

ForenSeq™ mtDNA Control Region Kit Reference Guide

VEROGEN PROPRIETARY
Document # VD2019001 Rev. A
June 2019



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Table of Contents

Revision History	4
Introduction	5
DNA Input Recommendations	7
Protocol Introduction	8
Tips and Techniques.....	9
Library Prep Workflow.....	10
Amplify and Tag Targets	11
Enrich Targets	15
Purify Libraries	18
Normalize Libraries (Bead-Based Procedure)	21
Normalize Libraries (Manual Quantification Procedure)	24
Pool Libraries.....	25
Denature and Dilute Libraries (Bead-Normalized Procedure).....	26
Denature and Dilute Libraries (Manual Quant Procedure).....	28
Supporting Kit Information.....	30
Acronyms.....	33
Consumables and Equipment	34
Technical Assistance	37

Revision History

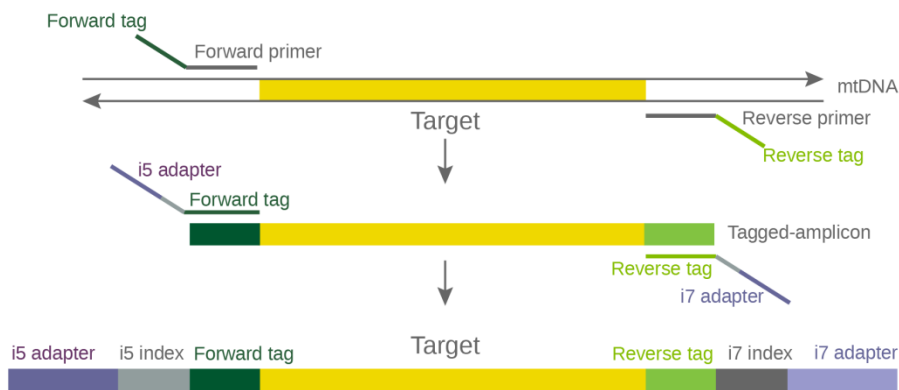
Document #	Date	Description of Change
VD2019001 Rev. A	June 2019	Initial release.

Introduction

This protocol explains how to prepare DNA libraries using reagents provided in the Verogen ForenSeq™ mtDNA Control Region Kit to type, database, casework or research samples for sequencing on the MiSeq FGx instrument.

Two reactions are set up for each sample to support efficient amplification of overlapping amplicons, targeting the human mitochondrial control region. Overlapping amplicons enable complete coverage of the control region. A primer mix containing tagged oligos for each target sequence is mixed with the mtDNA sample. PCR cycles link the tags to copies of each target to form DNA templates consisting of the regions of interest flanked by universal primer sequences. The tags are used to attach indexed adapters, which are then amplified using PCR, purified, pooled into a single tube, and then sequenced. The index sequences allow the sequencing system to separate and isolate the data generated from each sample.

Figure 1 ForenSeq mtDNA Control Region Prep Overview



The mtDNA Control Region Kit enables analysis of the control region of the mitochondrial genome. mtDNA samples ranging from high-quality single source to difficult samples may be assessed. Integrated indexing supports sequencing of up to 48 samples per MiSeq FGx micro flow cell run. The mtDNA Control Region Kit applies the paired-end read capability and high data quality of your Verogen MiSeq FGx sequencing system.

The ForenSeq mtDNA Control Region Kit offers:

- ▶ Two tiled primer mixes for balanced coverage of the control region.
- ▶ Small amplicon size for efficient amplification of mtDNA from degraded samples.
- ▶ Library generation allows for simultaneous preparation of up to 48 samples generating libraries of PCR products within a single plate. Each library is a collection of amplified DNA fragments from a single sample.
- ▶ Two methods are available for library normalization:
 - ▶ Bead-based normalization is the standard method for normalization. This method is ideal for high-sample throughput and can be automated.
 - ▶ Quantification-based normalization is available for low-sample throughput. In addition, quantification-based normalization can improve sequence sample representation for low-input samples (<2pg), which results in lower overall library yield.

Figure 2 Schematic of Tiled Amplicons for Complete Coverage Across the Control Region



- ▶ Overlapping primary amplicons (< 150 bp) for complete coverage under primer binding sites
- ▶ 3 bp spacing between primary amplicons to mitigate end of read trimming during analysis
- ▶ 18 primer pairs comprised of 122 primers to efficiently amplify degenerate bases within the mitochondrial genome

DNA Input Recommendations

It is important to quantify the input gDNA and assess the DNA quality before beginning the mtDNA Control Region Kit protocol. Follow these DNA input recommendations:

- ▶ 50 pg of human genomic DNA (gDNA) input is recommended per reaction. Two reactions are set up for each sample for a total input of 100 pg gDNA per sample.
- ▶ Use a fluorometric-based method or qPCR for quantification.
- ▶ The ForenSeq mtDNA Control Region Kit protocol is compatible with extracted mtDNA from hair, bone, tooth and buccal samples.
 - ▶ If using hair, successful profiles can be obtained from 0.5 cm of hair shaft.
 - ▶ The assay can accommodate up to 12 μ l per sample to support low input samples.

Protocol Introduction

When planning a sequencing run, refer to the supported maximum sequencing sample plexity on the MiSeq FGx® instrument as described in the following table.

Table 1 Maximum Sequencing Plexity by MiSeq FGx Flow Cell Type

Primer Mix	Standard Flow Cell	Micro Flow Cell
Control Region	> 48	48

- ▶ Create a sample sheet to record the positions of each sample and index adapter. For more information, see the *ForenSeq Universal Analysis Software Reference Guide* (document # VD2019002).
- ▶ 64 index combinations with 8 unique pairs to mitigate index cross talk (<0.05%) when combining high quality and low quality samples in the same sequencing run.
- ▶ Follow the protocol in the order shown using the specified volumes and incubation parameters.
- ▶ Processing fewer than eight samples at the same time, including positive and negative controls, can cause pipetting inaccuracy due to the small volumes used when preparing the master mix.
- ▶ Confirm kit contents and make sure that you have the required equipment and consumables. For more information, see *Supporting Kit Information* on page 30.

Tips and Techniques

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

Avoiding Cross-Contamination

- ▶ We recommend designating a separate workspace for mitochondrial DNA 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 row* and *each column*.
- ▶ Remove unused index adapter tubes from the working area.
- ▶ Set up mtPCR1 (copy and tag) in a pre-PCR environment.

Sealing the Plate

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

Mixing

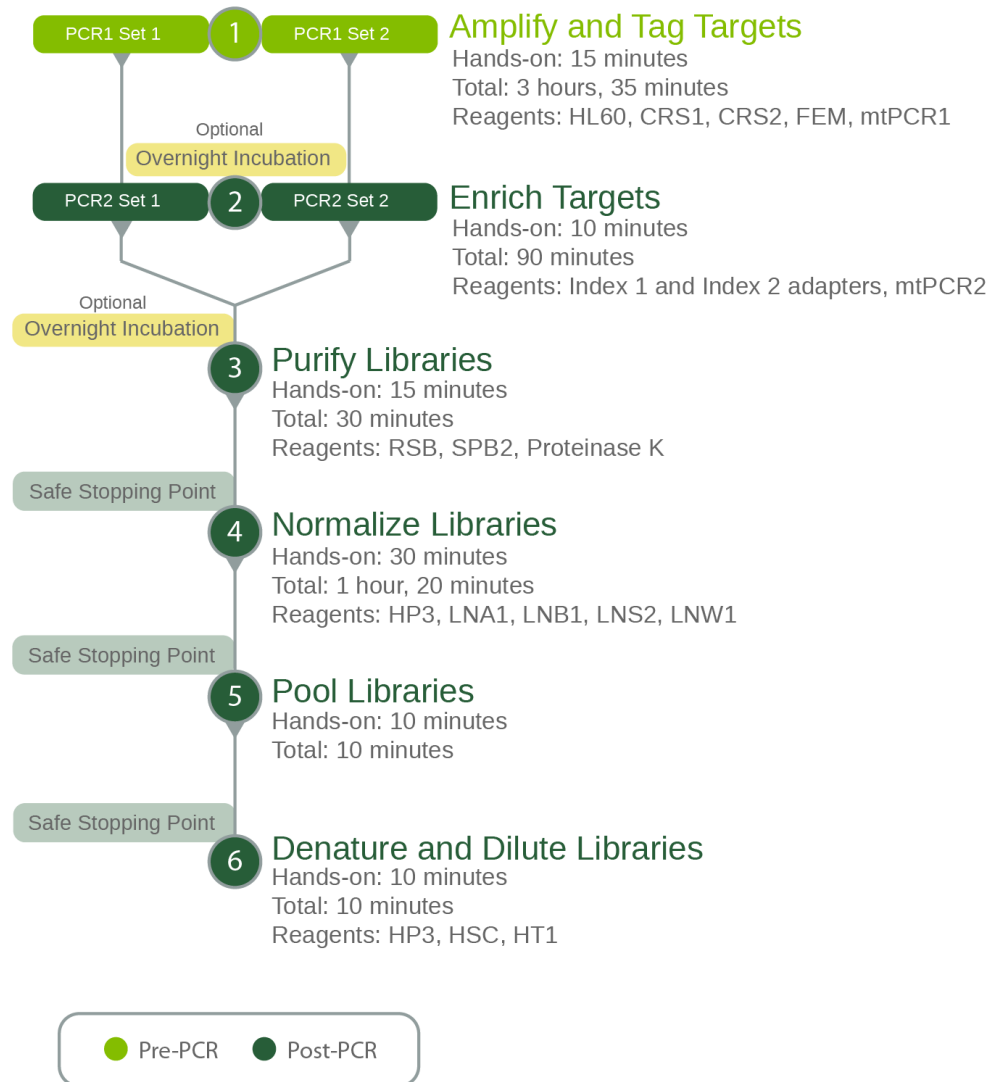
- ▶ Always centrifuge plates and tubes briefly after mixing.
- ▶ Do not centrifuge plates and tubes containing magnetic beads, except when indicated in the protocol.

Plate Transfers

- ▶ When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- ▶ If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
- ▶ For information about mtPCR2 plate transfer, see *Transfer from ForenSeq Sample Plate to Purification Bead Plate* on page 19.

Library Prep Workflow

Figure 3 mtDNA Control Region Kit Workflow



Amplify and Tag Targets

This process amplifies and tags regions specific to DNA sequences of the mitochondrial control region, using a ForenSeq oligonucleotide primer mix.

This protocol requires Control DNA HL60 and a negative PCR amplification control (nuclease-free water) in each preparation. If these controls are not included, troubleshooting support is limited.



NOTE

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

Consumables

- ▶ HL60 (Control DNA)
- ▶ CRS1 (Control Region Set 1)
- ▶ CRS2 (Control Region Set 2)
- ▶ FEM (ForenSeq Enzyme Mix)
- ▶ mtPCR1 (mtPCR1 Reaction Mix)
- ▶ 1.5 ml microcentrifuge tubes (2)
- ▶ 96-well 0.3 ml PCR plate, skirted or semiskirted
- ▶ DNA input:
 - ▶ Purified gDNA (100 pg per sample)
(Split across two mtPCR1 reactions: Set 1 and Set 2)
 - ▶ Hair (DNA extracted from 0.5–2 cm of hair shaft; Gallimore JM, McElhoe JA, Holland MM, (2018) Assessing heteroplasmic variant drift in the mtDNA control region of human hairs using an MPS approach *Forensic Science International: Genetics* 32 7-17)
 - ▶ For bone and teeth up to 12 µl of sample can be used to maximize input
- ▶ Microseal 'A' film
- ▶ Nuclease-free water
- ▶ [Optional] RNase/DNase-free eight-tube strip and caps



NOTE

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

About Reagents

- ▶ Dispense mtPCR1 slowly to avoid creating air bubbles.

Preparation



CAUTION

Failure to use the thermal ramping mode for your thermal cycler can have an adverse effect on results. See ramping modes for selected *Thermal Cyclers* on page 35.

1. Prepare the following consumables.

Item	Storage	Instructions
CRS1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.
CRS2	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.
FEM	-25°C to -15°C	Thaw at room temperature. Return to storage after use. Do not vortex. Flick tube to mix, and then centrifuge.
mtPCR1	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge.
HL60	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Do not vortex. Flick tube to mix, and then centrifuge.

2. Create a sample sheet to record the positions of each sample and index adapter.
3. Save the following mtPCR1 program on the thermal cycler in the post-amplification area. (See page 35 for recommended settings for the thermal cyclers supported in this protocol.) Total reaction volume for mtPCR1 is 15 µl.

- ▶ Choose the preheat lid option and set to 100°C
- ▶ 98°C for 3 minutes
- ▶ 8 cycles of:
 - ▶ 96°C for 45 seconds
 - ▶ 80°C for 10 seconds
 - ▶ 54°C for 2 minutes, with specified ramping mode
 - ▶ 66°C for 1.5 minutes, with specified ramping mode
- ▶ 10 cycles of:
 - ▶ 96°C for 30 seconds
 - ▶ 68°C for 2 minutes, with specified ramping mode
- ▶ 68°C for 10 minutes
- ▶ Hold at 10°C



NOTE

The mtPCR1 program takes approximately 3.5 hours and can be run overnight.

4. Label tubes and plates as follows:
 - ▶ Master Mix CRS1—1.5 ml microcentrifuge tube
 - ▶ Master Mix CRS2—1.5 ml microcentrifuge tube
 - ▶ ForenSeq Sample Plate—PCR plate
 - ▶ Control DNA Dilutions—2 x 1.5 ml microcentrifuge tubes

Procedure

1. Quantify gDNA using a fluorometric-based method or qPCR.
2. Dilute 100 pg purified gDNA input material to 8.33 pg/μl with nuclease-free water.
3. Create a master mix for eight or more reactions in the Master Mix CRS1 tube. Multiply each reagent volume by the number of reactions being prepared. Make 10% extra reagent for overage. (9 μl master mix required per sample.)
 - ▶ mtPCR1 (3.7 μl per sample) for example, eight reactions require 32.6 μl
 - ▶ FEM (0.3 μl per sample) for example, eight reactions require 2.64 μl
 - ▶ CRS1 (5.0 μl per sample) for example, eight reactions require 44 μl
4. Pipette to mix and then centrifuge briefly.
5. Create a master mix for eight or more reactions in the Master Mix CRS2 tube. Multiply each reagent volume by the number of reactions being prepared. Make 10% extra reagent for overage. (9 μl master mix required per sample.)
 - ▶ mtPCR1 (3.7 μl per sample) for example, eight reactions require 32.6 μl
 - ▶ FEM (0.3 μl per sample) for example, eight reactions require 2.64 μl
 - ▶ CRS2 (5.0 μl per sample) for example, eight reactions require 44 μl
6. Pipette to mix and then centrifuge briefly.
7. If processing more than eight samples, evenly distribute the master mix into each well of an eight-tube strip, and then use a multichannel pipette to dispense.
8. Add 9 μl master mix to each well of the ForenSeq Sample Plate.

Figure 4: ForenSeq Sample Plate Setup for 48 samples

	1	2	3	4	5	6	7	8	9	10	11	12
	Set 1 Primer Mix						Set 2 Primer Mix					
	R713	R714	R716	R717	R718	R719	R713	R714	R716	R717	R718	R719
A501	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41
A502	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42
A503	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43
A504	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44
A505	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45
A506	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46
A507	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Sample 47	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Sample 47
A508	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Sample 48	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Sample 48

- ▶ R7 = Index 1 (i7) A5 = Index 2 (i5)
 - ▶ Set up Primer Set 1 on the first half of the ForenSeq Sample Plate.
 - ▶ Set up Primer Set 2 on the second half of the ForenSeq Sample Plate.
 - ▶ If processing fewer than 48 samples, divide the ForenSeq Sample Plate as appropriate based on the number of samples being processed.
9. Dilute 2 μl HL60 with 198 μl nuclease-free water in a new 1.5 ml microcentrifuge tube. Gently flick the tube and then centrifuge briefly.
 10. To further dilute HL60, combine 5 μl diluted HL60 from step 9 with 55 μl nuclease-free water in a new 1.5 ml microcentrifuge tube. Gently flick the tube and then

mtPCR1—mtPCR1 Reaction Mix
 FEM—ForenSeq Enzyme Mix
 CRS1—Control Region Set 1
 CRS2—Control Region Set 2
 HL60—Control DNA

centrifuge briefly.

11. Add 6 μ l diluted HL60 (as a positive template control) to the appropriate wells according to the sample sheet.
12. Add 6 μ l nuclease-free water (as a negative PCR amplification control) to the appropriate wells according to the sample sheet.
13. Add 6 μ l diluted purified gDNA (8.33 pg/ μ l) sample to the appropriate wells according to the sample sheet. Pipette to mix.
14. Seal the plate with Microseal 'A' and centrifuge at 1000 \times g for 30 seconds.
15. Transport to the post-PCR area.
16. Place the plate on the 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 sequences required for cluster generation on the MiSeq FGx flow cell.

The index adapters tag DNA with a unique combination of index sequences that allow data from each tagged library to be separated during later analysis. Remember to use the same i5/i7 index adapter combination for the two primer set reactions set up per sample. See Figure 4 for ForenSeq Sample Plate setup.



NOTE

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

Consumables

- ▶ ForenSeq Index Plate Fixture
- ▶ Index 1 (i7) adapters and green tube caps
- ▶ Index 2 (i5) adapters and white tube caps
- ▶ mtPCR2 (mtPCR2 Reaction Mix)
- ▶ 1.7 ml microcentrifuge tubes (1 per index adapter tube)
- ▶ Microseal 'A' film

About Reagents

- ▶ If processing more than eight libraries at the same time, evenly distribute mtPCR2 to each well of an eight-tube strip, and then use a multichannel pipette to dispense.
- ▶ Dispense mtPCR2 slowly to avoid creating air bubbles.

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
Index adapters (i5 and i7)	-25°C to -15°C	Only remove adapters being used. Thaw at room temperature for 20 minutes. Vortex each tube to mix. Centrifuge briefly using a 1.7 ml Eppendorf tube.
mtPCR2	-25°C to -15°C	Thaw at room temperature. Invert the tube to mix.

2. Save the following mtPCR2 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 1.5 minutes
- ▶ 68°C for 10 minutes
- ▶ Hold at 10°C



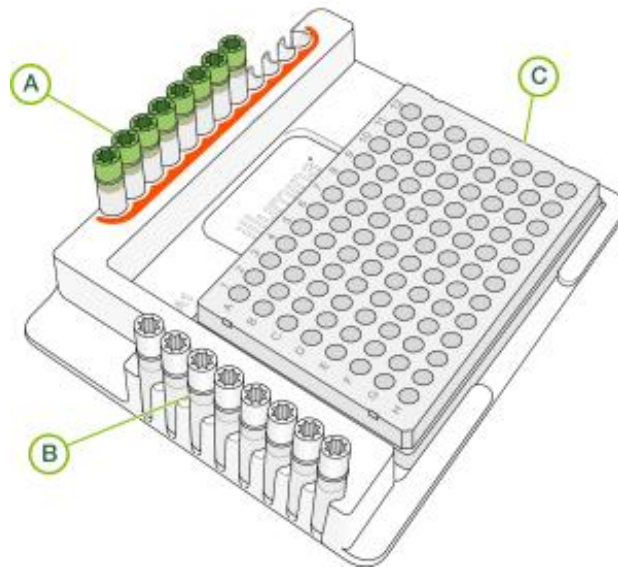
NOTE

The mtPCR2 program takes approximately 1 hour and can be run overnight.

Procedure

1. Centrifuge the ForenSeq Sample Plate at $1000 \times g$ for 30 seconds.
2. Arrange Index 1 (i7) adapters in columns 1–6 of the ForenSeq Index Plate Fixture.
3. Arrange Index 2 (i5) adapters in rows A–H of the ForenSeq Index Plate Fixture.
4. Place the plate on the ForenSeq Index Plate Fixture.

Figure 5 ForenSeq Index Plate Fixture (96 libraries)



- A. Columns 1–6: Index 1 (i7) adapters (green caps)
- B. Rows A–H: Index 2 (i5) adapters (white caps)
- C. ForenSeq Sample Plate

5. Using a multichannel pipette, add 4 μl Index 1 (i7) adapters to each column. Replace the caps on i7 adapter tubes with new green caps.
6. Using a multichannel pipette, add 4 μl Index 2 (i5) adapters to each row. Replace the caps on i5 adapter tubes with new white caps.



NOTE

Use the same i7 and i5 combination for CRS1 and CRS2 reactions set up for each sample according to the ForenSeq Sample Plate set up.

7. Vortex mtPCR2 and then centrifuge briefly.
8. Add 27 μl mtPCR2 to each well.
9. Seal the plate with Microseal 'A' and centrifuge at $1000 \times g$ for 30 seconds.
10. Place the plate on the preprogrammed thermal cycler and run the mtPCR2 program.

mtPCR2—mtPCR2 Reaction Mix
CRS1—Control Region Set 1
CRS2—Control Region Set 2

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 SPB2/ProK (Sample Purification Beads 2 with Proteinase K) to purify the amplified libraries from the other reaction components.

Consumables

- ▶ RSB (Resuspension Buffer)
- ▶ SPB2 (Sample Purification Beads 2)
- ▶ ProK (Proteinase K)
- ▶ 96-well 0.3 ml PCR plate, skirted or semiskirted
- ▶ 96-well midi plates
- ▶ Freshly prepared 80% ethanol (EtOH)
- ▶ 2 ml microcentrifuge tube
- ▶ Microseal 'B' adhesive seals
- ▶ RNase/DNase-free reagent reservoirs

About Reagents

- ▶ Before first use of SPB2, add 25 μ l ProK.
Mark the checkbox on the SPB2 label to indicate the addition of ProK.
- ▶ Vortex SPB2/ProK before each use.
- ▶ Vortex SPB2/ProK frequently to make sure that beads are evenly distributed.
- ▶ Aspirate and dispense SPB2/ProK slowly due to the viscosity of the solution.

Preparation



NOTE

To ensure optimal performance and library yield, make sure that the SPB2/ProK beads are brought to room temperature fully before use.

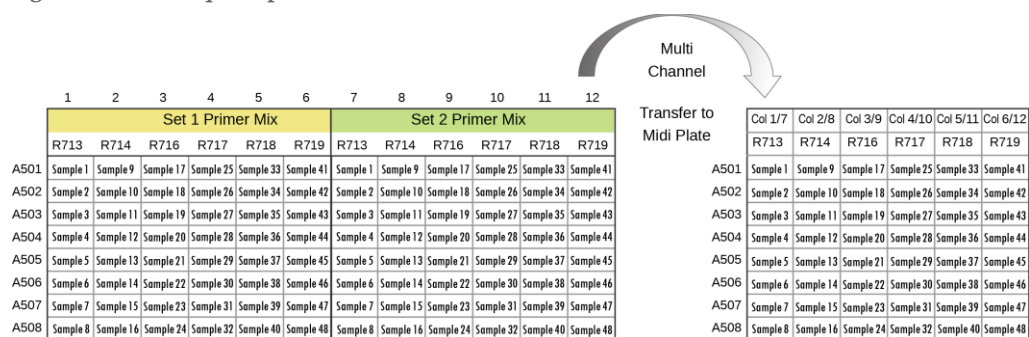
1. Prepare the following consumables.

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB2/ProK	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2. Label plates as follows:
 - ▶ Purification Bead Plate—midi plate
 - ▶ Purified Library Plate—PCR plate

Transfer from ForenSeq Sample Plate to Purification Bead Plate

Figure 6 ForenSeq Sample Plate to Purification Bead Plate



- ▶ Transfer 45 μ l of each set into the corresponding well/column of the Purification Bead Plate containing 90 μ l SPB2/ProK.
 - ▶ Transfer odd-numbered columns on the ForenSeq Sample Plate to odd-numbered columns on the Purification Bead Plate.
 - ▶ Transfer even-numbered columns on the ForenSeq Sample Plate to even-numbered columns on the Purification Bead Plate.

Procedure

1. Verify that ProK has been added to SPB2 (Tube label should be marked).
2. Vortex and then invert the SPB2/ProK tube several times to mix.
3. Prepare SPB2/ProK according to the number of libraries that you are preparing.

Number of Libraries	Instructions
< 16	Add 95 μ l SPB2/ProK \times the number of samples to a 1.7 ml microcentrifuge tube.
16–48	Add [95 μ l SPB2/ProK \times (the number of libraries/8)] + 5 μ l SPB2/ProK to each well of a column of a new midi plate or reagent reservoir.

4. Add 90 μ l SPB2/ProK to each well of the Purification Bead Plate according to the plate transfer layout.
5. Centrifuge the ForenSeq Sample Plate at 1000 \times g for 30 seconds.
6. Transfer 45 μ l of each primer set to the corresponding well/column of the Purification Bead Plate according to the ForenSeq Sample Plate sample setup.



NOTE

Ensure that set 1 and set 2 reactions for the same sample are combined into the appropriate well of the Purification Bead Plate.

7. Seal the plate with Microseal 'B' and shake at 1800 rpm for 2 minutes.
8. Incubate at room temperature for 5 minutes.
9. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
10. Remove and discard all supernatant from each well.
11. Wash two times as follows:
 - a. Add 200 μ l freshly prepared 80% EtOH to each well.
 - b. Incubate on the magnetic stand for 30 seconds.
 - c. Remove and discard all supernatant from each well.
12. Leave the plate on the magnetic stand and use a 20 μ l pipette to remove residual EtOH from each well.
13. Remove the plate from the magnetic stand.
14. Add 52.5 μ l RSB to each well.
15. Seal the plate with Microseal 'B' and shake at 1800 rpm for 2 minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 2 minutes.
16. Incubate at room temperature for 2 minutes.
17. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
18. Transfer 50 μ l to the corresponding well of the Purified Library Plate.
19. Seal the plate with Microseal "B" and centrifuge at 1000 \times g for 30 seconds.

Safe Stopping Point

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

SPB2—Sample Purification Beads 2
ProK—Proteinase K
RSB—Resuspension Buffer

Normalize Libraries Option 1: Bead-Based Procedure

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

Consumables

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



WARNING

This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. LNA1 and LNW1 contain β -mercaptoethanol; prolonged exposure can be toxic to the nervous system and cause organ damage.

Perform this procedure in a hood or well-ventilated area if desired. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and unused contents in accordance with the governmental safety standards for your region. For more information, see how to access safety data sheets (SDSs) in *Technical Assistance* on page 37.

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

About Reagents

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

Preparation

1. Prepare the following consumables.

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

2. Label tubes and plates as follows:
 - ▶ 0.1 N HP3—1.5 ml microcentrifuge tube or a 2.0 ml microcentrifuge tube
 - ▶ LNA1/LNB1 Master Mix—1.5 ml microcentrifuge tube or 15 ml conical tube
 - ▶ Normalization Working Plate—midi plate
 - ▶ Normalization Library Plate—PCR plate
3. Dedicate separate hazardous waste disposal containers for liquids and solids.

Procedure

1. Create a master mix in the LNA1/LNB1 Master Mix tube.
 - ▶ LNA1 (46.8 µl per sample) for example, eight reactions require 374 µl.
 - ▶ LNB1 (8.5 µl per sample) for example, eight reactions require 68 µl.
2. Vortex and then invert the tube several times to mix.
3. Pour into a reagent reservoir.
4. Transfer 45 µl to each well of the Normalization Working Plate that will contain a library according to the sample sheet.
5. To clear beads that might have aspirated, place the Purified Library Plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
6. Transfer 20 µl from each well of the Purified Library Plate to the corresponding well of the Normalization Working Plate.
7. Seal the plate with Microseal 'B' and shake at 1800 rpm for 30 minutes.
8. While the plate is shaking, perform the following steps:
 - a. Prepare the 0.1 N HP3 tube, as follows:
 - ▶ Nuclease-free water (33.3 µl per sample)
For example, eight reactions require 266.4 µl.
 - ▶ HP3 (1.8 µl per sample) For example, eight reactions require 14.4 µl.
 - ▶ Invert the tube several times to mix.
 - ▶ Set aside.
 - b. Add 30 µl LNS2 to each well of the Normalization Library Plate that will contain a library according to the sample sheet.

HP3—2 N NaOH
 LNA1—Library Normalization Additives 1
 LNB1—Library Normalization Beads 1
 LNS2—Library Normalization Storage Buffer 2

9. Immediately after the Normalization Working Plate has finished shaking, place the Normalization Working Plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
10. Remove and discard all supernatant from each well.
11. Remove the plate from the magnetic stand.
12. Wash two times with 45 μ l LNW1 as follows:
 - a. Add 45 μ l LNW1 to each well.
 - b. Seal the plate with Microseal 'B' and shake at 1800 rpm for 5 minutes.
 - c. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
 - d. Remove and discard all supernatant from each well.
13. Remove the plate from the magnetic stand.
14. Seal the plate with Microseal 'B' and centrifuge at $1000 \times g$ for 30 seconds.
15. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
16. Use a 20 μ l pipette to remove residual supernatant from each well.
17. Remove the plate from the magnetic stand.
18. Add 32 μ l freshly prepared 0.1 N HP3 (from step 8a) to each well.
19. Seal the plate with Microseal 'B' and shake at 1800 rpm for 5 minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 5 minutes.
20. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
21. Transfer 30 μ l to the corresponding well of the Normalization Library Plate (from step 8b). Pipette to mix.
22. Seal the plate with Microseal 'B' and centrifuge at $1000 \times g$ 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.

Go to Page 25 to Pool Normalized Libraries

Normalize Libraries Option 2: Manual Quantification Procedure

Perform the following procedure to quantify libraries and check library quality. Accurately quantifying DNA libraries ensures optimum cluster densities on the flow cell.

Consumables

- ▶ RSB (Resuspension Buffer)
- ▶ 96-well PCR plate
- ▶ 1.7 ml microcentrifuge tubes

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.

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

Procedure

1. Quantify the library using a fluorometric method, such as QuantiFluor® ONE (recommended), AccuClear, PicoGreen, or Qubit.
2. Calculate the volume of RSB required to adjust the library concentration to 0.75 ng/μl as follows:
 - a. Use the formula $C1V1=C2V2$, where C1 is the result of the library quantification, V1 is 4 μl, and C2 is 0.75 ng/μl to calculate the value for V2.
 - b. Calculate the amount of RSB (V2 – 4 μl) required to adjust the concentration of each library to 0.75 ng/μl.
 - c. Dilute NTC to 0.75 ng/μl, if reading is greater than 0.75 ng/μl.
3. Add the required volume of RSB to the corresponding well of the Normalized Library Plate. If a library is ≥ 42 ng/μl, transfer the RSB to a 1.7 ml tube.
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 tube.

Safe Stopping Point

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

Go to Page 25 to Pool Normalized Libraries

Pool Libraries

This process combines equal volumes of normalized library to create a pool of libraries that are sequenced together on the same MiSeq FGx flow cell. Make a separate pool for each normalization method, if Option 1 was used for some samples and Option 2 was used for other samples.

Consumables

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

Preparation



NOTE

For recommendations on supported maximum pooling numbers, see Table 1 on page 8.

1. Determine which libraries to pool for sequencing.
2. Label the tube Pooled Normalized Libraries.

Procedure

1. Transfer 5 μ l of each library to a new eight-tube strip.
2. Store the Normalized Library Plate in the post-PCR area at -25°C to -15°C for up to 30 days.
3. Transfer the contents of each eight-tube strip to the Pooled Normalized Libraries tube.
4. Vortex and then centrifuge briefly.

Safe Stopping Point

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

Option 1 Bead-Normalized Procedure: Denature and Dilute Libraries

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



NOTE

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

Consumables

- ▶ ForenSeq mtDNA Control Region Kit contents:
 - ▶ HP3 (2 N NaOH)
 - ▶ HSC (Human Sequencing Control)
- ▶ 1.5 ml microcentrifuge tubes (2)
- ▶ MiSeq FGx (micro or standard flow cell) Reagent Kit contents:
 - ▶ HT1 (Hybridization Buffer)
 - ▶ Reagent cartridge
- ▶ Nuclease-free water
- ▶ Pooled Bead-Normalized Libraries

About Reagents

- ▶ Follow the instructions to prepare the reagent cartridge in the *MiSeq FGx Instrument Reference Guide* (document # VD2018006).

Preparation

1. Prepare the following consumables.

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

2. Preheat the heat block to 96°C.
3. Label tubes as follows:
 - ▶ HSC denaturation
 - ▶ Denatured Normalized Libraries

Procedure

1. Create an HSC denaturation reaction in the HSC denaturation tube.
 - ▶ HSC (2 μ l)
 - ▶ HP3 (2 μ l)
 - ▶ Nuclease-free water (36 μ l)
2. Vortex and then centrifuge briefly.
3. Incubate at room temperature for 5 minutes.
4. Add 600 μ l HT1 to the Denatured Normalized Libraries tube.
5. Place the Pooled Normalized Libraries on the 96°C heat block for 2 minutes.
6. Transfer 5 μ l from the Pooled Normalized Libraries tube to the Denatured Normalized Libraries tube. Pipette to mix.
7. Cap the Pooled Normalized Libraries tube and store at -25°C to -15°C for up to 30 days.

Exceeding 30 days in storage can result in a significant reduction of cluster density.

8. Transfer 2 μ l HSC mixture to the Denatured Normalized Libraries tube. Pipette to mix.

Do not store HSC denaturation long-term, as significant reduction of cluster density may be observed.

9. Vortex and then centrifuge briefly.
10. Immediately load the entire contents onto the reagent cartridge.

For more information, see instructions in the *MiSeq FGx Instrument Reference Guide* (document # VD2018006).

HSC—Human Sequencing Control
HP3—2 N NaOH
HT1—Hybridization Buffer

Option 2 Manual Quant Procedure: Denature and Dilute Libraries

This process denatures and dilutes manual quant-normalized libraries in HT1 (Hybridization Buffer) and adds HSC (Human Sequencing Control) to the libraries in preparation for sequencing.

The denaturation steps in this procedure ensure that the concentration of NaOH is not more than 1 mM in the final solution after diluting with HT1. Higher concentrations of NaOH in the library can inhibit library hybridization to the flow cell and decrease cluster density.



NOTE

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

Consumables

- ▶ ForenSeq mtDNA Control Region Kit contents:
 - ▶ HP3 (2 N NaOH)
 - ▶ HSC (Human Sequencing Control)
- ▶ 1.5 ml microcentrifuge tubes (3)
- ▶ MiSeq FGx (micro or standard) Reagent Kit contents:
 - ▶ HT1 (Hybridization Buffer)
 - ▶ Reagent cartridge
- ▶ Nuclease-free water
- ▶ Pooled Manual Quant-Normalized Libraries

About Reagents

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

Preparation

1. Prepare the following consumables.

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

2. Label tubes as follows:
 - ▶ HSC denaturation
 - ▶ 20 pM Denatured Normalized Libraries
 - ▶ 6 pM Denatured Normalized Libraries
3. Prepare a fresh dilution of NaOH using the following volumes in a microcentrifuge tube:
 - ▶ Laboratory-grade water (90 µl)
 - ▶ HP3 (10 µl)

The result is 0.1 ml of 0.2 N NaOH.

4. Invert the tube several times to mix.



NOTE

Use the fresh dilution within **12 hours**.

Procedure

1. Create an HSC denaturation reaction in the HSC denaturation tube.
 - ▶ HSC (2 μ l)
 - ▶ HP3 (2 μ l)
 - ▶ Nuclease-free water (36 μ l)
2. Vortex and then centrifuge briefly.
3. Incubate at room temperature for 5 minutes.
4. Combine the following volumes in the 20 pM Denatured Normalized Libraries tube:
 - ▶ 0.75 ng/ μ l normalized library pool (5 μ l)
 - ▶ 0.2 N NaOH (5 μ l)
5. Vortex briefly and then centrifuge at 280 \times g for 1 minute.
6. Incubate at room temperature for 5 minutes.
7. Add 990 μ l HT1 to the tube containing denatured library.

The result is 1 ml of a 20 pM denatured library.
8. Dilute the 20 pM library to 6 pM using the following volumes:
 - ▶ 20 pM library (180 μ l)
 - ▶ HT1 (418 μ l)
 - ▶ HSC denaturation (2 μ l)
9. Vortex to mix and then centrifuge briefly.
10. Immediately load the entire contents onto the reagent cartridge. For more information, see instructions in the *MiSeq FGx Instrument Reference Guide* (document # VD2018006).

HSC—Human Sequencing Control
HP3—2 N NaOH
HT1—Hybridization Buffer

Supporting Kit Information

Kit Contents

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

Kit Name	Catalog #	Number of Reactions
ForenSeq™ mtDNA Control Region Kit, 48 reactions	TG-650-1001	48

Pre-PCR Box 1

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

Quantity (48 rxn) TG-650-1001	Reagent	Cap Insert Color	Description	Storage Temperature
1	HL60	Black	Control DNA	2°C to 8°C
1	CRS1	Blue	Control Region Set 1	2°C to 8°C
1	CRS2	Red	Control Region Set 2	2°C to 8°C

Pre-PCR Box 2

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

Quantity (48 rxn) TG-650-1001	Reagent	Cap Insert Color	Description	Storage Temperature
2	mtPCR1	Green	mtPCR1 Reaction Mix	-25°C to -15°C
1	FEM	Yellow	ForenSeq Enzyme Mix	-25°C to -15°C

Post-PCR Box 3

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

Quantity (48 rxn) TG-650-1001	Reagent	Cap Insert Color	Description	Storage Temperature
1	LNA1	—	Library Normalization Additives 1	-25°C to -15°C
1	LNS2	—	Library Normalization Storage Buffer 2	-25°C to -15°C
1	LNW1	—	Library Normalization Wash 1	-25°C to -15°C
1	HP3	Orange	HP3 2 N NaOH	-25°C to -15°C
2	mtPCR2	Purple	mtPCR2 Reaction Mix	-25°C to -15°C
1	HSC	Pink	Human Seq Control	-25°C to -15°C
1	A501	—	A501 Index Adapter	-25°C to -15°C
1	A502	—	A502 Index Adapter	-25°C to -15°C
1	A503	—	A503 Index Adapter	-25°C to -15°C
1	A504	—	A504 Index Adapter	-25°C to -15°C

Quantity (48 rxn) TG-650-1001	Reagent	Cap Insert Color	Description	Storage Temperature
1	A505	—	A505 Index Adapter	-25°C to -15°C
1	A506	—	A506 Index Adapter	-25°C to -15°C
1	A507	—	A507 Index Adapter	-25°C to -15°C
1	A508	—	A508 Index Adapter	-25°C to -15°C
1	R713	—	R713 Index Adapter	-25°C to -15°C
1	R714	—	R714 Index Adapter	-25°C to -15°C
1	R716	—	R716 Index Adapter	-25°C to -15°C
1	R717	—	R717 Index Adapter	-25°C to -15°C
1	R718	—	R718 Index Adapter	-25°C to -15°C
1	R719	—	R719 Index Adapter	-25°C to -15°C
1	R720	—	R720 Index Adapter	-25°C to -15°C
1	R721	—	R721 Index Adapter	-25°C to -15°C
1	—	—	i7 Index Tube Caps, Green	-25°C to -15°C
1	—	—	i5 Index Tube Caps, White	-25°C to -15°C

Post-PCR Box 4

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

Quantity (48 rxn) TG-650-1001	Reagent	Cap Insert Color	Description	Storage Temperature
1	LNB1	White	Library Normalization Beads 1	2°C to 8°C
1	RSB	—	Resuspension Buffer	2°C to 8°C
1	SPB2	—	Sample Purification Beads 2	2°C to 8°C
1	Pro K	—	Proteinase K	2°C to 8°C

Index Sequences

The kit contains the following index adapter sequences.

Index 1 (i7)

Index 1 (i7)	Sequence
R713	AGTCAAAT
R714	AGTTCCAT
R716	CCGTCCAT
R717	GTAGAGAT
R718	GTCCGCAT
R719	GTGAAAAT
R720	GTGGCCAT
R721	GTTTCGAT

Index 2 (i5)

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

Acronyms

Acronym	Definition
HL60	Control DNA
R7XX	i7 Index Adapter
A50X	i5 Index Adapter
CRS1	Control Region Set 1
CRS2	Control Region Set 2
FEM	ForenSeq Enzyme Mix
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
mtPCR1	mtPCR1 Reaction Mix
mtPCR2	mtPCR2 Reaction Mix
ProK	Proteinase K
RSB	Resuspension Buffer
SPB2	Sample Purification Beads 2

Consumables and Equipment

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

Consumables

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

Consumable	Supplier
1.5 ml microcentrifuge tubes	General lab supplier
1.7 ml microcentrifuge tubes	General lab supplier
2.0 ml microcentrifuge tubes	General lab supplier
15 ml conical tube	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
96-well 0.3 ml semiskirted PCR plates	Eppendorf Twin-Tec, part # 951020303 or VWR, part # 89136-706
96-well storage plates, round well, 0.8 ml ('midi' plate)	Fisher Scientific, part # AB-0859
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
MiSeq disposable wash tube	Verogen, part # MS-102-9999
Microseal 'A' film	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MiSeq FGx Reagent Kit	Verogen, catalog # TG-143-1001 or
MiSeq FGx Reagent Micro Kit	Verogen, catalog # TG-143-1002
Nuclease-free water	General lab supplier
RNase/DNase-free eight-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	Labcor, part # 730-001
QuantiFluor® ONE dsDNA System	Promega catalog # E4870, E4871
Thin-walled 0.5 ml PCR tubes	Axygen cantalog # PCR-05-C

Equipment

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

Equipment	Supplier/Description	Pre-PCR	Post-PCR
Heat Block	General lab supplier		X
96-well thermal cycler (with heated lid)	See the list of thermal cyclers.		X
Benchtop microcentrifuge	General lab supplier	X	X
ForenSeq Index Plate Fixture	Verogen, catalog # FC-451-1001		X
Magnetic stand-96	Life Technologies, part # AM10027		X
Microplate centrifuge	General lab supplier	X	X
Multichannel pipette 8 channel p20	General lab supplier		X
Multichannel pipette 8 channel p200	General lab supplier		X
1.5 ml 96-well heating system	General lab supplier		X
High-speed thermal mixers; select one: • BioShake iQ • BioShake XP	Q instruments, catalog #: • 1808-0506 • 1808-0505		X
Quantus™ Fluorometer	Promega catalog # EE6150	X	
Vortexer	General lab supplier	X	X

Thermal Cyclers

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

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

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

Technical Assistance

For technical assistance, contact Verogen Technical Support.

Table 2 General Information

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

Safety data sheets (SDSs)

► For MiSeq FGx sequencing kit safety data sheets, visit www.verogen.com/sds.

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

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