Verogen PrepStation for ForenSeq MainstAY Product Line with Enhanced PCR1 Buffer System

Reference Guide

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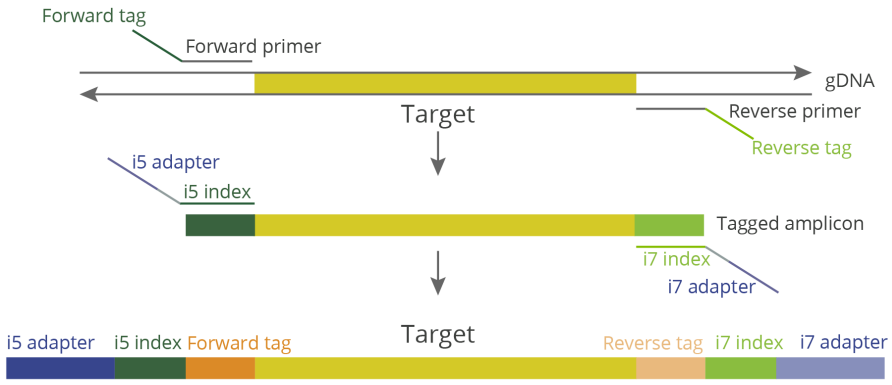
Introduction

The ForenSeq® MainstAY Kits generate dual-indexed libraries for sequencing. Each sample is combined with a primer mix that contains a pair of tagged oligos for each target sequence. PCR cycles link the tags to copies of each target, forming DNA templates consisting of the regions of interest flanked by universal primer binding sequences. The tags are then used to attach index adapters and the resulting library is amplified, purified, and pooled for sequencing.

The ForenSeq MainstAY Kits can be used with the standard PCR1 buffer that is available with the kits or the ForenSeq Enhanced PCR1 Buffer System (ePCR1) that is available as an add-on kit. Additionally, the kits may be used with the Verogen PrepStation for automated library preparation, or with a manual library preparation protocol.

This Reference Guide describes the protocol for using the ForenSeq MainstAY Kits with the ForenSeq Enhanced PCR1 Buffer System and the Verogen PrepStation.

Figure 1 Assay overview



Kit Features

The ForenSeq MainstAY Kits offer the following features:

- A concentrated primer mix that allows for increased input volume.
  - The base primer mix is DNA Primer Mix C (DPMC). ForenSeq MainstAY SE includes a second primer mix, DNA Primer Mix D (DPMD).
- Simultaneous preparation of up to 48 libraries using the Verogen PrepStation or 96 libraries prepared manually. Each library is a collection of amplified DNA fragments from one sample.
- Amplification of 27 autosomal short tandem repeat (aSTR) and 25 Y-STR markers in one reaction and sequencing of up to 96 libraries in one sequencing run.

— ForenSeq MainstAY SE includes all the markers in ForenSeq MainstAY, with the addition of marker SE33. See *Loci Detected with DPMC and DPMD* (on page 46) for more information.

The ForenSeq Enhanced PCR1 Buffer System offers the following features:

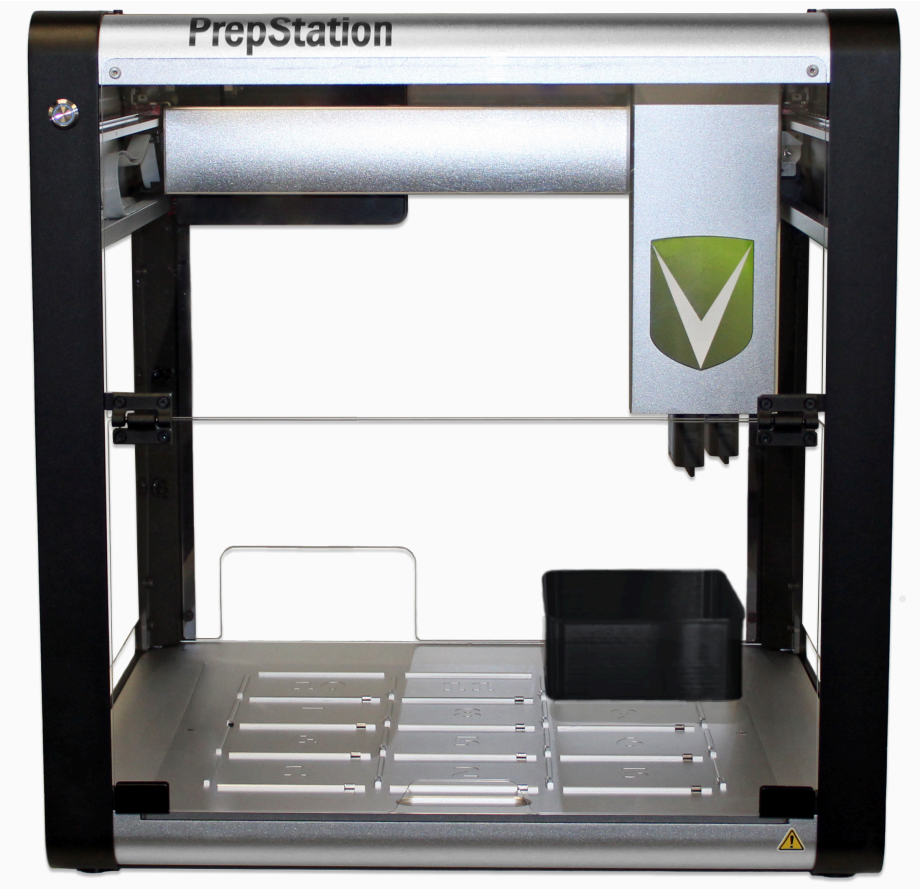
- An optimized buffer system that supports the simultaneous amplification of 96 challenging samples in the presence of high concentrations of inhibitors, such as humic acid.

Verogen PrepStation

The Verogen 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 Verogen'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 Verogen's PrepStation application on a networked computer.

Figure 2 PrepStation instrument

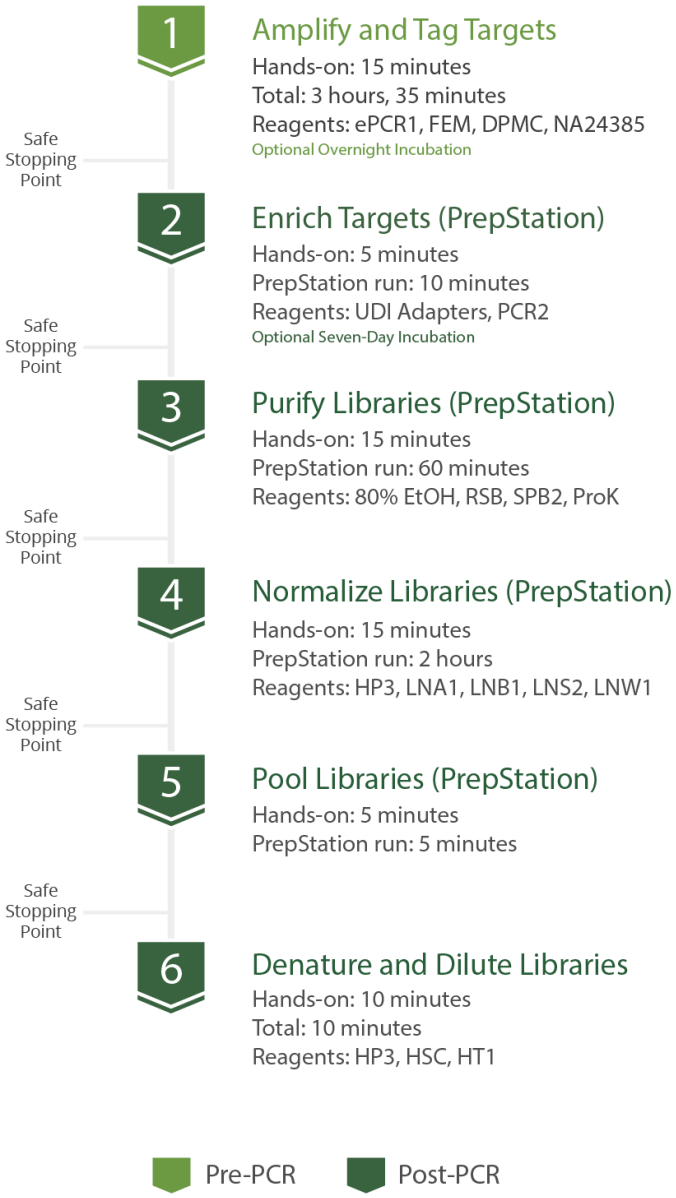


Protocol Steps

Figure 3 illustrates the steps to prepare libraries using the Enhanced PCR1 (ePCR1) buffer on the Verogen PrepStation, including hands-on times, PrepStation runtimes, and reagents. Safe stopping points are marked between steps.

NOTE: PrepStation runtimes listed below assume a run consisting of 48 samples (the maximum number that can be processed in a single run). See *Number of Samples* (on page 10) for more information.

Figure 3 Overview of the ForenSeq MainstAY protocol with the Enhanced PCR1 (ePCR1) buffer on the Verogen PrepStation



DNA Input Recommendations

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

Controls

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

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

Acronyms

Acronym	Definition
DNL	Diluted Normalized Libraries
DPMC	DNA Primer Mix C
DPMD	DNA Primer Mix D (To be used only with MainstAY SE Kit)
ePCR1	Enhanced PCR1 Reaction Mix
gDNA	Genomic DNA
FEM	Enzyme Mix
FSP	ForenSeq Sample Plate
HP3	2 N NaOH
HSC	Human Sequencing Control
HT1	Hybridization Buffer
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNS2	Library Normalization Storage Buffer 2
LNW1	Library Normalization Wash 1
NA24385	NA24385 Positive Amplification Control DNA
NLP	Normalized Library Plate
NWP	Normalization Working Plate
PBP	Purification Bead Plate
PCR2	PCR2 Reaction Mix
PLP	Purified Library Plate
PNL	Pooled Normalized Libraries

Acronym	Definition
ProK	Proteinase K
RSB	Resuspension Buffer
SPB2	Sample Purification Beads 2
UDI	Unique Dual Indexes

Additional Resources

This guide provides comprehensive information about the use of the ForenSeq MainstAY Kits with the ForenSeq Enhanced PCR1 (ePCR1) Buffer System on the Verogen PrepStation, including detailed protocol instructions. To download additional kit documentation and access the latest versions, visit the Documentation page[1] on Verogen's website.

Resource	Description
Verogen PrepStation for ForenSeq MainstAY with the Enhanced PCR1 Buffer System Materials List (document # VD2022029)	Lists the consumables and equipment needed to perform the protocol.

[1]https://verogen.com/documentation/

Protocol

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Introduction

This chapter describes the ForenSeq MainstAY protocol with step-by-step instructions to prepare libraries for sequencing using the ForenSeq Enhanced PCR1 (ePCR1) Buffer System on the Verogen PrepStation. For an overview of the protocol with reagents and durations for each step, see *Protocol Steps* (on page 7).

Before starting, confirm kit contents and make sure that you have the necessary reagents, consumables, and equipment. For a list of items, see *Materials* (on page 37).

Number of Samples

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

Up to 96 samples can be pooled for a sequencing run with a MiSeq FGx Reagent Micro Kit **as long as no duplicate UDIs are used**.

Cleaning the PrepStation

To avoid sample-to-sample contamination between protocols, Verogen 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 *Verogen PrepStation Preventative and Maintenance Guide (document # VD2023001)*.

Tips and Techniques

Protocol Continuity

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

Plate Setup

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

> The ***Universal Analysis Software v2.0 Reference Guide (document # VD2019002)*** provides detailed information on sample sheets and input of sample information.

Preventing Cross-Contamination

- Set up the *Amplify and Tag Targets* (below) process in a pre-PCR environment. Perform all other processes in a post-PCR environment.
- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each well***.
- When processing smaller sample batches of less than 96 libraries at a time, prevent aerosolization by resealing the utilized, pierced UDI wells with Microseal "B" adhesive seal. Trim excess seal with a scissors.
- Do not apply Microseal "B" to unused UDI wells as this will impact the integrity of the single-use pierceable foil.

Sealing the Plate

- Apply a microseal to cover the plate and seal with a rubber roller. After each use, discard seals from plates.
- Use Microseal 'A' pressure film for thermal cycling. 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.

Amplify and Tag Targets

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

Consumables

- NA24385 (NA24385 Positive Amplification Control DNA)
- DPMC (DNA Primer Mix C) or DPMD (DNA Primer Mix D)

- FEM (Enzyme Mix)
- ePCR1 (Enhanced PCR1 Reaction Mix)
- 1.5 ml LoBind microcentrifuge tubes (2)
- 96-well PCR plate, semiskirted
- Input gDNA
- Microseal 'A' film
- Nuclease-free water
- [Optional] RNase/DNase-free 8-tube strip and caps

About Reagents

- Use PCR1 for standard samples and ePCR1 for inhibited samples.
- ***ePCR1 should not be used with crude lysates or FTA card punches.***
- Do not vortex NA24385, FEM, or input gDNA.
- For information on DPMC and DPMD, see *Loci Detected with DPMC and DPMD* (on page 46).

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
NA24385	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert three times to mix, and then centrifuge briefly.
DPMC or DPMD	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	Remove from storage immediately before use, and then return to storage immediately after use.
ePCR1	-25°C to -15°C	Thaw at room temperature for 30 minutes. Vortex to mix, and then centrifuge briefly.

2. Save the following PCR1 program on the thermal cycler in the post-amplification area. See Table 1 for ramp modes.
   - Choose the preheat lid option. See Table 1 for lid temperatures.
   - 98°C for 3 minutes
   - 8 cycles of:
     - 96°C for 45 seconds
     - 80°C for 30 seconds
     - 54°C for 2 minutes, with applicable ramp mode
     - 68°C for 2 minutes, with applicable ramp mode
   - 10 cycles of:
     - 96°C for 30 seconds
     - 68°C for 3 minutes, with applicable ramp mode
   - 68°C for 10 minutes
   - Hold at 10°C

Table 1 Thermal Cycler lid temperature and ramp modes

Thermal Cycler	Temperature Mode	Lid Temperature	Ramp Mode	Vessel Type
ABI LTI thermal cycler 9700	9600 emulation	Heated	8%	Polypropylene plates and tubes
Bio-Rad	Calculated	Heated, constant at 100°C	0.2°C per second	Polypropylene plates and tubes
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	2%	Plate
Proflex 96-well PCR System	Not applicable	Heated, constant at 105°C	0.2°C per second	Polypropylene plates and tubes
QIAmplifier 96-well thermal cycler	Standard	Heated, constant at 100°C	0.1°C per second	Polypropylene plates
Veriti 96-well thermal cycler	Standard	Heated, constant at 105°C	4%	Polypropylene plates and tubes

The PCR1 program takes ~3.5 hours and can be run overnight. (The duration for the amplification will depend on the thermal cycler used.)

3. Label a new PCR plate FSP (for ForenSeq Sample Plate).
4. Label a new 1.5 ml tube per your input type:

Input Type	Label
Purified DNA	Master Mix

Procedure

Purified DNA

1. Using nuclease-free water, dilute 1 ng purified DNA input to 0.125 ng/µl.
2. In the Master Mix tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.
   - ePCR1 (4.7 µl)
   - FEM (0.3 µl)
   - DPMC or DPMD (2 µl)

   For example, for eight samples prepare 61.6 µl master mix: 41.4 µl ePCR1, 2.6 µl FEM, and 17.6 µlDPMC or DPMD.
3. Pipette to mix, and then cap and centrifuge briefly.
4. [Optional] Evenly distribute the master mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.
5. Add 7 µl master mix to each well of the FSP.
6. In a new 1.5 ml tube, combine the following volumes to dilute NA24385:
   - NA24385 (2 µl)
   - Nuclease-free water (158 µl)
7. Cap and gently invert three times to mix, and then centrifuge briefly.

8. Add 8 µl diluted NA24385 to at least one well of the FSP as a positive amplification control.
9. Pipette to mix.
10. Add 8 µl nuclease-free water to at least one well of the FSP as a negative amplification control.
11. Pipette to mix.
12. Add 8 µl 0.125 ng/µl DNA to each well of the FSP.
13. Seal and centrifuge at 1000 × g for 30 seconds.
14. Place on the preprogrammed thermal cycler and run the PCR1 program.
15. Unless you are stopping, proceed to *Enrich Targets (Post 1 – Enrichment)* (below).

SAFE STOPPING POINT

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

Enrich Targets (*Post 1 – Enrichment*)

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

Consumables

- PCR2 (PCR2 Reaction Mix)
- UDI (Unique Dual Index) plate (UDI0001–UDI0096)
- Microseal 'A' film
- [Optional] Microseal "B" film
- Opentrons 200 µl filter tips
- Opentrons 20 µl filter tips
- Thermo Scientific Abgene 0.8mL reagent midi plates
- Trash bags (Hommaly)

About Reagents

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

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
UDI plate	-25°C to -15°C	Thaw at room temperature, place on a plate shaker to mix, and then centrifuge at 1000 × g for 30 seconds.
PCR2 Reaction Mix	-25°C to -15°C	Thaw at room temperature for 20 minutes, and then invert to mix.

2. Save the following PCR2 program on the thermal cycler:

- Choose the preheat lid option and set to 100°C
- 98°C for 30 seconds
- 15 cycles of:
  - 98°C for 20 seconds
  - 66°C for 120 seconds
- 68°C for 10 minutes
- Hold at 10°C

Total program time is ~46 minutes. (The duration for the amplification will depend on the thermal cycler used.)

3. Remove the clear plastic lid (**do not remove the foil seal cover**) from the UDI plate and discard appropriately.
4. Label a new midi plate as "Reagent Midi Plate".
5. Prepare the reagent midi plate by aliquoting reagents into wells as specified in Table 2 and Table 3 below. (Blank columns indicate wells that should be left empty.)

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 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												PCR2 Reaction Mix
B												PCR2 Reaction Mix
C												PCR2 Reaction Mix
D												PCR2 Reaction Mix
E												PCR2 Reaction Mix
F												PCR2 Reaction Mix
G												PCR2 Reaction Mix
H												PCR2 Reaction Mix

Table 3 Reagent volumes, *Post 1 – Enrichment*

Reagent	Midi plate column	# of reactions	Volume per well
PCR2 Reaction Mix	Column 12	8	35 µl
		16	70 µl
		24	105 µl
		32	140 µl
		40	175 µl
		48	210 µl

Procedure

1. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
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 UDI 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 lefthand 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.

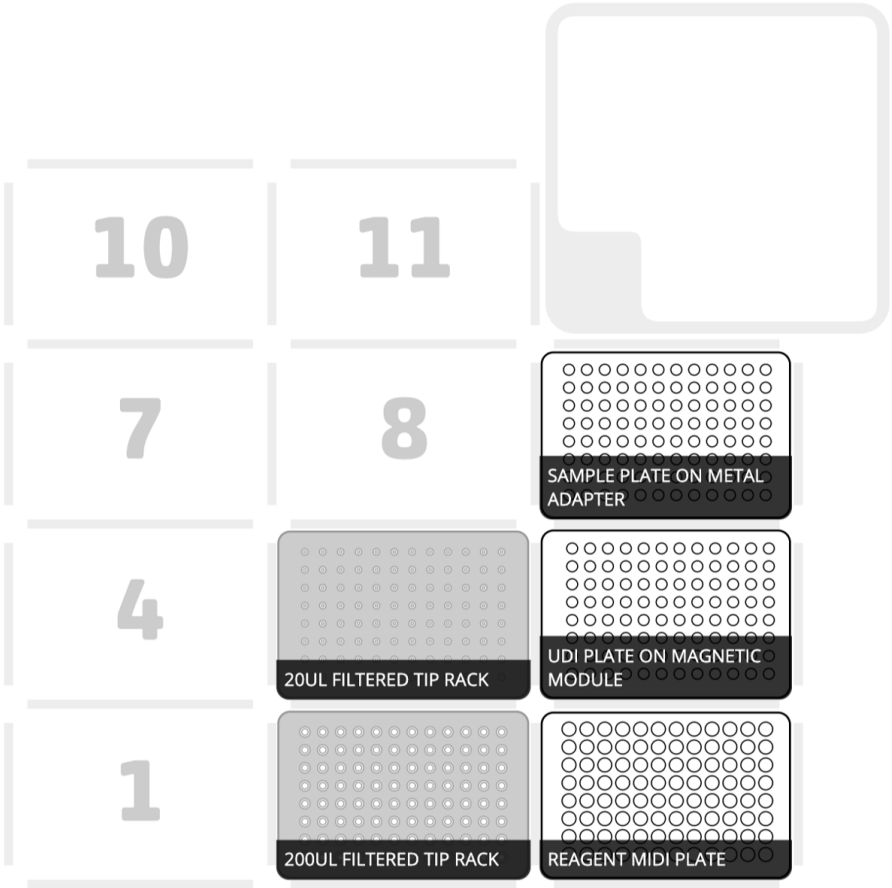
   NOTE: If a window appears prompting you to apply stored labware offset data, select Apply stored data.

   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 4 below).

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 UDI plate on top of the magnetic module and secure it by screwing in the ***small*** clamp.
- Ensure that the foil seal remains on the UDI plate. (The instrument will pierce the foil.)
- Ensure that lids have been removed from the tip racks.

Figure 4 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.

IMPORTANT: Carefully observe the following while performing the labware position check:

- 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 ***do not adjust the Z-axis (Up and Down) for other labware***.
- 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.

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 seconds.
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°C to 8°C for up to 7 days. Alternatively, leave on the thermal cycler overnight.

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

Purify Libraries (*Post 2 – Purification*)

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

Consumables

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

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
SPB2	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex for **≥ *1 minute*** and invert to mix.
ProK	-25°C to -15°C	Remove from storage immediately before use, and then return to storage immediately after use.

2. Label plates as follows:

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

3. Make the SPB2 Master Mix as follows.

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

4. Prepare the reagent midi plate by aliquoting additional reagents into wells as specified in Table 4 and Table 5 below. (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	PCR2 Reaction Mix
B					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
C					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
D					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
E					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
F					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
G					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
H					RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix

Table 5 Reagent volumes, *Post 2 – Purification*

Reagent	Midi plate column	# of reactions	Volume per well
RSB	Column 5	8	70 µl
		16	140 µl
		24	210 µl
		32	280 µl
		40	350 µl
		48	420 µl
SPB2	Column 6	8	54 µl
		16	108 µl
		24	162 µl
		32	216 µl
		40	270 µl
		48	325 µl
80% EtOH*	Columns 8, 10	8	300 µl
		16	500 µl
		24	650 µl
	Columns (8, 10) & (9, 11)	32	(650 µl) & (300 µl)
		40	(650 µl) & (500 µl)
		48	(650 µl) & (650 µl)

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

Procedure

1. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
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.

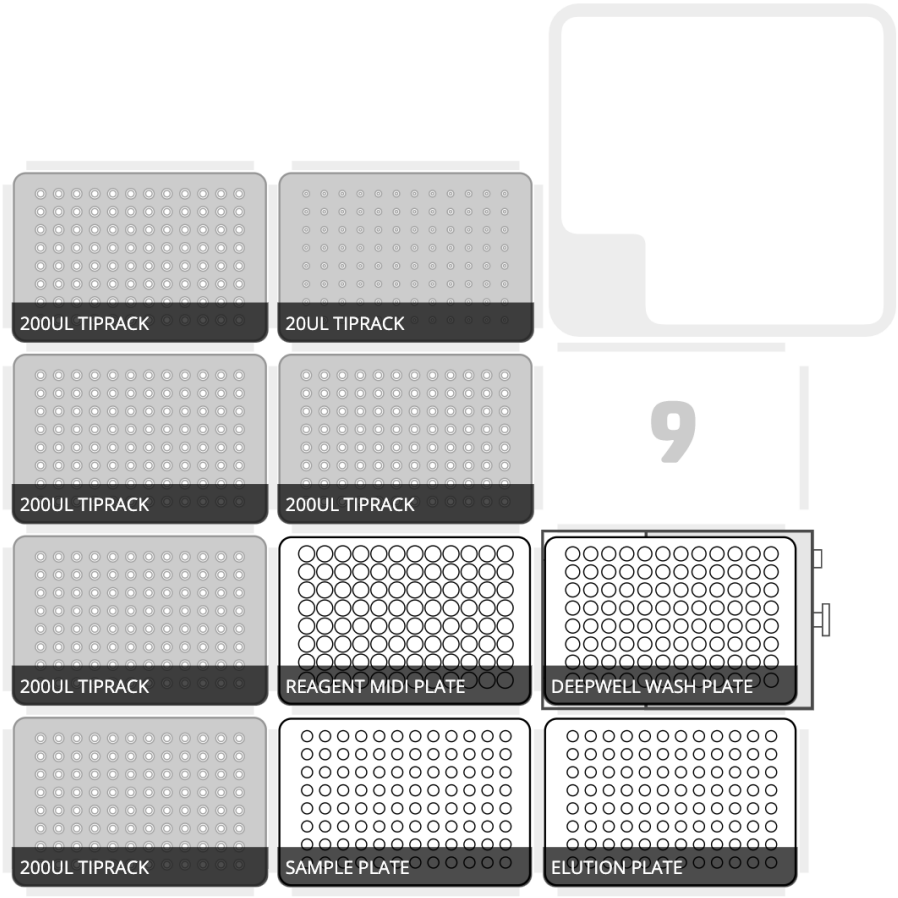
> **NOTE:** If a window appears prompting you to apply stored labware offset data, select Apply stored data.

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 below).

> **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 5 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*

Number 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 performing the labware position check:

- 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 ***do not adjust the Z-axis (Up and Down) for other labware***.
- 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 that offset data. You may select Apply stored data to apply the same offsets to the current run. (Whether you select this option or not, Verogen 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 seconds.
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.

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
- 96-well PCR plate, skirted or semiskirted
- PVC reagent reservoir
- Microseal 'B' film
- Opentrons 200 µl filter tips
- Opentrons 20 µl filter tips
- Thermo Scientific Abgene 0.8mL 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.

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

Preparation

1. Prepare the following consumables:

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

2. Label vessels as follows.

Vessel	Label
1.5 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 below. Do not add overage.

Table 7 LNA1/LNB1 Master Mix

	Volumes to add	
# of reactions	LNA1	LNB1
8	379 µl	69 µl
16	758 µl	138 µl
24	1138 µl	208 µl
32	1517 µl	275 µl
40	1896 µl	344 µl
48	2275 µl	413 µl

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	
# of reactions	Nuclease-free water	HP3
8	381 µl	21 µl
16	762 µl	42 µl
24	1142 µl	62 µl
32	1523 µl	83 µl
40	1904 µl	104 µl
48	2285 µl	125 µl

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: 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.

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	PCR2 Reaction Mix
B	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
C	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
D	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
E	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
F	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
G	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix
H	LNS2	0.1 N HP3	LNW1	LNA1/LNB1	RSB	SPB2		EtOH	EtOH	EtOH	EtOH	PCR2 Reaction Mix

Table 10 Reagent volumes, *Post 3 – Normalization*

Reagent	Midi plate column	# of reactions	Volume per well
LNS2	Column 1	8	36 µl
		16	72 µl
		24	108 µl
		32	144 µl
		40	180 µl
		48	216 µl
0.1 N HP3	Column 2	8	40 µl
		16	80 µl
		24	120 µl
		32	160 µl
		40	200 µl
		48	240 µl
LNW1	Column 3	8	100 µl
		16	200 µl
		24	300 µl
		32	400 µl
		40	500 µl
		48	600 µl
LNA1/LNB1 Master Mix	Column 4	8	56 µl
		16	112 µl
		24	168 µl
		32	224 µl
		40	280 µl
		48	336 µl

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.

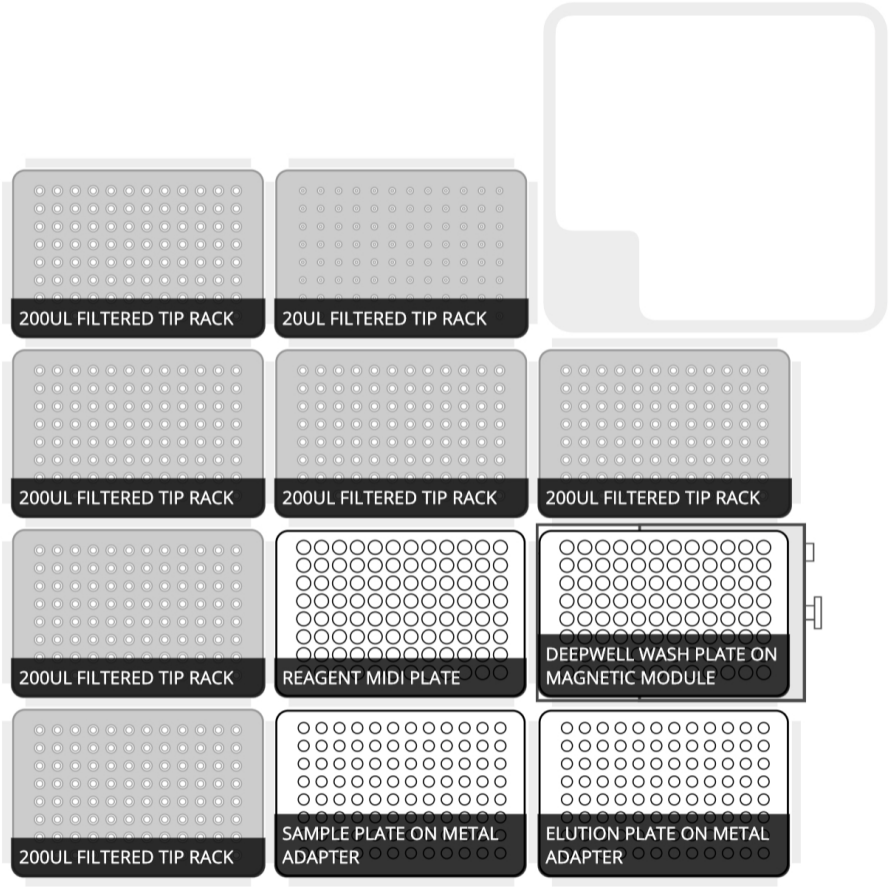
> **NOTE:** If a window appears prompting you to apply stored labware offset data, select Apply stored data.

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 below).

> **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 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 3 – Normalization* protocol



NOTE: The deck map in the application may show more filtered 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*

Number of reactions	Deck slots requiring 200 µl filtered tip racks
8	1
16	1, 4
24	1, 4, 7
32	1, 4, 7, 10
40	1, 4, 7, 10, 8
48	1, 4, 7, 10, 8, 9

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 performing the labware position check:

- 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 ***do not adjust the Z-axis (Up and Down) for other labware***.
- 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 that offset data. You may select Apply stored data to apply the same offsets to the current run. (Whether you select this option or not, Verogen 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. Centrifuge the sealed plate at 1000 × g for 30 seconds.
3. 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.

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, semiskirted
- Microseal 'B' film
- Opentrons 200 µl filter tips
- Opentrons 20 µl filter tips
- Trash bags (Hommaly)

Preparation

1. Select libraries to pool for sequencing.
   For recommendations, see *Number of Samples* (on page 10).
2. Label vessels 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 seconds.
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 pool 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.

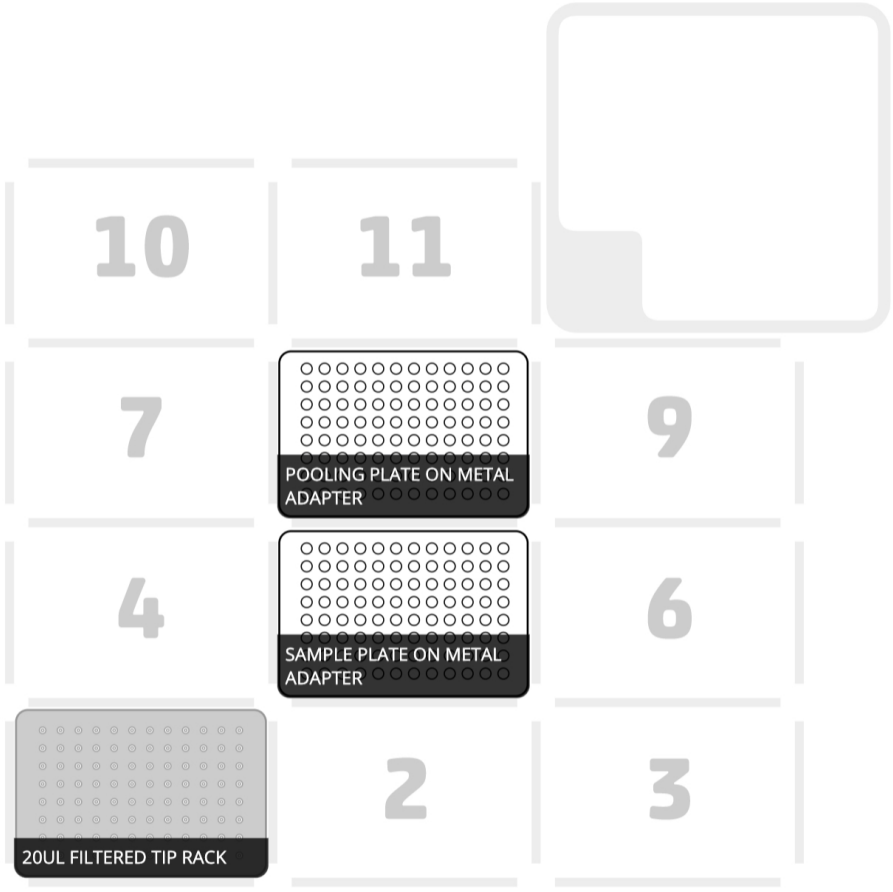
      NOTE: If a window appears prompting you to apply stored labware offset data, select Apply stored data.

   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 below).

      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 rack.

Figure 7 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 performing the labware position check:

- 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 ***do not adjust the Z-axis (Up and Down) for other labware***.
- 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 that offset data. You may select Apply stored data to apply the same offsets to the current run. (Whether you select this option or not, Verogen 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. Centrifuge the sealed plate at 1000 × g for 30 seconds.
4. Transfer libraries from each well of the 8-tube strip 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, cap the tube 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.)

Denature and Dilute Libraries

This process dilutes libraries to the loading concentration, adds a sequencing control, and uses a heat-based method to denature the libraries for sequencing.

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

Consumables

- HP3 (2 N NaOH)
- HSC (Human Sequencing Control)
- MiSeq FGx Reagent Kit contents:
  — HT1 (Hybridization Buffer)

  - Reagent cartridge
- Nuclease-free water
- 1.5 ml LoBind microcentrifuge tubes (2)

Preparation

1. Prepare the following consumables:

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

2. Preheat the microheating system to 96°C.
3. Label two new 1.5 ml tubes:
   - Denatured HSC
   - DNL for Denatured Normalized Libraries

Procedure

1. In the Denatured HSC tube, combine the following volumes:

   Strictly follow the order of adding the reagents.

   - HSC (2 µl)
   - HP3 (2 µl)
   - Nuclease-free water (36 µl)
2. Pipette gently to mix. Cap and centrifuge briefly to mix.
3. Incubate at room temperature for 5 minutes.
4. Add 600 µl HT1 to the DNL tube.
5. Place the PNL tube in the preheated microheating system and incubate for 2 minutes.

6. Immediately transfer an appropriate volume of libraries from the PNL tube to the DNL tube. Refer to Table 12 below.

   This step assumes that you may choose to sequence libraries from multiple PrepStation runs, or a mix of libraries prepared via PrepStation and libraries prepared manually, together in a single MiSeq FGx flow cell.

Table 12 Library volumes

Number of libraries	PrepStation library volume	Manual library volume	Total library volume
96 PrepStation, 0 manual	18 µl	n/a	18 µl
48 PrepStation, 48 manual	9 µl	6 µl	15 µl
0 PrepStation, 96 manual*	n/a	12 µl	12 µl

*This row included for comparison only.

7. Pipette to mix.
8. Cap the PNL tube and store at -25°C to -15°C for ≤ 30 days.
   Exceeding 30 days significantly reduces cluster density.
9. Add 4 µl denatured HSC to the DNL tube.
   You can store the denatured HSC at room temperature for ≤ 1 day.
10. Pipette to mix.
11. Cap and vortex to mix, and then centrifuge briefly.
12. Immediately transfer the entire volume to the reagent cartridge per instructions in the *MiSeq FGx Sequencing System Reference Guide (document # VD2018006)*.

Materials

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Kit Contents and Storage

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

Kit Name	Part #
ForenSeq MainstAY Kit (96 Reactions)	V16000142
ForenSeq MainstAY Kit (384 Reactions)	V16000128
ForenSeq MainstAY SE Kit (96 Reactions)	V16000183
ForenSeq Enhanced PCR1 Buffer System (96 Reactions)	V16000137

All reagents in a box are shipped at the same temperature. When a reagent has a different storage temperature than most other reagents in the box, you can initially store the reagent at the same temperature as the other reagents. After first use, store the reagent at the indicated temperature. See *Post-PCR Box 2* (below).

ForenSeq MainstAY Kit (96 Reactions) (V16000142)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
2	FEM	Enzyme Mix	Yellow	-25°C to -15°C
2	PCR1	PCR1 Reaction Mix	Green	-25°C to -15°C

Post-PCR Box 2

Quantity	Reagent	Description	Cap	Storage
1	HP3	2 N NaOH	Orange	-25°C to -15°C
1	HSC	Human Sequencing Control	Pink	-25°C to -15°C
1	LNA1	Library Normalization Additives 1	Clear	-25°C to -15°C
1	LNS2	Library Normalization Storage Buffer 2	Clear	Room temperature*
2	LNW1	Library Normalization Wash 1	Clear	2°C to 8°C*
2	PCR2	PCR2 Reaction Mix	Purple	-25°C to -15°C
1	UDI Plate	Unique Dual Index	Clear	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 3

Quantity	Reagent	Description	Cap	Storage
1	LNB1	Library Normalization Beads 1	White	2°C to 8°C
1	RSB	Resuspension Buffer	Green	2°C to 8°C
1	SPB2	Sample Purification Beads 2	Red	2°C to 8°C

Pre-PCR Box 4

Quantity	Reagent	Description	Cap	Storage
1	NA24385	NA24385 Positive Amplification Control DNA	Black	2°C to 8°C
2	DPMC	DNA Primer Mix C	White	2°C to 8°C

ForenSeq MainstAY Kit (384 Reactions) (V16000128)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
8	FEM	Enzyme Mix	Yellow	-25°C to -15°C
8	PCR1	PCR1 Reaction Mix	Green	-25°C to -15°C

Post-PCR Box 2

Quantity	Reagent	Description	Cap	Storage
3	HP3	2 N NaOH	Orange	-25°C to -15°C
1	HSC	Human Sequencing Control	Pink	-25°C to -15°C
4	LNA1	Library Normalization Additives 1	Clear	-25°C to -15°C
4	LNS2	Library Normalization Storage Buffer 2	Clear	Room temperature*
8	LNW1	Library Normalization Wash 1	Clear	2°C to 8°C*
8	PCR2	PCR2 Reaction Mix	Purple	-25°C to -15°C
1	UDI Plate	Unique Dual Index	Clear	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 3

Quantity	Reagent	Description	Cap	Storage
4	LNB1	Library Normalization Beads 1	White	2°C to 8°C
1	RSB	Resuspension Buffer	Green	2°C to 8°C

Quantity	Reagent	Description	Cap	Storage
2	SPB2	Sample Purification Beads 2	Red	2°C to 8°C

Pre-PCR Box 4

Quantity	Reagent	Description	Cap	Storage
4	NA24385	NA24385 Positive Amplification Control DNA	Black	2°C to 8°C
8	DPMC	DNA Primer Mix C	White	2°C to 8°C

ForenSeq MainstAY SE Kit (96 Reactions) (V16000183)

Pre-PCR Box 1

Quantity	Reagent	Description	Cap	Storage
2	FEM	Enzyme Mix	Yellow	-25°C to -15°C
2	PCR1	PCR1 Reaction Mix	Green	-25°C to -15°C

Post-PCR Box 2

Quantity	Reagent	Description	Cap	Storage
1	HP3	2 N NaOH	Orange	-25°C to -15°C
1	HSC	Human Sequencing Control	Pink	-25°C to -15°C
1	LNA1	Library Normalization Additives 1	Clear	-25°C to -15°C
1	LNS2	Library Normalization Storage Buffer 2	Clear	Room temperature*
2	LNW1	Library Normalization Wash 1	Clear	2°C to 8°C*
2	PCR2	PCR2 Reaction Mix	Purple	-25°C to -15°C
1	UDI Plate	Unique Dual Index	Clear	-25°C to -15°C

* Shipped at -25°C to -15°C

Post-PCR Box 3

Quantity	Reagent	Description	Cap	Storage
1	LNB1	Library Normalization Beads 1	White	2°C to 8°C
1	RSB	Resuspension Buffer	Green	2°C to 8°C
1	SPB2	Sample Purification Beads 2	Red	2°C to 8°C

Pre-PCR Box 4

Quantity	Quantity	Reagent	Description	Cap	Storage
1	4	NA24385	NA24385 Positive Amplification Control DNA	Black	2°C to 8°C
2	8	DPMD	DNA Primer Mix D	White	2°C to 8°C

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

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

Consumables and Equipment

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

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

Consumables

Consumable	Supplier
1.5 ml LoBind microcentrifuge tubes	VWR, catalog # 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 # AB-0765 (individually sealed)
96-well twin.tec PCR plates, semiskirted	One of the following suppliers: • Eppendorf, catalog # 951020303 • VWR, catalog # 89136-706
96-well twin.tec PCR plate, skirted, 150 µl	Eppendorf, catalog # 951020401
Ethyl alcohol, pure	Sigma-Aldrich, catalog # E7023
Microseal 'A' sealing film	Bio-Rad, catalog # MSA5001
Microseal 'B' sealing film, adhesive, optical	Bio-Rad, catalog # MSB1001
MiSeq FGx Reagent Micro Kit	Verogen part # 20021681

Consumable	Supplier
Multichannel reagent reservoirs, PVC, disposable	VWR, catalog # 89094-688
Nuclease-free water	General lab supplier
Reagent reservoirs	Corning, catalog # MTS-11-8-C-R-S
RNase/DNase-free 8-tube strips and caps	General lab supplier
Waste bags	Verogen, part # V16000208

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
Computer with PrepStation and Opentrons applications installed[1]	User-supplied; installation and setup supported by your Verogen FAS		X
Heating system, 96-well, 1.5 ml	General lab supplier		X
Magnetic module for PrepStation instrument[2]	Opentrons (SKU: 999-00098)		X
Metal PCR plate adapters[2]	Opentrons (SKU: 999-00028)		X
Microplate centrifuge	General lab supplier	X	X
P20 8-channel pipette arm (GEN2)[2,3]	Opentrons (SKU: 999-00005)		X
P300 8-channel pipette arm (GEN2)[2,4]	Opentrons (SKU: 999-00006)		X
PrepStation instrument	Verogen, part # V16000192		X
Rubber roller	General lab supplier	X	X
Thermal cycler, 96-well with heated lid	See *Thermal Cyclers* (on the next page)		X
Vortexer	General lab supplier	X	X
[Optional] 10 µl pipettes	General lab supplier	X	X

[1] 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.

[2] This item is included with your purchase of the PrepStation instrument (Verogen, part # V16000192), and does not need to be purchased separately. Replacement parts may be purchased from Opentrons as listed.

[3] Installed on left side of the PrepStation instrument.

[4] Installed on 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	Gradient S, Simulated Tube	Heated	Plate
QIAmplifier 96-well thermal cycler	Standard	Heated, constant at 100°C	Polypropylene plates
Proflex 96-well PCR System	Not applicable	Heated, constant at 105°C	Polypropylene plates and tubes
Veriti 96-well thermal cycler	Standard	Heated, constant at 105°C	Polypropylene plates and tubes

* Only gold heat blocks are supported.

Index Adapter Sequences

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

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

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0001	ATCACGAT	AGCGCTAG	A1
UDI0002	CGATGTAT	GATATCGA	A2
UDI0003	TTAGGCAT	CGCAGACG	A3
UDI0004	TGACCAAT	TATGAGTA	A4
UDI0005	ACAGTGAT	AGGTGCGT	A5
UDI0006	GCCAATAT	GAACATAC	A6
UDI0007	CAGATCAT	ACATAGCG	A7
UDI0008	ACTTGAAT	GTGCGATA	A8

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0009	GATCAGAT	CCAACAGA	A9
UDI0010	TAGCTTAT	TTGGTGAG	A10
UDI0011	GGCTACAT	CGCGGTTC	A11
UDI0012	CTTGTAAT	TATAACCT	A12
UDI0013	AGTCAAAT	AAGGATGA	B1
UDI0014	AGTTCCAT	GGAAGCAG	B2
UDI0015	ATGTCAAT	TCGTGACC	B3
UDI0016	CCGTCCAT	CTACAGTT	B4
UDI0017	GTAGAGAT	ATATTCAC	B5
UDI0018	GTCCGCAT	GCGCCTGT	B6
UDI0019	GTGAAAAT	ACTCTATG	B7
UDI0020	GTGGCCAT	GTCTCGCA	B8
UDI0021	GTTTCGAT	AAGACGTC	B9
UDI0022	CGTACGAT	GGAGTACT	B10
UDI0023	GAGTGGAT	ACCGGCCA	B11
UDI0024	GGTAGCAT	GTTAATTG	B12
UDI0025	ACTGATAT	AACCGCGG	C1
UDI0026	ATGAGCAT	GGTTATAA	C2
UDI0027	ATTCCTAT	CCAAGTCC	C3
UDI0028	CAAAAGAT	TTGGACTT	C4
UDI0029	CAACTAAT	CAGTGGAT	C5
UDI0030	CACCGGAT	TGACAAGC	C6
UDI0031	CACGATAT	CTAGCTTG	C7
UDI0032	CACTCAAT	TCGATCCA	C8
UDI0033	CAGGCGAT	CCTGAACT	C9
UDI0034	CATGGCAT	TTCAGGTC	C10
UDI0035	CATTTTAT	AGTAGAGA	C11
UDI0036	CCAACAAT	GACGAGAG	C12
UDI0037	CGGAATAT	AGACTTGG	D1
UDI0038	CTAGCTAT	GAGTCCAA	D2
UDI0039	CTATACAT	CTTAAGCC	D3

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0040	CTCAGAAT	TCCGGATT	D4
UDI0041	GACGACAT	CTGTATTA	D5
UDI0042	TAATCGAT	TCACGCCG	D6
UDI0043	TACAGCAT	ACTTACAT	D7
UDI0044	TATAATAT	GTCCGTGC	D8
UDI0045	TCATTCAT	AAGGTACC	D9
UDI0046	TCCCGAAT	GGAACGTT	D10
UDI0047	GTTCCAAT	AATTCTGC	D11
UDI0048	ACCTTGGC	GGCCTCAT	D12
UDI0049	ATATCTCG	ATCTTAGT	E1
UDI0050	GCGCTCTA	GCTCCGAC	E2
UDI0051	AACAGGTT	ATACCAAG	E3
UDI0052	GGTGAACC	GCGTTGGA	E4
UDI0053	CAACAATG	CTTCACGG	E5
UDI0054	TGGTGGCA	TCCTGTAA	E6
UDI0055	AGGCAGAG	AGAATGCC	E7
UDI0056	GAATGAGA	GAGGCATT	E8
UDI0057	TGCGGCGT	CCTCGGTA	E9
UDI0058	CATAATAC	TTCTAACG	E10
UDI0059	GATCTATC	ATGAGGCT	E11
UDI0060	AGCTCGCT	GCAGAATC	E12
UDI0061	CGGAACTG	CACTACGA	F1
UDI0062	TAAGGTCA	TGTCGTAG	F2
UDI0063	TTGCCTAG	ACCACTTA	F3
UDI0064	CCATTCGA	GTTGTCCG	F4
UDI0065	ACACTAAG	ATCCATAT	F5
UDI0066	GTGTCGGA	GCTTGCGC	F6
UDI0067	TTCCTGTT	AGTATCTT	F7
UDI0068	CCTTCACC	GACGCTCC	F8
UDI0069	GCCACAGG	CATGCCAT	F9
UDI0070	ATTGTGAA	TGCATTGC	F10

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
UDI0071	ACTCGTGT	ATTGGAAC	F11
UDI0072	GTCTACAC	GCCAAGGT	F12
UDI0073	CAATTAAC	CGAGATAT	G1
UDI0074	TGGCCGGT	TAGAGCGC	G2
UDI0075	AGTACTCC	AACCTGTT	G3
UDI0076	GACGTCTT	GGTTCACC	G4
UDI0077	TGCGAGAC	CATTGTTG	G5
UDI0078	CATAGAGT	TGCCACCA	G6
UDI0079	ACAGGCGC	CTCTGCCT	G7
UDI0080	GTGAATAT	TCTCATTC	G8
UDI0081	AACTGTAG	ACGCCGCA	G9
UDI0082	GGTCACGA	GTATTATG	G10
UDI0083	CTGCTTCC	GATAGATC	G11
UDI0084	TCATCCTT	AGCGAGCT	G12
UDI0085	AGGTTATA	CAGTTCCG	H1
UDI0086	GAACCGCG	TGACCTTA	H2
UDI0087	CTCACCAA	CTAGGCAA	H3
UDI0088	TCTGTTGG	TCGAATGG	H4
UDI0089	TATCGCAC	CTTAGTGT	H5
UDI0090	CGCTATGT	TCCGACAC	H6
UDI0091	GTATGTTC	AACAGGAA	H7
UDI0092	ACGCACCT	GGTGAAGG	H8
UDI0093	TACTCATA	CCTGTGGC	H9
UDI0094	CGTCTGCG	TTCACAAT	H10
UDI0095	TCGATATC	ACACGAGT	H11
UDI0096	CTAGCGCT	GTGTAGAC	H12

Amplicon Information

Loci Detected with DPMC and DPMD 46

Loci Detected with DPMC and DPMD

The following tables list loci detected with DPMC and DPMD.

- Amplicon lengths exclude 120 bp for adapter sequences.
- Amplicon start and end positions are the one-base endpoints of the entire amplicon, including the sequence that matches primers on the hg19 human reference genome.
- Amelogenin is a genetic marker that confirms the biological sex of the biological sample donor. The size range is 106–112 bp and the control DNA is male.
- All the markers except SE33 are present in both DPMC and DPMD. SE33 is only present in DPMD.

Autosomal STRs

Locus	Repeats	Amplicon Length (bp)	Chromo-some	NA24385 Control Alleles
D1S1656	7–21.3	133–192	1	13,14
TPOX	4–16	61–109	2	8,8
D2S441	7–17	137–177	2	11,15
D2S1338	10–33.1	110–203	3	22,4
D3S1358	8–22	138–194	3	15,16
D4S2408	8–13	98–118	4	8,9
FGA	12.2–53	150–312	4	20,23
D5S818	4–20	98–162	5	11,12
CSF1PO	5–17	72–120	5	10,12
SE33[1]	3-39.2	190-336	6	17,24.2
D6S1043	8–26	154–226	6	11,14
D7S820[2]	5–21.1	118–183	7	11,12
D8S1179	6–20	82–138	8	13,16
D9S1122	8–15	104–132	9	12,12
D10S1248	7–20	124–176	10	14,16
TH01	3–14	96–140	11	9,9.3
vWA	11–26	135–195	12	16,18
D12S391	13–28	229–289	12	22,22

Locus	Repeats	Amplicon Length (bp)	Chromo-some	NA24385 Control Alleles
D13S317	5–17	138–186	13	11,13
PentaE	5–28.4	362–481	15	10,18
D16S539	4–17	132–184	16	11,11
D17S1301	9–15	130–154	17	11,11
D18S51	6–40	136–272	18	13,16
D19S433	4–27	148–240	19	14,16.2
D20S482	9–17	125–157	20	14,14
D21S11	12–41.2	147–265	21	30,31.2
PentaD	1.1–19	209–298	21	9,12
D22S1045	8–19	201–245	22	16,16

[1] DPMD only.

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

Y-STRs

Locus	Repeats	Amplicon Length (bp)	Chromosome	NA24385 Control Alleles
DYF387S1	30–44	207–263	Y	38,40
DYS19	9–19	269–309	Y	14
DYS385a-b	7–28	232–316	Y	16,18
DYS389I	9–17	236–268	Y	13
DYS389II	24–34	283–323	Y	29
DYS390	17–28	290–334	Y	25
DYS391	5–16	119–163	Y	10
DYS392	6–17	318–362	Y	13
DYS393	9–18	108–144	Y	12
DYS437	10–18	194–226	Y	14
DYS438	6–16	129–179	Y	11
DYS439	6–17	167–211	Y	10
DYS448	14–26	330–402	Y	20
DYS460	7–14	348–376	Y	11
DYS481	17–32	129–174	Y	26

Locus	Repeats	Amplicon Length (bp)	Chromosome	NA24385 Control Alleles
DYS505	9–15	162–186	Y	13
DYS522	8–17	298–334	Y	12
DYS533	7–17	186–226	Y	12
DYS549	10–14	210–226	Y	12
DYS570	10–26	142–206	Y	18
DYS576	10–25	163–223	Y	18
DYS612	26–33	275–296	Y	37
DYS635	15–30	242–302	Y	21
DYS643	7–15	141–181	Y	9
Y-GATA-H4	8–15	159–187	Y	10

Technical Support

For technical assistance, contact Verogen Technical Support.

	Contact Information
Address	11111 Flintkote Avenue San Diego CA 92121 USA
Website	www.qiagen.com www.qiagen.com/support/technical-support (or visit www.qiagen.com)
Email	techservice-na@qiagen.com (North America)
Telephone	+1.833.837.6436 toll-free (North America) +1.858.285.4101 tel +44 (0) 208 054 8706 (United Kingdom)

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

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

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Verogen

+1.833.837.6436 toll-free
(North America)

+1.858.285.4101
(outside North America)

techsupport@verogen.com

www.verogen.com