ForenSeq Imagen Kit with the Enhanced PCR1 Buffer System

Reference Guide

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Revision History

Document #	Date	Description of Change
VD2022011 Rev. A	August 2022	Initial release

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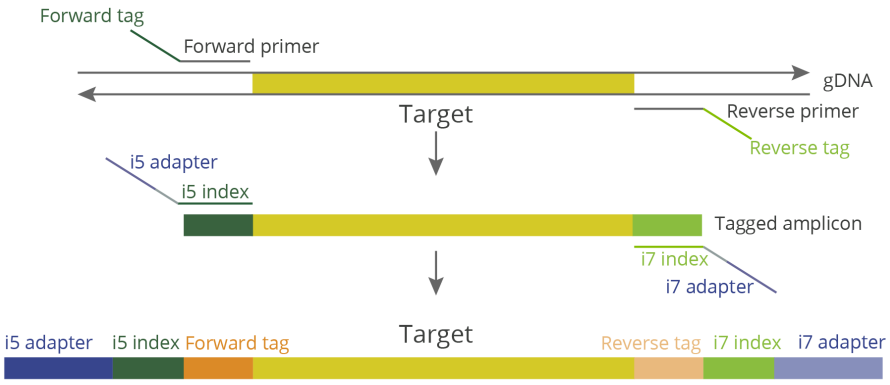
Overview

Introduction

The ForenSeq® Imagen Kit generates 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 Imagen Kit can be used with the standard PCR1 (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 Imagen Kit with the ForenSeq Enhanced PCR1 Buffer System.

Figure 1 Assay overview



Kit Features

The ForenSeq Imagen Kit offers the following features:

- A concentrated primer mix that allows for increased input volume.
- Simultaneous preparation of up to 96 libraries. Each library is a collection of amplified DNA fragments from one sample.
- Two primer mixes, either of which can be used for generation of DNA libraries based on user preferences:
  - DPME – Amplifies SNP targets for the prediction skin, hair, and eye color, also referred to as *external visible characteristics* (EVC).
  - DPMF (Geo) – Amplifies SNP targets for the prediction of EVCs in DPME and bio-geographical ancestry (BGA) markers.

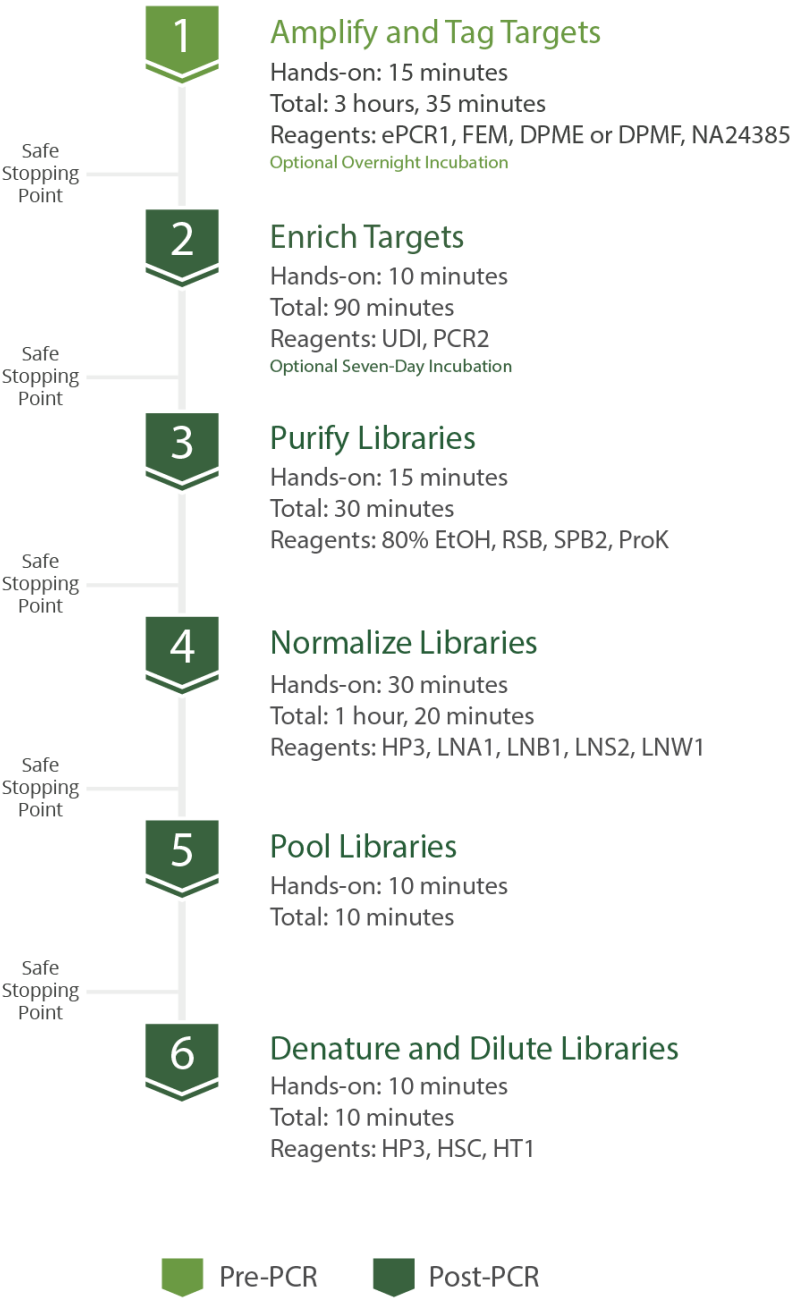
ForenSeq Enhanced PCR1 Buffer System offers the following features:

- 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 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 Imagen protocol with the Enhanced PCR1 (ePCR1) 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 NA24385 Positive Amplification Control DNA (NA24385) for use as the positive template control and the negative amplification control is nuclease-free water. The protocol includes instructions to prepare each control.

Additional Resources

This guide provides comprehensive information on the ForenSeq Imagen 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 Imagen Kit with Enhanced PCR1 Buffer System Checklist (document # VD2022013)	Provides concise protocol instructions for the experienced user.
ForenSeq Imagen Kit with Enhanced PCR1 Buffer System Materials List (document # VD2022012)	Lists the consumables and equipment needed to perform the protocol.

Protocol

Introduction

This chapter describes the ForenSeq Imagen 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 23).

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.

Reference the following table to determine the maximum number of libraries to pool for a run, depending on MiSeq FGx® reagent kit.

Reagent Kit	Maximum Number of Libraries
MiSeq FGx Reagent Micro Kit	96

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 *Universal Analysis Software v2.0 Reference Guide (document # VD2022014)* provides detailed information on sample sheets and input of sample information.

Preventing Cross-Contamination

- Set up the *Amplify and Tag Targets* (next 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***.
- When processing smaller sample batches of less than 96 libraries at a time, prevent aerosolization by resealing the utilized, pierced UDI wells with Microseal "B" adhesive seal. Trim excess seal with a scissors.
- Do not apply Microseal "B" to unused UDI wells as this will impact the integrity of the single-use pierceable foil.

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.
- 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 SNPs to tag and amplify the input gDNA.

Consumables

- NA24385 (NA24385 Positive Amplification Control DNA)
- DPME (DNA Primer Mix E) or DPMF (Geo) (DNA Primer Mix F)
- 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.***
- Do not vortex NA24385, FEM, or input gDNA.
- For information on DPME and DPMF (Geo), see *Loci Detected with DPME and DPMF (Geo)* (page 30).

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
NA24385	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert three times to mix, and then centrifuge briefly.
FEM	-25°C to -15°C	Remove from storage immediately before use, and then return to storage immediately after use.
DPME or DPMF (Geo)	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.
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 1 for ramp modes.
   - Choose the preheat lid option. See Table 1 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 1 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
QIAmplifier 96-well thermal cycler	Standard	Heated, constant at 100°C	0.1°C per second	Polypropylene plates
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. (The duration for the amplification will depend on the thermal cycler used.)

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 0.125 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)
   - DPME or DPMF (Geo) (2 µl)

For example, for eight samples prepare 61.6 µl master mix: 41.4 µl ePCR1, 2.6 µl FEM, and 17.6 µl DPME or DPMF (Geo).

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 7 µl master mix to each well of the FSP.
6. In a new 1.7 ml tube, combine the following volumes to dilute NA24385:
   - NA24385 (2 µl)
   - Nuclease-free water (158 µl)
7. Cap and gently invert three times to mix, and then centrifuge briefly.
8. Add 8 µl diluted NA24385 to at least one well of the FSP as a positive amplification control.
9. Pipette to mix.
10. Add 8 µl nuclease-free water to at least one well of the FSP as a negative amplification control.
11. Pipette to mix.
12. Add 8 µl 0.125 ng/µl DNA to each well of the FSP.
13. Seal and centrifuge at 1000 × g for 30 seconds.
14. Place on the preprogrammed thermal cycler and run the PCR1 program.
15. Unless you are stopping, proceed to *Enrich Targets* (next page).

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 the UDI (Unique Dual Index) adapters and sequences required for cluster generation. The UDI adapters tag DNA with a unique combination of sequences that identify each sample for analysis.

Consumables

- PCR2 (PCR2 Reaction Mix)
- UDI (Unique Dual Index) plate (UDI0001–UDI0096)
- Microseal 'A' film
- [Optional] Microseal "B" film

About Reagents

- Dispense PCR2 slowly to prevent bubbles.
- Each well of the UDI plate is single-use.
- The row and column labels are only visible from the underside of the UDI plate. Raise the plate overhead to check the labels.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
UDI plate	-25°C to -15°C	Thaw at room temperature.
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 120 seconds
   - 68°C for 10 minutes
   - Hold at 10°C

   Total program time is ~46 minutes. (The duration for the amplification will depend on the thermal cycler used.)
3. Remove the protective cover from the UDI plate and discard appropriately.
4. Centrifuge at 1000 × g for 30 seconds.

Procedure

1. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
2. Using a new pipette tip for each well, pierce the foil covering of the UDI plate and transfer 8 µl UDI adapter to each sample well in the FSP. See Table 2 for the UDI Adapter Plate layout.
3. Briefly centrifuge PCR2, and then pipette to mix.

4. [Optional] Evenly distribute PCR2 among each tube of an 8-tube strip. Use a multichannel pipette to dispense.
5. Add 27 µl PCR2 to each well of the FSP.
6. Pipette to mix.
7. Seal and centrifuge at 1000 × g for 30 seconds.
8. Place on the preprogrammed thermal cycler and run the PCR2 program.

Table 2 UDI Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI0001	UDI0002	UDI0003	UDI0004	UDI0005	UDI0006	UDI0007	UDI0008	UDI0009	UDI0010	UDI0011	UDI0012
B	UDI0013	UDI0014	UDI0015	UDI0016	UDI0017	UDI0018	UDI0019	UDI0020	UDI0021	UDI0022	UDI0023	UDI0024
C	UDI0025	UDI0026	UDI0027	UDI0028	UDI0029	UDI0030	UDI0031	UDI0032	UDI0033	UDI0034	UDI0035	UDI0036
D	UDI0037	UDI0038	UDI0039	UDI0040	UDI0041	UDI0042	UDI0043	UDI0044	UDI0045	UDI0046	UDI0047	UDI0048
E	UDI0049	UDI0050	UDI0051	UDI0052	UDI0053	UDI0054	UDI0055	UDI0056	UDI0057	UDI0058	UDI0059	UDI0060
F	UDI0061	UDI0062	UDI0063	UDI0064	UDI0065	UDI0066	UDI0067	UDI0068	UDI0069	UDI0070	UDI0071	UDI0072
G	UDI0073	UDI0074	UDI0075	UDI0076	UDI0077	UDI0078	UDI0079	UDI0080	UDI0081	UDI0082	UDI0083	UDI0084
H	UDI0085	UDI0086	UDI0087	UDI0088	UDI0089	UDI0090	UDI0091	UDI0092	UDI0093	UDI0094	UDI0095	UDI0096

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.

NOTE: When processing less than 96 libraries, reseal only the utilized or pierced UDI plate wells with Microseal "B". Do not seal unpierced or unused UDI wells as this will impact the integrity of the single-use pierceable foil. The remaining UDIs can be utilized with subsequent library preparations.

Purify Libraries

This process combines purification beads with an enzyme to purify the amplified libraries from other reaction components.

Consumables

- RSB (Resuspension Buffer)
- SPB2 (Sample Purification Beads 2)
- Freshly prepared 80% ethanol (EtOH)
- Proteinase K
- 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
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
SPB2	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex for **≥ *1 minute*** and invert to mix.
ProK	-25°C to -15°C	Remove from storage immediately before use, and then return to storage immediately after use.

2. Label plates as follows.

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

3. Make the SPB2 Master Mix as follows.

SPB2 Beads Master Mix	SPB2	ProK
for 96 samples	5.50 ml	25.0 µl
for 64 samples	3.50 ml	16.4 µl
for 48 samples	2.70 ml	12.5 µl
for 32 samples	1.75 ml	8.2 µl
for 24 samples	1.30 ml	6.2 µl

Procedure

1. Add 45 µl SPB2 Master Mix to each well of the PBP.
2. Centrifuge the sealed FSP at 1000 × g for 30 seconds.

NOTE: If needed, transfer the appropriate volume into a 2 ml tube before dispensing 45 µl to each well.

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 (~5 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. With a 20 µl pipette, remove residual EtOH from each well.
12. Remove from the magnetic stand.
13. Add 52.5 µl RSB to each well.
14. Seal and shake at 1800 rpm for 2 minutes.
15. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.
16. Incubate at room temperature for 2 minutes.
17. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
18. Transfer 50 µl supernatant from each well of the PBP to the corresponding well of the PLP and discard the PBP plate appropriately.
19. 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
- Two each of either of the following tubes:
  - 1.7 ml microcentrifuge tube
  - 15 ml conical 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
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.

Item	Storage	Instructions
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	0.1 N HP3
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.
   - Store the remaining buffer at the appropriate temperature. See *Post-PCR Box 2* (page 23).
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 8).
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
- 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:

Strictly follow the order of adding the reagents.

   - 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 600 µl HT1 to the DNL tube.

5. Place the PNL tube in the preheated microheating system and incubate for 2 minutes.
6. Immediately transfer 8 µl library from the PNL tube to the DNL tube.
7. Pipette to mix.
8. Cap the PNL tube and store at -25°C to -15°C for ≤ 30 days.
   Exceeding 30 days significantly reduces cluster density.
9. Add 4 µl denatured HSC to the DNL tube.
   You can store the denatured HSC at room temperature for ≤ 1 day.
10. Pipette to mix.
11. Cap and vortex to mix, and then centrifuge briefly.
12. 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 Imagen Kit (96 Reactions)	V16000189
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. See *Post-PCR Box 2* (below).

ForenSeq Imagen Kit (96 Reactions) (V16000142)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
2	FEM	Enzyme Mix	Yellow	-25°C to -15°C
2	PCR1	PCR1 Reaction Mix	Green	-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*
2	LNW1	Library Normalization Wash 1	Clear	2°C to 8°C*
2	PCR2	PCR2 Reaction Mix	Purple	-25°C to -15°C
1	UDI Plate	Unique Dual Index	Clear	-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	Green	2°C to 8°C
1	SPB2	Sample Purification Beads 2	Red	2°C to 8°C

Pre-PCR Box 4

Quantity	Reagent	Description	Cap	Storage
1	NA24385	NA24385 Positive Amplification Control DNA	Black	2°C to 8°C
2	DPME	DNA Primer Mix E	Gray	2°C to 8°C
2	DPMF (Geo)	DNA Primer Mix F	Orange	2°C to 8°C

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

Box 1

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

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
96-well twin.tec PCR plates, semiskirted	One of the following suppliers: • Eppendorf, catalog # 951020303 • VWR, catalog # 89136-706

Consumable	Supplier
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
MiSeq FGx Reagent Micro Kit	Verogen part # 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 pipettes	General lab supplier	X	X
200 µl pipettes	General lab supplier	X	X
1000 µl pipettes	General lab supplier	X	X
20 µl multichannel pipettes (8-channel)	General lab supplier	X	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] 10 µl pipettes	General lab supplier	X	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*	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
QIAmplifier 96-well thermal cycler	Standard	Heated, constant at 100°C	Polypropylene plates
Proflex 96-well PCR System	Not applicable	Heated, constant at 105°C	Polypropylene plates and tubes
Veriti 96-well thermal cycler	Standard	Heated, constant at 105°C	Polypropylene plates and tubes

* Only gold heat blocks are supported.

Index Adapter Sequences

The following table lists the 8 bp sequences for the Unique Dual Index (UDI) adapters included in the ForenSeq Imagen Kit. Each adapter combines an Index 1 (i7) and Index 2 (i5) sequence.

- Index 1 adapter:
  `CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA`
- Index 2 adapter:
  `/5Biosg/AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT`

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0001	ATCACGAT	AGCGCTAG	A1
UDI0002	CGATGTAT	GATATCGA	A2
UDI0003	TTAGGCAT	CGCAGACG	A3
UDI0004	TGACCAAT	TATGAGTA	A4
UDI0005	ACAGTGAT	AGGTGCGT	A5
UDI0006	GCCAATAT	GAACATAC	A6
UDI0007	CAGATCAT	ACATAGCG	A7
UDI0008	ACTTGAAT	GTGCGATA	A8
UDI0009	GATCAGAT	CCAACAGA	A9
UDI0010	TAGCTTAT	TTGGTGAG	A10
UDI0011	GGCTACAT	CGCGGTTC	A11
UDI0012	CTTGTAAT	TATAACCT	A12

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0013	AGTCAAAT	AAGGATGA	B1
UDI0014	AGTTCCAT	GGAAGCAG	B2
UDI0015	ATGTCAAT	TCGTGACC	B3
UDI0016	CCGTCCAT	CTACAGTT	B4
UDI0017	GTAGAGAT	ATATTCAC	B5
UDI0018	GTCCGCAT	GCGCCTGT	B6
UDI0019	GTGAAAAT	ACTCTATG	B7
UDI0020	GTGGCCAT	GTCTCGCA	B8
UDI0021	GTTTCGAT	AAGACGTC	B9
UDI0022	CGTACGAT	GGAGTACT	B10
UDI0023	GAGTGGAT	ACCGGCCA	B11
UDI0024	GGTAGCAT	GTTAATTG	B12
UDI0025	ACTGATAT	AACCGCGG	C1
UDI0026	ATGAGCAT	GGTTATAA	C2
UDI0027	ATTCCTAT	CCAAGTCC	C3
UDI0028	CAAAAGAT	TTGGACTT	C4
UDI0029	CAACTAAT	CAGTGGAT	C5
UDI0030	CACCGGAT	TGACAAGC	C6
UDI0031	CACGATAT	CTAGCTTG	C7
UDI0032	CACTCAAT	TCGATCCA	C8
UDI0033	CAGGCGAT	CCTGAACT	C9
UDI0034	CATGGCAT	TTCAGGTC	C10
UDI0035	CATTTTAT	AGTAGAGA	C11
UDI0036	CCAACAAT	GACGAGAG	C12
UDI0037	CGGAATAT	AGACTTGG	D1
UDI0038	CTAGCTAT	GAGTCCAA	D2
UDI0039	CTATACAT	CTTAAGCC	D3
UDI0040	CTCAGAAT	TCCGGATT	D4
UDI0041	GACGACAT	CTGTATTA	D5
UDI0042	TAATCGAT	TCACGCCG	D6

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0043	TACAGCAT	ACTTACAT	D7
UDI0044	TATAATAT	GTCCGTGC	D8
UDI0045	TCATTCAT	AAGGTACC	D9
UDI0046	TCCCGAAT	GGAACGTT	D10
UDI0047	GTTCCAAT	AATTCTGC	D11
UDI0048	ACCTTGGC	GGCCTCAT	D12
UDI0049	ATATCTCG	ATCTTAGT	E1
UDI0050	GCGCTCTA	GCTCCGAC	E2
UDI0051	AACAGGTT	ATACCAAG	E3
UDI0052	GGTGAACC	GCGTTGGA	E4
UDI0053	CAACAATG	CTTCACGG	E5
UDI0054	TGGTGGCA	TCCTGTAA	E6
UDI0055	AGGCAGAG	AGAATGCC	E7
UDI0056	GAATGAGA	GAGGCATT	E8
UDI0057	TGCGGCGT	CCTCGGTA	E9
UDI0058	CATAATAC	TTCTAACG	E10
UDI0059	GATCTATC	ATGAGGCT	E11
UDI0060	AGCTCGCT	GCAGAATC	E12
UDI0061	CGGAACTG	CACTACGA	F1
UDI0062	TAAGGTCA	TGTCGTAG	F2
UDI0063	TTGCCTAG	ACCACTTA	F3
UDI0064	CCATTCGA	GTTGTCCG	F4
UDI0065	ACACTAAG	ATCCATAT	F5
UDI0066	GTGTCGGA	GCTTGCGC	F6
UDI0067	TTCCTGTT	AGTATCTT	F7
UDI0068	CCTTCACC	GACGCTCC	F8
UDI0069	GCCACAGG	CATGCCAT	F9
UDI0070	ATTGTGAA	TGCATTGC	F10
UDI0071	ACTCGTGT	ATTGGAAC	F11
UDI0072	GTCTACAC	GCCAAGGT	F12

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0073	CAATTAAC	CGAGATAT	G1
UDI0074	TGGCCGGT	TAGAGCGC	G2
UDI0075	AGTACTCC	AACCTGTT	G3
UDI0076	GACGTCTT	GGTTCACC	G4
UDI0077	TGCGAGAC	CATTGTTG	G5
UDI0078	CATAGAGT	TGCCACCA	G6
UDI0079	ACAGGCGC	CTCTGCCT	G7
UDI0080	GTGAATAT	TCTCATTC	G8
UDI0081	AACTGTAG	ACGCCGCA	G9
UDI0082	GGTCACGA	GTATTATG	G10
UDI0083	CTGCTTCC	GATAGATC	G11
UDI0084	TCATCCTT	AGCGAGCT	G12
UDI0085	AGGTTATA	CAGTTCCG	H1
UDI0086	GAACCGCG	TGACCTTA	H2
UDI0087	CTCACCAA	CTAGGCAA	H3
UDI0088	TCTGTTGG	TCGAATGG	H4
UDI0089	TATCGCAC	CTTAGTGT	H5
UDI0090	CGCTATGT	TCCGACAC	H6
UDI0091	GTATGTTC	AACAGGAA	H7
UDI0092	ACGCACCT	GGTGAAGG	H8
UDI0093	TACTCATA	CCTGTGGC	H9
UDI0094	CGTCTGCG	TTCACAAT	H10
UDI0095	TCGATATC	ACACGAGT	H11
UDI0096	CTAGCGCT	GTGTAGAC	H12

Amplicon Information

Loci Detected with DPME and DPMF (Geo)

The following tables list loci detected with DPME and DPMF (Geo).

Imagen SNPs List with Positive Control (NA24385) Genotype

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs16891982	Eye Color, Skin Color, Hair Color	Autosomal	G/G
rs28777	Hair Color, Skin Color	Autosomal	A/A
rs12203592	Eye Color, Skin Color, Hair Color	Autosomal	C/C
rs4959270	Hair Color	Autosomal	A/C
rs683	Hair Color, Skin Color	Autosomal	A/A
rs10756819	Skin Color	Autosomal	A/G
rs1042602	Hair Color, Skin Color	Autosomal	A/C
rs1393350	Eye Color, Skin Color	Autosomal	G/G
rs1126809	Skin Color	Autosomal	G/G
rs12821256	Hair Color, Skin Color	Autosomal	T/T
rs12896399	Eye Color, Skin Color	Autosomal	T/G
rs2402130	Hair Color, Skin Color	Autosomal	A/A
rs17128291	Skin Color	Autosomal	G/A
rs1545397	Skin Color	Autosomal	A/A
rs1800414	Skin Color	Autosomal	T/T
rs1800407	Eye Color, Skin Color, Hair Color	Autosomal	C/C
rs12441727	Skin Color	Autosomal	G/G
rs1470608	Skin Color	Autosomal	G/G
rs1129038	Skin Color	Autosomal	T/T
rs12913832	Eye Color, Skin Color, Hair Color	Autosomal	G/G
rs2238289	Skin Color	Autosomal	A/A
rs6497292	Skin Color	Autosomal	A/A

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs1667394	Skin Color	Autosomal	T/T
rs1426654	Skin Color	Autosomal	A/A
rs3114908	Skin Color	Autosomal	C/C
rs3212355	Skin Color	Autosomal	C/C
rs312262906	Hair Color	Autosomal	C/C
rs1805005	Hair Color	Autosomal	G/G
rs1805006	Hair Color, Skin Color	Autosomal	C/C
rs2228479	Hair Color, Skin Color	Autosomal	G/G
rs11547464	Hair Color, Skin Color	Autosomal	G/G
rs1805007	Hair Color, Skin Color	Autosomal	C/C
rs201326893_Y152OCH	Hair Color	Autosomal	C/C
rs1110400	Hair Color, Skin Color	Autosomal	T/T
rs1805008	Hair Color, Skin Color	Autosomal	C/C
rs885479	Hair Color, Skin Color	Autosomal	A/A
rs1805009	Hair Color	Autosomal	G/G
rs8051733	Skin Color	Autosomal	A/A
rs6059655	Skin Color	Autosomal	G/G
rs6119471	Skin Color	Autosomal	C/C
rs2378249	Hair Color, Skin Color	Autosomal	A/A
rs9785983	Biological Sex	Y	A
rs13304869	Biological Sex	Y	G
rs9786433	Biological Sex	Y	G
rs9785846	Biological Sex	Y	G
rs13304992	Biological Sex	Y	G
rs13305939	Biological Sex	Y	A
rs9786113	Biological Sex	Y	A
rs28651585	Biological Sex	Y	A
rs34141256	Biological Sex	Y	G
rs9785999	Biological Sex	Y	T
rs13305838	Biological Sex	Y	C
rs13305443	Biological Sex	Y	G

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs13305670	Biological Sex	Y	A
rs7474433	Biological Sex	Y	C

Imagen Geo SNPs List with Positive Control (NA24385) Genotype

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs3737576	Biogeographical Ancestry	Autosomal	T/T
rs7554936	Biogeographical Ancestry	Autosomal	T/T
rs2814778	Biogeographical Ancestry	Autosomal	T/T
rs798443	Biogeographical Ancestry	Autosomal	A/A
rs1876482	Biogeographical Ancestry	Autosomal	G/G
rs1834619	Biogeographical Ancestry	Autosomal	G/G
rs3827760	Biogeographical Ancestry	Autosomal	A/A
rs260690	Biogeographical Ancestry	Autosomal	A/A
rs6754311	Biogeographical Ancestry	Autosomal	C/C
rs10497191	Biogeographical Ancestry	Autosomal	C/C
rs1919550	Biogeographical Ancestry	Autosomal	A/A
rs12498138	Biogeographical Ancestry	Autosomal	G/G
rs4833103	Biogeographical Ancestry	Autosomal	C/C
rs1229984	Biogeographical Ancestry	Autosomal	C/C
rs3811801	Biogeographical Ancestry	Autosomal	G/G
rs7657799	Biogeographical Ancestry	Autosomal	T/T
rs870347	Biogeographical Ancestry	Autosomal	A/A
rs16891982	Biogeographical Ancestry, Eye Color, Skin Color, Hair Color	Autosomal	G/G
rs28777	Hair Color, Skin Color	Autosomal	A/A
rs7722456	Biogeographical Ancestry	Autosomal	T/T
rs12203592	Eye Color, Skin Color, Hair Color	Autosomal	C/C
rs4959270	Hair Color	Autosomal	A/C
rs192655	Biogeographical Ancestry	Autosomal	A/A
rs3823159	Biogeographical Ancestry	Autosomal	A/A
rs917115	Biogeographical Ancestry	Autosomal	C/T
rs1462906	Biogeographical Ancestry	Autosomal	C/C

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs6990312	Biogeographical Ancestry	Autosomal	T/G
rs2196051	Biogeographical Ancestry	Autosomal	G/A
rs1871534	Biogeographical Ancestry	Autosomal	G/G
rs683	Hair Color, Skin Color	Autosomal	A/A
rs10756819	Skin Color	Autosomal	A/G
rs3814134	Biogeographical Ancestry	Autosomal	A/A
rs4918664	Biogeographical Ancestry	Autosomal	A/A
rs174570	Biogeographical Ancestry	Autosomal	C/C
rs1042602	Hair Color, Skin Color	Autosomal	A/C
rs1393350	Eye Color, Skin Color	Autosomal	G/G
rs1126809	Skin Color	Autosomal	G/G
rs1079597	Biogeographical Ancestry	Autosomal	C/C
rs12821256	Hair Color, Skin Color	Autosomal	T/T
rs2238151	Biogeographical Ancestry	Autosomal	C/T
rs671	Biogeographical Ancestry	Autosomal	G/G
rs7997709	Biogeographical Ancestry	Autosomal	T/T
rs1572018	Biogeographical Ancestry	Autosomal	C/T
rs2166624	Biogeographical Ancestry	Autosomal	G/G
rs7326934	Biogeographical Ancestry	Autosomal	G/G
rs9522149	Biogeographical Ancestry	Autosomal	C/C
rs12896399	Eye Color, Skin Color	Autosomal	T/G
rs2402130	Hair Color, Skin Color	Autosomal	A/A
rs17128291	Skin Color	Autosomal	G/A
rs200354	Biogeographical Ancestry	Autosomal	G/G
rs1545397	Skin Color	Autosomal	A/A
rs1800414	Biogeographical Ancestry, Skin Color	Autosomal	T/T
rs1800407	Eye Color, Skin Color, Hair Color	Autosomal	C/C
rs12441727	Skin Color	Autosomal	G/G
rs1470608	Skin Color	Autosomal	G/G
rs1129038	Skin Color	Autosomal	T/T

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs12913832	Biogeographical Ancestry, Eye Color, Skin Color, Hair Color	Autosomal	G/G
rs2238289	Skin Color	Autosomal	A/A
rs6497292	Skin Color	Autosomal	A/A
rs1667394	Skin Color	Autosomal	T/T
rs12439433	Biogeographical Ancestry	Autosomal	G/A
rs735480	Biogeographical Ancestry	Autosomal	T/T
rs1426654	Biogeographical Ancestry, Skin Color	Autosomal	A/A
rs3114908	Skin Color	Autosomal	C/C
rs459920	Biogeographical Ancestry	Autosomal	C/T
rs3212355	Skin Color	Autosomal	C/C
rs312262906	Hair Color	Autosomal	C/C
rs1805005	Hair Color	Autosomal	G/G
rs1805006	Hair Color, Skin Color	Autosomal	C/C
rs2228479	Hair Color, Skin Color	Autosomal	G/G
rs11547464	Hair Color, Skin Color	Autosomal	G/G
rs1805007	Hair Color, Skin Color	Autosomal	C/C
rs201326893_Y152OCH	Hair Color	Autosomal	C/C
rs1110400	Hair Color, Skin Color	Autosomal	T/T
rs1805008	Hair Color, Skin Color	Autosomal	C/C
rs885479	Hair Color, Skin Color	Autosomal	A/A
rs1805009	Hair Color	Autosomal	G/G
rs8051733	Skin Color	Autosomal	A/A
rs4411548	Biogeographical Ancestry	Autosomal	T/C
rs2593595	Biogeographical Ancestry	Autosomal	A/A
rs17642714	Biogeographical Ancestry	Autosomal	T/A
rs4471745	Biogeographical Ancestry	Autosomal	G/G
rs11652805	Biogeographical Ancestry	Autosomal	T/T
rs2042762	Biogeographical Ancestry	Autosomal	T/T
rs7226659	Biogeographical Ancestry	Autosomal	G/G
rs3916235	Biogeographical Ancestry	Autosomal	C/T

SNP ID	SNP Predictive Capability	SNP Locus Type	Genotype
rs4891825	Biogeographical Ancestry	Autosomal	A/G
rs7251928	Biogeographical Ancestry	Autosomal	C/A
rs6059655	Skin Color	Autosomal	G/G
rs6119471	Skin Color	Autosomal	C/C
rs2378249	Hair Color, Skin Color	Autosomal	A/A
rs310644	Biogeographical Ancestry	Autosomal	C/T
rs2024566	Biogeographical Ancestry	Autosomal	A/A
rs9785983	Biological Sex	Y	A
rs13304869	Biological Sex	Y	G
rs9786433	Biological Sex	Y	G
rs9785846	Biological Sex	Y	G
rs13304992	Biological Sex	Y	G
rs13305939	Biological Sex	Y	A
rs9786113	Biological Sex	Y	A
rs28651585	Biological Sex	Y	A
rs34141256	Biological Sex	Y	G
rs9785999	Biological Sex	Y	T
rs13305838	Biological Sex	Y	C
rs13305443	Biological Sex	Y	G
rs13305670	Biological Sex	Y	A
rs7474433	Biological Sex	Y	C

Technical Support

For technical assistance, contact Verogen Technical Support.

	Contact Information
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Email	techsupport@verogen.com
Telephone	+1.833.837.6436 toll-free (North America) +1.858.285.4101 tel +44 (0) 208 054 8706 (United Kingdom)

Safety data sheets (SDS)—Available for download from verogen.com/product-documentation.

Product documentation—Available for download from verogen.com/product-documentation.

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