ForenSeq MainstAY Kit

Reference Guide

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Revision History

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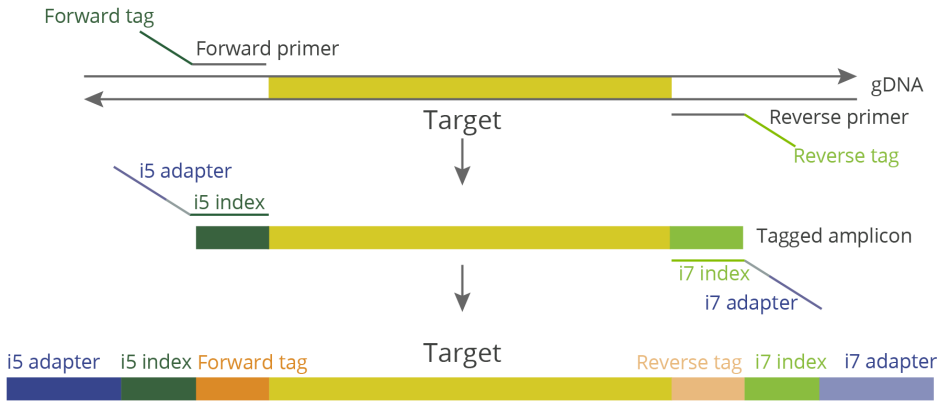
Introduction

The ForenSeq® MainstAY Kit generates 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 sequences. The tags are then used to attach index adapters and the resulting library is amplified, purified, and pooled for sequencing.

Figure 1 Assay overview



Kit Features

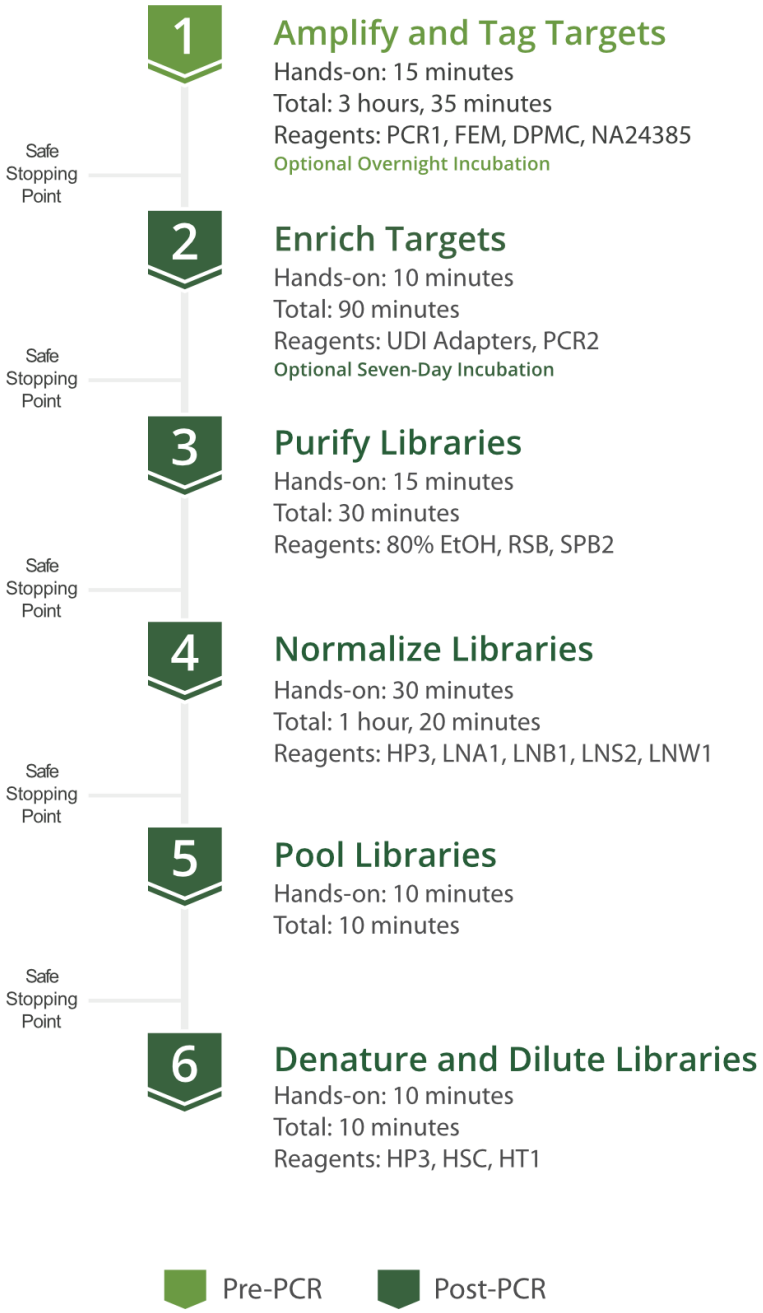
The ForenSeq MainstAY Kit offers the following features:

- A concentrated primer mix that allows for increased input volume.
- Simultaneous preparation of up to 96 libraries in one plate. Each library is a collection of amplified DNA fragments from one sample.
- Amplification of autosomal short tandem repeat (aSTR) and Y-STR markers in one reaction and sequencing of up to 96 libraries in one run.

Protocol Steps

The following diagram lists the steps to prepare libraries with hands-on times, total times, and reagents. Safe stopping points are marked between steps.

Figure 2 Overview of the ForenSeq MainstAY protocol



DNA Input Recommendations

Use 1 ng 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 crude lysate from buccal swabs and FTA card stains:

- For crude lysate, use 2 µl input material per sample. See *Consumables* on page 25 for recommended lysis buffers.
- For FTA paper, use a 1.2 mm FTA punch card for each sample.

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.

Additional Resources

This guide provides comprehensive information on the ForenSeq MainstAY Kit with detailed protocol instructions. Visit the Documentation page on the Verogen website to download additional kit documentation and access the latest versions.

Resource	Description
ForenSeq MainstAY Kit Checklist (document # VD2021016)	Provides concise protocol instructions for the experienced user.
ForenSeq MainstAY Kit Materials List (document # VD2021017)	Lists the consumables and equipment needed to perform the protocol.

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Introduction

This chapter describes the ForenSeq MainstAY protocol with step-by-step instructions to prepare libraries for sequencing. For an overview of the protocol with reagents and durations for each step, see *Protocol Steps* on page 6.

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 23.

Number of Samples

Process at least eight samples at a time, including positive and negative amplification controls. Preparing master mixes for fewer than eight samples can introduce pipetting inaccuracies due to small volumes.

Reference the following table to determine the maximum number of libraries to pool for a run, depending on MiSeq FGx® reagent kit.

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.

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* 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.
- Aspirate and dispense beads slowly due to viscosity.
- Do not centrifuge plates and tubes containing beads, except when indicated.
- Vortex beads before use and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- 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 (~2 minutes).

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)
- FEM (Enzyme Mix)
- PCR1 (PCR1 Reaction Mix)
- 1.7 ml microcentrifuge tubes (2)

- 96-well PCR plate, semiskirted
- Input gDNA
- Microseal 'A' film
- Nuclease-free water
- [FTA card] 1X TBE Buffer (100 µl per FTA card punch)
- [FTA card] Microseal 'B' film
- [Optional] RNase/DNase-free 8-tube strip and caps

About Reagents

- Do not vortex NA24385, FEM, or input gDNA.
- For information on DPMC, see *Loci Detected with DPMC* on page 31.

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.
1X TBE Buffer*	-25°C to -15°C	Thaw at room temperature. Invert three times to mix, and then centrifuge briefly.
FEM	-25°C to -15°C	Remove from storage immediately before use, and then return to storage immediately after use.
DPMC	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.
PCR1	-25°C to -15°C	Thaw at room temperature for 30 minutes. Vortex to mix, and then centrifuge briefly.

* For FTA cards only

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 and set to 100°C
   - 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 Ramp modes

Thermal Cycler	Ramp Mode
ABI LTI thermal cycler 9700	8%
Bio-Rad	0.2°C per second
Eppendorf Mastercycler Pro S	2%
Proflex 96-well PCR System	0.2°C per second
Veriti 96-well thermal cycler	4%

The PCR1 program takes ~3.5 hours and can be run overnight.

3. Label a new PCR plate FSP for ForenSeq Sample Plate.
4. Label a new 1.7 ml tube per your input type:

Input Type	Label
Crude lysate	Master Mix
FTA card	FTA Master Mix
Purified DNA	Master Mix

Procedure

Purified DNA

1. Using nuclease-free water, dilute 1 ng purified DNA input to 0.125 ng/µl.
2. In the Master Mix tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.
   - PCR1 (4.7 µl)
   - FEM (0.3 µl)
   - DPMC (2 µl)

   For example, for eight samples prepare 61.6 µl master mix: 41.4 µl PCR1, 2.6 µl FEM, and 17.6 µl DPMC.
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 1.7 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. Add 8 µl nuclease-free water to at least one well of the FSP as a negative amplification control.
10. Add 8 µl 0.125 ng/µl DNA to each well of the FSP, and then pipette to mix.
11. Seal and centrifuge at 1000 × g for 30 seconds.
12. Place on the preprogrammed thermal cycler and run the PCR1 program.
13. Unless you are stopping, proceed to *Enrich Targets* on page 14.

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 (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, 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 1.7 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* on page 14.

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.

FTA Card

1. Place a 1.2 mm FTA card punch into each well of the FSP.
2. Add 100 µl 1X TBE Buffer.
3. Place on a PCR tube storage rack.
4. Seal with microseal 'B' film and shake at 1800 rpm for 2 minutes.
5. Centrifuge at 1000 × g for 30 seconds.
6. Remove and discard all supernatant.
7. Add the following volumes to each control well of the FSP:
   - PCR1 (4.7 µl)
   - FEM (0.3 µl)
   - DPMC (2 µl)
8. In a 1.7 ml tube, combine the following volumes to dilute NA24385:
   - NA24385 (2 µl)
   - Nuclease-free water (158 µl)
9. Cap and gently invert three times to mix, and then centrifuge briefly.
10. Add 8 µl diluted NA24385 to the positive amplification control wells.
11. Pipette to mix.
12. Add 8 µl nuclease-free water to the negative amplification control wells.
13. Pipette to mix.
14. In the FTA Master Mix tube, combine the following volumes. Multiple each volume by the number of samples and add 10% for overage.
    - PCR1 (4.7 µl)
    - FEM (0.3 µl)
    - DPMC (2 µl)
    - Nuclease-free water (8 µl)

    For example, for eight samples prepare 61.6 µl master mix: 41.4 µl PCR1, 2.6 µl FEM, and 17.6 µl DPMC.
15. Pipette to mix, and then seal and centrifuge briefly.
16. [Optional] Evenly distribute FTA Master Mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.
17. Add 15 µl FTA Master Mix to each well of the FSP that contains an FTA card punch.
18. Seal with microseal 'A' film, and then centrifuge at 1000 × g for 30 seconds.
19. Place on the preprogrammed thermal cycler and run the PCR1 program.
20. Unless you are stopping, proceed to *Enrich Targets* on page 14.

SAFE STOPPING POINT

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

Enrich Targets

This process amplifies the DNA and adds 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

About Reagents

- Dispense 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.
PCR2	-25°C to -15°C	Thaw at room temperature for 20 minutes, and then invert to mix.

2. Save the following PCR2m 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.
3. Remove the protective cover from the UDI plate and discard appropriately.

4. Centrifuge at 1000 × g for 30 seconds

Procedure

1. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
2. Using a new pipette tip for each well, pierce the foil covering the UDI plate and transfer 8 µl UDI adapter to each sample well in the FSP. For details on the UDI Adapter Plate Layout, Refer to Table 2.
3. Briefly centrifuge PCR2, and then pipette to mix.
4. [Optional] Evenly distribute PCR2 among each tube of an 8-tube strip. Use a multichannel pipette to dispense.
5. Add 27 µl PCR2 to each well of the FSP.
6. Pipette to mix.
7. Seal and centrifuge at 1000 × g for 30 seconds.
8. Place on the preprogrammed thermal cycler and run the PCR2m program.

Table 2 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°C to 8°C for up to 7 days. Alternatively, leave on the thermal cycler overnight.

NOTE: When processing less 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.

Purify Libraries

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

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

Procedure

1. Add 45 µl SPB2 to each well of the PBP.
2. Centrifuge the sealed FSP at 1000 × g for 30 seconds.
3. Transfer 45 µl reaction from each well of the FSP to the corresponding well of the PBP.
4. Discard the FSP plate.
5. Seal the PBP and shake at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait until the liquid is transparent (~5 minutes).
8. Remove and discard all supernatant.
9. Keep on the magnetic stand and wash as follows.
    a. Add 200 µl fresh 80% EtOH to each well.
    b. Incubate for 30 seconds.
    c. Remove and discard all supernatant.
10. Wash a **second** time.

11. With a 20 µl pipette, remove residual EtOH from each well.
12. Remove from the magnetic stand.
13. Add 52.5 µl RSB to each well.
14. Seal and shake at 1800 rpm for 2 minutes.
15. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 minutes.
16. Incubate at room temperature for 2 minutes.
17. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
18. Transfer 50 µl supernatant from each well of the PBP to the corresponding well of the PLP.
19. Seal and centrifuge at 1000 × g for 30 seconds.

SAFE STOPPING POINT

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

Normalize Libraries

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:
  - 1.7 ml microcentrifuge tube
  - 15 ml conical tube
- 96-well midi plate
- 96-well PCR plate, skirted or semiskirted
- PVC reagent reservoir
- Microseal 'B' film

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, see the safety data sheets (SDS) at verogen.com/product-documentation.**

Preparation

1. Prepare the following consumables:

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

2. Label vessels as follows.

Vessel	Label
1.7 ml tube or 15 ml conical tube	0.1 N HP3
1.7 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. Dedicate separate hazardous waste disposal containers for liquids and solids.

Procedure

1. In the LNA1/LNB1 Master Mix tube, combine the following volumes. Multiply each volume by the number of samples, but do not add overage.
   - LNA1 (46.8 µl)
   - LNB1 (8.5 µl)

   For example, for eight samples, combine 374.4 µl LNA1 and 68 µl LNB1.
2. Vortex, and then invert several times to mix.
3. Transfer the entire volume to a reagent reservoir.
4. Add 45 µl LNA1/LNB1 Master Mix to each sample well of the NWP.
5. To clear any beads that might have aspirated, place the PLP on the magnetic stand and wait until the liquid is clear (~2 minutes).

6. Transfer 20 µl supernatant from each well of the PLP to the corresponding well of the NWP.
7. Seal the NWP and shake at 1800 rpm for 30 minutes.
8. While the plate is shaking, perform steps 9–11 to save time later in the process.
9. In the 0.1 N HP3 tube, combine the following volumes. Multiply each volume by the number of samples, but do not add overage.
   - Nuclease-free water (33.3 µl)
   - HP3 (1.8 µl)

   For example, eight samples require 266.4 µl nuclease-free water and 14.4 µl HP3.
10. Invert several times to mix, and then set aside.
11. Add 30 µl LNS2 to each sample well of the NLP.
12. Immediately after shaking, place the NWP on the magnetic stand and wait until the liquid is clear (~2 minutes).
13. Remove and discard all supernatant.
14. Remove from the magnetic stand.
15. Wash as follows.
    a. Add 45 µl LNW1 to each well.
    b. Seal and shake at 1800 rpm for 5 minutes.
    c. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
    d. Remove and discard all supernatant.
    e. Remove from the magnetic stand.
16. Wash a **second** time.
17. Seal and centrifuge at 1000 × g for 30 seconds.
18. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
19. With a 20 µl pipette, remove residual LNW1 from each well.
20. Remove from the magnetic stand.
21. Add 32 µl freshly prepared 0.1 N HP3 to each well.
22. Seal and shake at 1800 rpm for 5 minutes.
23. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 5 minutes.
24. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
25. Transfer 30 µl supernatant from the NWP to the corresponding well of the NLP.
26. Pipette to mix.
27. Seal and centrifuge at 1000 × g for 30 seconds.

SAFE STOPPING POINT

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

Pool Libraries

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

- 1.7 ml microcentrifuge tube
- RNase/DNase-free 8-tube strip and caps
- Microseal 'B' film

Preparation

1. Select libraries to pool for sequencing.
   For recommendations, see *Number of Samples* on page 8.
2. Label the 1.7 ml tube PNL for Pooled Normalized Libraries.

Procedure

1. Transfer 5 µl of each library to a new 8-tube strip.
2. Seal the NLP and store in the post-PCR area at -25°C to -15°C for ≤ 30 days.
3. Transfer libraries from each well of the 8-tube strip to the PNL tube.
4. Cap and vortex to mix, and then centrifuge briefly.

SAFE STOPPING POINT

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

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
- 1.7 ml 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.

2. Preheat the microheating system to 96°C.
3. Label two new 1.7 ml tubes:
   - Denatured HSC
   - DNL for Denatured Normalized Libraries

Procedure

1. In the Denatured HSC tube, combine the following volumes:
   - HSC (2 µl)
   - HP3 (2 µl)
   - Nuclease-free water (36 µl)
2. Cap and vortex to mix, and then centrifuge briefly.
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 12 µl library from the PNL tube to the DNL tube.
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)*.

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Kit Contents and Storage

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

Kit Name	Part #
ForenSeq MainstAY Kit (96 Reactions)	V16000142
ForenSeq MainstAY Kit (384 Reactions)	V16000128

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.

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	UDI0001-UDI0096	ForenSeq UDI Adapter Plate 1	Not applicable	-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
4	UDI0001-UDI0096	ForenSeq UDI Adapter Plate 1	Not applicable	-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

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
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tube	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
96-well deep well storage plates (midi plates)	Fisher Scientific, part # AB-0859
96-well twin.tec PCR plates, semiskirted	One of the following suppliers: • Eppendorf, catalog # 951020303 • VWR, catalog # 89136-706
96-well twin.tec PCR plate, skirted, 150 µl	Eppendorf, catalog # 951020401
Ethyl alcohol, pure	Sigma-Aldrich, catalog # E7023
Microseal 'A' sealing film	Bio-Rad, catalog # MSA5001
Microseal 'B' sealing film, adhesive, optical	Bio-Rad, catalog # MSB1001

Consumable	Supplier
One of the following kits: • MiSeq FGx Reagent Kit • MiSeq FGx Reagent Micro Kit	Verogen part #: • 15066817 • 20021681
Multichannel reagent reservoirs, PVC, disposable	Labcor, part # 730-001
Nuclease-free water	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
[Crude lysate] One of the following kits: • QuickExtract DNA Extraction Solution • SwabSolution Kit	The corresponding supplier: • Epicentre, catalog # QE09050 • Promega, catalog # DC8271
[FTA card] 1X TBE Buffer	General lab supplier
[FTA card] PCR Tube Storage Racks	VWR, catalog # 80086-074

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
ForenSeq Index Plate Fixture	Verogen, part # 15055269		X
Heating system, 96-well, 1.5 ml	General lab supplier		X
Magnetic stand-96	Life Technologies, part # AM10027		X
Microplate centrifuge	General lab supplier	X	X
Thermal cycler, 96-well with heated lid	See *Thermal Cyclers*		X
Thermoshaker, one of the following: • BioShake iQ • BioShake XP	QInstruments, item #: • 1808-0506 • 1808-0505		X
Rubber roller	General lab supplier	X	X
Vortexer	General lab supplier	X	X
[Optional] 10 µl pipettes	General lab supplier	X	X

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
Proflex 96-well PCR System	Not applicable	Heated, constant at 105°C	Polypropylene plates and tubes
Veriti 96-well thermal cycler	Standard	Heated, constant at 105°C	Polypropylene plates and tubes

* Only gold heat blocks are supported.

Index Adapter Sequences

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

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

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

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

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
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
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

Index Name	Index 1 Bases	Index 2 Bases	Plate well location
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
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

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Loci Detected with DPMC

The following tables list loci detected with DPMC.

- 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 gender of the biological sample donor. The size range is 106–112 bp and the control DNA is male.

Autosomal STRs

Locus	Repeats	Amplicon Length (bp)	Chromosome	NA24385 Control Alleles
D1S1656	7-21.3	133-192	1	12,13
TPOX	4-16	61-109	2	11,11
D2S441	7-17	137-177	2	10,14
D2S1338	10-33.1	110-203	3	22,25
D3S1358	8-22	138-194	3	17,18
D4S2408	8-13	98-118	4	9,9
FGA	12.2-53	150-312	4	20,23
D5S818	4-20	98-162	5	12,12
CSF1PO	5-17	72-120	5	12,12
D6S1043	8-26	154-226	6	12,20
D7S820*	5-21.1	118-183	7	8,11
D8S1179	6-20	82-138	8	14,15
D9S1122	8-15	104-132	9	12,12
D10S1248	7-20	124-176	10	13,15
TH01	3-14	96-140	11	6,9.3
vWA	11-26	135-195	12	16,19

Locus	Repeats	Amplicon Length (bp)	Chromosome	NA24385 Control Alleles
D12S391	13-28	229-289	12	18,23
D13S317	5-17	138-186	13	9,11
PentaE	5-28.4	362-481	15	7,14
D16S539	4-17	132-184	16	9,13
D17S1301	9-15	130-154	17	11,12
D18S51	6-40	136-272	18	16,18
D19S433	4-27	148-240	19	13,14
D20S482	9-17	125-157	20	14,15
D21S11	12-41.2	147-265	21	29,31.2
PentaD	1.1-19	209-298	21	12,13
D22S1045	8-19	201-245	22	16,16

* 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	37,38
DYS19	9-19	269-309	Y	14
DYS385a-b	7-28	232-316	Y	13,16
DYS389I	9-17	236-268	Y	14
DYS389II	24-34	283-323	Y	31
DYS390	17-28	290-334	Y	24
DYS391	5-16	119-163	Y	10
DYS392	6-17	318-362	Y	13
DYS393	9-18	108-144	Y	
DYS437	10-18	194-226	Y	14
DYS438	6-16	129-179	Y	9
DYS439	6-17	167-211	Y	12
DYS448	14-26	330-402	Y	19

Locus	Repeats	Amplicon Length (bp)	Chromosome	NA24385 Control Alleles
DYS460	7-14	348-376	Y	11
DYS481	17-32	129-174	Y	22
DYS505	9-15	162-186	Y	11
DYS522	8-17	298-334	Y	12
DYS533	7-17	186-226	Y	12
DYS549	10-14	210-226	Y	13
DYS570	10-26	142-206	Y	17
DYS576	10-25	163-223	Y	18
DYS612	26-33	275-296	Y	29
DYS635	15-30	242-302	Y	21
DYS643	7-15	141-181	Y	10
Y-GATA-H4	8-15	159-187	Y	11

Technical Support

For technical assistance, contact Verogen Technical Support.

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

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

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