October 2025

ForenSeq® mtDNA Control Region Kit Handbook for PrepStation

For mtDNA Control Region Library Preparation using the PrepStation automation solution

Sample to Insight

Table of Contents

Kit Contents

ForenSeq mtDNA Control Region Kit	
Catalog number	V16000085
No. of reactions	48

Reagent	Description	Cap	Quantity	Storage temperature (°C)
Pre-PCR Box 1				
CRS1	Control Region Set 1	Blue	1	2–8
CRS2	Control Region Set 2	Red	1	2–8
HL60	Control DNA HL60	Black	1	2–8

Reagent	Description	Cap	Quantity	Storage temperature (°C)
Pre-PCR Box 2				
FEM	ForenSeq Enzyme Mix	Yellow	1	–25 to –15
mtPCR1	mtPCR1 Reaction Mix	Green	2	–25 to –15

Reagent	Description	Cap	Quantity	Storage temperature (°C)
Post-PCR Box 3				
A501	A501 Index Adapter	White	1	–25 to –15
A502	A502 Index Adapter	White	1	–25 to –15
A503	A503 Index Adapter	White	1	–25 to –15
A504	A504 Index Adapter	White	1	–25 to –15
A505	A505 Index Adapter	White	1	–25 to –15
A506	A506 Index Adapter	White	1	–25 to –15
A508	A508 Index Adapter	White	1	–25 to –15
HP3	2 N NaOH	Orange	1	–25 to –15
HSC	Human Sequencing Control	Pink	1	–25 to –15
LNA1	Library Normalization Additives 1	Clear	1	–25 to –15
LNS2	Library Normalization Storage Buffer 2	Clear	1	15–30*
LNW1	Library Normalization Wash 1	Clear	1	–25 to –15
mtPCR2	mtPCR2 Reaction Mix	Purple	2	–25 to –15
R713	R713 Index Adapter	Green	1	–25 to –15
R714	R714 Index Adapter	Green	1	–25 to –15
R716	R716 Index Adapter	Green	1	–25 to –15
R717	R717 Index Adapter	Green	1	–25 to –15
R718	R718 Index Adapter	Green	1	–25 to –15
R719	R719 Index Adapter	Green	1	–25 to –15
R720	R720 Index Adapter	Green	1	–25 to –15
R721	R721 Index Adapter	Green	1	–25 to –15
Not applicable	i7 Index Tube Caps	Green	1	15–30*
Not applicable	i5 Index Tube Caps	White	1	15–30*

* Shipped at –25°C to –15°C

Reagent	Description	Cap	Quantity	Storage temperature (°C)
Post-PCR Box 4				
LNB1	Library Normalization Beads 1	White	1	2–8
ProK	Proteinase K	Clear	1	2–8
RSB	Resuspension Buffer	Purple	1	2–8
SPB2	Sample Purification Beads 2	Red	1	2–8

Shipping and Storage

The ForenSeq mtDNA Whole Genome Kit is shipped in 4 boxes. When you receive the kit, promptly store reagents at the indicated temperatures in “Kit Contents” on page 4.

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 (see Post-PCR Box 3 in “Kit Contents”, previous section). After first use, store the reagent at the indicated temperature.

Under these conditions, the components are stable until the expiration date. The expiration date for the product is provided on the label and will vary based on the date of manufacture of the kit.

Intended Use

The ForenSeq mtDNA Whole Genome Kit is intended for molecular biology applications in forensic, human identity, and paternity testing. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend that all users of QIAGEN® products adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

Each lot of ForenSeq mtDNA Control Region Kits is tested against predetermined specifications to ensure consistent product quality.

Introduction

The ForenSeq® mtDNA Control Region Kit prepares up to 48 paired-end, dual-indexed libraries for sequencing and analysis of the control region of the human mitochondrial genome (mtGenome). The protocol divides each sample into 2 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 binding sequences. Index adapters then attach to the tags. The resulting libraries are amplified, purified, and pooled into one tube for sequencing.

This handbook describes the protocol for using the ForenSeq mtDNA Control Region Kit with the Opentrons PrepStation.

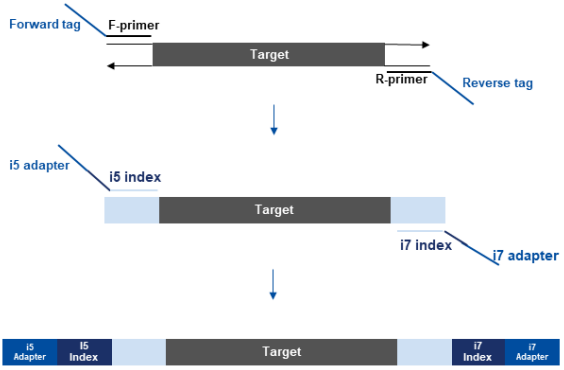


Figure 1. Assay overview.

Kit features

The ForenSeq mtDNA Whole Genome Kit offers the following features:

- An efficient protocol that 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 <150 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 mtDNA control region. Eighteen primer pairs composed of 122 primers efficiently amplify degenerate bases.

Figure 2. Tiled amplicons for complete coverage.

Opentrons PrepStation

The Opentrons PrepStation is a high-precision liquid handler that includes preprogrammed protocols to prepare libraries for sequencing. Optimized for use with the MiSeq® FGx Sequencing System and QIAGEN's Universal Analysis Software, the PrepStation enables a streamlined, automated workflow for enriching targets and purifying, normalizing, and pooling libraries.

The PrepStation platform consists of an eleven-slot deck layout that includes one designated deck slot for a magnetic module and allocated space for labware waste. Automation protocols are controlled and executed through Opentrons PrepStation application on a networked computer.

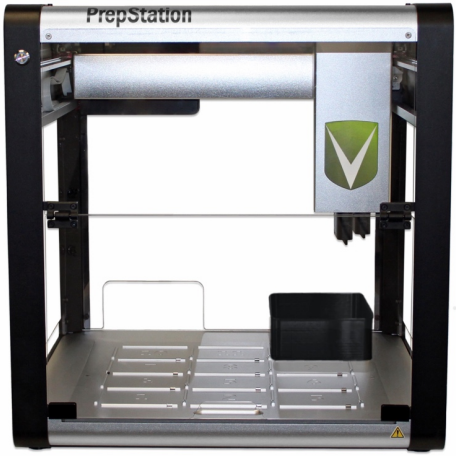


Figure 3. PrepStation instrument.

Description of protocols

The following table lists the steps to prepare libraries using the ForenSeq mtDNA Control Region Kit on the Opentrons PrepStation including hands-on times, Opentrons PrepStation runtimes, and reagents. Safe stopping points are marked between steps.

Note: Runtimes listed below assume a protocol consisting of 48 samples (the maximum number that can be prepared at one time). See "Number of samples" on page 20 for more information.

Step	Hands-on time	Total time	Reagents
Amplify and tag targets*	15 min	3 h 35 min	CRS1, CRS2, FEM, HL60, mtPCR1
Enrich targets†	5 min	14 min	Index 1 Adapters, Index 2 Adapters, mtPCR2
Purify libraries†	15 min	1 h 15 min	80% EtOH, ProK, RSB, SPB2
Safe stopping point	–	–	–
Normalize libraries†	15 min	2 h	HP3, LNA1, LNB1, LNS2, LNW1
Safe stopping point	–	–	–
Pool libraries†	5 min	7 min	–
Safe stopping point	–	–	–
Denature and dilute libraries†	10 min	10 min	HP3, HSC, HT1

* Pre-PCR

† Post-PCR

Abbreviations

A50X	I5 Index Adapter
CRS1	Control Region Set 1
CRS2	Control Region Set 2
FEM	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

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

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 alternative consumables and equipment.

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
TruSeq™ Index Plate Fixture	Illumina (cat. no. 15028344)		
Computer with PrepStation and Opentrons applications installed*	User-supplied; Installation and setup supported by your QIAGEN FAS		X

Equipment	Supplier	Pre-PCR	Post-PCR
Heating system, 96-well, 1.5 mL	General lab supplier		X
Magnetic module for PrepStation instrument†	Opentrons (SKU: 999-00098)		X
Metal PCR plate adapters†	Opentrons (SKU: 999-00028)		X
Microplate centrifuge	General lab supplier	X	X
P20 8-channel pipette arm (GEN2)†‡	Opentrons (SKU: 999-00005)		X
P300 8-channel pipette arm (GEN2)†§	Opentrons (SKU: 999-00006)		X
PrepStation instrument	Opentrons (cat. no. 991-00210)		X
Rubber roller	General lab supplier	X	X
Thermal cycler, 96-well with heated lid	See "Thermal cyclers" (below)		X
Vortexer	General lab supplier	X	X
[Optional] 10 µL pipettes	General lab supplier	X	X

* The Opentrons application is supported on the following operating systems: Windows 10 or later, macOS 10.10 or later, Ubuntu 12.04 or later. Minimum hardware requirements: 64-bit processor, 512 MB of RAM, 300 MB of free hard drive space for installation.

† This item is included with your purchase of the PrepStation instrument (Opentrons, part no. V16000192), and does not need to be purchased separately. Replacement parts may be purchased from Opentrons as listed.

‡ Installed on the left side of the PrepStation instrument.

§ Installed on the right side of the PrepStation instrument.

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	Standard	Heated	Plate
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 the fast block (gold) is supported.

† Only the standard block is supported.

Consumables

Consumable	Supplier
1.5 mL LoBind microcentrifuge tubes	VWR, cat. no. 80077-230 or 80077-232
15 mL conical tube	General lab supplier
20 µL barrier pipette tips	General lab supplier
200 µL barrier pipette tips	General lab supplier
20 µL filter tips	Opentrons (SKU: 999-00099)
200 µL filter tips	Opentrons (SKU: 999-00081)
96-well deep well storage plates (midi plates)	Fisher Scientific, part no. AB-0765 (individually sealed)
96-well twin.tec PCR plates, semiskirted	One of the following suppliers: • Eppendorf, catalog no. 951020303 • VWR, catalog no. 89136-706
96-well twin.tec PCR plate, skirted, 150 µL	Eppendorf, catalog no. 951020401
Ethyl alcohol, pure	Sigma-Aldrich, catalog no. E7023
Microseal 'A' sealing film	Bio-Rad, catalog no. MSA5001
Microseal 'B' sealing film, adhesive, optical	Bio-Rad, catalog no. MSB1001
MiSeq FGx Reagent Micro Kit	QIAGEN, catalog no. 20021681
Multichannel reagent reservoirs, PVC, disposable	VWR, catalog no. 89094-688
Nuclease-free water	General lab supplier
Reagent reservoirs	Corning, catalog no. MTS-11-8-C-R-S
RNase/DNase-free 8-tube strips and caps	General lab supplier
Waste bags	QIAGEN, catalog no. V16000208

Important Notes

DNA input recommendations

QIAGEN recommends using an 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 the input using a fluorometric-based method or qPCR and assess quality.

The kit is also compatible with crude lysate from buccal swabs. Use 2 µL input material per sample. See "Consumables" on the previous page for recommended lysis buffers.

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.

Number of samples

The PrepStation can prepare libraries in multiples of 8 samples at a time, up to a maximum of 48 samples at a time, including positive and negative amplification controls.

Plan a maximum plexity of 48 for a MiSeq FGx® micro flow cell, which is provided in the MiSeq FGx Reagent Micro Kit. Plexity is the number of libraries pooled for a run. For example, if you pool 8 libraries, the plexity is 8.

Index adapter strategy

The kit includes 24 index adapters that support either 6 unique pairs or 64 total combinations. When preparing samples of varying input amounts and quality, using 8 unique index adapter pairs mitigates cross-talk.

The following table provides example index adapter combinations for 8 reactions 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
Reference sample 2	R717	A504
Reference sample 3	R718	A505
Reference sample 4	R719	A506
Positive control	R720	A507
Negative control	R721	A508

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.

Note: The *Universal Analysis Software User Guide for ForenSeq Imagen Kit Module* (Verogen document no. VD2022014) should be referenced.

Preventing cross-contamination

- Designate a separate workspace for mtDNA extraction and analysis from nuclear DNA extraction and analysis.
- Set up the Amplify and Tag Targets 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**.
- Remove unused index adapter tubes from the working area.

Sealing the plate

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

Handling beads

- For optimal performance and yield, confirm that beads are at room temperature before use.
- Vortex beads thoroughly before use. Resuspended beads are evenly distributed and homogenous in color.
- Aspirate and dispense beads slowly due to viscosity when preparing a run.
- Do not centrifuge plates and tubes containing beads, except when indicated.

Cleaning PrepStation

To avoid sample-to-sample contamination between protocols, QIAGEN recommends cleaning the PrepStation at the end of each protocol.

Cleaning the PrepStation between protocols

- Remove all labware from the deck. Dispose of consumed labware.
- Empty the trash bin and replace the bag.
- To make sure the deck is clean and free of spills, clean any exposed surfaces on the deck using 70% ethanol and let them dry.

Preventative maintenance of PrepStation

For information about preventative maintenance of PrepStation, refer to ***Opentrons OT-2 Liquid Handler Manual***.

Protocol

This chapter describes the ForenSeq mtDNA Control Region protocol with step-by-step instructions to prepare libraries on the Opentrons PrepStation for sequencing. For an overview of the protocol with reagents and durations for each step, see "Description of protocols" on page 14.

Before starting, confirm kit contents and make sure that you have the necessary reagents, consumables, and equipment. For a list of items, see "Equipment and Reagents to Be Supplied by User" on page 16.

Protocol step 1: Amplify and tag targets

This process uses an oligonucleotide primer mix to amplify and tag regions specific to DNA sequences in the mitochondrial control region.

Consumables

- CRS1 (Control Region Set 1)
- CRS2 (Control Region Set 2)
- FEM (ForenSeq Enzyme Mix)
- HL60 (Control DNA HL60)
- mtPCR1 (mtPCR1 Reaction Mix)
- 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.

Item	Storage temperature (°C)	Instructions
CRS1	2–8	Let stand for 30 min to bring to room temperature. Vortex to mix, and then centrifuge.
CRS2	2–8	Let stand for 30 min to bring to room temperature. Vortex to mix, and then centrifuge.
FEM	–25 to –15	Remove from storage immediately before use, and then return to storage immediately after use.
HL60	2–8	Let stand for 30 min to bring to room temperature. Flick to mix, and then centrifuge.
mtPCR1	–25 to –15	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.

2. Save the following mtPCR1 program on the thermal cycler in the post-amplification area. See Table 1 for ramp modes.
    - Choose the preheat lid option and set to 100°C.
    - Reaction volume is 15 µL .

- 98°C for 3 min
- 8 cycles of
  - 96°C for 45 s
  - 80°C for 10 s
  - 54°C for 2 min with applicable ramp mode
  - 66°C for 1.5 min with applicable ramp mode
- 10 cycles of
  - 96°C for 30 s
  - 68°C for 2 min with applicable ramp mode
- 68°C for 3 min
- Hold at 10°C.

Table 1. Ramp modes

Thermal cycler	Ramp mode
ABI LTI thermal cycler 9700 9600 emulation mode	8%
Bio-Rad	0.2°C/s
Eppendorf Mastercycler Pro S	2%
Proflex 96-well PCR System	0.2°C/s
QIAamplifier 96 thermal cycler	0.1°C/s
Veriti 96-well thermal cycler	4%

Total program time is approximately 3.5 h and can continue overnight.

3. Label tubes and the plate as follows.

Vessel	Label
1.7 mL microcentrifuge tube	Master Mix CRS1
1.7 mL microcentrifuge tube	Master Mix CRS2
1.7 mL microcentrifuge tube	Control DNA Dilution 1
1.7 mL microcentrifuge tube	Control DNA Dilution 2
96-well PCR plate	mtDNA CR Sample Plate

Procedure

1. Using nuclease-free water, dilute 100 pg gDNA to a volume of ≥12 µL at 8.33 pg/µL. Gently pipet to mix.
2. In the Master Mix CRS1 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)
   - CRS1 (5 µL)

   For example, for 48 samples prepare 475.1 µL CRS1 Master Mix: 195.3 µL mtPCR1, 15.8 µL FEM, and 264 µL CRS1.
3. In the Master Mix CRS2 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)
   - CRS2 (5 µL)

For example, for 48 samples prepare 475.1 µL CRS2 Master Mix: 195.3 µL mtPCR1, 15.8 µL FEM, and 264 µL CRS2.

4. Pipet each master mix to mix, and then cap and centrifuge briefly.
5. **Optional**: For >16 samples:
    a. Transfer CRS1 Master Mix to an 8-tube strip, evenly distributing between tubes.
    b. Transfer CRS2 Master Mix to an 8-tube strip, evenly distributing between tubes.
6. Using a multichannel pipette, add master mixes to the mtDNA CR Sample Plate:
    a. Divide the plate into 2 even sections depending on the number of samples. See Figure 4 for an example.
    b. Add 9 µL CRS1 Master Mix to each well in the first section.
    c. Add 9 µL CRS2 Master Mix to each well in the second section.

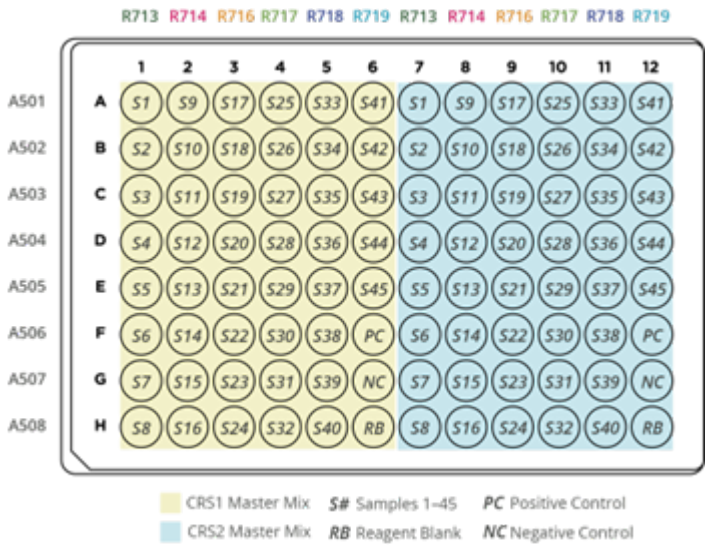


Figure 4. Example plate setup for 16 samples.

This step sets up the plate for samples and controls divided into 2 sets. A subsequent step splits each sample and control into 2 reactions: one for the CRS1 set and one for the CRS2 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 pipet 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 pipet to mix, and then cap and centrifuge briefly.

8. Add the reagent blank:

   a. Add 6 µL reagent blank to one well of the CRS1 set.

   b. Add 6 µL reagent blank to the corresponding well of the CRS2 set.

   c. Pipet to mix.

9. Divide each sample between the 2 sets:

   a. Add 6 µL 8.33 pg/µL gDNA to one well of the CRS1 set.

   b. Add 6 µL 8.33 pg/µL gDNA to the corresponding well of the CRS2 set.

   c. Pipet to mix.

10. Add the positive amplification control:

    a. Add 6 µL 8.33 pg/µL HL60 to one well of the CRS1 set.

    b. Add 6 µL 8.33 pg/µL HL60 to the corresponding well of the CRS2 set.

    c. Pipet to mix.

11. Add the negative amplification control:

    a. Add 6 µL nuclease-free water to one well of the CRS1 set.

    b. Add 6 µL nuclease-free water to the corresponding well of the CRS2 set.

    c. Pipet to mix.

12. Seal with microseal 'A' film, and then centrifuge at 1000 × *g* for 30 s.

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 2–8°C for up to 2 days. Alternatively, leave the thermal cycler on overnight.

Protocol step 2: Enrich targets (Post 1 – Enrichment)

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.

Consumables

- mtPCR2 (mtPCR2 Reaction Mix)
- Index 1 Adapters (R7XX) and green caps
- Index 2 Adapters (A50X) and white caps
- ForenSeq Index Plate Fixture
- 1.7 mL microcentrifuge tubes (1 per index adapter tube)
- 96-well PCR plate
- Microseal 'A' film
- Opentrons 200 µL filter tips
- Opentrons 20 µL filter tips
- Thermo Scientific Abgene 0.8 mL reagent midi plates
- Trash bags (Hommaly)

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.
- If not running 48 samples, seal the CDI plate using Seal B after use and store in –20°C.

Preparation

1. Prepare the following consumables.

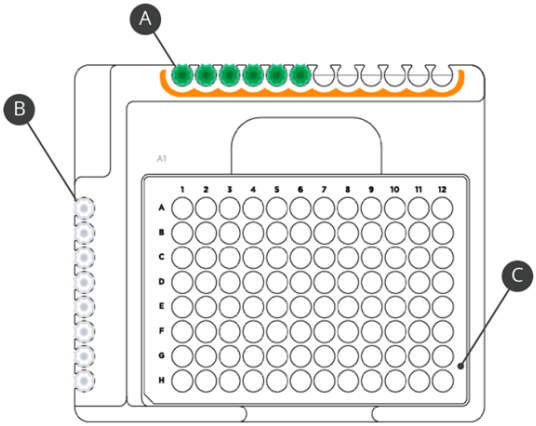
Item	Storage temperature (°C)	Instructions
Index adapters	–25 to –15	Remove only the index adapters being used. Thaw at room temperature for 20 min. Vortex each tube to mix, and then centrifuge briefly.
mtPCR2	–25 to –15	Thaw at room temperature for 20 min, and then invert to mix.

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 s
   - 15 cycles of:
     - 98°C for 20 s
     - 66°C for 30 s
     - 68°C for 1.5 min
   - 68°C for 10 min
   - Hold at 10°C

   Total program time is approximately 45 minutes.
3. If using a fresh ForenSeq mtDNA Control Region kit, then take the room temp, vortexed, and spun-down Index adapters and add them to a ForenSeq Index Plate Fixture according to the image below.

Note: If not using a fresh kit, take out the previously stored CDI Plate, shake, and spin down before use.

4. Label a new PCR plate as "CDI Plate" and place it in the center of the fixture.



(A) Index i7 Adapter tubes in columns 1–6. **(B)** Index i5 Adapter tubes in rows A–H. **(C)** CDI Plate.

Transfer 18 µL of each index to each library across their corresponding rows or columns as in the table below. The total volume in each well should be 36 µL.

		R713	R714	R716	R717	R718	R719
		1	2	3	4	5	6
A501	A	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A502	B	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A503	C	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A504	D	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A505	E	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A506	F	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A507	G	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL
A508	H	36 µL	36 µL	36 µL	36 µL	36 µL	36 µL

5. Label a new midi plate as "Reagent Midi Plate".
6. Prepare the reagent midi plate by aliquoting reagents into wells as specified in Table 2 and Table 3 on the next page. (Blank columns indicate wells that should be left empty.)

   Note: While aliquoting reagents into the midi plate, ensure that reagents settle to the bottom of the wells.

   Note: Reagent volumes must correspond to the number of reactions that will be processed in the run.

   Important: You may optionally seal the unused wells (Columns 1 to 11) to keep them clean for use in future steps. (Use microseal "B" to seal.)

Table 2. Reagent midi plate layout, Post 1 – Enrichment

	1	2	3	4	5	6	7	8	9	10	11	12
A	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
B	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
C	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
D	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
E	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
F	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
G	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix
H	–	–	–	–	–	–	–	–	–	–	–	mtPCR2 Reaction Mix

Table 3. Reagent volumes, Post 1 – Enrichment

Reagent	Midi plate column	No. of reactions	Volume per well (µL)
mtPCR2 Reaction Mix	Column 12	8	65
		16	130
		24	195
		32	260
		40	325
		48	390

Procedure

1. Centrifuge the sealed FSP at 1000 × *g* for 30 s.
2. Create a "Verogen" protocol set using the PrepStation application:

a. Launch the PrepStation application.

b. Select **Add Protocol Set**.

c. Choose the appropriate Sample Count and CDI Start Position, then select **Add Protocol Set**.

d. After a few seconds, navigate back to the Protocol Sets screen by selecting the arrow at the top left. The new protocol set will now appear on the Protocol Sets screen.

3. Run the "Post 1 – Enrichment" protocol on the PrepStation instrument:

   a. Launch the Opentrons application.

   b. Select **Protocols** from the left-hand menu. Your new protocols will appear under All Protocols.

   c. Select the appropriate protocol to enrich targets. (The name of the protocol will include "Post 1 – Enrichment".) A screen with details about the protocol appears.

   d. Select **Run Protocol**. A sidebar listing available PrepStation instruments appears.

   e. Select the instrument you want to use, then select **Proceed to setup**. A screen with further setup instructions appears.

   f. Select the **+** button next to Labware Setup. A drop-down window displaying the deck map for this protocol opens.

      Note: If a window appears prompting you to apply stored labware offset data, select Apply stored data.

   g. If the trash bin is not already empty, empty it and replace the bag.

   h. Load reagents and other labware onto the deck as shown in the deck map (also shown in Figure 5 on the next page).

      Note: Carefully observe the following while loading labware:

- Load labware from back to front. (Load deck slot 9, then 6, then 3.)
- Ensure that labware are oriented such that labels are facing you.
- Ensure that all labware are seated securely inside their deck slots, and not resting on any dividers between deck slots.
- Place the CDI plate on top of the magnetic module and secure it by screwing in the small clamp.
- Ensure that the foil seal remains on the CDI plate. (The instrument will pierce the foil.) If seal B, remove seal before placing on deck.
- Ensure that lids have been removed from the tip racks.

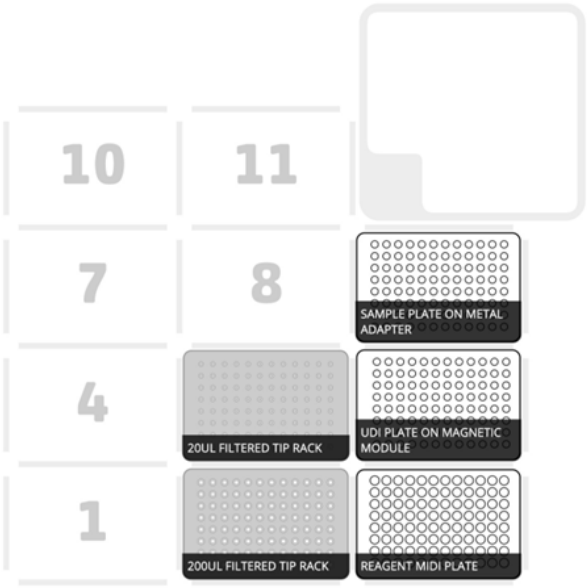


Figure 5. Deck map for *Post 1 – Enrichment protocol.*

i. When you have finished loading labware, select **Run Labware Position Check**. The application will guide you through a workflow to verify that all labware is correctly placed.

j. When you have completed the labware position check, select **Proceed to Run**.

k. Select **Start Run**. The instrument begins to perform the protocol.

4. Once the run has completed, seal the FSP using microseal 'A'. Centrifuge the sealed plate at 1000 × *g* for 30 s.

5. Place the FSP on the preprogrammed thermal cycler and run the PCR2 program.

6. Clean the PrepStation instrument:

   a. Remove all labware from the deck.

   b. Dispose of consumed labware.

   c. Empty the trash bin and replace the bag.

   d. To make sure the deck is clean and free of spills, clean any exposed surfaces on the deck using 70% ethanol and let them dry.

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

Note: The remaining unused CDIs can be utilized with subsequent library preparations.

Protocol step 3: Purify libraries (Post 2 – Purification)

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

Consumables

- RSB (Resuspension Buffer)
- ProK (Proteinase K)
- SPB2 (Sample Purification Beads 2)
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate
- Microseal 'B' film
- Opentrons 200 µL filter tips
- Opentrons 20 µL filter tips
- Thermo Scientific Abgene 0.8 mL reagent midi plates
- Trash bags (Hommaly)

About reagents

Aspirate and dispense SPB2/ProK slowly due to viscosity.

Preparation

1. Prepare the following consumables.

Item	Storage temperature (°C)	Instructions
ProK	2–8	Let stand for 30 min to bring to room temperature. Invert to mix, and then centrifuge.
RSB	2–8	Let stand for 30 min to bring to room temperature. Vortex and invert to mix.
SPB2	2–8	Let stand for 30 min to bring to room temperature. Vortex for ≥1 min and invert to mix.

2. Label plates as follows.

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

3. Prepare the SPB2 tube for a first or subsequent use.
   - For first-time use, add 25 µL ProK to the SPB2 tube. Select the checkbox on the SPB2 label to indicate the addition.
   - For subsequent use, make sure the checkbox on the SPB2 label is selected.
4. Prepare the reagent midi plate by aliquoting additional reagents into wells as specified in Table 4 and Table 5. (Blank columns indicate wells that should be left empty. Shaded columns indicate wells with reagents from an earlier protocol step.)

   Note: Observe the following practices.

   - While aliquoting reagents into the midi plate, ensure that reagents settle to the bottom of the wells.

- Reagent volumes must correspond to the number of reactions that will be processed in the run.
- You may optionally seal the unused wells (Columns 1 to 4) to keep them clean for use in future steps. (Use microseal 'B' to seal.)

Table 4. Reagent midi plate layout, Post 2 – Purification

	1	2	3	4	5	6	7	8	9	10	11	12
A					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
B					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
C					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
D					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
E					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
F					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
G					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
H					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix

Table 5. Reagent volumes, *Post 2 – Purification*

Reagent	Midi plate column	No. of reactions	Volume per well (µL)
mtPCR2 Reaction Mix	Column 12	8	65
		16	130
		24	195
		32	260
		40	325
		48	390
SPB2	Column 6	8	
		16	
		24	
		32	
		40	
		48	
80% EtOH*	Columns 8, 10	8	
		16	
		24	
	Columns 8, 10 and Columns 9, 11	32	
		40	
		48	

* If you are processing 24 reactions or fewer, leave columns 9 and 11 empty.

Procedure

1. Centrifuge the sealed FSP at 1000 × *g* for 30 s.
2. Run the Post 2 – Purification protocol on the PrepStation instrument:

a. Launch the Opentrons application.

b. Select **Protocols** from the lefthand menu.

c. Select the appropriate protocol to purify libraries. (The name of the protocol will include "Post 2 – Purification".) A screen with details about the protocol appears.

d. Select **Run Protocol**. A sidebar listing available PrepStation instruments appears.

e. Select the instrument you want to use, then select **Proceed to setup**. A screen with further setup instructions appears.

f. Select the **+** button next to Module Setup. A drop-down window displaying the deck map for this protocol opens.

g. Place the magnetic module as shown in the deck map. Ensure the module is plugged in and connected to the PrepStation USB port.

   Note: When the module is on and connected, a green checkmark with "Connected" will appear in the deck map.

h. Select the **+** button next to Labware Setup, or select **Proceed to labware setup**. A drop-down window displaying the deck map for this protocol opens.

   Note: If a window appears prompting you to apply stored labware offset data, select **Apply stored data**.

i. If the trash bin is not already empty, empty it and replace the bag.

j. Load reagents and other labware onto the deck as shown in the deck map (also shown in Figure 5).

   Note: Carefully observe the following while loading labware:

   - Load labware from back to front. (Load deck slot 10, then 7, then 4, then 1.)
   - Ensure that labware are oriented such that labels are facing you.

- Ensure that all labware are seated securely inside their deck slots, and not resting on any dividers between deck slots.
- Place the PBP plate on top of the magnetic module (while being careful not to place it on any of the small corner ridges) and secure it by screwing in the large clamp.
- Ensure that lids have been removed from the tip racks.

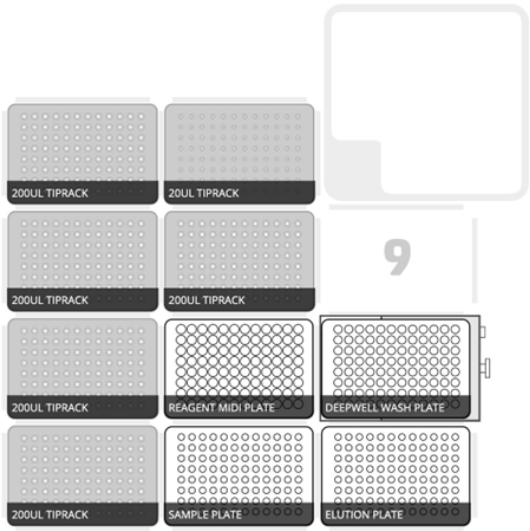


Figure 6. Deck map for *Post 2 – Purification* protocol.

Note: The deck map in the application may show more tip racks than you need, depending on the number of reactions you are processing. See Table 6 to identify which tip racks are necessary.

Table 6. Tip racks required for *Post 2 – Purification*

No. of reactions	Deck slots requiring 200 µL filtered tip racks
8	1
16	1, 4
24	1, 4, 7
32	1, 4, 7
40	1, 4, 7, 10
48	1, 4, 7, 10, 8

k. When you have finished loading labware, select **Run Labware Position Check**. The application will guide you through a workflow to verify that all labware is correctly placed.

   Important: Carefully observe the following while loading labware.

   - During this workflow, you may select **Reveal Jog Controls** to create offsets that adjust how the instrument moves to each labware in the X, Y, and Z directions.
   - You can create offsets along any axis for tip racks, but if Z heights are adjusted, make sure to test prior to using with samples and reagents.
   - Each pipette **arm** should be adjusted vertically (Z-axis) to be as level as possible, and horizontally (X- and Y-axes) to be as close as possible to the center of each tip when viewed from the front or side.

- Each pipette **tip** should be adjusted horizontally (X- and Y-axes) to be as close as possible to the center of each well when viewed from the front or side.

Note: If you applied labware offsets during a previous protocol run, a window appears showing the offset data. You may select **Apply stored data** to apply the same offsets to the current run. (Whether you select this option or not, QIAGEN recommends that you still calibrate the offsets as described above.)

l. When you have completed the labware position check, select **Proceed to Run**.

m. Select **Start Run**. The instrument begins to perform the protocol.

3. Once the run has completed, seal the PLP using microseal 'B'. Centrifuge the sealed plate at 1000 × *g* for 30 s.

4. Clean the PrepStation instrument:

   a. Remove all labware from the deck.

   b. Dispose of consumed labware.

   c. Empty the trash bin and replace the bag.

   d. To make sure the deck is clean and free of spills, clean any exposed surfaces on the deck using 70% ethanol and let them dry.

⏸ **Safe stopping point**. If you are stopping, seal the plate and store at –25°C to –15°C for up to 1 year.

Protocol step 4: Normalize libraries (*Post 3 – Normalization*)

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:
    - 1.5 mL LoBind microcentrifuge tube
    - 15 mL conical tube
- PVC reagent reservoir
- 96-well PCR plate
- Microseal 'B' film
- Opentrons 200 µL filter tips
- Opentrons 20 µL filter tips
- Thermo Scientific Abgene 0.8 mL reagent midi plates
- Trash bags (Hommaly)

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.

Important: 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, refer to **qiagen.com/safety** where you can find safety data sheets (SDS).

Preparation

1. Prepare the following consumables.

Item	Storage temperature (°C)	Instructions
HP3	–25 to –15	Thaw at room temperature for 30 min. Vortex to mix, and then centrifuge briefly.
LNA1	–25 to –15	Thaw at room temperature for 30 min. Vortex with intermittent inversion.
LNB1	2–8	Let stand for 30 min to bring to room temperature. Vortex for ≥1 min, inverting 5 times every 15 s. Pipet to mix until the bead pellet at the bottom is resuspended.
LNW1	2–8	Let stand for 30 min to bring to room temperature.
LNS2	15–30	Remove from storage.

2. Label plates as follows.

Vessel	Label
1.5 mL tube or 15 mL conical tube	0.1 N HP3
1.5 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. Prepare a hazardous waste disposal container.

4. In the LNA1/LNB1 Master Mix tube, combine volumes of reagents as specified in Table 7. Do not add overage.

Table 7. LNA1/LNB1 Master Mix

	Volumes to add (µL)	
No. of reactions	**LNA1**	**LNB1**
8	590	107
16	1180	214

5. Vortex, and then invert several times to mix.
6. In the 0.1 N HP3 tube, combine volumes of reagents as specified in Table 8 below. Do not add overage.

Table 8. 0.1 N HP3

	Volumes to add (µL)	
No. of reactions	**Nuclease-free water**	**HP3**
8	570	31
16	1140	62

7. Invert several times to mix, and then set aside.
8. Prepare the reagent midi plate by aliquoting additional reagents into wells as specified in Table 9 and Table 10 below. (Blank columns indicate wells that should be left empty. Shaded columns indicate wells with reagents from an earlier protocol step.)

   Note: While aliquoting reagents into the midi plate, ensure that reagents settle to the bottom of the wells.

   Note: Reagent volumes must correspond to the number of reactions that will be processed in the run.

Table 9. Reagent midi plate layout, *Post 3 – Normalization*

	1	2	3	4	5	6	7	8	9	10	11	12
A	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
B	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
C	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
D	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
E	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
F	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
G	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix
H	LNS2	0.1 N HP3	LNW1	LNA1/ LNB1	RSB	SPB2	–	EtOH	EtOH	EtOH	EtOH	mtPCR2 Reaction Mix

Table 10. Reagent volumes, *Post 3 – Normalization*

Reagent	Midi plate column	No. of reactions	Volume per well (µL)
LNS2	Column 1	8	36
		16	72

Table 10. Reagent volumes, Post 3 – Normalization (continued)

Reagent	Midi plate column	No. of reactions	Volume per well (µL)
0.1 N HP3	Column 2	8	60
		16	120
LNW1	Column 3	8	100
		16	200
LNA1/LNB1 Master Mix	Column 4	8	75
		16	150

Procedure

1. Run the "Post 3 – Normalization" protocol on the PrepStation instrument:
    a. Launch the Opentrons application.
    b. Select **Protocols** from the lefthand menu.
    c. Select the appropriate protocol to normalize libraries. (The name of the protocol will include "Post 3 – Normalization".) A screen with details about the protocol appears.
    d. Select **Run Protocol**. A sidebar listing available PrepStation instruments appears.
    e. Select the instrument you want to use, then select **Proceed to setup**. A screen with further setup instructions appears.
    f. Select the **+** button next to Module Setup. A drop-down window displaying the deck map for this protocol opens.
    g. Place the magnetic module as shown in the deck map. Ensure the module is plugged in and connected to the PrepStation USB port.

Note: When the module is on and connected, a green checkmark with "Connected" will appear in the deck map.

h. Select the **+** button next to Labware Setup, or select **Proceed to labware setup**. A drop-down window displaying the deck map for this protocol opens.

   Note: If a window appears prompting you to apply stored labware offset data, select **Apply stored data**.

i. If the trash bin is not already empty, empty it and replace the bag.

j. Load reagents and other labware onto the deck as shown in the deck map (also shown in Figure 6).

   Note: Carefully observe the following while loading labware:

   - Load labware from back to front. (Load deck slot 10, then 7, then 4, then 1.)
   - Ensure that labware are oriented such that labels are facing you.
   - Ensure that all labware are seated securely inside their deck slots, and not resting on any dividers between deck slots.
   - Place the NWP plate on top of the magnetic module (while being careful not to place it on any of the small corner ridges) and secure it by screwing in the large clamp.
   - Ensure that lids have been removed from the tip racks.

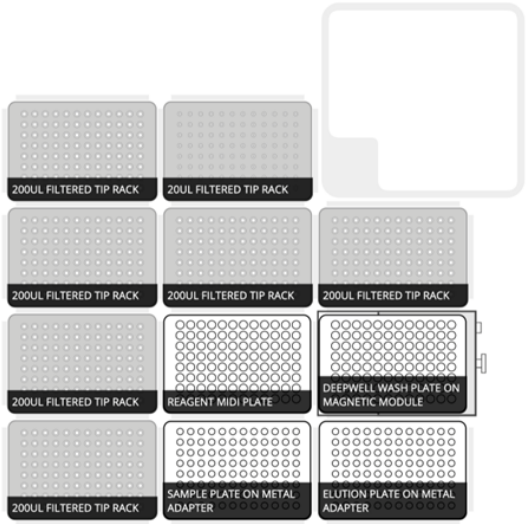


Figure 7. Deck map for *Post 3 – Normalization* protocol.

Note: The deck map in the application may show more tip racks than you need, depending on the number of reactions you are processing. See Table 11 to identify which tip racks are necessary.

Table 11. Tip racks required for *Post 3 – Normalization*

No. of reactions	Deck slots requiring 200 µL filtered tip racks
8	1
16	1, 4

k. When you have finished loading labware, select **Run Labware Position Check**. The application will guide you through a workflow to verify that all labware is correctly placed.

Important: Carefully observe the following while loading labware.

- During this workflow, you may select **Reveal Jog Controls** to create offsets that adjust how the instrument moves to each labware in the X, Y, and Z directions.
- You can create offsets along any axis for tip racks, but if Z heights are adjusted, make sure to test prior to using with samples and reagents.
- Each pipette **arm** should be adjusted vertically (Z-axis) to be as level as possible, and horizontally (X- and Y-axes) to be as close as possible to the center of each tip when viewed from the front or side.
- Each pipette **tip** should be adjusted horizontally (X- and Y-axes) to be as close as possible to the center of each well when viewed from the front or side.

Note: If you applied labware offsets during a previous protocol run, a window appears showing the offset data. You may select **Apply stored data** to apply the same offsets to the current run. (Whether you select this option or not, QIAGEN recommends that you still calibrate the offsets as described above.)

l. When you have completed the labware position check, select **Proceed to Run**.

m. Select **Start Run**. The instrument begins to perform the protocol.

2. Once the run has completed, seal the NLP using microseal 'B'. Centrifuge the sealed plate at 1000 × *g* for 30 s.

3. Clean the PrepStation instrument:

   a. Remove all labware from the deck.

   b. Dispose of consumed labware.

   c. Empty the trash bin and replace the bag.

   d. To make sure the deck is clean and free of spills, clean any exposed surfaces on the deck using 70% ethanol and let them dry.

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

Protocol step 5: Pool libraries (*Post 4 – Pooling*)

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

- 1.5 mL LoBind microcentrifuge tube
- RNase/DNase-free 8-tube strip and caps
- 96-well PCR plate
- Microseal 'B' film
- Opentrons 200 µL filter tips
- Opentrons 20 µL filter tips
- Trash bags (Hommaly)

Preparation

1. Select libraries to pool for sequencing.
2. Label plates as follows.

Vessel	Label
1.5 mL tube	PNL for Pooled Normalized Libraries
PCR plate	Pooling Plate

Procedure

1. Centrifuge the sealed NLP at 1000 × *g* for 30 s.
2. Run the "Post 4 – Pooling" protocol on the PrepStation instrument:
    a. Launch the Opentrons application.
    b. Select **Protocols** from the lefthand menu.
    c. Select the appropriate protocol to normalize libraries. (The name of the protocol will include "Post 4 – Pooling".) A screen with details about the protocol appears.
    d. Select **Run Protocol**. A sidebar listing available PrepStation instruments appears.
    e. Select the instrument you want to use, then select **Proceed to setup**. A screen with further setup instructions appears.
    f. Select the **+** button next to Labware Setup, or select **Proceed to labware setup**. A drop-down window displaying the deck map for this protocol opens.

    Note: If a window appears prompting you to apply stored labware offset data, select **Apply stored data**.
    g. If the trash bin is not already empty, empty it and replace the bag.
    h. Load labware onto the deck as shown in the deck map (also shown in Figure 7).

    Note: Carefully observe the following while loading labware:

    - Load labware from back to front. (Load deck slot 8, then 5, then 1.)
    - Ensure that labware are oriented such that labels are facing you.
    - Ensure that all labware are seated securely inside their deck slots, and not resting on any dividers between deck slots.
    - Ensure that lids have been removed from the tip racks.

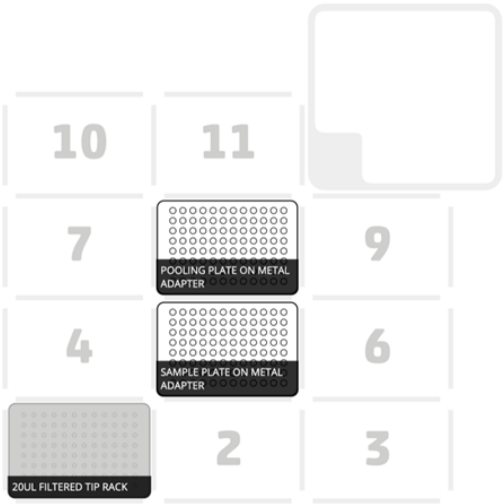


Figure 8. Deck map for *Post 4 – Pooling* protocol.

i. When you have finished loading labware, select **Run Labware Position Check**. The application will guide you through a workflow to verify that all labware is correctly placed.

Important: Carefully observe the following while loading labware.

- During this workflow, you may select **Reveal Jog Controls** to create offsets that adjust how the instrument moves to each labware in the X, Y, and Z directions.
- You can create offsets along any axis for tip racks, but if Z heights are adjusted, make sure to test prior to using with samples and reagents.
- Each pipette **arm** should be adjusted vertically (Z-axis) to be as level as possible, and horizontally (X- and Y-axes) to be as close as possible to the center of each tip when viewed from the front or side.

- Each pipette **tip** should be adjusted horizontally (X- and Y-axes) to be as close as possible to the center of each well when viewed from the front or side.

Note: If you applied labware offsets during a previous protocol run, a window appears showing the offset data. You may select **Apply stored data** to apply the same offsets to the current run. (Whether you select this option or not, QIAGEN recommends that you still calibrate the offsets as described above.)

j. When you have completed the labware position check, select **Proceed to Run**.

k. Select **Start Run**. The instrument begins to perform the protocol.

3. Once the run has completed, seal the NLP using microseal 'B'. Centrifuge the sealed plate at 1000 × *g* for 30 s.
4. Transfer libraries from each well of the Pooling Plate to the PNL tube.
5. Cap and vortex to mix, and then centrifuge briefly.
6. Clean the PrepStation instrument:
   a. Remove all labware from the deck.
   b. Dispose of consumed labware in the appropriate **hazardous** waste receptacles.
   c. Empty the trash bin and replace the bag.
   d. To make sure the deck is clean and free of spills, clean any exposed surfaces on the deck using 70% ethanol and let them dry.

⏸ **Safe stopping point**. If you are stopping, seal the plate and store at –25°C to –15°C for up to 30 days.

Removing the Verogen Protocol Set from Opentrons

To complete the PrepStation component of the library prep protocol, remove the Verogen protocol set from the Opentrons application as follows:

1. Launch the PrepStation application.
2. Select the protocol set you want to remove from the Opentrons application.
3. Select **Remove Protocol Set**.

The protocol set is removed from the Opentrons application. (If you still see the protocol set in the Opentrons application, refresh the Protocols page by navigating to another page and back, or by relaunching the application.)

Protocol step 6: Denature and dilute libraries

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

Consumables

- HP3 (2 N NaOH)
- HSC (Human Sequencing Control)
- MiSeq FGx Reagent Micro 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 no. VD2018006).

2. Prepare the following consumables.

Item	Storage temperature (°C)	Instructions
HP3	–25 to –15	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
HSC	–25 to –15	Let stand for 30 min to bring to room temperature. Invert to mix, and then centrifuge.
HT1	–25 to –15	Thaw at room temperature, and then vortex to mix.

3. Preheat the heat block to 96°C.
4. Label 2 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. Pipet gently to mix. Cap and centrifuge briefly to mix.
3. Incubate at room temperature for 5 min.
4. Add 600 µL HT1 to the Denatured Normalized Libraries tube.
5. Place the Pooled Normalized Libraries tube on the preheated heat block and incubate for 2 min.

6. Immediately transfer 5 µL library from the Pooled Normalized Libraries tube to the Denatured Normalized Libraries tube.
7. Pipet 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. Pipet 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 no. VD2018006).

Technical Support

For more information, see also the Frequently Asked Questions page at our Technical Support Center: (**www.qiagen.com/FAQ/FAQList.aspx**). The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

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Product	Contents	Cat. no.
ForenSeq mtDNA Control Region Kit (48)	Prepares up to 48 paired-end, dual-indexed libraries for sequencing and analysis of the control region of the human mitochondrial genome (mtGenome).	V16000085
	Related product	
MiSeq FGx Reagent Micro Kit	Supports up to 5 million paired-end reads for deep sequencing or high-throughput sample processing	20021681

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Document Revision History

Date	Changes
10/2025	Initial release

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