September 2024

# ForenSeq® Imagen Kit Handbook for PrepStation

For Imagen Library Preparation using the PrepStation automation solution

Sample to Insight

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# Kit Contents

| ForenSeq Imagen Kit<br>Catalog number<br>No. of reactions | | | V16000189<br>96 | |
|---|---|---|---|---|
| **Reagent** | **Description** | **Cap** | **Quantity** | **Storage temperature (°C)** |
| **Pre-PCR Box 1** | | | | |
| FEM | ForenSeq Enzyme Mix | Yellow | 8 | –25 to –15 |
| PCR1 | PCR1 Reaction Mix | Green | 9 | –25 to –15 |
| **Post PCR Box 2** | | | | |
| HP3 | 2 N NaOH | Orange | 1 | –25 to –15 |
| HSC | Human Sequencing Control | Pink | 1 | –25 to –15 |
| LNA1 | Library Normalization Additives 1 | Clear | 1 | –25 to –15 |
| LNS2 | Library Normalization Storage Buffer 2 | Clear | 1 | Room temperature* |
| LNW1 | Library Normalization Wash 1 | Clear | 2 | 2–8* |
| PCR2 | PCR2 Reaction Mix | Purple | 2 | –25 to –15 |
| UDI Plate | Unique Dual Index | Clear | 1 | –25 to –15 |
| **Post PCR Box 3** | | | | |
| LNB1 | Library Normalization Beads 1 | White | 1 | 2–8 |
| RSB | Resuspension Buffer | Green | 1 | 2–8 |
| SPB2 | Sample Purification Beads 2 | Red | 1 | 2–8 |
| **Pre-PCR Box 4** | | | | |
| NA24385 | NA24385 Positive Amplification Control DNA | Black | 1 | 2–8 |
| DPME | DNA Primer Mix E | Gray | 2 | 2–8 |
| DPMF (Geo) | DNA Primer Mix F | Orange | 2 | 2–8 |

* Shipped at –25°C to –15°C

# Shipping and Storage

The ForenSeq Imagen Kit is shipped in 4 boxes. When you receive the kit, promptly store reagents at the indicated temperatures.

- Box 1 is shipped at –25°C to –15°C. Upon receipt, all components in Box 1 should be stored immediately at –25°C to –15°C in a constant-temperature freezer.
- Box 2 is shipped at –25°C to –15°C. Upon receipt, all components in Box 2 should be stored immediately at –25°C to –15°C in a constant-temperature freezer.
- Box 3 is shipped at 2–8°C. Upon receipt, all components in Box 3 should be stored immediately at 2–8°C in a constant-temperature refrigerator.
- Box 4 is shipped at 2–8°C. Upon receipt, all components in Box 4 should be stored immediately at 2–8°C in a constant-temperature refrigerator.

When a reagent has a different storage temperature than most other reagents in the box, you can initially store the reagent at the same temperature as the other reagents (see Post-PCR Box 2 in "Kit Contents", previous section). After first use, store the reagent at the indicated temperature.

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

# Intended Use

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

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

# Safety Information

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

# Quality Control

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

# Introduction

The ForenSeq® Imagen Kit prepares up to 96 paired-end, 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 Imagen Kit can be used with the standard PCR1 buffer that is available with the kit or the ForenSeq Enhanced PCR1 Buffer System (ePCR1) that is available as an add-on kit. Additionally, the kit may be used with the PrepStation for automated library preparation, or with a manual library preparation protocol.

This handbook summarizes the protocol for using the ForenSeq Imagen Kit with the standard PCR1 buffer and the PrepStation.

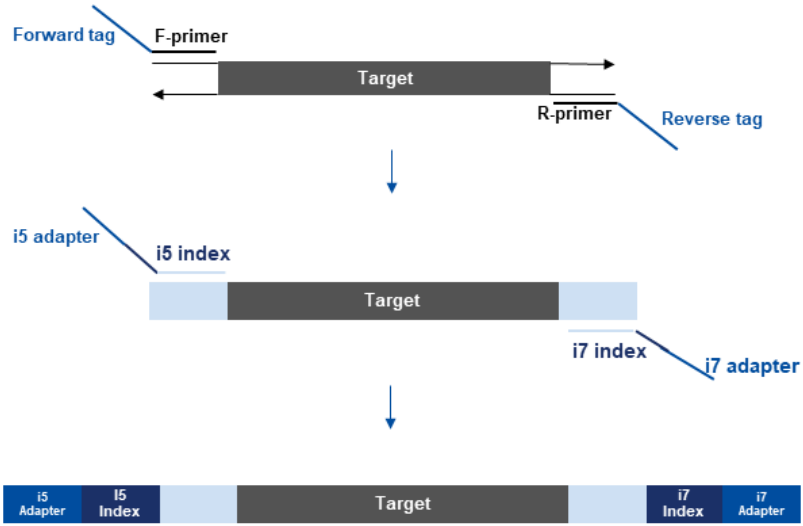


**Figure 1. Assay overview.**

## Kit features

The ForenSeq Imagen Kit offers the following features:

- Two concentrated primer mixes, either of which can be used for generation of DNA libraries based on user preferences:
  - DPME – Amplifies SNP targets for the prediction of skin, hair, and eye color, also referred to as external visible characteristics (EVC).
  - DPMF (Geo) – Amplifies SNP targets for the prediction of EVCs in DPME and bio-geographical ancestry (BGA) markers.
- Simultaneous preparation of up to 48 libraries using the PrepStation. Each library is a collection of amplified DNA fragments from one sample.
- Amplification of 55 (DPME primer mix) or 107 (DPMF primer mix) SNPs in one reaction and sequencing of up to 96 libraries in one sequencing run.

## Opentrons PrepStation

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

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

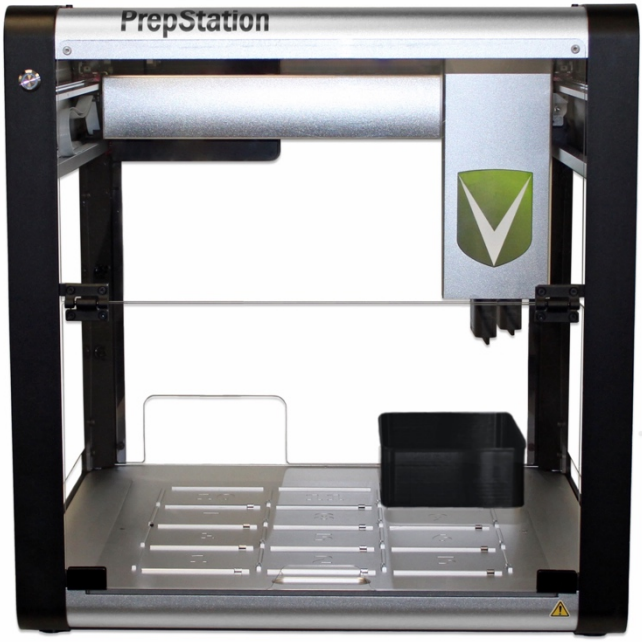


**Figure 2. PrepStation instrument.**

## Description of protocols

The following table lists the steps to prepare libraries on the PrepStation including hands-on times, runtimes, and reagents. Safe stopping points are marked between steps.

**Note**: Runtimes listed below assume a protocol consisting of 48 samples (the maximum number that can be prepared at one time). See "Number of samples" on page 15 for more information.

| Step | Hands-on time | Total time | Reagents |
|---|---|---|---|
| Amplify and tag targets* | 15 min | 3 h 35 min | NA24385, DPME or DPMF, FEM, PCR1 |
| Enrich targets† | 10 min | 90 min | UDI Adapters, PCR2 |
| Purify libraries† | 15 min | 30 min | 80% EtOH, RSP, SPB2 |
| Safe stopping point | – | – | – |
| Normalize libraries† | 30 min | 1 h 20 min | HP3, LNA1, LNB1, LNS2, LNW1 |
| Safe stopping point | – | – | – |
| Pool libraries† | 10 min | 10 min | – |
| Safe stopping point | – | – | – |
| Denature and dilute libraries† | 10 min | 10 min | HP3, HSC, HT1 |

* Pre-PCR

† Post-PCR

# Acronyms

| Abbreviations | Definition |
|---|---|
| DPME | DNA Primer Mix E |
| DPMF | DNA Primer Mix F |
| 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 |
| PLP | Purified Library Plate |
| PNL | Pooled Normalized Libraries |
| RSB | Resuspension Buffer |
| SPB2 | Sample Purification Beads 2 |
| UDI | Unique Dual Index |

# Equipment and Reagents to Be Supplied by User

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

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

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

## Equipment

| Equipment | Supplier | Pre-PCR | Post-PCR |
|---|---|---|---|
| 20 µL pipettes | General lab supplier | X | X |
| 200 µL pipettes | General lab supplier | X | X |
| 1000 µL pipettes | General lab supplier | X | X |
| 20 µL multichannel pipettes (8-channel) | General lab supplier | X | X |
| 200 µL multichannel pipettes (8-channel) | General lab supplier | | X |
| Benchtop microcentrifuge | General lab supplier | X | X |
| Computer with PrepStation and Opentrons applications installed | Installation and setup supported by Opentrons | | X |
| Heating system, 96-well, 1.5 mL | General lab supplier | | X |
| Magnetic module for PrepStation instrument | Opentrons (SKU: 999-00098) | | X |
| Metal PCR plate adapters | Opentrons (SKU: 999-00028) | | X |

| Equipment | Supplier | Pre-PCR | Post-PCR |
|---|---|---|---|
| Microplate centrifuge | General lab supplier | X | X |
| P20 8-channel pipette arm (GEN2)* | Opentrons (SKU: 999-00005) | | X |
| P300 8-channel pipette arm (GEN2)† | Opentrons (SKU: 999-00006) | | X |
| PrepStation instrument | Opentrons (SKU: 991-00210) | | X |
| Rubber roller | General lab supplier | X | X |
| Thermal cycler, 96-well with heated lid | See "Thermal cyclers" (below) | | X |
| Vortexer | General lab supplier | X | X |
| [Optional] 10 µL pipettes | General lab supplier | X | X |

* Installed on the left side of the PrepStation instrument.

† Installed on the right side of the PrepStation instrument.

## Thermal cyclers

The following table lists supported thermal cyclers with recommended settings. If your laboratory has an unlisted thermal cycler, evaluate the thermal cycler before performing the protocol.

| Thermal cycler | Temperature mode | Lid temperature |
|---|---|---|
| ABI LTI thermal cycler 9700* | 9600 emulation | Heated |
| Bio-Rad | Calculated | Heated, constant at 100°C |
| Eppendorf Mastercycler Pro S | Gradient S, Simulated Tube | Heated |
| QIAmplifier 96-well thermal cycler | Standard | Heated, constant at 100°C |
| Proflex 96-well PCR System | Not applicable | Heated, constant at 105°C |
| Veriti 96-well thermal cycler | Standard | Heated, constant at 105°C |

* Only gold heat blocks are supported

# Consumables

| Consumable | Supplier |
|---|---|
| 1.5 mL LoBind microcentrifuge tubes | VWR, cat. no. 80077-230 |
| 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 | QIAGEN, 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 (for PrepStation waste) | General lab supplier |

# Important Notes

## DNA input recommendations

- Use 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 compatible with isolated, purified genomic DNA.
- It is not recommended to use FTA card punches with the use of the PrepStation, due to the possibility of the punch preventing PrepStation to efficiently pipette during the Purify Libraries step.

### Controls

- Each preparation must include at least one positive amplification control and at least one negative amplification control. If these controls are not included, troubleshooting support is limited.
- The kit includes NA24385 Positive Amplification Control DNA (NA24385) for use as the positive template control and the negative amplification control is nuclease-free water. The protocol includes instructions to prepare each control.

## Number of samples

- The PrepStation can prepare libraries in multiples of 8 samples at a time, up to a maximum of 48 samples. This includes any 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.

- If planning to pool and sequence 96 samples simultaneously with the use of the PrepStation, it is recommended to use UDI columns 1–6 for the first 48 samples, and use UDI columns 7–12 for the second 48 samples. This involves choosing 1 for the UDI Start Position on the PrepStation application for the first 48 samples, and choosing 7 for the UDI Start Position for the second 48 samples.
- Reference the following table to determine the maximum number of libraries to pool for a run.

| Reagent kit | Maximum number of libraries |
|---|---|
| MiSeq FGx Reagent Micro Kit | 96 |

# Tips and techniques

## Protocol continuity

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

## Plate setup

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

**Note**: The *Universal Analysis Software User Guide for ForenSeq Imagen Kit Module* (Verogen document # VD2022014) should be referenced.

## Preventing cross-contamination

- Set up the Amplify and Tag Targets process in a pre-PCR environment. Perform all other processes in a post-PCR environment.
- When adding or transferring samples, change tips between **each sample**.
- When adding adapters or primers, change tips between **each well**.
- When processing smaller sample batches of fewer than 96 libraries at a time, prevent aerosolization by resealing the utilized, pierced UDI wells with Microseal 'B' adhesive seal. Trim excess seal with 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.
- 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 before use for at least 1 min, and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogeneous in color.
- Aspirate and dispense beads slowly due to viscosity.

- Do not centrifuge plates and tubes containing beads, except when indicated.
- Do not let beads sit in reservoirs, and do not reuse reservoirs for beads.
- If beads aspirate into pipette tips during supernatant removal, dispense back to the plate on the magnetic stand and wait until the liquid is clear (around 2 min).

## Index adapter sequences

The following table lists the 8 bp sequences for the Unique Dual Index (UDI) adapters included in the ForenSeq Imagen 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 |

| Index name | Index 1 bases | Index 2 bases | Plate well location |
|---|---|---|---|
| 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 |

| Index name | Index 1 bases | Index 2 bases | Plate well location |
|---|---|---|---|
| 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 |
| 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 |

| Index name | Index 1 bases | Index 2 bases | Plate well location |
|---|---|---|---|
| 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 |
| UDI0079 | ACAGGCGC | CTCTGCCT | G7 |

| Index name | Index 1 bases | Index 2 bases | Plate well location |
|---|---|---|---|
| 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 |

# Protocol

This chapter describes the ForenSeq Imagen protocol with step-by-step instructions to prepare libraries for sequencing using the standard PCR1 buffer on the Opentrons PrepStation. For an overview of the protocol with reagents and durations for each step, see “Description of protocols” on page 9.

Before starting, confirm kit contents and make sure that you have the necessary reagents, consumables, and equipment. For a list of items, see "Equipment and Reagents to Be Supplied by User" on page 12.

## Cleaning the PrepStation

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

### Cleaning the PrepStation between protocols

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

### Preventative maintenance of PrepStation

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

## Protocol step 1: Amplify and tag targets

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

### Consumables

- FEM (ForenSeq Enzyme Mix)
- DPME (DNA Primer Mix E) or DPMF (Geo) (DNA Primer Mix F)
- PCR1 (PCR1 Reaction Mix)
- NA24385 (NA24385 Positive Amplification Control DNA)
- Input gDNA
- Nuclease-free water
- 96-well PCR plate, semi-skirted
- 1.5 mL LoBind microcentrifuge tubes (2)
- Microseal 'A' film
- **Optional**: RNase/DNase-free 8-tube strip and caps

### About reagents

- Use PCR1 for standard samples.
- Do not vortex NA24385, FEM, or input gDNA.
- Prepare fresh dilutions of NA24385 every time.
- For information on DPME and DPMF (geo), see Loci Detected with DPME and DPMF (Geo).

## Preparation

1. Prepare the following consumables:

| Item | Storage temperature (°C) | Instructions |
|---|---|---|
| NA24385 | 2–8 | Let stand for 30 min to bring to room temperature. Invert 3 times to mix, and then centrifuge briefly. |
| PCR1 | –25 to –15 | Thaw at room temperature. Vortex to mix, and then centrifuge briefly. |
| FEM | –25 to –15 | Thaw at room temperature, and then centrifuge briefly. Return to storage immediately after use. |
| DPME or DPMF (Geo) | 2–8 | Let stand for 30 min to bring to room temperature. Vortex to mix, and then centrifuge. |

2. Save the following Imagen PCR1 program on the thermal cycler in the post-amplification area. See Table 1 for lid temperatures and ramp modes.
    - Choose the preheat lid option and set to applicable temperature.
    - 98°C for 3 min
    - 8 cycles of:
        - 96°C for 45 s
        - 80°C for 30 s
        - 54°C for 2 min with applicable ramp mode
        - 68°C for 90 s with applicable ramp mode
    - 10 cycles of:
        - 96°C for 30 s
        - 68°C for 3 min, with applicable ramp mode

- 68°C for 10 min
- Hold at 10°C

**Table 1. 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/s | 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 100°C | 0.2°C/s | Polypropylene plates and tubes |
| QIAamplifier 96-Well Thermal Cycler | Standard | Heated, constant at 100°C | 0.1°C/s | Polypropylene plates |
| Veriti 96-Well Thermal Cycler | Standard | Heated, constant at 105°C | 4% | Polypropylene plates and tubes |

The Imagen PCR1 program takes approximately 3.5 h 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 according to 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. Gently pipette to mix.
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)
   - DPME or DPMF (2 µL)
   - FEM (0.3 µL)

   As an example, for 8 samples, prepare 61.6 µL Master Mix with 41.4 µL PCR1, 2.6 µL FEM, and 17.6 µL DPME or DPMF.
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 sample well of the ForenSeq Sample Plate (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 3 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 for 30 s at 100 × *g*.
14. Place on the preprogrammed thermal cycler and run the PCR1 program.
15. Unless you are stopping, proceed to "Protocol step 2: Enrich targets (Post 1 – Enrichment)".

**Safe stopping point**

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

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

This process amplifies the DNA and adds 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 0.8 mL reagent midi plates
- Trash bags

### About reagents

- Pipette PCR2 buffer 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 prior to putting the plate onto the PrepStation dock.

## Preparation

1. Prepare the following consumables:

| Item | Storage temperature (°C) | Instructions |
|---|---|---|
| UDI plate | –25 to –15 | Thaw at room temperature, place on a plate shaker to mix, and then centrifuge for 30 s at 100 × *g*. |
| PCR2 | –25 to –15 | Thaw at room temperature for 20 min, and then invert to mix. |

2. Save the following Imagen PCR2 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C.
   - 98°C for 30 s
   - 15 cycles of:
     - 98°C for 20 s
     - 66°C for 120 s
   - 68°C for 10 min
   - Hold at 10°C

   Total program time is approximately 46 min. (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. Centrifuge for 30 s at 100 × *g*.
5. Label a new midi plate as "Reagent Midi Plate". This plate will be used by the PrepStation.
6. Prepare the Reagent Midi Plate by aliquoting reagents into wells as specified in Table 2 and Table 3. (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. (See Table 3.)
- 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 | No. of reactions | Volume per well (µL) |
|---|---|---|---|
| PCR2 Reaction Mix | Column 12 | 8 | 35 |
| | | 16 | 70 |
| | | 24 | 105 |
| | | 32 | 140 |
| | | 40 | 175 |
| | | 48 | 210 |

## Procedure

1. Centrifuge the sealed FSP for 30 s at 100 × *g*.
2. Create a Verogen protocol set using the PrepStation application:
   a. Launch the PrepStation application.
   b. Select **Add Protocol Set**.
   c. Choose the appropriate Assay, either **ForenSeq Imagen** (for use with primer mix DPME) or **Forensic Imagen Geo** (for use with primer mix DPMF). Then select the Sample Count and UDI Start Position (listed as column numbers).
   d. Select **Add Protocol Set** after choosing the Assay, Sample Count, and UDI Start Position.
   e. After a few seconds, navigate back to the Protocol Sets screen by selecting the green 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.

    i. It may be necessary to select **Reanalyze protocol** in the top right corner if there appears to be a "Protocol analysis failure".

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 dropdown 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 3).

    **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 seal remains on the UDI plate. (The instrument will pierce the foil.)
- Ensure that lids have been removed from the tip racks.

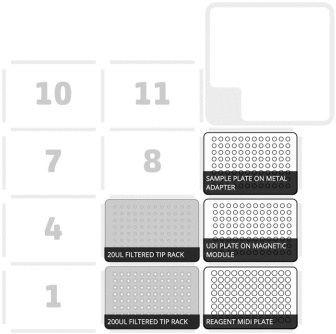


**Figure 3. Deck map for Post 1 - Enrichment protocol.**

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 for 30 s at 100 × *g*.
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.

**Table 4. UDI plate layout**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | UDI0001 | UDI0002 | UDI0003 | UDI0004 | UDI0005 | UDI0006 | UDI0007 | UDI0008 | UDI0009 | UDI0010 | UDI0011 | UDI0012 |
| B | UDI0013 | UDI0014 | UDI0015 | UDI0016 | UDI0017 | UDI0018 | UDI0019 | UDI0020 | UDI0021 | UDI0022 | UDI0023 | UDI0024 |
| C | UDI0025 | UDI0026 | UDI0027 | UDI0028 | UDI0029 | UDI0030 | UDI0031 | UDI0032 | UDI0033 | UDI0034 | UDI0035 | UDI0036 |
| D | UDI0037 | UDI0038 | UDI0039 | UDI0040 | UDI0041 | UDI0042 | UDI0043 | UDI0044 | UDI0045 | UDI0046 | UDI0047 | UDI0048 |
| E | UDI0049 | UDI0050 | UDI0051 | UDI0052 | UDI0053 | UDI0054 | UDI0055 | UDI0056 | UDI0057 | UDI0058 | UDI0059 | UDI0060 |
| F | UDI0061 | UDI0062 | UDI0063 | UDI0064 | UDI0065 | UDI0066 | UDI0067 | UDI0068 | UDI0069 | UDI0070 | UDI0071 | UDI0072 |
| G | UDI0073 | UDI0074 | UDI0075 | UDI0076 | UDI0077 | UDI0078 | UDI0079 | UDI0080 | UDI0081 | UDI0082 | UDI0083 | UDI0084 |
| H | UDI0085 | UDI0086 | UDI0087 | UDI0088 | UDI0089 | UDI0090 | UDI0091 | UDI0092 | UDI0093 | UDI0094 | UDI0095 | UDI0096 |

### Safe stopping point

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

**Note**: When processing fewer than 96 libraries, 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.

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

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

### Consumables

- RSB (Resuspension Buffer)
- SPB2 (Sample Purification Beads 2)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plate
- 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 0.8 mL reagent midi plates
- Trash bags

## Preparation

1. Prepare the following consumables:

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

2. Label plates as follows.

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

3. Prepare the reagent midi plate by aliquoting additional reagents into wells as specified in Table 5 and Table 6. (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. (See Table 6.)
   - 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 5. 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 6. Reagent volumes, Post 2 Purification**

| Reagent | Midi plate column | No. of reactions | Volume per well (µL) |
|---|---|---|---|
| RSB | Column 5 | 8 | 70 |
| | | 16 | 140 |
| | | 24 | 210 |
| | | 32 | 280 |
| | | 40 | 350 |
| | | 48 | 420 |
| SPB2 | Column 6 | 8 | 54 |
| | | 16 | 108 |
| | | 24 | 162 |
| | | 32 | 216 |
| | | 40 | 270 |
| | | 48 | 325 |
| 80% EtOH* | Columns 8, 10 | 8 | 300 |
| | | 16 | 500 |
| | | 24 | 650 |
| | Columns (8, 10) and (9, 11) | 32 | 650 and 300 |
| | | 40 | 650 and 500 |
| | | 48 | 650 and 650 |

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

## Procedure

1. Centrifuge the sealed FSP for 30 s at 100 × *g*.
2. Run the Post 2 – Purification protocol on the PrepStation instrument:
    a. Launch the Opentrons application.
    b. Select **Protocols** from the left-hand menu.
    c. Select the appropriate protocol to purify libraries. (The name of the protocol will include "Post 2 Purification".) A screen with details about the protocol appears.
    d. Select **Run Protocol**. A sidebar listing available PrepStation instruments appears.
    e. Select the instrument you want to use, then select **Proceed to setup**. A screen with further setup instructions appears.
    f. Select the **+** button next to Module Setup. A drop-down window displaying the deck map for this protocol opens.
    g. Place the magnetic module as shown in the deck map. Ensure the module is plugged in and connected to the PrepStation USB port.

        **Note**: When the module is on and connected, a green checkmark with "Connected" will appear in the deck map.
    h. Select the **+** button next to Labware Setup or select **Proceed to labware setup**. A drop-down window displaying the deck map for this protocol opens.

        **Note**: If a window appears prompting you to apply stored labware offset data, select **Apply stored data**.
    i. If the trash bin is not already empty, empty it and replace the bag.
    j. Load reagents and other labware onto the deck as shown in the deck map (also shown in Figure 5).

**Note**: Carefully observe the following while loading labware:

- Load labware from back to front. (Load deck slot 10, then 7, then 4, then 1.)
- Ensure that labware are oriented such that labels are facing you.
- Ensure that all labware are seated securely inside their deck slots, and not resting on any dividers between deck slots.
- Place the PBP plate on top of the magnetic module (be careful to avoid placing 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.

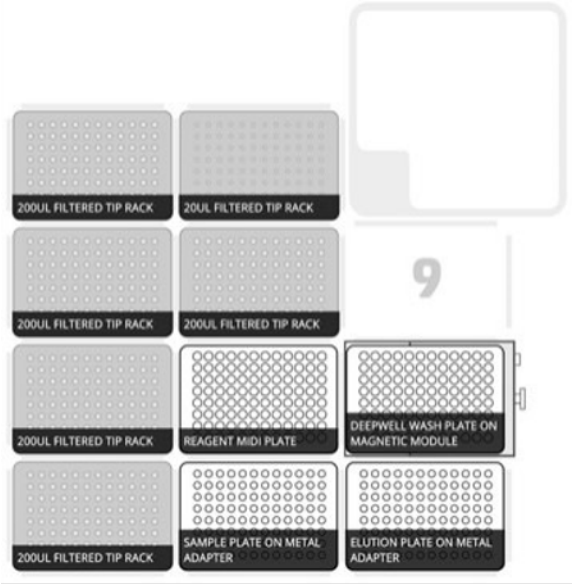


**Figure 4. Deck map for Post 2 – Purification protocol.**

**Note**: The deck map in the application may show more tip racks than you may need, depending on the number of reactions you are processing. See Table 7 to identify which tip racks are necessary.

**Table 7. Tip racks required for Post 2 – Purification**

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

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

**Important**: Carefully observe the following while 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, QIAGEN recommends that you still calibrate the offsets as described above.)

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

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

3. Once the run has completed, seal the PLP using Microseal 'B'. Centrifuge the sealed plate for 30 s at 100 × *g*.

4. Clean the PrepStation instrument:

   a. Remove all labware from the deck.

   b. Dispose of consumed labware.

   c. Empty the trash bin and replace the bag.

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

### Safe stopping point

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

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

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

### Consumables

- HP3 (2 N NaOH)
- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNS2 (Library Normalization Storage Buffer 2)
- LNW1 (Library Normalization Wash 1)
- Nuclease-free water
- Two each of either of the following tubes:
    - 1.5 mL microcentrifuge tube
    - 15 mL conical tube
- 96-well midi plate
- 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 0.8 mL reagent midi plates
- Trash bags

## About reagents

The volumes combined in the LNA1/LNB1 Master Mix tube and the 0.1 N HP3 tube include overage, so calculating additional overage is not necessary.

**Important**: This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

## Preparation

1. Prepare the following consumables:

| Item | Storage temperature (°C) | Instructions |
|---|---|---|
| HP3 | –25 to –15 | Thaw at room temperature for ≥30 min. Vortex to mix, and then centrifuge briefly. |
| LNA1 | –25 to –15 | Thaw at room temperature for ≥30 min. Vortex with intermittent inversion |
| LNB1 | 2–8 | Let stand for 30 min to equilibrate to room temperature. Vortex for at least 1 min, inverting 5 times every 15 s. Pipette to mix until the bead pellet at the bottom is resuspended. |
| LNW1 | 2–8 | Let stand for 30 min to equilibrate to room temperature. |
| LNS2 | 15–30 | 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 for all waste generated in the normalization step.
4. In the LNA1/LNB1 Master Mix tube, combine volumes of reagents as specified in Table 8 below. Do not add overage.

**Table 8. LNA1/LNB1 Master Mix**

| | Volume to add (µL) | |
|---|---|---|
| No. of reactions | LNA1 | LNB1 |
| 8 | 379 | 69 |
| 16 | 758 | 138 |
| 24 | 1138 | 208 |
| 32 | 1517 | 275 |
| 40 | 1896 | 344 |
| 48 | 2275 | 413 |

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 9 on the next page. Do not add overage.

**Table 9. LNA1/LNB1 Master Mix**

| | Volume to add (µL) | |
|---|---|---|
| **No. of reactions** | **Nuclease-free water** | **HP3** |
| 8 | 381 | 21 |
| 16 | 762 | 42 |
| 24 | 1142 | 62 |
| 32 | 1523 | 83 |
| 40 | 1904 | 104 |
| 48 | 2285 | 125 |

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 Figure 5 and Table 10. (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.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| B | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| C | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| D | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| E | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| F | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| G | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |
| H | LNS2 | 0.1 N HP3 | LNW1 | LNA1/LN B1 | RSB | SPB2 | – | EtOH | EtOH | EtOH | EtOH | PCR2 Reaction Mix |

**Figure 5. Reagent midi plate layout, Post 3 – Normalization.**

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

| Reagent | Midi plate column | No. of reactions | Volume per well (µL) |
|---|---|---|---|
| LNS2 | Column 1 | 8 | 36 |
| | | 16 | 72 |
| | | 24 | 108 |
| | | 32 | 144 |
| | | 40 | 180 |
| | | 48 | 216 |

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

| Reagent | Midi plate column | No. of reactions | Volume per well (µL) |
|---|---|---|---|
| 0.1 N HP3 | Column 2 | 8 | 40 |
| | | 16 | 80 |
| | | 24 | 120 |
| | | 32 | 160 |
| | | 40 | 200 |
| | | 48 | 240 |
| LNW1 | Column 3 | 8 | 100 |
| | | 16 | 200 |
| | | 24 | 300 |
| | | 32 | 400 |
| | | 40 | 500 |
| | | 48 | 600 |
| LNA1/LNB1 Master Mix | Column 4 | 8 | 56 |
| | | 16 | 112 |
| | | 24 | 168 |
| | | 32 | 224 |
| | | 40 | 280 |
| | | 48 | 336 |

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

        **Note**: Carefully observe the following while loading labware:

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

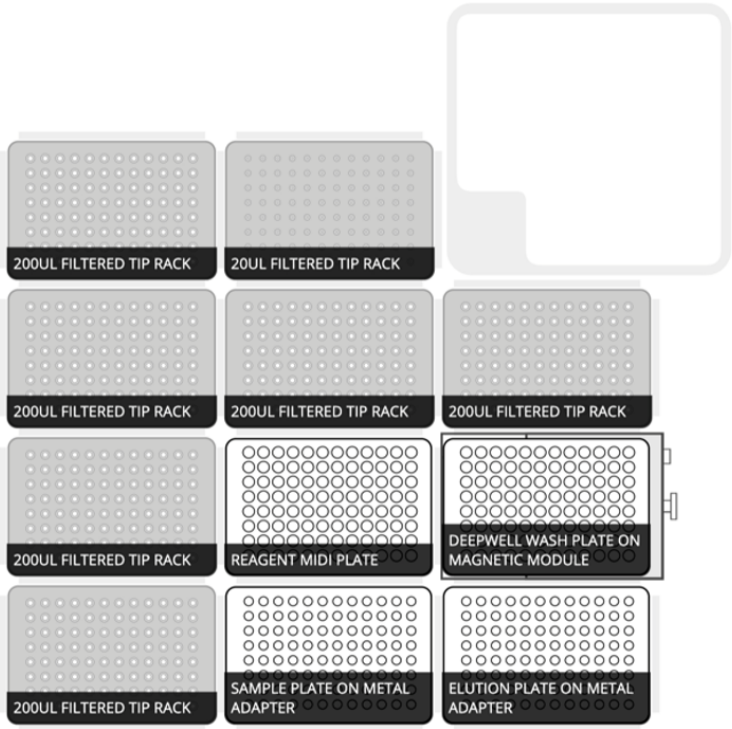


**Figure 6. Deck map for Post 3 – Normalization protocol.**

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

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

| No. of reactions | Deck slots requiring 200 µL filtered tip racks |
|---|---|
| 8 | 1 |
| 16 | 1, 4 |
| 24 | 1, 4, 7 |
| 32 | 1, 4, 7, 10 |
| 40 | 1, 4, 7, 10, 8 |
| 48 | 1, 4, 7, 10, 8, 9 |

j. 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; if you do, QIAGEN recommends that you still calibrate the offsets as described above.

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

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

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

2. Once the run has completed, seal the NLP. Centrifuge the sealed plate for 30 s at 100 × *g*.

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.

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

This process combines equal volumes of each normalized library to create a pool of libraries that are sequenced together on the same flow cell.

### Consumables

- 1.5 mL LoBind microcentrifuge tube
- RNase/DNase-free 8-tube strip and caps
- Microseal 'B' film
- Opentrons 200 µL filter tips
- Opentrons 20 µL

### Preparation

1. Select libraries to pool for sequencing. For recommendations, see "Number of samples" on page 15.
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 for 30 s at 100 × *g*.
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. Load labware onto the deck as shown in the deck map (also shown in Figure 7).

   **Note**: Carefully observe the following while loading labware:

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

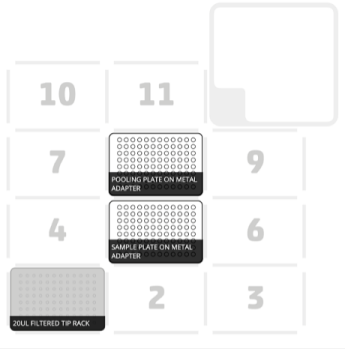


**Figure 7. Deck map for Post 4 – Pooling protocol.**

h. 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; if you do, QIAGEN recommends that you still calibrate the offsets as described above.

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

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

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

3. Once the run has completed, seal the NLP. Centrifuge the sealed plate for 30 s at 100 × *g*.
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 Verogen Protocol Set from Opentrons

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

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

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

## Protocol step 6: Denature and dilute libraries

This process 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 microcentrifuge tubes (2)

## Preparation

1. Prepare the following consumables:

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

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

## Procedure

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

   **Important**: 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 min.

4. Add 600 µL HT1 to the DNL tube.
5. Place the PNL tube in the preheated microheating system and incubate for 2 min.
6. Immediately transfer an appropriate volume of libraries from the PNL tube to the DNL tube. Refer to Table 12.

**Table 12. Library volumes**

| No. of libraries | PrepStation library volume (µL) | Manual library volume (µL) | Total library volume (µL) |
|---|---|---|---|
| 96 PrepStation, 0 manual | 12 | n/a | 12 |
| 48 PrepStation, 48 manual | 5 | 4 | 9 |
| 0 PrepStation, 96 manual* | n/a | 8 | 8 |

* This row is 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).

# Technical Support

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

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| ForenSeq Imagen Kit (96) | Prepares up to 96 paired-end, dual-indexed libraries for sequencing and analysis of phenotypic and biogeographical ancestry information. | V16000189 |
| | **Related product** | |
| MiSeq FGx Micro Reagent Kit | Supports up to 5 million paired-end reads for deep sequencing or high-throughput sample processing | 15066817 |

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

# Document Revision History

| Date | Changes |
|---|---|
| 09/2024 | Initial release |

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