ForenSeq mtDNA Whole Genome Kit Reference Guide

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Overview

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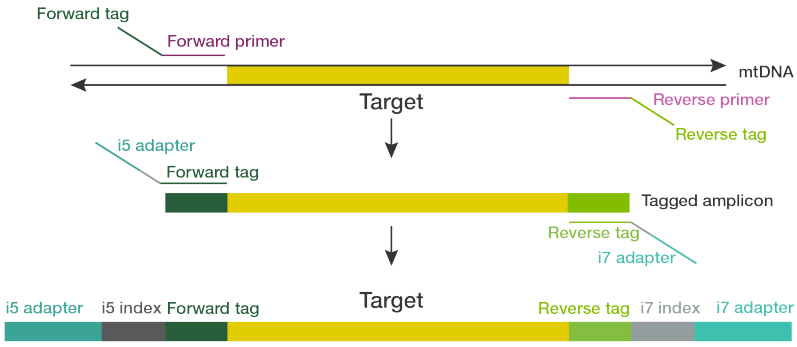
Introduction

The Verogen ForenSeq™ mtDNA Whole Genome Kit prepares up to 48 paired-end, dual-indexed libraries for sequencing and analysis of the entire human mitochondrial genome (mtGenome). The protocol divides each sample into two PCR pools, a strategy that promotes efficient amplification of overlapping amplicons to allow complete coverage.

A primer mix containing tagged oligos for each target sequence mixes with each sample. PCR cycles link the tags to copies of each target, forming DNA templates consisting of regions of interest flanked by universal primer sequences. Index adapters then attach to the tags. The resulting libraries are amplified, purified, and pooled into one tube for sequencing.

Figure 1 Assay Overview



Kit Features

The ForenSeq mtDNA Whole Genome Kit offers the following features:

- Efficient protocol simultaneously prepares all libraries in one plate. Each library is a collection of tagged, amplified DNA fragments from one sample.
- Flexible input requirements allow preparation of libraries from samples that range from high-quality, single-source to degraded.
- Two options for library normalization support high-throughput workflows that can be automated or low-throughput workflows for low-input samples.
- A small amplicon size of < 210 bp with a minimum overlap of 3 bp improves recovery of mitochondrial DNA (mtDNA) from degraded samples.
- Two tiled primer mixes provide balanced coverage of the whole mtGenome. More than 500 primers efficiently amplify degenerate bases.

Figure 2 Tiled Amplicons for Complete Coverage

DNA Input Recommendations

The ForenSeq mtDNA Whole Genome Kit is optimized for input of 100 pg human genomic DNA (gDNA) per sample. Each sample is ***divided into two 50 pg reactions*** early in the protocol. Before starting the protocol, quantify input DNA using a fluorometric-based method or qPCR and assess quality.

The kit is compatible with mtDNA extracted from hair, bone, tooth, and buccal samples. For hair, use DNA extracted from 0.5–2 cm of hair shaft.

Controls

Each preparation must include HL60 (positive template control) and a negative amplification control. If these controls are not included, troubleshooting support is limited.

The kit provides HL60 and the negative amplification control is nuclease-free water. The protocol includes instructions to prepare each control and a reagent blank. Like the input DNA, each control is divided into two reactions.

Additional Resources

This guide provides comprehensive information on the ForenSeq mtDNA Whole Genome Kit with detailed protocol instructions. Visit the Documentation page on the Verogen website to download additional kit documentation and access the latest versions.

Resource	Description
ForenSeq mtDNA Whole Genome Kit Checklist (document # VD2020007)	Provides concise protocol instructions for the experienced user.
ForenSeq mtDNA Whole Genome Kit Materials (document # VD2020008)	Lists consumables and equipment needed to complete the protocol.

Protocol

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Introduction

This chapter describes the ForenSeq mtDNA Whole Genome protocol with step-by-step instructions to prepare libraries for sequencing. Before starting, confirm kit contents and make sure that you have the required consumables and equipment. For a list of items, see *Supporting Information* on page 24.

Number of Samples

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

Plan on sequencing a maximum of 16 libraries on a standard MiSeq FGx® flow cell, which is provided in the MiSeq FGx Reagent Kit. You can prepare the full 48 libraries and split them between three flow cells, if desired.

Index Adapter Strategy

The kit includes 24 index adapters that support either eight unique pairs or 64 total combinations. When preparing samples of varying input amounts and quality, using eight ***unique*** index adapter pairs mitigates cross-talk.

The following table provides example index adapter combinations to help with sensitivity for low-input samples, especially those in the presence of high-input samples.

Sample Type	Index 1 (i7) Adapter	Index 2 (i5) Adapter
Casework sample 1	R713	A501
Casework sample 2	R714	A502
Reference sample 1	R716	A503

Sample Type	Index 1 (i7) Adapter	Index 2 (i5) Adapter
Reference sample 2	R717	A503
Reference sample 3	R718	A503
Reference sample 4	R719	A503
Positive control	R720	A504
Reagent blank	R721	A505
Negative control	R721	A506

Purification Options

The protocol includes two purification processes: the first is required and the second is optional. Verogen recommends the optional purification when preparing libraries from samples with ≤ 20 pg total input gDNA to enhance sensitivity and improve the performance of low-input samples.

Normalization Methods

The protocol includes two methods for library normalization. Libraries must be normalized for sequencing, so choose the method that best suits your input and throughput needs:

- **Bead-based normalization**—The standard normalization method. Use this method when processing high-input samples of similar input, amount, and type. This method is optimized for high-sample throughput and can be automated.
- **Quantification-based normalization**—An alternative normalization method. Use this method for low-input samples and samples of varying inputs, such as when combining reference samples and casework samples. This method is optimized for low-sample throughput and can improve sequencing representation for low-input samples with ≤ 20 pg total input.

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

- Avoid using wells along the edge of the plate when preparing < 48 samples.
- Create a sample sheet to record the positions of each sample, control, and index adapter.
  - Reference the sample sheet during the protocol to ensure proper plate setup.
  - See the *ForenSeq Universal Analysis Software v2.0 Reference Guide (document # VD2019002)* for more information on sample sheets and inputting sample information.

Preventing Cross-Contamination

- Designate a separate workspace for mtDNA extraction and analysis from nuclear DNA extraction and analysis.
- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each well***.

- Remove unused index adapter tubes from the working area.
- Set up reactions for target amplification and tagging in the pre-PCR environment.

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. The seals are effective at -40°C to 110°C.

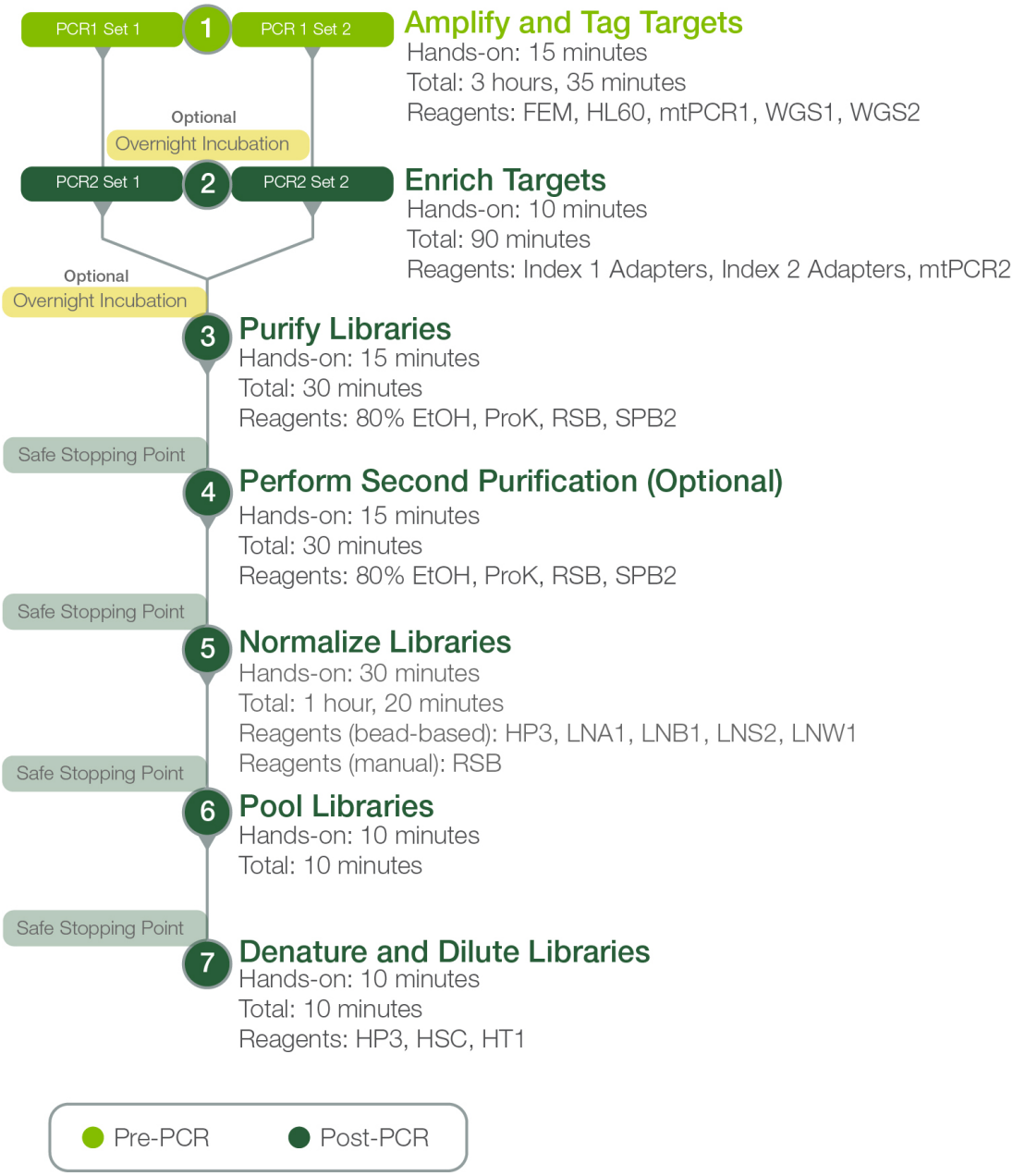
Handling Beads

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

Library Prep Diagram

The following diagram provides an overview of the ForenSeq mtDNA Whole Genome Kit protocol to prepare libraries. Safe stopping points are marked between steps.

Figure 3 Protocol Overview



Amplify and Tag Targets

This process uses an oligonucleotide primer mix to amplify and tag regions specific to DNA sequences in the whole mtGenome.

Consumables

- FEM (ForenSeq Enzyme Mix)
- HL60 (Control DNA HL60)
- mtPCR1 (mtPCR1 Reaction Mix)
- WGS1 (Whole Genome Mix Set 1)
- WGS2 (Whole Genome Mix Set 2)
- Input DNA
- Nuclease-free water
- 1.7 ml microcentrifuge tubes (4)
- 96-well PCR plate, skirted or semi-skirted
- Microseal 'A' film
- Microseal 'B' film
- [Optional] RNase/DNase-free 8-tube strips and caps (2)

About Reagents

- Dispense mtPCR1 slowly to prevent bubbles.
- Do not vortex FEM, HL60, or input DNA.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
FEM	-25°C to -15°C	Thaw at room temperature. Flick to mix, and then centrifuge. Return to storage immediately after use.
HL60	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Flick to mix, and then centrifuge.
mtPCR1	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge.
WGS1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.
WGS2	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.

2 Save the following mtPCR1 program on the thermal cycler in the post-amplification area. Reference Table 1 for ramp mode.
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 15 µl
   - 98°C for 3 minutes
   - 8 cycles of:
     - 96°C for 45 seconds
     - 80°C for 10 seconds
     - 54°C for 2 minutes, with applicable ramp mode
     - 66°C for 1.5 minutes, with applicable ramp mode
   - 10 cycles of:
     - 96°C for 30 seconds
     - 68°C for 2 minutes, with applicable ramp mode

- 68°C for 10 minutes
- Hold at 10°C

Table 1 Ramp Modes

Thermal Cycler	Ramp Mode
ABI LTI thermal cycler 9700	8%
Bio-Rad	0.2°C per second
Eppendorf Mastercycler Pro S	2%
Proflex 96-well PCR System	0.2°C per second
Veriti 96-well thermal cycler	4%

Total program time is ~3.5 hours and can continue overnight.

3 Label tubes and the plate as follows.

Vessel	Label
1.7 ml microcentrifuge tube	Master Mix WGS1
1.7 ml microcentrifuge tube	Master Mix WGS2
1.7 ml microcentrifuge tube	Control DNA Dilution 1
1.7 ml microcentrifuge tube	Control DNA Dilution 2
96-well PCR plate	mtDNA WG Sample Plate

Procedure

1 Using nuclease-free water, dilute 100 pg gDNA to a volume of ≥ 12 µl at 8.33 pg/µl. Gently pipette to mix.

2 In the Master Mix WGS1 tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.
- mtPCR1 (3.7 µl)
- FEM (0.3 µl)
- WGS1 (5 µl)

For example, for 16 samples prepare 158.4 µl WGS1 Master Mix: 65.1 µl mtPCR1, 5.3 µl FEM, and 88 µl WGS1.

3 In the Master Mix WGS2 tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.
- mtPCR1 (3.7 µl)
- FEM (0.3 µl)
- WGS2 (5 µl)

For example, for 16 samples prepare 158.4 µl WGS2 Master Mix: 65.1 µl mtPCR1, 5.3 µl FEM, and 88 µl WGS2.

4 Pipette each master mix to mix, and then cap and centrifuge briefly.

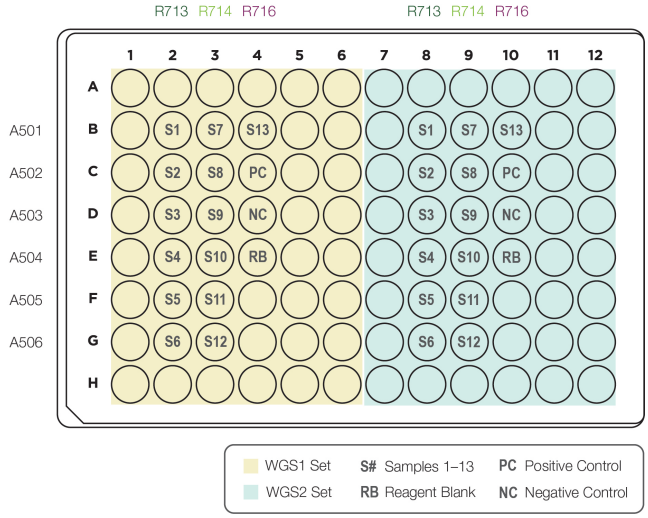
5 [Optional] For > 16 samples:

a Transfer WGS1 Master Mix to an 8-tube strip, evenly distributing between tubes.
b Transfer WGS2 Master Mix to an 8-tube strip, evenly distributing between tubes.

6 Using a multichannel pipette, add master mixes to the mtDNA WG Sample Plate:

a Divide the plate into two even sections depending on the number of samples. See Figure 4 for an example.
b Add 9 µl WGS1 Master Mix to each well in the first section.
c Add 9 µl WGS2 Master Mix to each well in the second section.

Figure 4 Example Plate Setup for 16 Samples



This step sets up the plate for samples and controls divided into two sets. A subsequent step splits each sample and control into two reactions: one for the WGS1 set and one for the WGS2 set. Both reactions must use the same i7 (R7XX) and i5 (A50X) index adapter combination.

7 Dilute 10 ng/µl HL60 stock:

a In the Control DNA Dilution 1 tube, combine the following volumes to prepare 100 pg/µl HL60:
- 10 ng/µl HL60 (2 µl)
- Nuclease-free water (198 µl)

b Gently pipette to mix, and then cap and centrifuge briefly.

c In the Control DNA Dilution 2 tube, combine the following volumes to prepare 8.33 pg/µl HL60:
- 100 pg/µl HL60 (5 µl)
- Nuclease-free water (55 µl)

d Gently pipette to mix, and then cap and centrifuge briefly.

8 Add the reagent blank:

a Add 6 µl reagent blank to one well of the WGS1 set.

b Add 6 µl reagent blank to the corresponding well of the WGS2 set.

c Pipette to mix.

9 Divide each sample between the two sets:

a Add 6 µl 8.33 pg/µl gDNA to one well of the WGS1 set.

b Add 6 µl 8.33 pg/µl gDNA to the corresponding well of the WGS2 set.

c Pipette to mix.

10 Add the positive amplification control:

a Add 6 µl 8.33 pg/µl HL60 to one well of the WGS1 set.

b Add 6 µl 8.33 pg/µl HL60 to the corresponding well of the WGS2 set.

c Pipette to mix.

11 Add the negative amplification control:

   a Add 6 µl nuclease-free water to one well of the WGS1 set.
   b Add 6 µl nuclease-free water to the corresponding well of the WGS2 set.
   c Pipette to mix.

12 Seal with microseal 'A' film and centrifuge at 1000 × g for 30 seconds.

13 Place on the preprogrammed thermal cycler and run the mtPCR1 program.

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

NOTE

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 green caps
- Index 2 Adapters (A50X) and white caps
- mtPCR2 (mtPCR2 Reaction Mix)
- ForenSeq Index Plate Fixture
- 1.7 ml microcentrifuge tubes (1 per index adapter tube)
- RNase/DNase-free 8-tube strip and caps
- Microseal 'A' film
- Microseal 'B' film

About Reagents

- Dispense mtPCR2 slowly to prevent bubbles.
- Centrifuge index adapter tubes in the 1.7 ml microcentrifuge tubes.
- Use the same i7 and i5 index adapter combination for both reactions set up for a sample.

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.
mtPCR2	-25°C to -15°C	Thaw at room temperature, and then invert to mix.

2 Save the following mtPCR2 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C

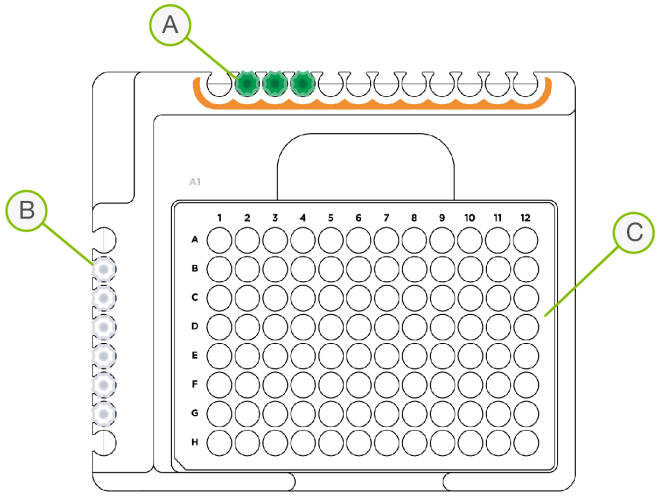
- Reaction volume is 50 µl
- 98°C for 30 seconds
- 15 cycles of:
  - 98°C for 20 seconds
  - 66°C for 30 seconds
  - 68°C for 1.5 minutes
- 68°C for 10 minutes
- Hold at 10°C

Total program time is ~45 minutes.

Procedure

1. Centrifuge the sealed mtDNA WG Sample Plate at 1000 × g for 30 seconds.
2. Arrange the index adapters in the ForenSeq Index Plate Fixture:
   a. Place an Index 1 Adapter tube (green cap) in each column corresponding to the WGS1 set.
   b. Place an Index 2 Adapter tube (white cap) in each row containing sample.
3. Place the mtDNA WG Sample Plate on the ForenSeq Index Plate Fixture.

Figure 5 Example Fixture Setup for 16 Samples



A Index 1 Adapter tubes in columns 2–4
B Index 2 Adapter tubes in rows B–G
C mtDNA WG Sample Plate

4. With a multichannel pipette, add Index 1 Adapters:
   a. Down columns in the WGS1 set, add 4 µl R7XX to each well.
   b. Move the Index 1 Adapter tubes to the columns corresponding to the WGS2 set. Keep the same sequence used for the WGS1 set.
   c. Down columns in the WGS2 set, add 4 µl R7XX to each well.
   d. Replace the cap on each Index 1 Adapter tube with a new green cap.
5. With a multichannel pipette, add Index 2 Adapters:
   a. Add 4 µl A50X across each row.
   b. Replace the cap on each Index 2 Adapter tube with a new white cap.

6 Invert mtPCR2 several times to mix, and then centrifuge briefly.

7 [Optional] Evenly divide mtPCR2 among each tube of an 8-tube strip. Use a multichannel pipette to dispense.

8 Add 27 µl mtPCR2 to each well.

9 Pipette to mix.

10 Seal with microseal 'A' film and centrifuge at 1000 × g for 30 seconds.

11 Place on the preprogrammed thermal cycler and run the mtPCR2 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 combines purification beads with an enzyme to purify the amplified libraries from other reaction components. If you plan to perform the optional second purification, this process is the first of two purifications.

Consumables

- ProK (Proteinase K)
- RSB (Resuspension Buffer)
- SPB2 (Sample Purification Beads 2)
- [Second purification] 96-well midi plates (2)
- [No second purification] The following plates:
  - 96-well midi plate
  - 96-well PCR plate, skirted or semiskirted
- Freshly prepared 80% ethanol (EtOH)
- 1.7 ml microcentrifuge tube
- 50 ml reservoir
- Microseal 'B' film

About Reagents

- Aspirate and dispense SPB2/ProK slowly due to viscosity.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
ProK	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert to mix, and then centrifuge.
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.

2 For each sample, prepare 440 µl fresh 80% EtOH from absolute ethanol.

3 If you are performing the second purification, label plates as follows.

Plate Type	Label
Midi	Purification Bead Plate
Midi	Purification Bead Plate 2

4 If you are ***not*** performing the second purification, label plates as follows.

Plate Type	Label
Midi	Purification Bead Plate
PCR	Purified Library Plate

5 Prepare the SPB2 tube for a first or subsequent use:
- For first-time use, add 25 µl ProK. Select the checkbox on the SPB2 label to indicate the addition.
- For a subsequent use, make sure the checkbox on the SPB2 label is selected.

6 Vortex and invert the SPB2/ProK tube several times to mix.

7 Prepare the appropriate volume of SPB2/ProK depending on how many samples you started the protocol with:

Number of Samples	Volume of SPB2/ProK (µl)	Instruction
≤ 15	95 × number of samples	Add the calculated volume to a 1.7 ml microcentrifuge tube.
≥ 16	100 × number of samples	Add the calculated volume to reservoir.

For example, for 16 samples add 1.6 ml SPB2/ProK to a reservoir.

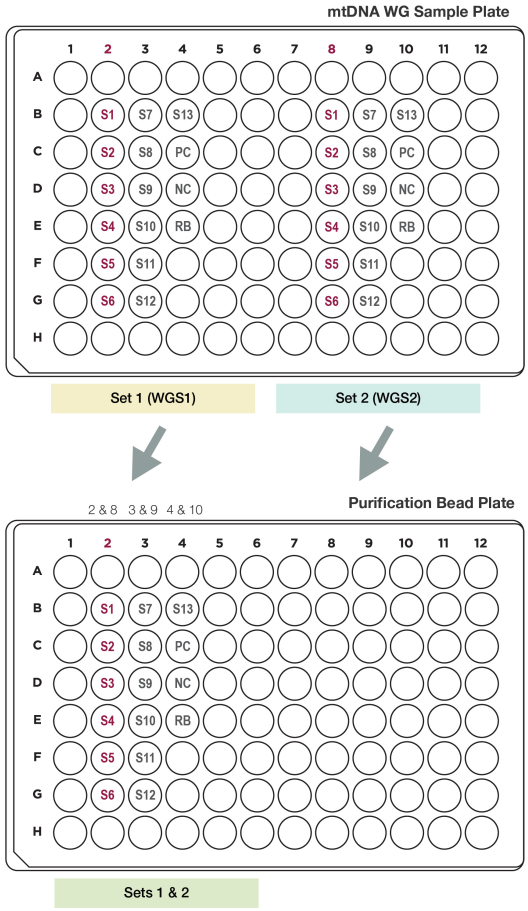
Procedure

1 Add 90 µl SPB2/ProK to each well of the Purification Bead Plate.

2 Centrifuge the sealed mtDNA WG Sample Plate at 1000 × g for 30 seconds.

3 With a multichannel pipette, transfer 45 µl each reaction from the mtDNA WG Sample Plate to the corresponding column of the Purification Bead Plate to combine each reaction set in one well. For example, transfer columns 2 and 8 of the mtDNA WG Sample Plate to column 2 of the Purification Bead Plate (see Figure 6). The resulting volume per well is 180 µl (90 µl sample + 90 µl SPB2/ProK).

Figure 6 Example Transfer for 16 Samples



4 Discard the mtDNA WG Sample Plate.

5 Seal the Purification Bead Plate 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 transparent (~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 (~2 minutes).

18 Transfer 50 µl supernatant from each well of the Purification Bead Plate to the applicable plate:
   - If you are performing the second purification, transfer to the Purification Bead Plate 2.
   - If you are not performing the second purification, transfer to the Purified Library Plate.

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.

Perform Second Purification (Optional)

This optional process repeats the preceding purification process, combining purification beads with an enzyme to further purify libraries. A second purification is highly beneficial for low-input samples (≤ 20 pg gDNA).

Consumables

- ProK (Proteinase K)
- RSB (Resuspension Buffer)
- SPB2 (Sample Purification Beads 2)
- Freshly prepared 80% ethanol (EtOH)
- 1.7 ml microcentrifuge tube
- 50 ml reservoir
- 96-well PCR plate, skirted or semi-skirted
- Microseal 'B' film

About Reagents

- Aspirate and dispense SPB2/ProK slowly due to viscosity.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
ProK	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert to mix, and then centrifuge.
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.

2 For each sample, prepare 440 µl fresh 80% EtOH from absolute ethanol. Alternatively, use 80% EtOH prepared within the day.

3 Label a new PCR plate Purified Library Plate.

4 Prepare the SPB2 tube for a first or subsequent use:
   - For first-time use, add 25 µl ProK. Select the checkbox on the SPB2 label to indicate the addition.
   - For a subsequent use, make sure the checkbox on the SPB2 label is selected.

5 Vortex and invert the SPB2/ProK tube several times to mix.

6 Prepare the appropriate volume of SPB2/ProK depending on the number of samples:

Number of Samples	Volume of SPB2/ProK (µl)	Instruction
≤ 15	55 × number of samples	Add the calculated volume to a 1.7 ml microcentrifuge tube.
≥ 16	60 × number of samples	Add the calculated volume to a reservoir.

Procedure

1 Add 50 µl SPB2/ProK to each sample well of the Purification Bead Plate 2.
Each well contains a volume of 100 µl.

2 Seal and shake at 1800 rpm for 2 minutes.

3 Incubate at room temperature for 5 minutes.

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

5 Remove and discard all supernatant.

6 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.

7 Wash a **second** time.

8 With a 20 µl pipette, remove residual EtOH from each well.

9 Remove from the magnetic stand.

10 Add 52.5 µl RSB to each well.

11 Seal and shake at 1800 rpm for 2 minutes.

12 If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.

13 Incubate at room temperature for 2 minutes.

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

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

16 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 Using a Bead-Based Method (Option 1)

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

Use a bead-based method to normalize libraries from high-input samples of similar input, amount, and type. This method is optimized for high-sample throughput and can be automated.

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
- 1.7 ml microcentrifuge tubes (3)
- 15 ml conical tube
- 50 ml reservoir
- 96-well midi plate
- 96-well PCR plate, skirted or semi-skirted
- Microseal 'B' film

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/sds.**

About Reagents

- The volumes to prepare LNA1/LNB1 Master Mix and 0.1 N HP3 include overage.

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 five times every 15 seconds. Pipette until the beads are fully 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 Hold LNA1 in front of a light. Make sure that no crystals are present and all precipitate has dissolved.

3 Make sure that LNS2 is fully resuspended with no pellet at the bottom of the tube. If necessary, use a 1000 µl pipette to fully resuspend.

4 Label tubes and plates as follows.

Vessel	Label
1.7 ml microcentrifuge tube	0.1 N HP3
1.7 ml microcentrifuge tube or 15 ml conical tube	LNA1/LNB1 Master Mix
Midi plate	Normalization Working Plate
PCR plate	Normalization Library Plate

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

Procedure

1. Vortex LNB1 to resuspend.
2. In the LNA1/LNB1 Master Mix tube, combine the following volumes without adding overage. Multiply each volume by the number of samples.
   - LNA1 (46.8 µl)
   - LNB1 (8.5 µl)
3. Cap and vortex the LNA1/LNB1 Master Mix tube, and then invert several times to mix.
4. Transfer all LNA1/LNB1 Master Mix to a reservoir.
5. Add 45 µl LNA1/LNB1 Master Mix to each sample well of the Normalization Working Plate.
6. Place the Purified Library Plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
7. Transfer 20 µl from each well of the Purified Library Plate to the corresponding well of the Normalization Working Plate.
   Each well contains a volume of 65 µl.
8. Seal the Purified Library Plate containing remaining libraries and store at -25°C to -15°C for up to 1 year from the date of purification.
9. Seal the Normalization Working Plate and shake at 1800 rpm for 30 minutes.
10. While the plate is shaking, perform steps 11–13 to save time later in the process.
11. In the 0.1 N HP3 tube, combine the following volumes without adding overage. Multiply each volume by the number of samples.
    - Nuclease-free water (33.3 µl)
    - HP3 (1.8 µl)
12. Invert several times to mix, and then set aside.
13. Add 30 µl LNS2 to each sample well of the Normalization Library Plate, and then set aside.
14. Immediately after shaking, place the Normalization Working Plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. Remove and discard all supernatant.
16. Wash as follows.
    a. Remove from the magnetic stand.
    b. Add 45 µl LNW1 to each well.
    c. Seal and shake at 1800 rpm for 5 minutes.
    d. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 5 minutes.
    e. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
    f. Remove and discard all supernatant.
17. Wash a **second** time.
18. Remove from the magnetic stand.
19. Seal and centrifuge at 1000 × g for 30 seconds.
20. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
21. With a 20 µl pipette, remove residual supernatant from each well.

22 Remove from the magnetic stand.

23 Add 32 µl 0.1 N HP3 to each well.

24 Seal and shake at 1800 rpm for 5 minutes.

25 If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 5 minutes.

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

27 Transfer 30 µl supernatant from the Normalized Working Plate to the Normalization Library Plate. Pipette to mix as you dispense.
The resulting volume is 60 µl per sample well.

28 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.

Normalize Libraries Using Manual Quantification (Option 2)

This process quantifies libraries and checks the quality. Accurate quantification helps ensure optimum cluster density on the flow cell and a similar number of read counts.

Use a manual quantification method to normalize libraries from low-input samples and samples of varying inputs. This method is optimized for low-sample throughput and can improve sequencing representation for low-input samples (≤ 20 pg gDNA).

Consumables

- RSB (Resuspension Buffer)
- 96-well PCR plate, skirted or semi-skirted
- 1.7 ml microcentrifuge tube

Preparation

1 Prepare the following consumable:

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.

2 Label a new 96-well PCR plate Quant Normalized Library Plate.

Procedure

1 Quantify the library using a fluorometric method, such as QuantiFluor ONE (recommended), AccuCleer, PicoGreen, or Qubit.

2 For libraries with a concentration > 0.75 ng/µl, calculate the volume of RSB required to dilute the library to 0.75 ng/µl as follows.

   a Use the formula $C_1V_1=C_2V_2$ to calculate the value for V_2, where:
      - C_1 is the library quantification result
      - V_1 is 4 µl undiluted library

- C_2 is 0.75 ng/µl
- V_2 is the final volume of diluted library

b Calculate the amount of RSB (V_2 - 4 µl) required to dilute each library to 0.75 ng/µl.

3 Add the calculated volume of RSB to the corresponding well of the Quant Normalized Library Plate. If a library is ≥ 42 ng/µl, add RSB to a 1.7 ml microcentrifuge tube instead.

4 Transfer 4 µl of each library from the Purified Library Plate to the corresponding well of the Normalized Library Plate or 1.7 ml microcentrifuge tube.
The result is a Normalized Library Plate or 1.7 ml microcentrifuge tube of 0.75 ng/uL libraries.

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 step combines equal volumes of normalized libraries to create a pool of libraries that are sequenced together on the same flow cell. Only pool libraries normalized with the same method.

Consumables

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

Preparation

1 Select ≤ 16 libraries to pool for sequencing.
Sixteenis the maximum number of ForenSeq mtDNA Whole Genome libraries the MiSeq FGx standard flow cell supports.

2 Label a new 1.7 ml microcentrifuge tube Pooled Normalized Libraries.

Procedure

1 Transfer 5 µl each library to a new 8-tube strip.

2 Seal the Normalized Library Plate and store in the post-PCR area at -25°C to -15°C for up to 30 days.

3 Transfer libraries from the 8-tube strip to the Pooled Normalized Libraries tube.

4 Cap and vortex to mix, and then centrifuge briefly.

5 Follow the applicable instructions to denature and dilute libraries:
- For bead-based normalized libraries, see *Denature and Dilute Bead-Normalized Libraries (Option 1)* on page 21.
- For manually normalized libraries, see *Denature and Dilute Manually Normalized Libraries (Option 2)* on page 22.

SAFE STOPPING POINT

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

Denature and Dilute Bead-Normalized Libraries (Option 1)

This process adds a sequencing control and uses a heat-based method to denature and dilute libraries normalized with the bead-based method.

Wait until you are ready to prepare sequencing reagents and set up the run before starting this process. Delays can impact template loading.

Consumables

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

Preparation

1. Prepare the reagent cartridge per instructions in the *MiSeq FGx Sequencing System Guide (document # VD2018006)*.
2. 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.

3. Preheat the heat block to 96°C.
4. Label two new 1.7 ml microcentrifuge tubes as follows.
   - Denatured HSC
   - 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. Cap and vortex to mix, and then centrifuge briefly.
3. Incubate at room temperature for 5 minutes.
4. Add 600 µl HT1 to the Denatured Normalized Libraries tube.
5. Incubate the Pooled Normalized Libraries tube on the preheated heat block for 2 minutes.
6. Immediately transfer 5 µl library from the Pooled Normalized Libraries tube to the Denatured Normalized Libraries tube.

7 Pipette to mix.

8 Cap the Pooled Normalized Libraries tube and store at -25°C to -15°C for ≤ 30 days.
Exceeding 30 days can significantly reduce cluster density.

9 Add 4 µl denatured HSC to the Denatured Normalized Libraries tube.
You can store 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 Guide (document # VD2018006)*.

Denature and Dilute Manually Normalized Libraries (Option 2)

This process adds a sequencing control and uses an NaOH-based method to denature and dilute libraries normalized with the manual quantification method. The denaturation process ensures that the concentration of NaOH in the final library does not exceed 1 mM. Higher concentrations can inhibit hybridization to the flow cell and decrease cluster density.

Wait until you are ready to prepare sequencing reagents and set up the run before starting this process. Delays can impact template loading.

Consumables

- HP3 (2 N NaOH)
- HSC (Human Sequencing Control)
- Reagent Kit contents:
  - HT1 (Hybridization Buffer)
  - Reagent cartridge
- Nuclease-free water
- Pooled manually quantified libraries
- 1.7 ml microcentrifuge tubes (4)

Preparation

1 Prepare the reagent cartridge per instructions in the *MiSeq FGx Sequencing System Guide (document # VD2018006)*.

2 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.

3 Label three new 1.7 ml microcentrifuge tubes as follows.
- 6 pM Denatured Normalized Libraries
- 20 pM Denatured Normalized Libraries
- Denatured HSC

4 In another new 1.7 ml microcentrifuge tube, combine the following volumes to prepare 0.1 ml 0.2 N NaOH:
   - Laboratory-grade water (90 µl)
   - HP3 (10 µl)

5 Invert the tube several times to mix. Use within ***12 hours***.
   Freshly prepared NaOH is essential to the denaturation process.

Procedure

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

   You can store denatured HSC at room temperature for ≤ 1 day.

2 Cap and vortex to mix, and then centrifuge briefly.

3 Incubate at room temperature for 5 minutes.

4 In the 20 pM Denatured Normalized Libraries tube, combine the following volumes:
   - 0.75 ng/µl normalized library pool (5 µl)
   - 0.2 N HP3 (5 µl)

5 Cap and vortex briefly.

6 Centrifuge at 280 × g for 1 minute.

7 Incubate at room temperature for 5 minutes.

8 Add 990 µl HT1 to the 20 pM Denatured Normalized Libraries tube.
   The result is 1 ml 20 pM denatured library.

9 In the 6 pM Denatured Normalized Libraries tube, combine the following volumes to dilute the 20 pM library to 6 pM:
   - 20 pM library (180 µl)
   - HT1 (416 µl)
   - Denatured HSC (4 µl)

10 Cap and vortex to mix, and then centrifuge briefly.

11 Immediately transfer the entire volume to the reagent cartridge per instructions in the *MiSeq FGx Sequencing System Guide (document # VD2018006)*.

Supporting Information

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Acronyms

Acronym	Definition
A50X	i5 Index Adapter
FEM	ForenSeq Enzyme Mix
HL60	Control DNA HL60
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
mtDNA	Mitochondrial DNA
mtGenome	Mitochondrial genome
mtPCR1	mtPCR1 Reaction Mix
mtPCR2	mtPCR2 Reaction Mix
NaOH	Sodium hydroxide
ProK	Proteinase K
R7XX	i7 Index Adapter
RSB	Resuspension Buffer
SPB2	Sample Purification Beads 2
WGS1	Whole Genome Mix Set 1
WGS2	Whole Genome Mix Set 2

Kit Contents and Storage

Make sure that you have the reagents identified in this section before starting the protocol. Use the reagents provided in the ForenSeq mtDNA Whole Genome Kit, without making substitutions. When you receive the kit, promptly store reagents at the indicated temperatures.

Kit Name	Verogen Part #
ForenSeq mtDNA Whole Genome Kit (48 reactions)	V16000086

ForenSeq mtDNA Whole Genome Kit (48 reactions) (V16000086)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
1	HL60	Control DNA HL60	Black	2°C to 8°C
1	WGS1	Whole Genome Mix Set 1	Blue	2°C to 8°C
1	WGS2	Whole Genome Mix Set 2	Red	2°C to 8°C

Pre-PCR Box 2

Quantity	Reagent	Description	Cap	Storage
1	FEM	ForenSeq Enzyme Mix	Yellow	-25°C to -15°C
2	mtPCR1	mtPCR1 Reaction Mix	Green	-25°C to -15°C

Post-PCR Box 3

Quantity	Reagent	Description	Cap	Storage
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	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	15°C to 30°C*
1	LNW1	Library Normalization Wash 1	Clear	-25°C to -15°C
2	mtPCR2	mtPCR2 Reaction Mix	Purple	-25°C to -15°C
1	R713	R713 Index Adapter	Green	-25°C to -15°C
1	R714	R714 Index Adapter	Green	-25°C to -15°C
1	R716	R716 Index Adapter	Green	-25°C to -15°C

Quantity	Reagent	Description	Cap	Storage
1	R717	R717 Index Adapter	Green	-25°C to -15°C
1	R718	R718 Index Adapter	Green	-25°C to -15°C
1	R719	R719 Index Adapter	Green	-25°C to -15°C
1	R720	R720 Index Adapter	Green	-25°C to -15°C
1	R721	R721 Index Adapter	Green	-25°C to -15°C
1	Not applicable	i7 Index Tube Caps	Green	15°C to 30°C*
1	Not applicable	i5 Index Tube Caps	White	15°C to 30°C*

* Shipped at -25°C to -15°C.

Post-PCR Box 4

Quantity	Reagent	Description	Cap	Storage
1	LNB1	Library Normalization Beads 1	White	2°C to 8°C
2	ProK	Proteinase K	Clear	2°C to 8°C
1	RSB	Resuspension Buffer	Clear	2°C to 8°C
2	SPB2	Sample Purification Beads 2	Clear	2°C to 8°C

Index Adapter Sequences

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

Index 1 (i7)

Index Name	Sequence
R713	AGTCAAAT
R714	AGTTCCAT
R716	CCGTCCAT
R717	GTAGAGAT
R718	GTCCGCAT
R719	GTGAAAAT
R720	GTGGCCAT
R721	GTTTCGAT

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.

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tubes	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
50 ml PVC reservoirs	VWR, catalog # 89094-688
96-well deepwell storage plates, 0.8 ml (midi plate)	Fisher Scientific, catalog # AB-0859
96-well twin.tec PCR Plate, semi-skirted, 250 µl	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, 200 proof for molecular biology, 500 ml (absolute alcohol)	MilliporeSigma, product # E7023
Microseal 'A' sealing film	Bio-Rad, catalog # MSA5001
Microseal 'B' sealing film, adhesive, optical	Bio-Rad, catalog # MSB1001
Reagent Kit	Verogen, part # 15066817
Nuclease-free water	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
QuantiFluor ONE dsDNA System*	Promega, catalog # E4870 or E4871
[Optional] Index Adapter Replacement Caps	Verogen, part # 15026762

* Or equivalent fluormetric method, such as AccuCleer, PicoGreen, or Qubit.

Equipment

Equipment	Supplier	Pre-PCR	Post-PCR
20 µl multichannel pipettes (8-channel)	General lab supplier	X	X
200 µl multichannel pipette (8-channel)	General lab supplier		X
Benchtop microcentrifuge	General lab supplier	X	X
ForenSeq Index Plate Fixture	Verogen, part # 15055269		X
Magnetic stand-96	Life Technologies, catalog # AM10027		X
Microcentrifuge tube heat block	General lab supplier		X
Microplate centrifuge	General lab supplier	X	X
Thermal cycler, 96-well with heated lid	See *Thermal Cyclers*		X
Thermoshaker, BioShake iQ or BioShake XP	QInstruments, item # 1808-0506 or 1808-0505		X
Quantus Fluorometer	Promega, catalog # E6150	X	X*
Vortexer	General lab supplier	X	X

* For normalization using the manual quantification method.

Thermal Cyclers

The following table lists the recommended settings for the thermal cycler. If your lab has a thermal cycler that is not listed, 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	Standard	Heated	Plate
Proflex 96-well PCR System	Not applicable	Heated, constant at 100°C	Polypropylene plates and tubes
Veriti 96-well thermal cycler[2]	Standard	Heated, constant at 100°C	Polypropylene plates and tubes

[1] Only the fast block (gold) is supported.

[2] Only the standard blocks is supported.

Technical Assistance

For technical assistance, contact Verogen Technical Support.

Table 2 Contact Information

Address	11111 Flintkote Avenue San Diego CA 92121 USA
Website	www.verogen.com
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/sds.

Product documentation—Available for download from verogen.com/support.

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