# Verogen PrepStation for ForenSeq MainstAY Product Line

## Reference Guide

VEROGEN PROPRIETARY
Document # VD2022026 Rev. A
September 2023

## Revision History

| Document # | Date | Description of Change |
|---|---|---|
| VD2022026 Rev. A | September 2023 | Initial release |

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# Overview



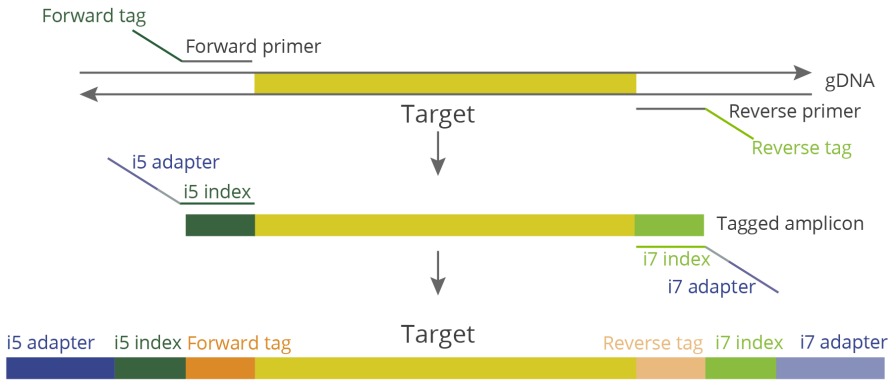
## Introduction

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 standard PCR1 buffer 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 in one plate 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.

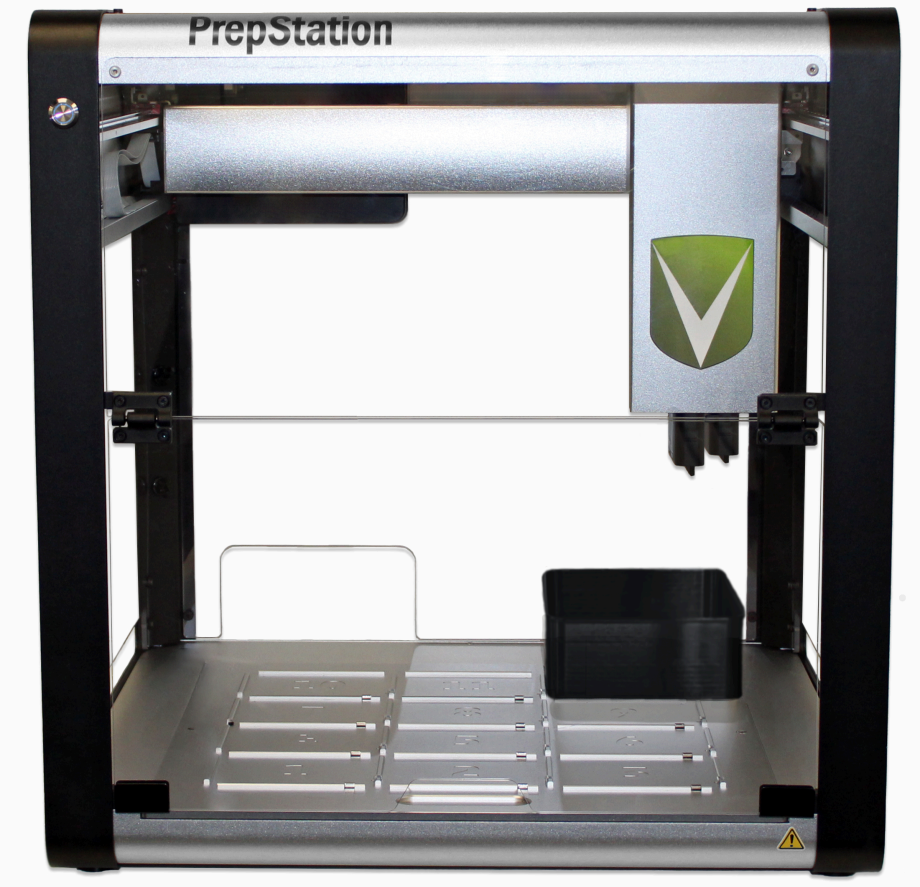
— ForenSeq MainstAY SE includes all the markers in ForenSeq MainstAY, with the addition of marker SE33. See *Loci Detected with DPMC and DPMD* (on page 46) for more information.

## 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

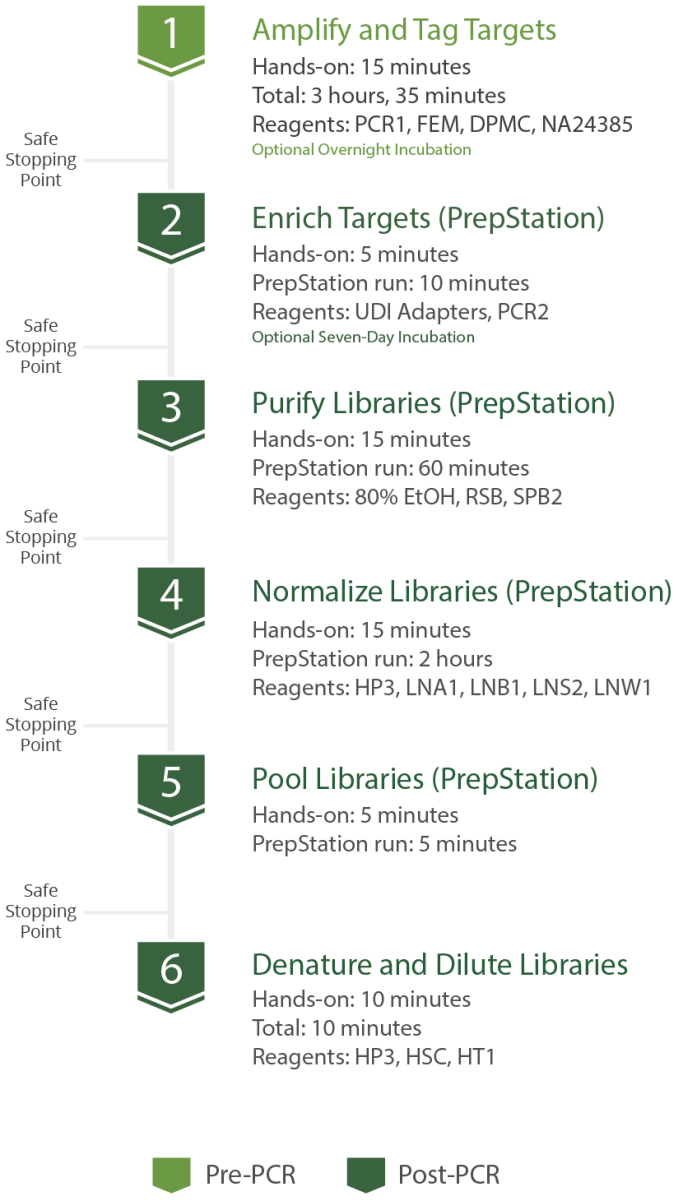


# Protocol Steps

Figure 3 illustrates the steps to prepare libraries using the standard PCR1 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 standard PCR1 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.

The kit is also compatible with crude lysate from buccal swabs. Use 2 µl input material per sample. See *Consumables* (on page 40) 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.

# 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) |
| 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 |
| PCR1 | PCR1 Reaction Mix |
| PCR2 | PCR2 Reaction Mix |

| Acronym | Definition |
|---|---|
| PLP | Purified Library Plate |
| PNL | Pooled Normalized Libraries |
| 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 standard PCR1 buffer 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 Product Line Materials List (document # VD2022025)* | Lists the consumables and equipment needed to perform the protocol. |

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

# Protocol



## Introduction

This chapter describes the ForenSeq MainstAY protocol with step-by-step instructions to prepare libraries for sequencing using the standard PCR1 buffer 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)
- PCR1 (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.
- 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. |
| PCR1 | -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 |
| Crude lysate | 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.
   - PCR1 (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 PCR1, 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)* (on the next page).

### SAFE STOPPING POINT

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

## Crude Lysate

1. In the Master Mix tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.
   - PCR1(4.7 µl)
   - FEM (0.3 µl)
   - DPMC or DPMD (2 µl)
   - Nuclease-free water (6 µl)

   For example, for eight samples prepare 114.4 µl master mix: 41.4 µl PCR1, 2.6 µl FEM, 17.6 µl DPMC or DPMD, and 52.8 µl nuclease-free water.
2. Pipette to mix, and then cap and centrifuge briefly.
3. [Optional] Evenly distribute the master mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.
4. Add 13 µl master mix to each well of the FSP.
5. In a new 1.5 ml tube, combine the following volumes to dilute NA24385:
   - NA24385 (2 µl)
   - Nuclease-free water (38 µl)
6. Cap and gently invert three times to mix, and then centrifuge briefly.
7. Add 2 µl diluted NA24385 to at least one well of the FSP as a positive template control.
8. Add 2 µl nuclease-free water to at least one well of the FSP as a negative template control.
9. Add 2 µl diluted crude lysate to each remaining well of the FSP.
10. Seal and centrifuge at 1000 × g for 30 seconds.
11. Place on the preprogrammed thermal cycler and run the PCR1 program.
12. Unless you are stopping, proceed to *Enrich Targets (Post 1 – Enrichment)* (on the next page).

### SAFE STOPPING POINT

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

# Enrich Targets (*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.

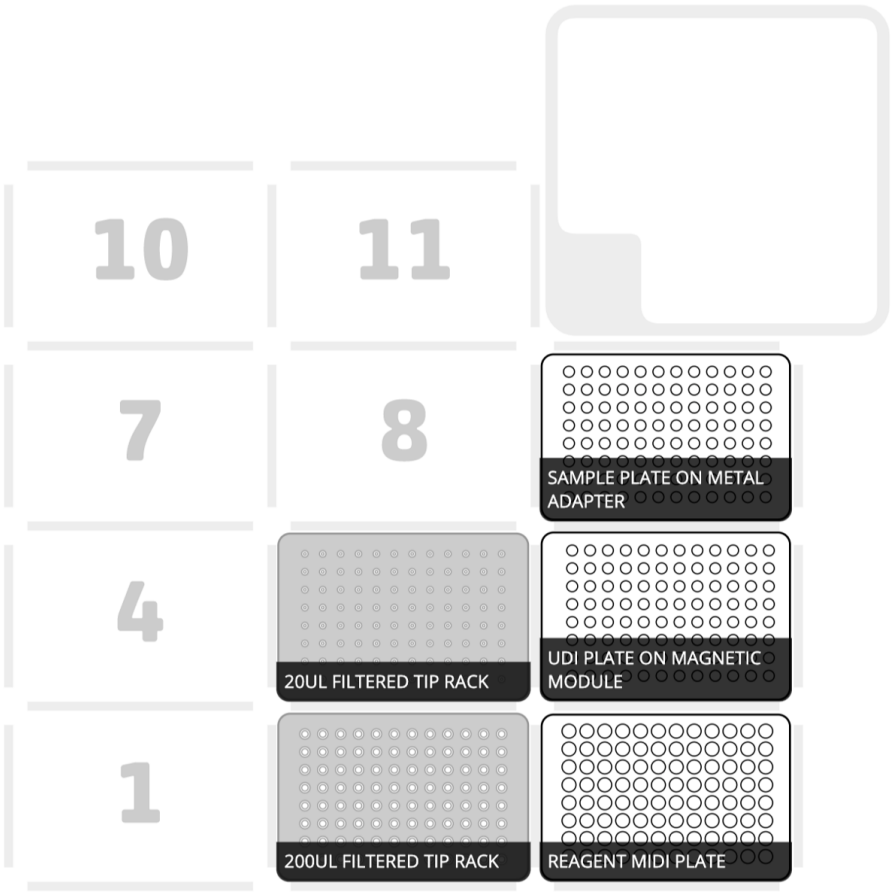
      **NOTE:** If a window appears prompting you to apply stored labware offset data, select Apply stored data.

   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)
- 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. |

2. Label plates as follows:

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

3. 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.

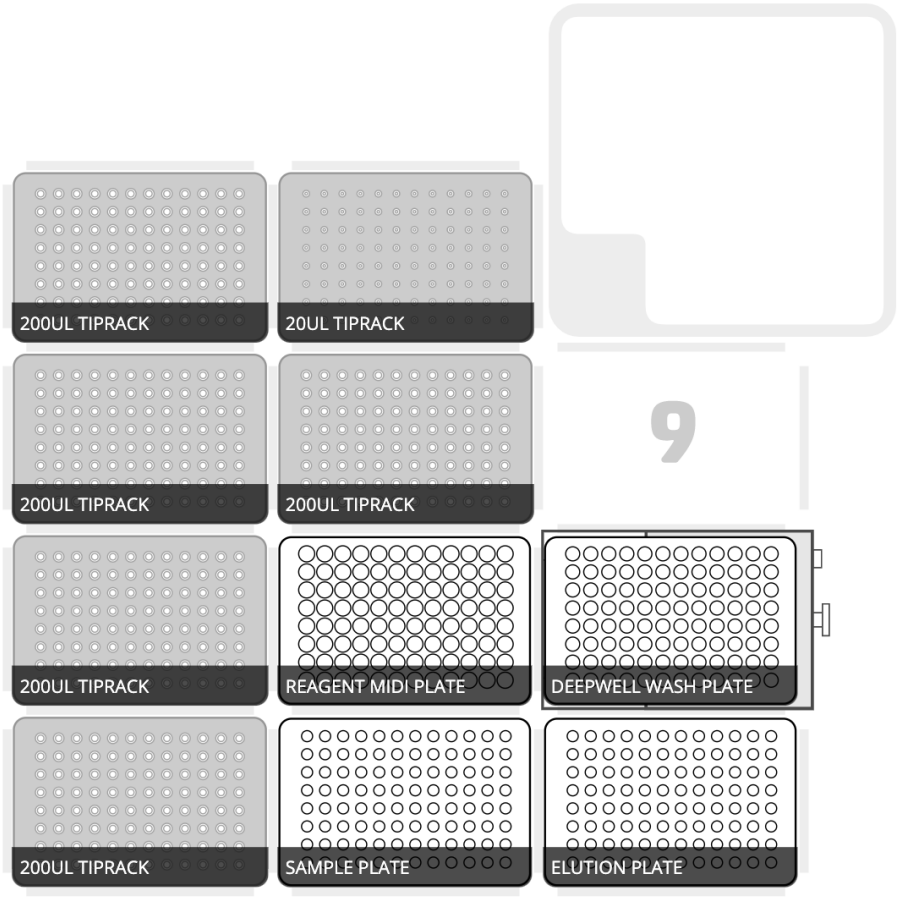
      **NOTE:** If a window appears prompting you to apply stored labware offset data, select Apply stored data.

   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.<br>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.

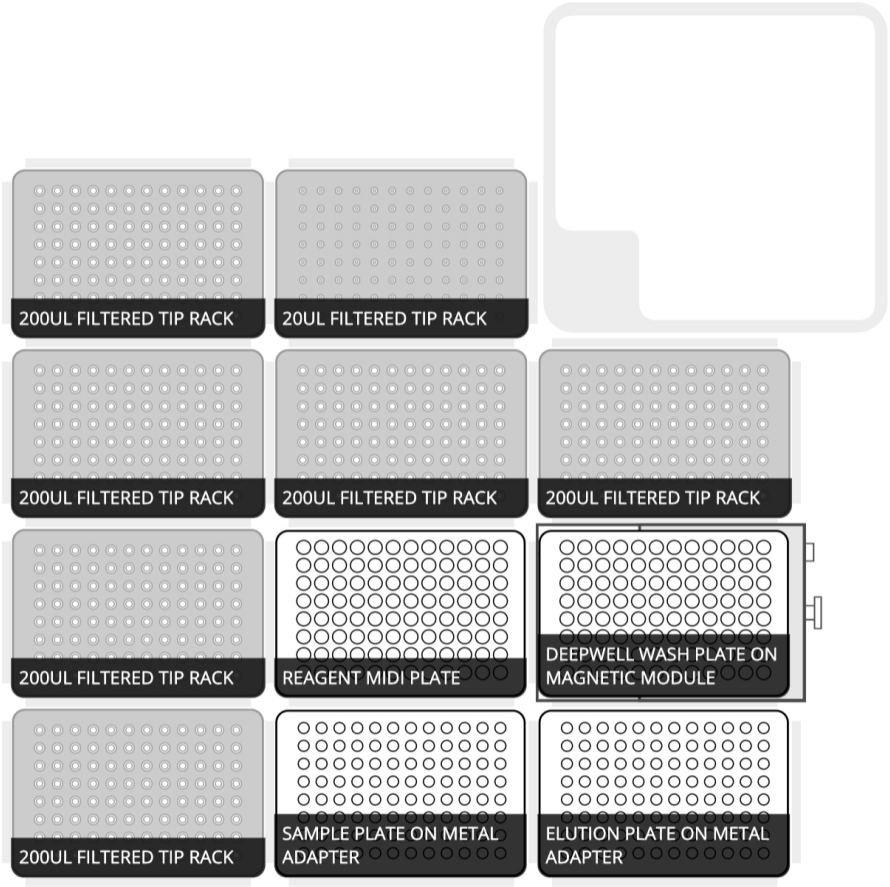
> **NOTE:** If a window appears prompting you to apply stored labware offset data, select Apply stored data.

**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.

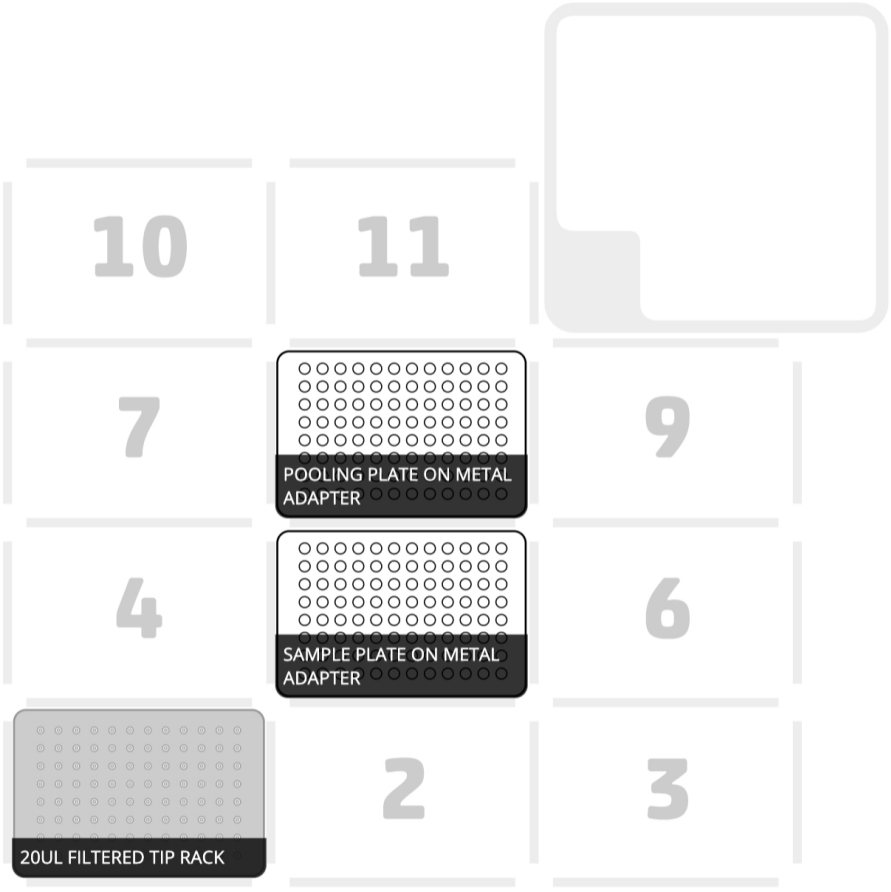
      **NOTE:** If a window appears prompting you to apply stored labware offset data, select Apply stored data.

   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



## 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 |

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 |
| 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 |

# 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:<br>• Eppendorf, catalog # 951020303<br>• 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 |
| 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 |

| Equipment | Supplier | Pre-PCR | Post-PCR |
|---|---|---|---|
| 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* (below) | | 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 |

| Thermal Cycler | Temperature Mode | Lid Temperature | Vessel Type |
|---|---|---|---|
| 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 |
| 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 |

| Index Name | Index 1 Bases | Index 2 Bases | Plate well location |
|---|---|---|---|
| UDI0017 | GTAGAGAT | ATATTCAC | B5 |
| UDI0018 | GTCCGCAT | GCGCCTGT | B6 |
| UDI0019 | GTGAAAAT | ACTCTATG | B7 |
| UDI0020 | GTGGCCAT | GTCTCGCA | B8 |
| UDI0021 | GTTTCGAT | AAGACGTC | B9 |
| UDI0022 | CGTACGAT | GGAGTACT | B10 |
| UDI0023 | GAGTGGAT | ACCGGCCA | B11 |
| UDI0024 | GGTAGCAT | GTTAATTG | B12 |
| UDI0025 | ACTGATAT | AACCGCGG | C1 |
| UDI0026 | ATGAGCAT | GGTTATAA | C2 |
| UDI0027 | ATTCCTAT | CCAAGTCC | C3 |
| UDI0028 | CAAAAGAT | TTGGACTT | C4 |
| UDI0029 | CAACTAAT | CAGTGGAT | C5 |
| UDI0030 | CACCGGAT | TGACAAGC | C6 |
| UDI0031 | CACGATAT | CTAGCTTG | C7 |
| UDI0032 | CACTCAAT | TCGATCCA | C8 |
| UDI0033 | CAGGCGAT | CCTGAACT | C9 |
| UDI0034 | CATGGCAT | TTCAGGTC | C10 |
| UDI0035 | CATTTTAT | AGTAGAGA | C11 |
| UDI0036 | CCAACAAT | GACGAGAG | C12 |
| UDI0037 | CGGAATAT | AGACTTGG | D1 |
| UDI0038 | CTAGCTAT | GAGTCCAA | D2 |
| UDI0039 | CTATACAT | CTTAAGCC | D3 |
| UDI0040 | CTCAGAAT | TCCGGATT | D4 |
| UDI0041 | GACGACAT | CTGTATTA | D5 |
| UDI0042 | TAATCGAT | TCACGCCG | D6 |
| UDI0043 | TACAGCAT | ACTTACAT | D7 |
| UDI0044 | TATAATAT | GTCCGTGC | D8 |
| UDI0045 | TCATTCAT | AAGGTACC | D9 |
| UDI0046 | TCCCGAAT | GGAACGTT | D10 |
| UDI0047 | GTTCCAAT | AATTCTGC | D11 |

| Index Name | Index 1 Bases | Index 2 Bases | Plate well location |
|---|---|---|---|
| UDI0048 | ACCTTGGC | GGCCTCAT | D12 |
| UDI0049 | ATATCTCG | ATCTTAGT | E1 |
| UDI0050 | GCGCTCTA | GCTCCGAC | E2 |
| UDI0051 | AACAGGTT | ATACCAAG | E3 |
| UDI0052 | GGTGAACC | GCGTTGGA | E4 |
| UDI0053 | CAACAATG | CTTCACGG | E5 |
| UDI0054 | TGGTGGCA | TCCTGTAA | E6 |
| UDI0055 | AGGCAGAG | AGAATGCC | E7 |
| UDI0056 | GAATGAGA | GAGGCATT | E8 |
| UDI0057 | TGCGGCGT | CCTCGGTA | E9 |
| UDI0058 | CATAATAC | TTCTAACG | E10 |
| UDI0059 | GATCTATC | ATGAGGCT | E11 |
| UDI0060 | AGCTCGCT | GCAGAATC | E12 |
| UDI0061 | CGGAACTG | CACTACGA | F1 |
| UDI0062 | TAAGGTCA | TGTCGTAG | F2 |
| UDI0063 | TTGCCTAG | ACCACTTA | F3 |
| UDI0064 | CCATTCGA | GTTGTCCG | F4 |
| UDI0065 | ACACTAAG | ATCCATAT | F5 |
| UDI0066 | GTGTCGGA | GCTTGCGC | F6 |
| UDI0067 | TTCCTGTT | AGTATCTT | F7 |
| UDI0068 | CCTTCACC | GACGCTCC | F8 |
| UDI0069 | GCCACAGG | CATGCCAT | F9 |
| UDI0070 | ATTGTGAA | TGCATTGC | F10 |
| UDI0071 | ACTCGTGT | ATTGGAAC | F11 |
| UDI0072 | GTCTACAC | GCCAAGGT | F12 |
| UDI0073 | CAATTAAC | CGAGATAT | G1 |
| UDI0074 | TGGCCGGT | TAGAGCGC | G2 |
| UDI0075 | AGTACTCC | AACCTGTT | G3 |
| UDI0076 | GACGTCTT | GGTTCACC | G4 |
| UDI0077 | TGCGAGAC | CATTGTTG | G5 |
| UDI0078 | CATAGAGT | TGCCACCA | G6 |

| Index Name | Index 1 Bases | Index 2 Bases | Plate well location |
|---|---|---|---|
| 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

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<br>San Diego CA 92121 USA |
| **Website** | www.qiagen.com<br><br>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)<br>+1.858.285.4101 tel<br>+44 (0) 208 054 8706 (United Kingdom) |

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

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Verogen is a dedicated developer of human identification products for sequencing and analysis of forensic genomic samples. Working closely with the forensics community, Verogen places exceptional value on flexible, scalable solutions that deliver results when you need them most.

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