ForenSeq DNA Signature Prep Kit with ForenSeq Enhanced PCR1 Buffer System

Reference Guide

VEROGEN PROPRIETARY
Document # VD2021046 Rev. A
January 2022

Revision History

Document #	Date	Description of Change
VD2021046 Rev. A	January 2022	Initial release

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

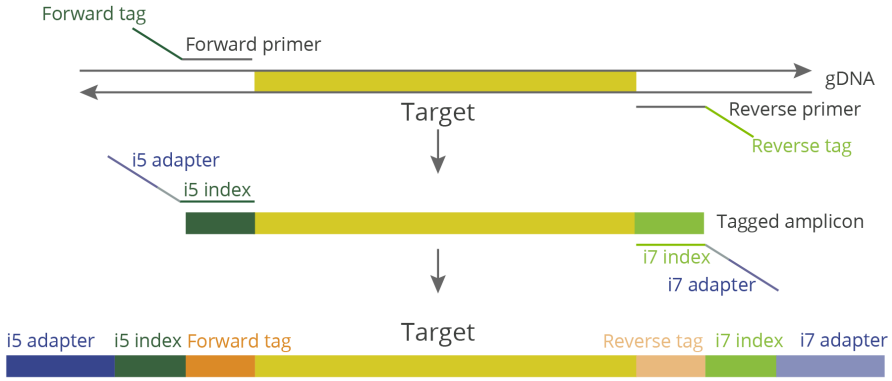
This product includes 2800M Control DNA manufactured by Promega Corporation for distribution by Verogen, Inc.

Overview

Introduction

The ForenSeq® DNA Signature Prep Kit generates 384 dual-indexed libraries for sequencing. Each sample is combined with a primer mix that contains a pair of tagged oligos for each target sequence. PCR cycles link the tags to copies of each target, forming DNA templates consisting of the regions of interest flanked by universal primer binding sequences. The tags are then used to attach index adapters and the resulting library is amplified, purified, and pooled for sequencing. The ForenSeq DNA Signature Prep Kit can be used with the standard PCR1 buffer that is available with the kit or an enhanced buffer system (ePCR1) that is available as an add-on kit. This Reference Guide summarizes the protocol for using the ForenSeq DNA Signature Prep Kit with the ForenSeq Enhanced PCR1 Buffer System.

Figure 1 Assay overview



Kit Features

The ForenSeq DNA Signature Prep Kit offers the following features:

- Simultaneous preparation of up to 96 libraries. Each library is a collection of amplified DNA fragments from one sample.
- Amplify short tandem repeat (STR) and single-nucleotide polymorphism (SNP) amplicons from challenging samples in one reaction.
- Choose between two targeted primer mixes, DNA Primer Mix A (DPMA) or DNA Primer Mix B (DPMB), to prepare samples for databasing or casework.

ForenSeq Enhanced PCR1 Buffer System offers the following features:

- An optimized buffer system that support the simultaneously amplification of 96 challenging samples in the presence of high concentrations of inhibitors, such as humic acid.

Protocol Steps

The following diagram lists the steps to prepare libraries with challenging examples, using the enhanced PCR1 buffer, along with hands-on times, total times, and reagents. Safe stopping points are marked between steps.

Figure 2 Overview of the ForenSeq DNA Signature Prep protocol with the ForenSeq Enhanced PCR1 Reaction Mix

DNA Input Recommendations

Use 1 ng purified human genomic DNA (gDNA) as input. Before starting the protocol, quantify the input using a fluorometric-based method or qPCR and assess quality.

Controls

Each preparation must include at least one positive amplification control and at least one negative amplification control. If these controls are not included, troubleshooting support is limited.

The kit includes Control DNA 2800M (2800M) for use as the positive template control and the negative amplification control is nuclease-free water. The protocol includes instructions to prepare each control.

Acronyms

Acronym	Definition
DNL	Diluted Normalized Libraries
DPMA	DNA Primer Mix A
DPMB	DNA Primer Mix B
ePCR1	Enhanced PCR1 Reaction Mix
gDNA	Genomic DNA
FEM	Enzyme Mix
FSP	ForenSeq Sample Plate
HP3	2 N 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
2800M	Control DNA 2800M
NLP	Normalized Library Plate
NWP	Normalization Working Plate
PBP	Purification Bead Plate
PCR2	PCR2 Reaction Mix
PLP	Purified Library Plate
PNL	Pooled Normalized Libraries

Acronym	Definition
ProK	Proteinase K
RSB	Resuspension Buffer
SPB	Sample Purification Beads

Additional Resources

This guide provides comprehensive information on the use of ForenSeq DNA Signature Prep Kit with detailed protocol instructions for use of the ForenSeq Enhanced PCR1 Buffer System. Visit the Documentation page on the Verogen website to download additional kit documentation and access the latest versions.

Resource	Description
ForenSeq DNA Signature Prep Kit with Enhanced PCR1 Buffer System Checklist (document # VD2021047)	Provides concise protocol instructions for the experienced user.
ForenSeq DNA Signature Prep Kit with Enhanced PCR1 Buffer System Materials List (document # VD2021048)	Lists the consumables and equipment needed to perform the protocol.

Protocol

Introduction

This chapter describes the ForenSeq DNA Signature Prep protocol with step-by-step instructions to prepare libraries for sequencing using the ForenSeq Enhanced PCR1 buffer system. For an overview of the protocol with reagents and durations for each step, see *Protocol Steps* (page 6).

Before starting, confirm kit contents and make sure that you have the necessary reagents, consumables, and equipment. For a list of items, see *Materials* (page 22).

Number of Samples

Process at least eight samples at a time, including positive and negative amplification controls. Preparing master mixes for fewer than eight samples can introduce pipetting inaccuracies due to small volumes.

Use the following table to determine the maximum number of libraries to pool for a run, depending on primer mix, sample type, and MiSeq FGx® reagent kit. Casework recommendations are intended for samples where DNA mixtures are possible or challenging samples with < 1 ng gDNA available and partial degradation.

Table 1 Maximum number of libraries

Primer Mix	Sample Type	MiSeq FGx Reagent Micro Kit	MiSeq FGx Reagent Kit
DPMA	Database or reference	36	96
	Casework	12	32
DPMB	Database or reference	12	32
	Casework	12	32

Primer Mixes

The kit includes two primer mixes: DPMA and DPMB. Both primer mixes detect identity informative SNPs (iiSNPs), autosomal STRs (aSTRs), and X- and Y-STRs. In addition to these targets, DPMB detects ancestry-informative SNPs (aiSNPs) and phenotypic-informative SNP (piSNPs).

The ForenSeq DNA Signature Prep Kit supports analysis of these SNPs and STRs from gDNA ranging from high-quality, single-source samples to difficult. One reaction with integrated indexing enables sequencing of up to 96 database samples using DPMA or 32 casework samples using DPMB in one run with the MiSeq FGx Reagent Kit.

Tips and Techniques

Protocol Continuity

- Follow the steps in the order indicated using the specified volumes and incubation parameters.
- Unless a safe stopping point is specified, proceed immediately to the next step.

Plate Setup

- Create a sample sheet to record the position of each sample, control, and index adapter.
- Reference the sample sheet throughout the protocol to ensure proper plate setup.

The *ForenSeq Universal Analysis Software v1.3 Reference Guide (document # VD2018007)* provides detailed information on sample sheets and input of sample information.

Preventing Cross-Contamination

- Set up the *Amplify and Tag Targets* (facing page) process in a pre-PCR environment. Perform all other processes in a post-PCR environment.
- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each well***.
- Do not reuse index adapter caps.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Apply a microseal to cover the plate and seal with a rubber roller. After each use, discard seals from plates.
- Use Microseal 'A' pressure film for thermal cycling. When using fewer than 96 wells, you can cut the film to size.
- Use Microseal 'B' adhesive film for shaking, centrifuging, and long-term storage. These seals are effective at -40°C to 110°C.

Handling Beads

- For optimal performance and yield, confirm that beads are at room temperature before use.
- Aspirate and dispense beads slowly due to viscosity.
- Do not centrifuge plates and tubes containing beads, except when indicated.
- Vortex beads before use and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- If beads aspirate into pipette tips during supernatant removal, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

Amplify and Tag Targets

This process uses an oligonucleotide primer mix with regions specific to the DNA sequences upstream and downstream of STRs and SNPs to tag and amplify the input gDNA.

Consumables

- 2800M (Control DNA 2800M)
- DPMA (DNA Primer Mix A) or DPMB (DNA Primer Mix B)
- FEM (Enzyme Mix)
- ePCR1 (Enhanced PCR1 Reaction Mix)
- 1.7 ml microcentrifuge tubes (2)
- 96-well PCR plate, semiskirted
- Input gDNA
- Microseal 'A' film
- Nuclease-free water
- [Optional] RNase/DNase-free 8-tube strip and caps

About Reagents

- Use PCR1 for standard samples and ePCR1 for inhibited samples.
- ePCR1 should not be used with crude lysates or FTA card punches.
- For information on DPMA and DPMB, see *Loci Detected with DPMA and DPMB* (page 29).

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
2800M	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert three times to mix, and then centrifuge briefly.
DPMA or DPMB	-25°C to -15°C	Thaw at room temperature. Invert three times to mix, and then centrifuge briefly.
FEM	-25°C to -15°C	Thaw at room temperature, and then centrifuge briefly. Return to storage immediately after use.
ePCR1	-25°C to -15°C	Thaw at room temperature for 30 minutes. Vortex to mix, and then centrifuge briefly.

2. Save the following PCR1 program on the thermal cycler in the post-amplification area. See Table 2 for ramp modes.
   - Choose the preheat lid option. See Table 2 for lid temperatures.
   - 98°C for 3 minutes
   - 8 cycles of:
     — 96°C for 45 seconds

  - 80°C for 30 seconds
  - 54°C for 2 minutes, with applicable ramp mode
  - 68°C for 2 minutes, with applicable ramp mode
- 10 cycles of:
  - 96°C for 30 seconds
  - 68°C for 3 minutes, with applicable ramp mode
- 68°C for 10 minutes
- Hold at 10°C

Table 2 Thermal Cycler lid temperature and ramp modes

Thermal Cycler	Temperature Mode	Lid Temperature	Ramp Mode	Vessel Type
ABI LTI thermal cycler 9700	9600 emulation	Heated	8%	Polypropylene plates and tubes
Bio-Rad	Calculated	Heated, constant at 100°C	0.2°C per second	Polypropylene plates and tubes
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	2%	Plate
Proflex 96-well PCR System	Not applicable	Heated, constant at 105°C	0.2°C per second	Polypropylene plates and tubes
Veriti 96-well thermal cycler	Standard	Heated, constant at 105°C	4%	Polypropylene plates and tubes

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

3. Label a new PCR plate FSP for ForenSeq Sample Plate.
4. Label a new 1.7 ml tube per your input type:

Input Type	Label
Purified DNA	Master Mix

Procedure

Purified DNA

1. Using nuclease-free water, dilute 1 ng purified DNA input to 0.2 ng/µl.
2. In the Master Mix tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.
   - ePCR1 (4.7 µl)
   - FEM (0.3 µl)
   - DPMA or DPMB (5 µl)

   For example, for eight samples prepare 88 µl master mix: 41.4 µl ePCR1, 2.6 µl FEM, and 44 µl DPMA or DPMB.
3. Pipette to mix, and then cap and centrifuge briefly.
4. [Optional] Evenly distribute the master mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.

5. Add 10 µl master mix to each well of the FSP.
6. In a new 1.7 ml tube, combine the following volumes to dilute 2800M:
   - 2800M (2 µl)
   - Nuclease-free water (98 µl)
7. Cap and gently invert three times to mix, and then centrifuge briefly.
8. Add 5 µl diluted 2800M to at least one well of the FSP as a positive amplification control.
9. Pipette to mix.
10. Add 5 µl nuclease-free water to at least one well of the FSP as a negative amplification control.
11. Pipette to mix.
12. Add 5 µl 0.2 ng/µl DNA to each well of the FSP.
13. Seal and centrifuge at 1000 × g for 30 seconds.
14. Transport to the post-PCR area.
15. Place on the preprogrammed thermal cycler and run the PCR1 program.
16. Unless you are stopping, proceed to *Enrich Targets* (below).

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 the sequences needed for cluster generation. The index adapters tag the DNA with a unique combination of sequences that identify each sample for analysis.

When preparing eight samples, you can perform this process using an 8-tube strip instead of the 96-well PCR plate.

Consumables

- Index 1 Adapters (R7XX) and orange caps
- Index 2 Adapters (A50X) and white caps
- PCR2 (PCR2 Reaction Mix)
- ForenSeq Index Plate Fixture
- Microseal 'A' film
- [Optional] 1.7 ml microcentrifuge tubes (1 per index adapter tube)

About Reagents

- Dispense PCR2 slowly to prevent bubbles.
- Centrifuge index adapter tubes in the 1.7 ml tubes, if necessary.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
Index adapters	-25°C to -15°C	Remove only the index adapters being used. Thaw at room temperature for 20 minutes. Vortex each tube to mix, and then centrifuge briefly.
PCR2	-25°C to -15°C	Thaw at room temperature for 20 minutes, and then invert to mix.

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

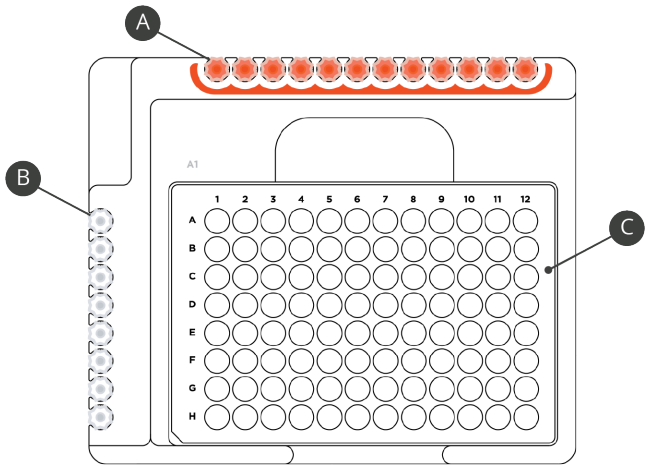
   Total program time is ~46 minutes.

Procedure

1. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
2. Place the Index 1 Adapter tubes (orange caps) in each column of the ForenSeq Index Plate Fixture.
3. Place the Index 2 Adapter tubes (white caps) in each row of the ForenSeq Index Plate Fixture.
4. Place the FSP on the ForenSeq Index Plate Fixture.

   Figure 3 shows the fixture setup with index adapter tubes and the FSP.

Figure 3 Fixture setup for 96 libraries



A Index 1 Adapter tubes in columns 1–12
B Index 2 Adapter tubes in rows A–H
C FSP

5. Using a multichannel pipette, add index adapters to the FSP:
   a. Add 4 µl R7XX down each column.

b. Replace the cap on each Index 1 Adapter tube with a new orange cap.
c. Add 4 µl A50X across each row.
d. Replace the cap on each Index 2 Adapter tube with a new white cap.

6. Vortex PCR2, and then centrifuge briefly.
7. [Optional] Evenly distribute PCR2 among each tube of an 8-tube strip. Use a multichannel pipette to dispense.
8. Add 27 µl PCR2 to each well.
9. Seal and centrifuge at 1000 × g for 30 seconds.
10. Place 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 purification beads to purify the amplified libraries from other reaction components.

Consumables

- RSB (Resuspension Buffer
- SPB (Sample Purification Beads)
- ProK (Proteinase K)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plate
- 96-well PCR plate, skirted or semiskirted
- PVC reagent reservoir
- Microseal 'B' film

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
ProK	-25°C to -15°C	Thaw at room temperature, and then centrifuge briefly. Return to storage immediately after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex for **≥ *1 minute*** and invert to mix.

2. Label plates as follows.

Plate Type	Label
Midi	PBP for Purification Bead Plate
PCR	PLP for Purified Library Plate

3. Calculate the appropriate volume for the SPB Master Mix:

SPB Beads Master Mix	SPB	ProK
for 96 samples	5.50 ml	25.0 ul
for 64 samples	3.50 ml	16.4 ul
for 48 samples	2.70 ml	12.5 ul
for 32 samples	1.75 ml	8.2 ul
for 24 samples	1.30 ml	6.2 ul

Procedure

1. Add 45 µl SPB Master Mix to each well of the PBP.
2. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
3. Transfer 45 µl reaction from each well of the FSP to the corresponding well of the PBP.
4. Discard the FSP plate.
5. Seal the PBP and shake at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
8. Remove and discard all supernatant.
9. Keep on the magnetic stand and wash as follows.
    a. Add 200 µl fresh 80% EtOH to each well.
    b. Incubate for 30 seconds.
    c. Remove and discard all supernatant.
10. Wash a **second** time.
11. Seal and centrifuge at 1000 × g for 30 seconds.
12. Place on the magnetic stand.
13. With a 20 µl pipette, remove residual EtOH from each well.
14. Remove from the magnetic stand.
15. Add 52.5 µl RSB to each well.
16. Seal and shake at 1800 rpm for 2 minutes.
17. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.
18. Incubate at room temperature for 2 minutes.
19. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
20. Transfer 50 µl supernatant from each well of the PBP to the corresponding well of the PLP.
21. Seal and 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 1 year.

Normalize Libraries

This process normalizes the concentration of each library for even representation without post-PCR quantification and individual normalization. Samples of varying types and input amounts achieve consistent cluster density, optimizing the resolution of each library in the pool.

Consumables

- HP3 (2 N NaOH)
- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNS2 (Library Normalization Storage Buffer 2)
- LNW1 (Library Normalization Wash 1)
- Nuclease-free water
- One of the following tubes:
  - 1.7 ml microcentrifuge tube
  - 15 ml conical tube
- 1.7 ml microcentrifuge tube
- 96-well midi plate
- 96-well PCR plate, skirted or semiskirted
- PVC reagent reservoir
- Microseal 'B' film

About Reagents

- The volumes combined in the LNA1/LNB1 Master Mix tube and the 0.1 N HP3 tube include overage, so calculating additional overage is not necessary.

Warning: This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. **For complete environmental, health, and safety information, see the safety data sheets (SDS) at verogen.com/product-documentation.**

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
HP3	-25°C to -15°C	Thaw at room temperature for ≥ 30 minutes. Vortex to mix, and then centrifuge briefly.
LNA1	-25°C to -15°C	Thaw at room temperature for ≥ 30 minutes. Vortex with intermittent inversion

Item	Storage	Instructions
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 vessels as follows.

Vessel	Label
1.7 ml tube or 15 ml conical tube	LNA1/LNB1 Master Mix
Midi plate	NWP for Normalization Working Plate
PCR plate	NLP for Normalization Library Plate

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

Procedure

1. In the LNA1/LNB1 Master Mix tube, combine the following volumes. Multiply each volume by the number of samples, but do not add overage.
   - LNA1 (46.8 µl)
   - LNB1 (8.5 µl)

   For example, for eight samples, combine 374.4 µl LNA1 and 68 µl LNB1.
2. Vortex, and then invert several times to mix.
3. Transfer the entire volume to a reagent reservoir.
4. Add 45 µl LNA1/LNB1 Master Mix to each sample well of the NWP.
5. To clear any beads that might have aspirated, place the PLP on the magnetic stand and wait until the liquid is clear (~2 minutes).
6. Transfer 20 µl supernatant from each well of the PLP to the corresponding well of the NWP.
7. Seal the NWP and shake at 1800 rpm for 30 minutes.
8. While the plate is shaking, perform steps 9–11 to save time later in the process.
9. In the 0.1 N HP3 tube, combine the following volumes. Multiply each volume by the number of samples, but do not add overage.
   - Nuclease-free water (33.3 µl)
   - HP3 (1.8 µl)

   For example, eight samples require 266.4 µl nuclease-free water and 14.4 µl HP3.
10. Invert several times to mix, and then set aside.
11. Add 30 µl LNS2 to each sample well of the NLP.
12. Immediately after shaking, place the NWP on the magnetic stand and wait until the liquid is clear (~2 minutes).
13. Remove and discard all supernatant.
14. Remove from the magnetic stand.
15. Wash as follows.

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

16. Wash a **second** time.
17. Seal and centrifuge at 1000 × g for 30 seconds.
18. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
19. With a 20 µl pipette, remove residual LNW1 from each well.
20. Remove from the magnetic stand.
21. Add 32 µl freshly prepared 0.1 N HP3 to each well.
22. Seal and shake at 1800 rpm for 5 minutes.
23. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 5 minutes.
24. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
25. Transfer 30 µl supernatant from the NWP to the corresponding well of the NLP.
26. Pipette to mix.
27. Seal and 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 each normalized library to create a pool of libraries that are sequenced together on the same flow cell.

Consumables

- 1.7 ml microcentrifuge tube
- RNase/DNase-free 8-tube strip and caps
- Microseal 'B' film

Preparation

1. Select libraries to pool for sequencing.
   For recommendations, see *Number of Samples* (page 9).
2. Label the 1.7 ml tube PNL for Pooled Normalized Libraries.

Procedure

1. Using a multichannel pipette, transfer 5 µl of each library to a new 8-tube strip.
2. Seal the NLP and store in the post-PCR area at -25°C to -15°C for ≤ 30 days.
3. Transfer libraries from each well of the 8-tube strip to the PNL tube.

4. Cap and vortex to mix, 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 libraries to the loading concentration, adds a sequencing control, and uses a heat-based method to denature the libraries for sequencing.

Start this process when you are ready to prepare sequencing reagents and set up the run. Delays can impact template loading.

Consumables

- HP3 (2 N NaOH)
- HSC (Human Sequencing Control)
- MiSeq FGx Reagent Kit contents:
  - HT1 (Hybridization Buffer)
  - Reagent cartridge
- Nuclease-free water
- Pooled libraries
- 1.7 ml microcentrifuge tubes (2)

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
HP3	-25°C to -15°C	Thaw at room temperature for ≥ 30 minutes, and then centrifuge briefly.
HSC	-25°C to -15°C	Let stand for 30 minutes to bring to room temperature. Invert to mix, and then centrifuge.
HT1	-25°C to -15°C	Thaw at room temperature, and then vortex to mix.
Reagent cartridge	-25°C to -15°C	Thaw in a water bath at room temperature.

2. Preheat the microheating system to 96°C.
3. Label two new 1.7 ml tubes:
   - Denatured HSC
   - DNL for Denatured Normalized Libraries

Procedure

1. In the Denatured HSC tube, combine the following volumes:
   - HSC (2 µl)
   - HP3 (2 µl)
   - Nuclease-free water (36 µl)

2. Pipette gently to mix. Cap and centrifuge briefly to mix.
3. Incubate at room temperature for 5 minutes.
4. Add 591 µl HT1 to the DNL tube.
5. Transfer 7 µl library from the PNL tube to the DNL tube.
6. Pipette to mix.
7. Cap the PNL tube and store at -25°C to -15°C for ≤ 30 days.
   Exceeding 30 days significantly reduces cluster density.
8. Add 4 µl denatured HSC to the DNL tube.
9. Pipette to mix.
   You can store the denatured HSC at room temperature for ≤ 1 day.
10. Cap and vortex to mix, and then centrifuge briefly.
11. Place on the preheated microheating system and incubate for 2 minutes.
12. Invert several times to mix.
13. Immediately cool on the benchtop cooler or in the ice-water bath for 5 minutes.
14. Immediately transfer the entire volume to the reagent cartridge per instructions in the *MiSeq FGx Sequencing System Reference Guide (document # VD2018006)*.

Materials

Kit Contents and Storage

Make sure that you have the reagents identified in this section before starting the protocol. When you receive the kit, promptly store reagents at the indicated temperatures.

Kit Name	Part #
ForenSeq DNA Signature Prep Kit (96 Reactions)	V16000023
ForenSeq DNA Signature Prep Kit (384 Reactions)	15066151
ForenSeq Enhanced PCR1 Buffer System	V16000137

All reagents in a box are shipped at the same temperature. When a reagent has a different storage temperature than most other reagents in the box, you can initially store the reagent at the same temperature as the other reagents. After first use, store the reagent at the indicated temperature.

ForenSeq DNA Signature Prep Kit (96 Reactions) (V16000023)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
1	2800M	Control DNA 2800M	Black	2°C to 8°C*
2	DPMA	DNA Primer Mix A	Blue	-25°C to -15°C
2	DPMB	DNA Primer Mix B	Red	-25°C to -15°C
2	FEM	Enzyme Mix	Yellow	-25°C to -15°C
2	PCR1	PCR1 Reaction Mix	Green	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 2

Quantity	Reagent	Description	Cap	Storage
1	HP3	2 N NaOH	Orange	-25°C to -15°C
1	HSC	Human Sequencing Control	Pink	-25°C to -15°C
1	LNA1	Library Normalization Additives 1	Clear	-25°C to -15°C
1	LNS2	Library Normalization Storage Buffer 2	Clear	Room temperature*

Quantity	Reagent	Description	Cap	Storage
2	LNW1	Library Normalization Wash 1	Clear	2°C to 8°C*
2	PCR2	PCR2 Reaction Mix	Purple	-25°C to -15°C
1	A501	A501 Index Adapter	White	-25°C to -15°C
1	A502	A502 Index Adapter	White	-25°C to -15°C
1	A503	A503 Index Adapter	White	-25°C to -15°C
1	A504	A504 Index Adapter	White	-25°C to -15°C
1	A505	A505 Index Adapter	White	-25°C to -15°C
1	A506	A506 Index Adapter	White	-25°C to -15°C
1	A507	A507 Index Adapter	White	-25°C to -15°C
1	A508	A508 Index Adapter	White	-25°C to -15°C
1	R701	R701 Index Adapter	Orange	-25°C to -15°C
1	R702	R702 Index Adapter	Orange	-25°C to -15°C
1	R703	R703 Index Adapter	Orange	-25°C to -15°C
1	R704	R704 Index Adapter	Orange	-25°C to -15°C
1	R705	R705 Index Adapter	Orange	-25°C to -15°C
1	R706	R706 Index Adapter	Orange	-25°C to -15°C
1	R707	R707 Index Adapter	Orange	-25°C to -15°C
1	R708	R708 Index Adapter	Orange	-25°C to -15°C
1	R709	R709 Index Adapter	Orange	-25°C to -15°C
1	R710	R710 Index Adapter	Orange	-25°C to -15°C
1	R711	R711 Index Adapter	Orange	-25°C to -15°C
1	R712	R712 Index Adapter	Orange	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 3

Quantity	Reagent	Description	Cap	Storage
1	LNB1	Library Normalization Beads 1	White	2°C to 8°C
1	RSB	Resuspension Buffer	Purple	2°C to 8°C
1	SPB	Sample Purification Beads	Red	2°C to 8°C

ForenSeq DNA Signature Prep Kit (384 Reactions) (15066151)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
2	2800M	Control DNA 2800M	Black	2°C to 8°C*
8	DPMA	DNA Primer Mix A	Blue	-25°C to -15°C
8	DPMB	DNA Primer Mix B	Red	-25°C to -15°C
8	FEM	Enzyme Mix	Yellow	-25°C to -15°C
8	PCR1	PCR1 Reaction Mix	Green	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 2

Quantity	Reagent	Description	Cap	Storage
3	HP3	2 N NaOH	Orange	-25°C to -15°C
1	HSC	Human Sequencing Control	Pink	-25°C to -15°C
4	LNA1	Library Normalization Additives 1	Clear	-25°C to -15°C
4	LNS2	Library Normalization Storage Buffer 2	Clear	Room temperature*
8	LNW1	Library Normalization Wash 1	Clear	2°C to 8°C*
8	PCR2	PCR2 Reaction Mix	Purple	-25°C to -15°C
1	A501	A501 Index Adapter	White	-25°C to -15°C
1	A502	A502 Index Adapter	White	-25°C to -15°C
1	A503	A503 Index Adapter	White	-25°C to -15°C
1	A504	A504 Index Adapter	White	-25°C to -15°C
1	A505	A505 Index Adapter	White	-25°C to -15°C
1	A506	A506 Index Adapter	White	-25°C to -15°C
1	A507	A507 Index Adapter	White	-25°C to -15°C
1	A508	A508 Index Adapter	White	-25°C to -15°C
1	R701	R701 Index Adapter	Orange	-25°C to -15°C
1	R702	R702 Index Adapter	Orange	-25°C to -15°C
1	R703	R703 Index Adapter	Orange	-25°C to -15°C
1	R704	R704 Index Adapter	Orange	-25°C to -15°C
1	R705	R705 Index Adapter	Orange	-25°C to -15°C

Quantity	Reagent	Description	Cap	Storage
1	R706	R706 Index Adapter	Orange	-25°C to -15°C
1	R707	R707 Index Adapter	Orange	-25°C to -15°C
1	R708	R708 Index Adapter	Orange	-25°C to -15°C
1	R709	R709 Index Adapter	Orange	-25°C to -15°C
1	R710	R710 Index Adapter	Orange	-25°C to -15°C
1	R711	R711 Index Adapter	Orange	-25°C to -15°C
1	R712	R712 Index Adapter	Orange	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 3

Quantity	Reagent	Description	Cap	Storage
4	LNB1	Library Normalization Beads 1	White	2°C to 8°C
1	RSB	Resuspension Buffer	Purple	2°C to 8°C
2	SPB	Sample Purification Beads	Red	2°C to 8°C

ForenSeq Enhanced PCR1 Buffer System (96 Reactions) (V16000137)

Quantity	Reagent	Description	Cap	Storage
1	ePCR1	ForenSeq Enhanced PCR1 Reaction Mix	Orange	-25°C to -15°C
1	ProK	Proteinase K	Clear	-25°C to -15°C

Index Adapter Sequences

The following tables list the 8 bp sequences for the index adapters included in the kit.

Index 1 (i7)

Index Name	Sequence
R701	ATCACGAT
R702	CGATGTAT
R703	TTAGGCAT
R704	TGACCAAT
R705	ACAGTGAT
R706	GCCAATAT

Index Name	Sequence
R707	CAGATCAT
R708	ACTTGAAT
R709	GATCAGAT
R710	TAGCTTAT
R711	GGCTACAT
R712	CTTGTAAT

Index 2 (i5)

Index Name	Sequence
A501	TGAACCTT
A502	TGCTAAGT
A503	TGTTCTCT
A504	TAAGACAC
A505	CTAATCGA
A506	CTAGAACA
A507	TAAGTTCC
A508	TAGACCTA

Consumables and Equipment

Make sure that you have the following user-supplied consumables and equipment before starting the protocol. These items supplement the library prep reagents and index adapters provided in the kit.

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

Consumables

Consumable	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 deep well storage plates (midi plates)	Fisher Scientific, part # AB-0859

Consumable	Supplier
96-well twin.tec PCR plates, semiskirted	One of the following suppliers: • Eppendorf, catalog # 951020303 • VWR, catalog # 89136-706
96-well twin.tec PCR plate, skirted, 150 µl	Eppendorf, catalog # 951020401
Ethyl alcohol, pure	Sigma-Aldrich, catalog # E7023
Microseal 'A' sealing film	Bio-Rad, catalog # MSA5001
Microseal 'B' sealing film, adhesive, optical	Bio-Rad, catalog # MSB1001
One of the following kits: • MiSeq FGx Reagent Kit • MiSeq FGx Reagent Micro Kit	Verogen part #: • 15066817 • 20021681
Multichannel reagent reservoirs, PVC, disposable	Labcor, part # 730-001
Nuclease-free water	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier

Equipment

Equipment	Supplier	Pre-PCR	Post-PCR
20 µl multichannel pipettes (8-channel)	General lab supplier		X
200 µl multichannel pipettes (8-channel)	General lab supplier		X
Benchtop microcentrifuge	General lab supplier	X	X
Heating system, 96-well, 1.5 ml	General lab supplier		X
Magnetic stand-96	Life Technologies, part # AM10027		X
Microplate centrifuge	General lab supplier	X	X
Thermal cycler, 96-well with heated lid	See *Thermal Cyclers* (below)		X
Thermoshaker, one of the following: • BioShake iQ • BioShake XP	QInstruments, item #: • 1808-0506 • 1808-0505		X
Rubber roller	General lab supplier	X	X
Vortexer	General lab supplier	X	X
[Optional] Benchtop cooler	VWR, catalog # 414004-286		X

Thermal Cyclers

The following table lists supported thermal cyclers with recommended settings. If your laboratory has an unlisted thermal cycler, evaluate the thermal cycler before performing the protocol.

Thermal Cycler	Temperature Mode	Lid Temperature	Vessel Type
ABI LTI thermal cycler 9700[1]	9600 emulation	Heated	Polypropylene plates and tubes
Bio-Rad	Calculated	Heated, constant at 100°C	Polypropylene plates and tubes
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate
Proflex 96-well PCR System[2]	Not applicable	Heated, constant at 105°C	Polypropylene plates and tubes
Veriti 96-well thermal cycler[2]	Standard	Heated, constant at 105°C	Polypropylene plates and tubes

[1] Only gold heat blocks are supported.

[2] Settings were verified after developmental validation of the ForenSeq DNA Signature Prep Kit.

Amplicon Information

Loci Detected with DPMA and DPMB

The following tables list loci detected with DPMA or DPMB. ***Loci in the piSNPs and aiSNPs are exclusive to*** DPMB. All other loci are detected with both primer mixes.

- Amplicon lengths exclude 120 bp for adapter sequences.
- Amplicon start and end positions are the one-base endpoints of the entire amplicon, including the sequence that matches primers on the hg19 human reference genome.
- Amelogenin is a genetic marker that confirms the gender of the biological sample donor. The size range is 106–112 bp and the control DNA is male.

Autosomal STRs

Locus	Repeats	Amplicon Length (bp)	Chromo-some	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[1]	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

Locus	Repeats	Amplicon Length (bp)	Chromo-some	2800M Control Alleles
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[2]	8–19	201–245	22	16,16

[1] Might include a low-level plus 0.1 base pair artifact with one T addition at the end of the STR repeat sequence of the parent allele. For example, 8,8.1 or 11,11.1.

[2] Interpret with caution. See *Interpreting Loci D22S1045* (page 37) for more information.

X-STRs

Locus	Repeats	Amplicon Length (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

Y-STRs

Locus	Repeats	Amplicon Length (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

Locus	Repeats	Amplicon Length (bp)	Chromosome	2800M Control Alleles
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 with caution. See *Interpreting Loci DYS392* (page 39) for more information.

Identity Informative SNPs

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs10495407	109	1	238439234	238439342	GG
rs1294331	85	1	233448359	233448443	GA
rs1413212	64	1	242806767	242806830	GG
rs1490413	98	1	4367256	4367353	AA
rs560681	90	1	160786641	160786730	AG
rs891700	115	1	239881850	239881964	AG
rs1109037	118	2	10085691	10085808	GG
rs12997453	100	2	182413195	182413294	AA

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs876724	119	2	114945	115063	CC
rs907100	115	2	239563542	239563656	CG
rs993934	120	2	124109120	124109239	CC
rs1355366	119	3	190806041	190806159	AG
rs1357617	120	3	961696	961815	AT
rs2399332	157	3	110300999	110301155	AC
rs4364205	98	3	32417576	32417673	GG
rs6444724	120	3	193207306	193207425	TT
rs1979255	102	4	190318007	190318108	GG
rs2046361	120	4	10968994	10969113	AA
rs279844	167	4	46329584	46329750	AT
rs6811238	120	4	169663541	169663660	GG
rs13182883	169	5	136633252	136633420	AG
rs159606	104	5	17374845	17374948	AA
rs251934	97	5	174778619	174778715	TT
rs338882	157	5	178690599	178690755	CC
rs717302	110	5	2879333	2879442	GG
rs13218440	170	6	12059928	12060097	AG
rs1336071	120	6	94537182	94537301	GG
rs214955	120	6	152697629	152697748	GG
rs727811	115	6	165045254	165045368	AA
rs321198	165	7	137029715	137029879	TT
rs6955448	120	7	4310285	4310404	CT
rs737681	120	7	155990742	155990861	TT
rs917118	109	7	4456953	4457061	CC
rs10092491	116	8	28411037	28411152	CT
rs2056277	104	8	139399038	139399141	CC
rs4606077	151	8	144656710	144656860	CT
rs763869	85	8	1375576	1375660	CT
rs1015250	117	9	1823702	1823818	GG

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs10776839	103	9	137417271	137417373	GG
rs1360288	119	9	128967994	128968112	CC
rs1463729	99	9	126881396	126881494	GA
rs7041158	115	9	27985907	27986021	CC
rs3780962	94	10	17193284	17193377	CC
rs735155	170	10	3374133	3374302	AA
rs740598	120	10	118506839	118506958	AG
rs826472	153	10	2406511	2406663	TT
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	TT
rs10773760	99	12	130761623	130761721	AG
rs2107612	103	12	888262	888364	AG
rs2111980	94	12	106328186	106328279	GG
rs2269355	65	12	6945881	6945945	CC
rs2920816	157	12	40862976	40863132	TT
rs1058083	76	13	100038193	100038268	AG
rs1335873	109	13	20901665	20901773	TT
rs1886510	116	13	22374646	22374761	CT
rs354439	170	13	106938320	106938489	TT
rs1454361	118	14	25850765	25850882	AT
rs4530059	170	14	104769099	104769268	GG
rs722290	101	14	53216686	53216786	GG
rs873196	114	14	98845506	98845619	CT
rs1528460	115	15	55210664	55210778	TT
rs1821380	118	15	39313343	39313460	GG
rs8037429	63	15	53616876	53616938	TT
rs1382387	89	16	80106318	80106406	GT

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs2342747	104	16	5868645	5868748	AG
rs430046	119	16	78016980	78017098	CC
rs729172	104	16	5606153	5606256	CC
rs740910	113	17	5706552	5706664	AA
rs8078417	143	17	80461847	80461989	CT
rs938283	98	17	77468433	77468530	TT
rs9905977	170	17	2919324	2919493	GG
rs1024116	98	18	75432317	75432414	AA
rs1493232	75	18	1127945	1128019	AA
rs1736442	153	18	55225698	55225850	GG
rs9951171	119	18	9749789	9749907	GG
rs576261	76	19	39559780	39559855	AC
rs719366	170	19	28463281	28463450	TT
rs1005533	158	20	39487066	39487223	AA
rs1031825	126	20	4447416	4447541	CC
rs1523537	117	20	51296076	51296192	CC
rs445251	119	20	15124865	15124983	CG
rs221956	97	21	43606933	43607029	CC
rs2830795	114	21	28608089	28608202	AA
rs2831700	79	21	29679639	29679717	AA
rs722098	101	21	16685561	16685661	AG
rs914165	156	21	42415865	42416020	AG
rs1028528	78	22	48362256	48362333	AG
rs2040411	68	22	47836378	47836445	AA
rs733164	120	22	27816711	27816830	AG
rs987640	120	22	33559450	33559569	AT

Phenotypic Informative SNPs

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Control Alleles
rs28777	92	5	33958916	33959007	AA
rs12203592	110	6	396273	396382	CC
rs4959270	161	6	457655	457815	AC
rs683	120	9	12709246	12709365	AC
rs1042602	113	11	88911659	88911771	AC
rs1393350	99	11	89010977	89011075	GG
rs12821256	119	12	89328278	89328396	CT
rs12896399	73	14	92773627	92773699	GG
rs2402130	120	14	92801169	92801288	AA
rs1800407	119	15	28230246	28230364	GG
N29insA	112	16	89985688	89985799	CC
rs1110400	173	16	89986044	89986216	TT
rs11547464	173	16	89986044	89986216	GG
rs1805005	213	16	89985774	89985986	GG
rs1805006	213	16	89985774	89985986	CC
rs1805007	173	16	89986044	89986216	CC
rs1805008	173	16	89986044	89986216	CC
rs1805009	227	16	89986484	89986710	GG
rs201326893_ Y152OCH	173	16	89986044	89986216	CC
rs2228479	213	16	89985774	89985986	GG
rs885479	173	16	89986044	89986216	GG
rs2378249	118	20	33218028	33218145	AA

Ancestry Informative SNPs

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Allele Control
rs2814778	120	1	159174650	159174769	AA
rs3737576	98	1	101709521	101709618	AA
rs7554936	106	1	151122413	151122518	CT

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Allele Control
rs10497191	101	2	158667153	158667253	CC
rs1834619	84	2	17901444	17901527	GG
rs1876482	120	2	17362526	17362645	CC
rs260690	115	2	109579681	109579795	AA
rs3827760	108	2	109513546	109513653	TT
rs6754311	98	2	136707920	136708017	CT
rs798443	84	2	7968221	7968304	AA
rs12498138	119	3	121459545	121459663	GG
rs1919550	117	3	121364112	121364228	AA
rs1229984	120	4	100239288	100239407	GG
rs3811801	114	4	100244261	100244374	CC
rs4833103	95	4	38815462	38815556	AC
rs7657799	116	4	105375396	105375511	TT
rs7722456	114	5	170202901	170203014	TT
rs870347	119	5	6844995	6845113	TT
rs16891982*	108	5	33951621	33951728	GG
rs192655	70	6	90518235	90518304	AG
rs3823159	119	6	136482701	136482819	AA
rs917115	71	7	28172543	28172613	TT
rs1462906	84	8	31896545	31896628	CC
rs1871534	71	8	145639652	145639722	CC
rs2196051	120	8	122124216	122124335	TT
rs6990312	111	8	110602270	110602380	GG
rs3814134	104	9	127267664	127267767	TT
rs4918664	168	10	94920962	94921129	AA
rs1079597	167	11	113296227	113296393	GG
rs174570	120	11	61597179	61597298	CC
rs2238151	113	12	112211753	112211865	CT
rs671	136	12	112241658	112241793	GG
rs1572018	116	13	41715225	41715340	AG

Locus	Amplicon Length (bp)	Chromosome	Amplicon Start Position	Amplicon End Position	2800M Allele Control
rs2166624	71	13	42579949	42580019	AG
rs7326934	96	13	49070482	49070577	GG
rs7997709	85	13	34847693	34847777	TT
rs9522149	119	13	111827125	111827243	CC
rs200354	165	14	99375246	99375410	GG
rs12439433	100	15	36219979	36220078	GG
rs1426654	92	15	48426457	48426548	AA
rs1800414	116	15	28196969	28197084	AA
rs735480	108	15	45152321	45152428	TT
rs12913832*	119	15	28365523	28365641	AG
rs459920	78	16	89730800	89730877	TT
rs11652805	119	17	62987113	62987231	TT
rs17642714	118	17	48726060	48726177	AT
rs2593595	102	17	41056210	41056311	TC
rs4411548	158	17	40658440	40658597	GG
rs4471745	67	17	53568849	53568915	GG
rs2042762	83	18	35277568	35277650	AA
rs3916235	120	18	67578894	67579013	AG
rs4891825	106	18	67867615	67867720	AG
rs7226659	149	18	40488180	40488328	GG
rs7251928	200	19	4077044	4077243	AA
rs310644	89	20	62159472	62159560	AA
rs2024566	88	22	41697312	41697399	AA

* Also used for phenotype prediction.

Interpreting Loci D22S1045

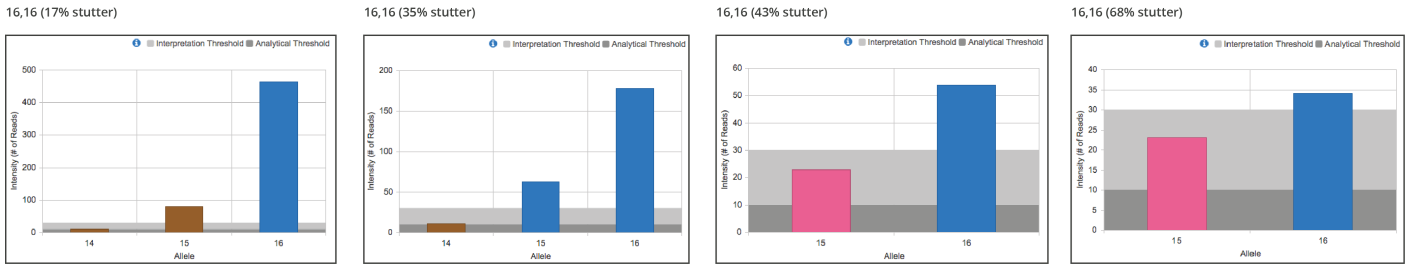
The following sections provide example interpretation methods to help interpret the aSTR locus D22S1045. Determine actual values and methods based on application and internal validation data.

Loci D22S1045 might indicate elevated n-1 repeat stutter, particularly with decreased marker coverage. Heterozygote imbalance might occur regardless of marker coverage. When determining the presence of a DNA mixture, consider multilocus genotype.

Data Trends for D22S1045

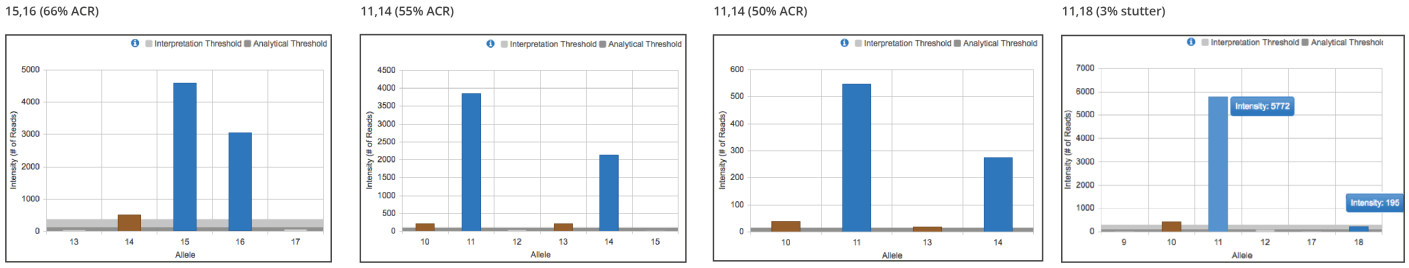
Elevated n-1 stutter can occur in low coverage situations, particularly for stutter in STR positions and lengths ≥ 15. Stutter percentages increase as coverage decreases, and in extreme cases can approach or surpass the read depth of the parent allele. The following figure shows progressively increasing n-1 stutter (15 position) as locus coverage decreases.

Figure 4 Increasing stutter



Heterozygote imbalance can occur at low or high locus coverage. Imbalance increases with a larger spread between allele lengths (for example, 11,18). The following figure shows progressively decreasing intralocus balance (allele count ratio [ACR]) as the allele number spread increases.

Figure 5 Decreasing intralocus balance



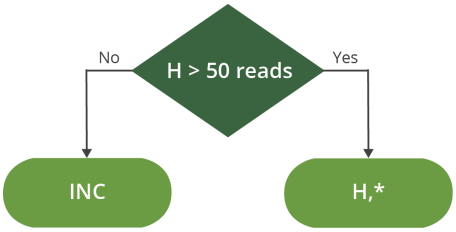
Genotype Determination at D22S1045

The following flowcharts illustrate example methods of genotype determination for loci D22S1045. The read values in each flowchart are intended as conservative examples that demonstrate an interpretation method using specific read level guidelines. Base actual values and methods on laboratory application and internal validation data.

One Typed Allele

In the following figure, H is the example allele, H is a true allele, and INC is an inconclusive result. An inconclusive result is a conservative conclusion that eliminates inadvertently typing stutter position. The asterisk (*) accounts for potential drop-out due to imbalance.

Figure 6 Flowchart for one typed allele

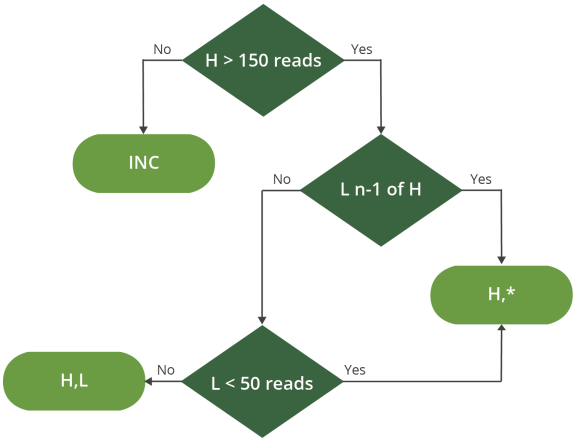


Two Typed Alleles

In the following figure, H is the allele with highest number of reads and L is the allele with lower number of reads. An inconclusive result (INC) is a conservative conclusion that eliminates the chance of accidentally typing stutter position when two potential alleles are present with < 150 available reads.

H,L indicates a true allele and L > 50 reads outside the n-1 position = obligate sister. H,* indicates a true allele, and L < 50 reads or L in the n-1 position might be elevated stutter.

Figure 7 Flowchart for two typed alleles



Interpreting Loci DYS392

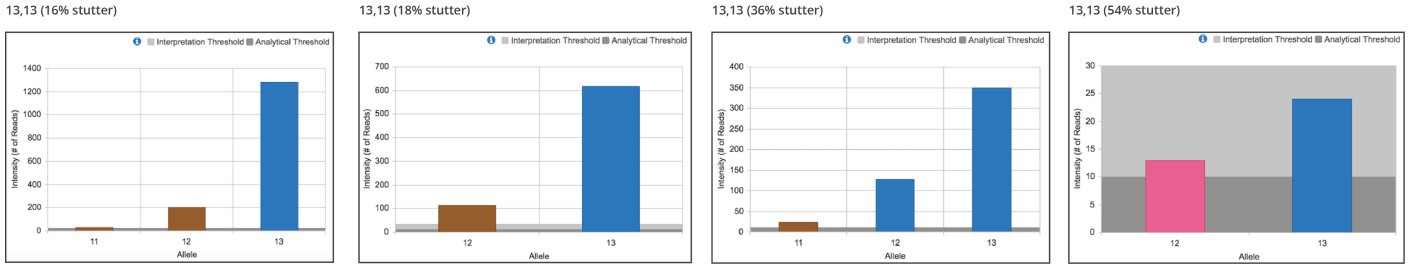
The following sections provide example interpretation methods to help interpret the Y-STR locus DYS392. Determine actual values and methods based on application and internal validation data.

Loci DYS392 might have indicate n-1 repeat stutter, particularly with decreased marker coverage. Consider multilocus genotype when determining the presence of a DNA mixture.

Data Trends for DYS392

Elevated n-1 stutter can occur when locus coverage is low. Stutter increases as coverage decreases, and in extreme cases can approach or surpass the read depth of the parent allele. The following figure shows progressively increasing n-1 stutter (12 position) as locus coverage decreases.

Figure 8 Increasing stutter



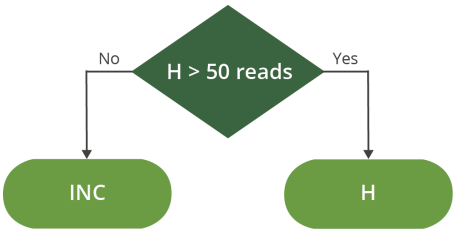
Genotype Determination at DYS392

The following flowcharts illustrate example methods of genotype determination for loci DYS392. The read values in each flowchart are intended as conservative examples that demonstrate an interpretation method using specific read level guidelines. Base actual values and methods on laboratory application and internal validation data.

One Typed Allele

In the following figure, H is the example allele, H is a true allele, and INC indicates an inconclusive result. An inconclusive result is a conservative conclusion that eliminates inadvertently typing stutter position.

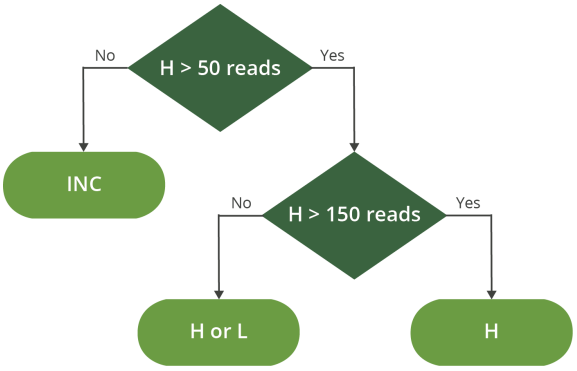
Figure 9 Flowchart for one typed allele



Stutter Position Typing

In the following figure, H is the allele with the highest number of reads and L is the allele with the lowest number of reads. INC is an inconclusive result and conservative conclusion to eliminate accidentally typing stutter position. A conclusion of H or L indicates the potential for either to be elevated stutter. A conclusion of H is a true allele, even with L at a high n-1 stutter percent.

Figure 10 Flowchart for n-1 stutter position typing with parent allele



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