours and can be run overnight.

4. Label tube and plates with a marker as follows.
   - [For Purified DNA or Crude lysate] Master Mix — 1.5 ml microcentrifuge tube
   - [For FTA card] FTA Master Mix — 1.5 ml microcentrifuge tube
   - FSP (ForenSeq Sample Plate) — PCR plate

Procedure for Purified DNA

1. Quantify gDNA using a fluorometric-based method or qPCR.
2. Dilute 1 ng purified DNA input material to 0.2 ng/µl with nuclease-free water.
3. Create a master mix for eight or more reactions in the Master Mix tube. Multiply each reagent volume by the number of reactions being prepared. Make 10% extra reagent for overage.
   - PCR1 (4.7 µl)
   - FEM (0.3 µl)
   - DPMA or DPMB (5.0 µl)
4. Pipette to mix and then centrifuge briefly.
5. If processing more than eight samples, evenly distribute the master mix into each well of an eight-tube strip, and then use a multichannel pipette to dispense.
6. Add 10 µl master mix to each well of the FSP plate.
7. Dilute 2 µl 2800M with 98 µl nuclease-free water in a new 1.5 ml microcentrifuge tube. Gently flick the tube and then centrifuge briefly.
8. Add 5 µl diluted 2800M as a positive template control to the appropriate well according to the sample sheet.
9. Add 5 µl nuclease-free water as a negative PCR amplification control to the appropriate well according to the sample sheet.

10 Add 5 µl diluted purified DNA (0.2 ng/µl) sample to each well according to the sample sheet. Pipette to mix.

11 Seal the plate and centrifuge at 1000 × g for 30 seconds.

12 Transport to the post-PCR area.

13 Place the plate on the thermal cycler and run the PCR1 program.

NOTE
Unless you are stopping, proceed to *Enrich Targets* on page 16.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Procedure for Crude Lysate

1 Quantify gDNA using a fluorometric-based method or qPCR.

2 Create a master mix for eight or more reactions in the Master Mix tube. Multiply each reagent volume by the number of reactions being prepared. Make 10% extra reagent for overage.
- PCR1 (4.7 µl)
- FEM (0.3 µl)
- DPMA or DPMB (5.0 µl)
- Nuclease-free water (3.0 µl)

3 Pipette to mix and then centrifuge briefly.

4 If processing more than eight samples, evenly distribute the master mix into each well of an eight-tube strip, and then use a multichannel pipette to dispense.

5 Add 13 µl master mix to each well of the FSP plate.

6 Dilute 2 µl 2800M with 38 µl nuclease-free water in a new 1.5 ml microcentrifuge tube.

7 Vortex the tube and then centrifuge briefly.

8 Add 2 µl diluted 2800M as a positive template control to the appropriate wells according to the sample sheet.

9 Add 2 µl nuclease-free water as a negative PCR amplification control to the appropriate wells according to the sample sheet.

10 Add 2 µl diluted crude lysate sample to each well.

11 Seal the plate and centrifuge at 1000 × g for 30 seconds.

12 Transport to the post-PCR area.

13 Place the plate on the thermal cycler and run the PCR1 program.

NOTE
Unless you are stopping, proceed to *Enrich Targets* on page 16.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Procedure for FTA Card Input Material

1. Quantify gDNA using a fluorometric-based method or qPCR.
2. Place a 1.2 mm FTA card punch into each well of the FSP plate according to the sample sheet.
3. Add 100 µl 1X TBE buffer.
4. Place on a PCR tube storage rack.
5. Shake at 1800 rpm for 2 minutes.
6. Centrifuge at 1000 × g for 30 seconds.
7. Remove and discard all supernatant.
8. Add the following reagents to each well of the FSP plate intended for positive and negative template controls:
   - PCR1 (4.7 µl)
   - FEM (0.3 µl)
   - DPMA or DMPB (5.0 µl)
9. Dilute 2 µl 2800M with 98 µl nuclease-free water in a new 1.5 ml microcentrifuge tube. Gently flick the tube and then centrifuge briefly.
10. Add 5 µl diluted 2800M as a positive template control to the appropriate wells containing reagents from step 8 according to the sample sheet. Pipette to mix.
11. Add 5 µl nuclease-free water as a negative PCR amplification control to the appropriate wells containing reagents from step 8 according to the sample sheet. Pipette to mix.
12. Create FTA sample master mix for eight or more reactions in the FTA Master Mix tube. Multiply each reagent volume by the number of reactions being prepared. Make 10% extra reagent for overage.
    - PCR1 (4.7 µl)
    - FEM (0.3 µl)
    - DPMA or DPMB (5.0 µl)
    - Nuclease-free water (5.0 µl)
13. Pipette to mix and then centrifuge briefly.
14. If processing more than eight samples, evenly distribute the master mix into each well of an eight-tube strip, and then use a multichannel pipette to dispense.
15. Add 15 µl FTA master mix to each well containing FTA punch in the FSP plate.
16. Seal the plate and centrifuge at 1000 × g for 30 seconds.
17. Transport to the post-PCR area.
18. Place the plate on the thermal cycler and run the PCR1 program.

NOTE
Unless you are stopping, proceed to *Enrich Targets* on page 16.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Enrich Targets

This process amplifies the DNA and adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for cluster amplification.

The index adapters tag DNA with a unique combination of index sequences, which allow data from each tagged library to be separated during later analysis.

NOTE
This procedure is described using a 96-well PCR plate. However, when processing eight libraries, it can be performed with an eight-tube strip.

Consumables

- ForenSeq Index Plate Fixture Kit
- Index 1 (i7) adapters and orange tube caps
- Index 2 (i5) adapters and white tube caps
- PCR2 (PCR2 Reaction Mix)
- 1.7 ml microcentrifuge tubes (1 per index adapter tube)
- Microseal 'A' film
- Microseal 'B' adhesive seal

NOTE
Use Microseal 'A' when sealing the plate before placing on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

About Reagents

- If processing more than eight libraries at the same time, evenly distribute PCR2 to each well of an eight-tube strip, and then use a multichannel pipette to dispense.
- Add PCR2 slowly to each well to avoid creating air bubbles.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
Index adapters (i5 and i7)	-25°C to -15°C	Only remove adapters being used. Thaw at room temperature for 20 minutes. Vortex each tube to mix. Centrifuge briefly using a 1.7 ml Eppendorf tube.
PCR2	-25°C to -15°C	Thaw at room temperature.

2 Save the following PCR2 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 98°C for 30 seconds
- 15 cycles of:
  - 98°C for 20 seconds
  - 66°C for 30 seconds
  - 68°C for 90 seconds
- 68°C for 10 minutes
- Hold at 10°C

Procedure

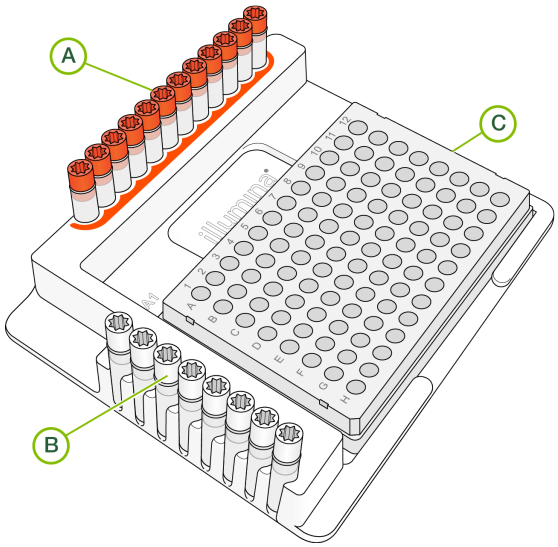
1 Centrifuge the FSP at 1000 × g for 30 seconds.

2 Arrange Index 1 (i7) adapters in columns 1–12 of the ForenSeq Index Plate Fixture.

3 Arrange Index 2 (i5) adapters in rows A–H of the ForenSeq Index Plate Fixture.

4 Place the plate on the ForenSeq Index Plate Fixture.

Figure 3 ForenSeq Index Plate Fixture (96 libraries)



A Columns 1–12: Index 1 (i7) adapters (orange caps)
B Rows A–H: Index 2 (i5) adapters (white caps)
C FSP plate

5 Using a multichannel pipette, add 4 µl Index 1 (i7) adapters to each column. Replace the caps on i7 adapter tubes with new orange caps.

6 Using a multichannel pipette, add 4 µl Index 2 (i5) adapters to each row. Replace the caps on i5 adapter tubes with new white caps.

7 Vortex PCR2 and then centrifuge briefly.

8 Add 27 µl PCR2 to each well.

9 Centrifuge at 1000 × g for 30 seconds.

10 Place the plate on the preprogrammed thermal cycler and run the PCR2 program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 7 days. Alternatively, leave on the thermal cycler overnight.

Purify Libraries

This process uses SPB (Sample Purification Beads) to purify the amplified libraries from the other reaction components.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- 96-well 0.3 ml PCR plate, skirted or semiskirted
- 96-well midi plates (2 plates, if processing 16–96 libraries)
- Freshly prepared 80% ethanol (EtOH)
- Microseal 'B' adhesive seals
- RNase/DNase-free reagent reservoirs (2 reservoirs, if processing more than 96 libraries)

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

NOTE

To ensure optimal performance and library yield, make sure that the SPB beads are brought to room temperature fully before use.

2 Label plates with a marker as follows.
- PBP (Purification Bead Plate) — midi plate
- PLP (Purified Library Plate) — PCR plate

Procedure

1 Prepare SPB according to the number of libraries you are preparing.

Number of Libraries	Procedure
< 16	Add 50 µl SPB × the number of libraries to a 1.7 ml microcentrifuge tube.
16–96	Add [50 µl SPB × (the number of libraries/8)] + 5 µl SPB to each well of a column of a new midi plate or reagent reservoir.
> 96	Add (50 µl SPB × the number of libraries) + 200 µl SPB to a multichannel reagent reservoir.

2 Add 45 µl SPB to each well of the PBP plate according to the sample sheet.

3 Centrifuge the FSP plate at 1000 × g for 30 seconds.

4 Transfer 45 µl to the corresponding well of the PBP plate, according to the sample sheet.

5 Seal the plate with Microseal 'B' and shake at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 5 minutes.

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

8 Remove and discard all supernatant from each well.

9 Wash two times as follows:

   a Add 200 µl freshly prepared 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

10 Centrifuge at 1000 × g for 30 seconds.

11 Place the plate on the magnetic stand.

12 Use a 20 µl pipette to remove residual EtOH from each well.

13 Remove the plate from the magnetic stand.

14 Add 52.5 µl RSB to each well.

15 Seal the plate with Microseal 'B' and shake at 1800 rpm for 2 minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 2 minutes.

16 Incubate at room temperature for 2 minutes.

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

18 Transfer 50 µl to the corresponding well of the PLP plate.

19 Centrifuge at 1000 × g for 30 seconds.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to one year.

Normalize Libraries

This process prepares DNA libraries for cluster generation to make sure that libraries of varying yields are equally represented within the sequencing run. This process assures that samples with varying input amounts or sample types achieve consistent cluster density to optimize the resolution of individual samples when pooled together. By normalizing the concentration of the libraries, while preserving the content of each library, post-PCR quantification and individual PCR product normalization are not necessary.

Consumables

- HP3 (2N-NaOH)
- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNS2 (Library Normalization Storage Buffer 2)
- LNW1 (Library Normalization Wash 1)
- 1.5 ml microcentrifuge tube
- One of the following:
  - 1.5 ml microcentrifuge tube
  - 15 ml conical tube
- 96-well 0.3 ml PCR plate, skirted or semiskirted
- 96-well midi plate
- Microseal 'B' adhesive seals
- Nuclease-free water
- RNase/DNase-free Reagent Reservoir

WARNING

This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin.

LNA1 and LNW1 contain ß-mercaptoethanol and prolonged exposure can be toxic to the nervous system and cause organ damage.

Perform this procedure in a hood or well-ventilated area if desired. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. For more information, see how to access safety data sheets (SDSs) in *Technical Assistance* on page 41.

Supernatant, excess LNA1/LNB1 Master Mix, and tips used to pipette LNA1 and LNB1 are hazardous waste. Discard in accordance with the governmental safety standards for your region.

About Reagents

- After vortexing, hold LNA1 in front of a light and make sure that no crystals are present and all precipitate has dissolved.
- After vortexing, make sure that LNB1 beads are well-resuspended and no pellet remains at the bottom of the tube.
- It is critical to resuspend the LNB1 bead pellet at the bottom of the tube. Use a 1000 µl pipette to make sure that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. Resuspension is essential for achieving consistent cluster density to optimize the resolution of individual libraries when pooled together.
- The library that remains in the PLP plate can be stored. Seal the PLP plate and store at -25°C to -15°C for up to 1 year.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
HP3	-25°C to -15°C	Thaw at room temperature.
LNA1	-25°C to -15°C	Thaw at room temperature. Vortex with intermittent inversion.
LNB1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex for at least 1 minute, inverting 5 times every 15 seconds. Pipette to mix until the bead pellet at the bottom is resuspended.
LNW1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
LNS2	15°C to 30°C	Remove from storage.

2 Label tubes and plates with a marker as follows.
- LNA1/LNB1 Master Mix — 1.5 ml microcentrifuge tube or 15 ml conical tube
- NWP (Normalization Working Plate) — midi plate
- NLP (Normalization Library Plate) — PCR plate

3 Dedicate separate hazardous waste disposal containers for liquids and solids.

Procedure

1 Create a master mix in the LNA1/LNB1 Master Mix tube.
- LNA1 (46.8 µl per sample) for example, eight reactions require 374 µl.
- LNB1 (8.5 µl per sample) For example, eight reactions require 68 µl.

2 Vortex and then invert the tube several times to mix.

3 Pour into a reagent reservoir.

4 Transfer 45 µl to each well of the NWP plate that will contain a library according to the sample sheet.

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

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

7 Seal the plate with Microseal 'B' and shake at 1800 rpm for 30 minutes.

8 While the plate is shaking, perform the following steps:

a Prepare 0.1 N HP3 in a new 1.5 ml microcentrifuge tube, as follows:
- Nuclease-free water (33.3 µl per sample) For example, eight reactions require 266.4 µl.
- HP3 (1.8 µl per sample) For example, eight reactions require 14.4 µl.
- Invert the tube several times to mix.
- Set aside.

b Add 30 µl LNS2 to each well of the NLP plate that will contain a library according to the sample sheet.

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

10 Remove and discard all supernatant from each well.

11 Remove the plate from the magnetic stand.

12 Wash two times with 45 µl LNW1 as follows:

   a Add 45 µl LNW1 to each well.
   b Seal the plate with Microseal 'B' and shake at 1800 rpm for 5 minutes.
   c Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
   d Remove and discard all supernatant from each well.

13 Remove the plate from the magnetic stand.

14 Centrifuge at 1000 × g for 30 seconds.

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

16 Use a 20 µl pipette to remove residual supernatant from each well.

17 Remove the plate from the magnetic stand.

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

19 Seal the plate with Microseal 'B' 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.

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

21 Transfer 30 µl to the corresponding well of the NLP plate. Pipette to mix.

22 Centrifuge at 1000 × g for 30 seconds.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Pool Libraries

This process combines equal volumes of normalized library to create a pool of libraries that are sequenced together on the same flow cell.

Consumables

- 1.5 ml microcentrifuge tube
- Microseal 'B' adhesive seal
- RNase/DNase-free eight-tube strip and caps

Preparation

1. Determine which libraries to pool for sequencing.

   NOTE
   For recommendations on supported maximum pooling numbers, see Table 1 on page 9.

2. Label the tube PNL to indicate Pooled Normalized Libraries.

Procedure

1. Transfer 5 µl of each library to a new eight-tube strip.
2. Store the plate in the post-PCR area at -25°C to -15°C for up to 30 days.
3. Transfer the contents of each well of the eight-tube strip to the PNL tube.
4. Vortex and then centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, cap the tube and store at -25°C to -15°C for up to 30 days.

Denature and Dilute Libraries

This process dilutes the libraries in HT1 (Hybridization Buffer), adds HSC (Human Sequencing Control), and heat denatures the libraries in preparation for sequencing.

NOTE
Perform this process immediately before loading the library onto the reagent cartridge to ensure efficient template loading on the flow cell.

Consumables

- ForenSeq DNA Signature Prep Kit contents:
  - HP3 (2N-NaOH)
  - HSC (Human Sequencing Control)
- 1.5 ml microcentrifuge tubes (2)
- MiSeq FGx Reagent Kit contents:
  - HT1 (Hybridization Buffer)
  - Reagent cartridge
- Nuclease-free water

About Reagents

- Follow *Prepare the Reagent Cartridge* instructions in the *MiSeq FGx Instrument Reference Guide (document # VD2018006)*.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
HP3	-25°C to -15°C	Thaw at room temperature.
HSC	-25°C to -15°C	Thaw at room temperature.
HT1	-25°C to -15°C	Thaw at room temperature.
Reagent cartridge	-25°C to -15°C	Thaw at room temperature.

2 Preheat the microheating system to 96°C.

3 Prepare either of the following:
- Remove a tube benchtop cooler from -25°C to -15°C storage or ice bucket.
- Prepare an ice-water bath by combining 3 parts ice and 1 part nuclease-free water.

4 Label tubes with a marker as follows:
- HSC mixture
- DNL to indicate Denatured Normalized Libraries.

Procedure

1 Create an HSC denaturation reaction in the HSC mixture tube.
- HSC (2 µl)
- HP3 (2 µl)
- Nuclease-free water (36 µl)

2 Vortex and then centrifuge briefly.

3 Incubate at room temperature for 5 minutes.

4 Add 591 µl HT1 to the DNL tube.

5 Transfer 7 µl from the PNL tube to the DNL tube. Pipette to mix.

6 Cap the PNL tube and store at -25°C to -15°C for up to 30 days. Exceeding 30 days in storage results in a significant reduction of cluster density.

7 Transfer 4 µl HSC mixture to the DNL tube. Pipette to mix. Do not store HSC mixture long term, which results in a significant reduction of cluster density.

8 Vortex and then centrifuge briefly.

9 Place on the 96°C microheating system for 2 minutes.

10 Invert the tube several times to mix.

11 Immediately place in the ice-water bath or on the -25°C to -15°C benchtop cooler for 5 minutes.

12 Immediately load the entire contents onto the reagent cartridge. For more information, see the *Load Sample Libraries Onto Cartridge* instructions in the *MiSeq FGx Instrument Reference Guide (document # VD2018006)*.

Supporting Kit Information

Kit Contents

Make sure that you have all the reagents identified in this section before starting the protocol.

Kit Name	Catalog #	Number of Reactions
ForenSeq DNA Signature Prep Kit, 384 reactions	TG-450-1001	384
ForenSeq DNA Signature Prep Kit, 96 reactions	TG-450-1002	96

Pre-PCR Box 1

Store each reagent at the temperature specified in the following table.

Quantity (384 rxn) TG-450-1001	Quantity (96 rxn) TG-450-1002	Reagent	Cap Insert Color	Description	Storage Temperature
2	1	2800M	Black	Control DNA 2800M	2°C to 8°C
8	2	PCR1	Green	PCR1 Reaction Mix	-25°C to -15°C
8	2	FEM	Yellow	Enzyme Mix	-25°C to -15°C
8	2	DPMA	Blue	DNA Primer Mix A	-25°C to -15°C
8	2	DPMB	Red	DNA Primer Mix B	-25°C to -15°C

Post-PCR Box 2

Store each reagent at the temperature specified in the following table.

Quantity (384 rxn) TG-450-1001	Quantity (96 rxn) TG-450-1002	Reagent	Cap Insert Color	Description	Storage Temperature
4	1	LNA1	—	Library Normalization Additives 1	-25°C to -15°C
4	1	LNS2	—	Library Normalization Storage Buffer 2	-25°C to -15°C
8	2	LNW1	—	Library Normalization Wash 1	-25°C to -15°C
3	1	HP3	Orange	HP3 2N-NaOH	-25°C to -15°C
8	2	PCR2	Purple	PCR2 Reaction Mix	-25°C to -15°C
1	1	HSC	Pink	Human Seq Control	-25°C to -15°C
1	1	A501	—	A501 Index Adapter	-25°C to -15°C
1	1	A502	—	A502 Index Adapter	-25°C to -15°C
1	1	A503	—	A503 Index Adapter	-25°C to -15°C
1	1	A504	—	A504 Index Adapter	-25°C to -15°C
1	1	A505	—	A505 Index Adapter	-25°C to -15°C
1	1	A506	—	A506 Index Adapter	-25°C to -15°C
1	1	A507	—	A507 Index Adapter	-25°C to -15°C
1	1	A508	—	A508 Index Adapter	-25°C to -15°C
1	1	R701	—	R701 Index Adapter	-25°C to -15°C
1	1	R702	—	R702 Index Adapter	-25°C to -15°C
1	1	R703	—	R703 Index Adapter	-25°C to -15°C

Quantity (384 rxn) TG-450-1001	Quantity (96 rxn) TG-450-1002	Reagent	Cap Insert Color	Description	Storage Temperature
1	1	R704	—	R704 Index Adapter	-25°C to -15°C
1	1	R705	—	R705 Index Adapter	-25°C to -15°C
1	1	R706	—	R706 Index Adapter	-25°C to -15°C
1	1	R707	—	R707 Index Adapter	-25°C to -15°C
1	1	R708	—	R708 Index Adapter	-25°C to -15°C
1	1	R709	—	R709 Index Adapter	-25°C to -15°C
1	1	R710	—	R710 Index Adapter	-25°C to -15°C
1	1	R711	—	R711 Index Adapter	-25°C to -15°C
1	1	R712	—	R712 Index Adapter	-25°C to -15°C
1	1	—	—	i7 Index Tube Caps, Orange	-25°C to -15°C
1	1	—	—	i5 Index Tube Caps, White	-25°C to -15°C

Post-PCR Box 3

Store each reagent at the temperature specified in the following table.

Quantity (384 rxn) TG-450-1001	Quantity (96 rxn) TG-450-1002	Reagent	Cap Insert Color	Description	Storage Temperature
4	1	LNB1	White	Library Normalization Beads 1	2°C to 8°C
1	1	RSB	—	Resuspension Buffer	2°C to 8°C
2	1	SPB	—	Sample Purification Beads	2°C to 8°C

Index Sequences

The kit contains the following index adapter sequences.

Index 1 (i7)

Index 1 (i7)	Sequence		Index 1 (i7)	Sequence
R701	ATCACGAT		R707	CAGATCAT
R702	CGATGTAT		R708	ACTTGAAT
R703	TTAGGCAT		R709	GATCAGAT
R704	TGACCAAT		R710	TAGCTTAT
R705	ACAGTGAT		R711	GGCTACAT
R706	GCCAATAT		R712	CTTGTAAT

Index 2 (i5)

Index 2 (i5)	Sequence
A501	TGAACCTT
A502	TGCTAAGT
A503	TGTTCTCT
A504	TAAGACAC
A505	CTAATCGA
A506	CTAGAACA
A507	TAAGTTCC
A508	TAGACCTA

Acronyms

Acronym	Definition
2800M	Control DNA 2800M
A7XX	i7 Index Adapter
A50X	i5 Index Adapter
DNL	Diluted Normalized Libraries
DPMA	DNA Primer Mix A
DPMB	DNA Primer Mix B
FEM	Enzyme Mix
FSP	ForenSeq Sample Plate
HP3	2N NaOH
HSC	Human Sequencing Control
HT1	Hybridization Buffer
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNS2	Library Normalization Storage Buffer 2
LNW1	Library Normalization Wash 1
NLP	Normalized Library Plate
NWP	Normalization Working Plate
PBP	Purification Bead Plate
PCR1	PCR1 Reaction Mix
PCR2	PCR2 Reaction Mix
PLP	Purified Library Plate
PNL	Pooled Normalized Libraries
RSB	Resuspension Buffer
SPB	Sample Purification Beads

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
1.5 ml microcentrifuge tubes	General lab supplier
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tube	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
96-well 0.3 ml semiskirted PCR plates	Eppendorf Twin-Tec, part # 951020303 or VWR, part # 89136-706
96-well storage plates, round well, 0.8 ml ('midi' plate)	Fisher Scientific, part # AB-0859
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
MiSeq disposable wash tube	Verogen, part # MS-102-9999
Microseal 'A' film	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MiSeq FGx Reagent Kit	Verogen, catalog # TG-143-1001 or Verogen, catalog # TG-143-1002
Nuclease-free water	General lab supplier
PCR tube storage rack (If using an FTA card as input material)	VWR, part # 80086-074
If using crude lysates as input material, select one: • QuickExtract DNA Extraction Solution • SwabSolution Kit	• Epicentre, catalog # QE09050 • Promega, catalog # DC8271
RNase/DNase-free eight-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	Labcor, part # 730-001

Equipment

Equipment	Supplier/Description	Pre-PCR	Post-PCR
1.5 ml tube benchtop cooler	VWR, catalog # 414004-286		X
96-well thermal cycler (with heated lid)	See *Thermal Cyclers* on page 31.		X
Benchtop microcentrifuge	General lab supplier	X	X
ForenSeq Index Plate Fixture	Verogen, catalog # FC-451-1001		X
Magnetic stand-96	Life Technologies, part # AM10027		X
Microplate centrifuge	General lab supplier	X	X
Multichannel pipette 8 channel p20	General lab supplier		X
Multichannel pipette 8 channel p200	General lab supplier		X
1.5 ml 96-well heating system	General lab supplier		X
High-speed thermal mixers; select one: • BioShake iQ • BioShake XP	Q instruments, catalog #: • 1808-0506 • 1808-0505		X
Vortexer	General lab supplier	X	X

Thermal Cyclers

The following table lists the recommended settings for the thermal cycler. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type	Ramp Mode
ABI LTI thermal cycler 9700*	9600 emulation	Heated	Polypropylene plates and tubes	8%
Bio-Rad	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes	0.2°C per second
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate	2%
Veriti 96-well thermal cycler**	Standard	Heated, Constant at 105°C	Polypropylene plates and tubes	4%
Proflex 96-well PCR System**	—	Heated, Constant at 105°C	Polypropylene plates and tubes	0.2°C per second

*For use with gold heat block only. Silver or aluminum heat blocks are not supported.

**Thermal cycler settings were verified after the developmental validation of the ForenSeq DNA Signature Prep Kit.

Loci

- The amplicon length does not include 120 bp for adapter sequences. The amplicon start and end positions are the 1-based endpoints of the entire amplicon including the sequence matching primers on the hg19 human reference genome.
- All loci in DNA Primer Mix A are also included in DNA Primer Mix B.
- SNP alleles are reported as described in dbSNP build 141.
- **Amelogenin**—A genetic marker that confirms the gender of the donor of the biological sample. Its size range is 106–112 bp and the control DNA is male.

Autosomal STRs

The following loci are detected using DNA Primer Mix A or DNA Primer Mix B.

Table 2 Autosomal STRs

Locus	Repeat Range (repeats)	Amplicon Length Range (bp)	Chromosome	2800M Control Alleles
D1S1656	7–21.3	133–192	1	12,13
TPOX	4–16	61–109	2	11,11
D2S441	7–17	137–177	2	10,14
D2S1338	10–33.1	110–203	3	22,25
D3S1358	8–22	138–194	3	17,18
D4S2408	8–13	98–118	4	9,9
FGA	12.2–53	150–312	4	20,23
D5S818	4–20	98–162	5	12,12
CSF1PO	5–17	72–120	5	12,12
D6S1043	8–26	154–226	6	12,20
D7S820**	5–21.1	118–183	7	8,11
D8S1179	6–20	82–138	8	14,15
D9S1122	8–15	104–132	9	12,12
D10S1248	7–20	124–176	10	13,15
TH01	3–14	96–140	11	6,9.3
vWA	11–26	135–195	12	16,19
D12S391	13–28	229–289	12	18,23
D13S317	5–17	138–186	13	9,11
PentaE	5–28.4	362–481	15	7,14
D16S539	4–17	132–184	16	9,13
D17S1301	9–15	130–154	17	11,12
D18S51	6–40	136–272	18	16,18
D19S433	4–27	148–240	19	13,14
D20S482	9–17	125–157	20	14,15
D21S11	12–41.2	147–265	21	29,31.2
PentaD	1.1–19	209–298	21	12,13
D22S1045*	8–19	201–245	22	16,16

* Interpret locus D22S1045 with caution. Elevated n-1 repeat stutter might be observed, particularly with decreased marker coverage. Heterozygote imbalance might be observed regardless of marker coverage. Consider multilocus genotype when determining the presence of a DNA mixture. For more information, see *Interpretation Examples for D22S1045 and DYS392* on page 38.

** A low-level plus .1 base pair artifact might be observed at locus D7S820 with a single T addition at the end of the STR repeat sequence of the parent allele (e.g., 8,8.1 or 11,11.1).

Identity Informative SNPs

The following loci are detected using DNA Primer Mix A or DNA Primer Mix B.

Table 3 Identity Informative SNPs

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs10495407	109	1	238439234	238439342	G
rs1294331	85	1	233448359	233448443	GA
rs1413212	64	1	242806767	242806830	G
rs1490413	98	1	4367256	4367353	A
rs560681	90	1	160786641	160786730	AG
rs891700	115	1	239881850	239881964	AG
rs1109037	118	2	10085691	10085808	G
rs12997453	100	2	182413195	182413294	A
rs876724	119	2	114945	115063	C
rs907100	115	2	239563542	239563656	CG
rs993934	120	2	124109120	124109239	C
rs1355366	119	3	190806041	190806159	AG
rs1357617	120	3	961696	961815	AT
rs2399332	157	3	110300999	110301155	AC
rs4364205	98	3	32417576	32417673	G
rs6444724	120	3	193207306	193207425	T
rs1979255	102	4	190318007	190318108	G
rs2046361	120	4	10968994	10969113	A
rs279844	167	4	46329584	46329750	AT
rs6811238	120	4	169663541	169663660	G
rs13182883	169	5	136633252	136633420	AG
rs159606	104	5	17374845	17374948	A
rs251934	97	5	174778619	174778715	T
rs338882	157	5	178690599	178690755	C
rs717302	110	5	2879333	2879442	G
rs13218440	170	6	12059928	12060097	AG
rs1336071	120	6	94537182	94537301	G
rs214955	120	6	152697629	152697748	G
rs727811	115	6	165045254	165045368	A
rs321198	165	7	137029715	137029879	T
rs6955448	120	7	4310285	4310404	CT
rs737681	120	7	155990742	155990861	T
rs917118	109	7	4456953	4457061	C
rs10092491	116	8	28411037	28411152	CT
rs2056277	104	8	139399038	139399141	C
rs4606077	151	8	144656710	144656860	CT
rs763869	85	8	1375576	1375660	CT
rs1015250	117	9	1823702	1823818	G
rs10776839	103	9	137417271	137417373	G
rs1360288	119	9	128967994	128968112	C
rs1463729	99	9	126881396	126881494	GA
rs7041158	115	9	27985907	27986021	C
rs3780962	94	10	17193284	17193377	T
rs735155	170	10	3374133	3374302	A

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs740598	120	10	118506839	118506958	AG
rs826472	153	10	2406511	2406663	T
rs964681	105	10	132698394	132698498	CT
rs10488710	118	11	115207134	115207251	CG
rs1498553	111	11	5708981	5709091	CT
rs2076848	118	11	134667502	134667619	AT
rs901398	90	11	11096173	11096262	T
rs10773760	99	12	130761623	130761721	AG
rs2107612	103	12	888262	888364	AG
rs2111980	94	12	106328186	106328279	G
rs2269355	65	12	6945881	6945945	C
rs2920816	157	12	40862976	40863132	T
rs1058083	76	13	100038193	100038268	AG
rs1335873	109	13	20901665	20901773	T
rs1886510	116	13	22374646	22374761	CT
rs354439	170	13	106938320	106938489	T
rs1454361	118	14	25850765	25850882	AT
rs4530059	170	14	104769099	104769268	G
rs722290	101	14	53216686	53216786	G
rs873196	114	14	98845506	98845619	CT
rs1528460	115	15	55210664	55210778	T
rs1821380	118	15	39313343	39313460	G
rs8037429	63	15	53616876	53616938	T
rs1382387	89	16	80106318	80106406	GT
rs2342747	104	16	5868645	5868748	AG
rs430046	119	16	78016980	78017098	C
rs729172	104	16	5606153	5606256	C
rs740910	113	17	5706552	5706664	A
rs8078417	143	17	80461847	80461989	CT
rs938283	98	17	77468433	77468530	T
rs9905977	170	17	2919324	2919493	G
rs1024116	98	18	75432317	75432414	A
rs1493232	75	18	1127945	1128019	A
rs1736442	153	18	55225698	55225850	G
rs9951171	119	18	9749789	9749907	G
rs576261	76	19	39559780	39559855	AC
rs719366	170	19	28463281	28463450	T
rs1005533	158	20	39487066	39487223	A
rs1031825	126	20	4447416	4447541	C
rs1523537	117	20	51296076	51296192	C
rs445251	119	20	15124865	15124983	CG
rs221956	97	21	43606933	43607029	C
rs2830795	114	21	28608089	28608202	A
rs2831700	79	21	29679639	29679717	A
rs722098	101	21	16685561	16685661	AG
rs914165	156	21	42415865	42416020	AG
rs1028528	78	22	48362256	48362333	AG
rs2040411	68	22	47836378	47836445	A
rs733164	120	22	27816711	27816830	AG
rs987640	120	22	33559450	33559569	AT

Y Haplotype Markers

The following loci are detected using DNA Primer Mix A or DNA Primer Mix B.

Table 4 Y Haplotype Markers

Locus	Repeat Range (repeats)	Amplicon Length Range (bp)	Chromosome	2800M Control Alleles
DYF387S1	30–44	207–263	Y	37,38
DYS19	9–19	269–309	Y	14
DYS385a-b	7–28	232–316	Y	13,16
DYS389I	9–17	236–268	Y	14
DYS389II	24–34	283–323	Y	31
DYS390	17–28	290–334	Y	24
DYS391	5–16	119–163	Y	10
DYS392*	6–17	318–362	Y	13
DYS437	10–18	194–226	Y	14
DYS438	6–16	129–179	Y	9
DYS439	6–17	167–211	Y	12
DYS448	14–26	330–402	Y	19
DYS460	7–14	348–376	Y	11
DYS481	17–32	129–174	Y	22
DYS505	9–15	162–186	Y	11
DYS522	8–17	298–334	Y	12
DYS533	7–17	186–226	Y	12
DYS549	10–14	210–226	Y	13
DYS570	10–26	142–206	Y	17
DYS576	10–25	163–223	Y	18
DYS612	26–33	275–296	Y	29
DYS635	15–30	242–302	Y	21
DYS643	7–15	141–181	Y	10
Y-GATA-H4	8–15	159–187	Y	11

* Interpret the locus DYS392 with caution. Elevated n-1 repeat stutter might be observed, particularly with decreased marker coverage. Consider multilocus genotype when determining the presence of a DNA mixture. For more information, see *Interpretation Examples for D22S1045 and DYS392* on page 38.

X Haplotype Markers

The following loci are detected using DNA Primer Mix A or DNA Primer Mix B.

Table 5 X Haplotype Markers

Locus	Repeat Range (repeats)	Amplicon Length Range (bp)	Chromosome	2800M Control Alleles
DXS10074	7–22	184–244	X	21
DXS10103	14–21	157–185	X	18
DXS10135	15.3–34	239–312	X	28
DXS7132	11–20	175–211	X	13
DXS7423	10–18	188–220	X	15
DXS8378	8–14	434–458	X	12
HPRTB	8–17	193–229	X	12

Phenotypic Informative SNPs

The following loci are detected when using DNA Primer Mix B. These loci are not present when using DNA Primer Mix A.

Table 6 Phenotypic Informative SNPs

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs28777	92	5	33958916	33959007	A
rs12203592	110	6	396273	396382	C
rs4959270	161	6	457655	457815	AC
rs683	120	9	12709246	12709365	AC
rs1042602	113	11	88911659	88911771	AC
rs1393350	99	11	89010977	89011075	G
rs12821256	119	12	89328278	89328396	CT
rs12896399	73	14	92773627	92773699	G
rs2402130	120	14	92801169	92801288	A
rs1800407	119	15	28230246	28230364	G
N29insA	112	16	89985688	89985799	C
rs1110400	173	16	89986044	89986216	T
rs11547464	173	16	89986044	89986216	G
rs1805005	213	16	89985774	89985986	G
rs1805006	213	16	89985774	89985986	C
rs1805007	173	16	89986044	89986216	C
rs1805008	173	16	89986044	89986216	C
rs1805009	227	16	89986484	89986710	G
rs201326893_Y152OCH	173	16	89986044	89986216	C
rs2228479	213	16	89985774	89985986	G
rs885479	173	16	89986044	89986216	G
rs2378249	118	20	33218028	33218145	A

Ancestry Informative SNPs

The following loci are detected when using DNA Primer Mix B. These loci are not present when using DNA Primer Mix A.

Table 7 Ancestry Informative SNPs

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs2814778	120	1	159174650	159174769	A
rs3737576	98	1	101709521	101709618	A
rs7554936	106	1	151122413	151122518	CT
rs10497191	101	2	158667153	158667253	C
rs1834619	84	2	17901444	17901527	G
rs1876482	120	2	17362526	17362645	C
rs260690	115	2	109579681	109579795	A
rs3827760	108	2	109513546	109513653	T
rs6754311	98	2	136707920	136708017	CT
rs798443	84	2	7968221	7968304	A
rs12498138	119	3	121459545	121459663	G

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs1919550	117	3	121364112	121364228	A
rs1229984	120	4	100239288	100239407	G
rs3811801	114	4	100244261	100244374	C
rs4833103	95	4	38815462	38815556	AC
rs7657799	116	4	105375396	105375511	T
rs7722456	114	5	170202901	170203014	T
rs870347	119	5	6844995	6845113	T
rs16891982*	108	5	33951621	33951728	G
rs192655	70	6	90518235	90518304	AG
rs3823159	119	6	136482701	136482819	A
rs917115	71	7	28172543	28172613	T
rs1462906	84	8	31896545	31896628	C
rs1871534	71	8	145639652	145639722	C
rs2196051	120	8	122124216	122124335	T
rs6990312	111	8	110602270	110602380	G
rs3814134	104	9	127267664	127267767	T
rs4918664	168	10	94920962	94921129	A
rs1079597	167	11	113296227	113296393	G
rs174570	120	11	61597179	61597298	C
rs2238151	113	12	112211753	112211865	CT
rs671	136	12	112241658	112241793	G
rs1572018	116	13	41715225	41715340	AG
rs2166624	71	13	42579949	42580019	AG
rs7326934	96	13	49070482	49070577	G
rs7997709	85	13	34847693	34847777	T
rs9522149	119	13	111827125	111827243	C
rs200354	165	14	99375246	99375410	G
rs12439433	100	15	36219979	36220078	G
rs1426654	92	15	48426457	48426548	A
rs1800414	116	15	28196969	28197084	A
rs735480	108	15	45152321	45152428	T
rs12913832*	119	15	28365523	28365641	AG
rs459920	78	16	89730800	89730877	T
rs11652805	119	17	62987113	62987231	T
rs17642714	118	17	48726060	48726177	AT
rs2593595	102	17	41056210	41056311	TC
rs4411548	158	17	40658440	40658597	G
rs4471745	67	17	53568849	53568915	G
rs2042762	83	18	35277568	35277650	A
rs3916235	120	18	67578894	67579013	AG
rs4891825	106	18	67867615	67867720	AG
rs7226659	149	18	40488180	40488328	G
rs7251928	200	19	4077044	4077243	A
rs310644	89	20	62159472	62159560	A
rs2024566	88	22	41697312	41697399	A

* Also used for phenotype prediction.

Interpretation Examples for D22S1045 and DYS392

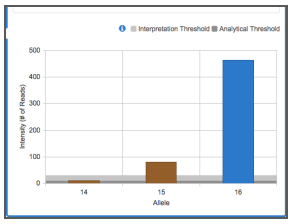
The following illustrations and example interpretation methods may assist with interpreting loci D22S1045 and DYS392. Actual values and methods may be determined based on a laboratory's application and internal validation data.

Locus D22S1045 Data Trends

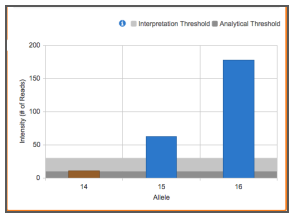
- Elevated n-1 stutter can occur in low coverage situations, particularly for stutter in STR positions/lengths ≥ 15. Stutter percentages increase as coverage decreases, and in extreme cases can approach, or surpass, the read depth of the parent allele.
- Heterozygote imbalance can occur at high or low locus coverage. Imbalance increases with a larger spread between allele lengths (e.g., 11,18).

Figure 4 Progressively increasing n-1 stutter (15 position) observed as locus coverage decreases

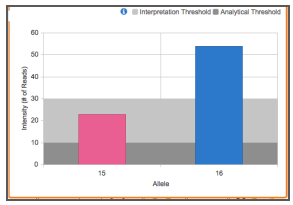
16,16 (17% stutter)



16,16 (35% stutter)



16,16 (43% stutter)



16,16 (68% stutter)

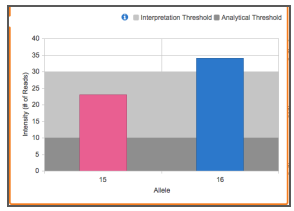
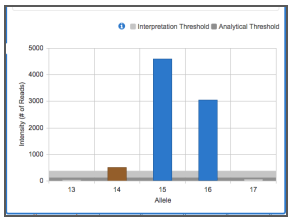
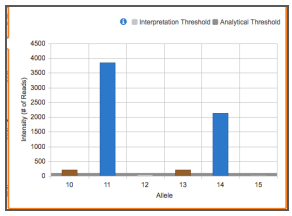


Figure 5 Progressively decreasing intralocus balance (allele count ratio (ACR)) as allele number spread increases

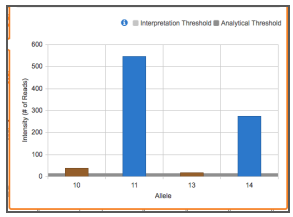
15,16 (66% ACR)



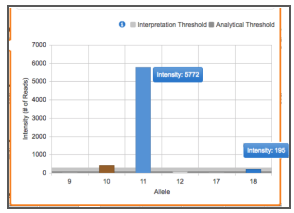
11,14 (55% ACR)



11,14 (50% ACR)



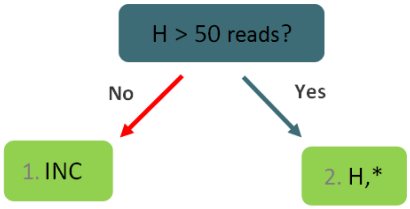
11,18 (3% ACR)



Decision Tree Methods for Genotype Determination at D22S1045

The following examples illustrate methods for genotype determination with one typed allele present (Figure 6) and two typed alleles present (Figure 7).

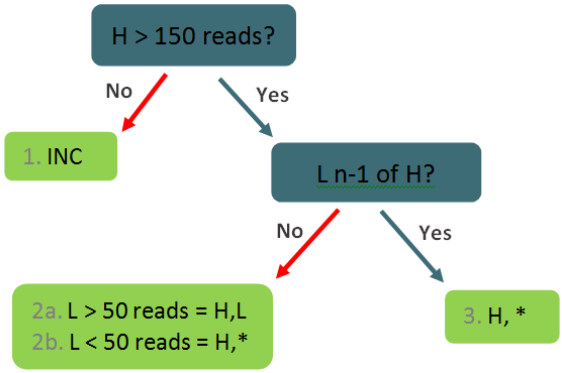
Figure 6 One Typed Allele Present



H=example allele

- 1. INC—An inconclusive result is a conservative conclusion used to eliminate chance of inadvertently typing stutter position when < 50 reads are available.
- 2. H,*—When > 50 reads, H is a true allele (not stutter); * accounts for potential drop-out due to imbalance.

Figure 7 Two Typed Alleles Present



H=allele with highest number of reads; L=allele with lower number of reads

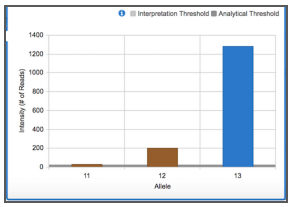
- 1. INC—An inconclusive result is a conservative conclusion used to eliminate chance of inadvertently typing stutter position when two potential alleles are present with < 150 reads available.
- 2a. H,L—H > 150=true allele (not stutter) and L > 50 reads outside of n-1 position=obligate sister.
- 2b. H,*—H > 150=true allele (not stutter) and L < 50 reads might be elevated stutter.
- 3. H,*—H > 150=true allele (not stutter) and L in n-1 position might be elevated stutter.

Locus DYS392 Data Trends

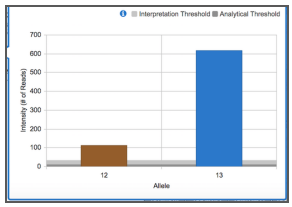
Elevated n-1 stutter can occur in low locus coverage situations. Stutter increases as coverage decreases and in extreme cases can approach, or surpass, the read depth of the parent allele.

Figure 8 Progressively increasing n-1 stutter (12 position) observed as locus coverage decreases

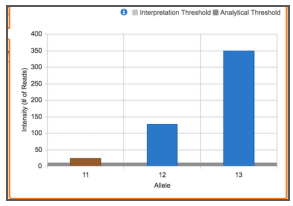
13,13 (16% stutter)



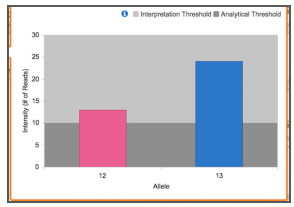
13,13 (18% stutter)



13,13 (36% stutter)



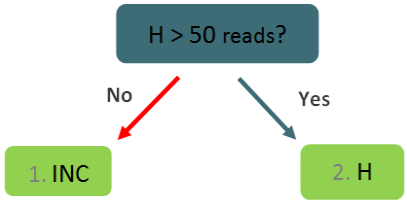
13,13 (54% stutter)



Decision Tree Methods for Genotype Determination at DYS392

The following examples illustrate methods for genotype determination with one typed allele present (Figure 9) and n-1 stutter position and parent allele present (Figure 10).

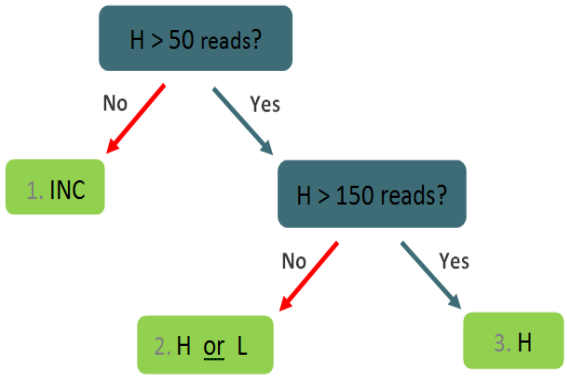
Figure 9 One Typed Allele Present



H=example allele

- 1. INC—An inconclusive result is a conservative conclusion used to eliminate chance of inadvertently typing stutter position when < 50 reads are available.
- 2. H—When > 50 reads, H is a true allele (not stutter).

Figure 10 n-1 Stutter Position Typing with Parent Allele



H=allele with highest number of reads; L=allele with lower number of reads

- 1. INC—An inconclusive result is a conservative conclusion used to eliminate chance of inadvertently typing stutter position when two potential alleles are present with < 50 reads available.
- 2. H or L—H < 150=potential for either H or L to be elevated stutter.
- 3. H—H > 150=H is a true allele, even with L at high n-1 stutter %.

NOTE
The decision tree read values are for example purposes only. These examples demonstrate an interpretation method for loci D22S1045 and DYS392 using specific read level guidelines. Actual values and methods for operational laboratories can be determined based on laboratory application and internal validation data observations.

NOTE
This method might be considered conservative for some laboratories. Additional data-informed decision points can be used in the decision tree methods, if desired.

Technical Assistance

For technical assistance, contact Verogen Technical Support.

Table 8 General Contact Information

Address	11111 Flintkote Avenue San Diego CA 92121 USA
Website	www.verogen.com
Email	techsupport@verogen.com
Phone	+1.833.837.6436 toll-free (North America) +1.858.285.4101 (outside North America)

Safety data sheets (SDSs)

- For MiSeq FGx sequencing kit safety data sheets, visit www.verogen.com/sds.
- For Research Use Only (RUO) sequencing reagent and Illumina library preparation kit safety data sheets, visit support.illumina.com/sds.

Product documentation—Available for download in PDF from the Verogen website. Go to www.verogen.com/support select the appropriate document.

Verogen
+1.833.837.6436 toll-free (North America)
+1.858.285.4101 (outside North America)
techsupport@verogen.com
www.verogen.com