illumina®

Nextera DNA Exome

Reference Guide

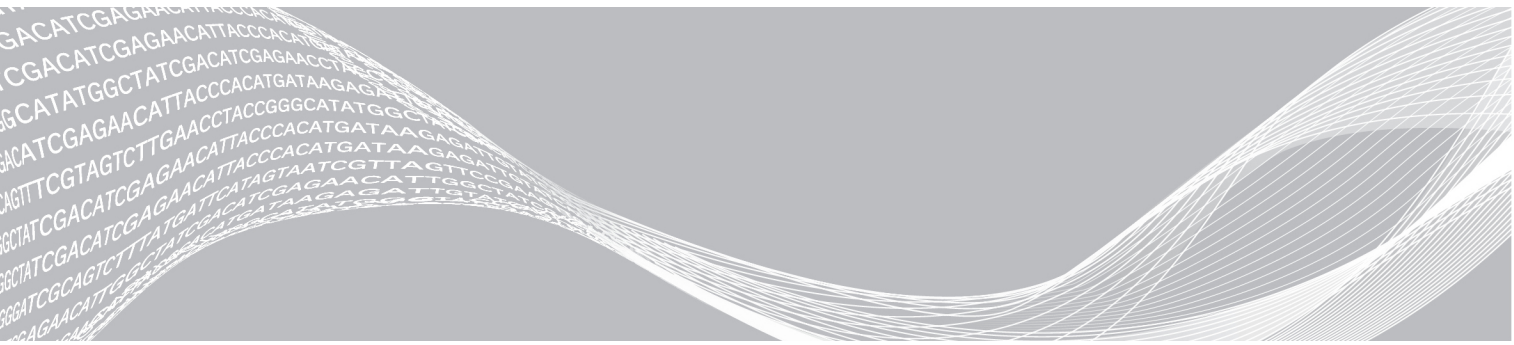

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Table of Contents

Chapter 1 Overview

Introduction

The Nextera DNA Exome workflow protocol explains how to prepare up to 96 indexed, paired-end libraries, followed by enrichment using exome probe panels and reagents provided in an Illumina® Nextera DNA Exome Kit. The libraries are prepared for subsequent cluster generation and DNA sequencing. The goal of this protocol is to fragment and add adapter sequences onto template DNA to generate indexed sequencing libraries that can be carried through enrichment for targeted resequencing applications.

The protocol offers:

- Excellent data quality with low input of 50 ng
- Fast and easy preparation of up to 96 enriched libraries in less than two days with minimal hands-on time

This protocol supports the following Illumina library prep kits:

- Nextera DNA Exome Kit (24 Samples)
- Nextera DNA Exome Kit (96 Samples)

Illumina-IDT Exome Enrichment Workflow

Nextera DNA Exome library prep and adapter components can be ordered separately to perform the Illumina-IDT Exome Enrichment workflow. If performing the Illumina-IDT Exome Enrichment workflow, the protocol documented in this guide can be followed through validation of libraries before hybridization and capture. Hybridization and capture continues following the IDT xGen hybridization capture protocol and reagents must be ordered separately through IDT. The libraries after IDT xGen hybridization and capture are ready for subsequent cluster generation and sequencing on Illumina platforms. For more information, see the Integrated DNA Technologies website.

DNA Input Recommendations

The Nextera DNA Exome protocol has been optimized for 50 ng of total gDNA.

Use a fluorometric-based method to quantify input gDNA specific for double-stranded DNA (dsDNA), such as QuantiFluor or Qubit, and run samples in triplicate for confident measurements.

- Use fluorometric-based methods for quantification, such as Qubit or PicoGreen to provide accurate quantification for dsDNA. UV spectrophotometric based methods, such as the Nanodrop, measures any nucleotides present in the sample including RNA, dsDNA, ssDNA, and free nucleotides, which can givean inaccurate measurement of gDNA.
- Make sure that the starting DNA does not contain more than 1 mM EDTA and is free of organic contaminants, such as phenol and ethanol. For more information, see *DNA Quantification* on page 37.
- DNA samples can contain substances that interfere with the Nextera tagmentation reaction and result in unexpected library insert sizes. To make sure that conditions are optimal before you begin library preparation, perform an optional sample cleanup, and then requantify the DNA samples.

Additional Resources

The following documentation is available for download from the Illumina website.

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
Nextera DNA Exome Checklist (document # 1000000040418)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
Nextera DNA Flex Library Prep Pooling Guide (document # 1000000031471)	Provides pooling guidelines for preparing libraries for Illumina sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
Sequencing Library qPCR Quantification Guide (part # 11322363)	Describes a qPCR method for quantifying sequencing by synthesis (SBS) libraries generated using the Illumina library preparation protocols.
BaseSpace help (help.basespace.illumina.com)	Provides information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.
Illumina Experiment Manager Guide (document # 15031335)	Provides information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
Local Run Manager Software Guide (document #100000002702)	Provides an overview of the Local Run Manager (LRM) software, instructions for using software features, and instructions for installing analysis modules on the instrument computer.
Hybridization capture of DNA Libraries using xGen Lockdown Probes and Reagents Protocol	Integrated DNA Technologies (IDT) protocol guide for use when performing the Illumina-IDT Exome Enrichment workflow. Provides information on performing hybridization through clean up of the amplified enriched library in the workflow.

Visit the Nextera DNA Exome workflow support page on the Illumina website for access to requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Chapter 2 Protocol

Introduction

This chapter describes the Nextera DNA Exome workflow protocol.

- Follow the protocol in the order described, using the specified volumes and incubation parameters.
- The protocol provides a single workflow with options for using plates or tubes as containers.
  - Differences for each option are designated with [Plate] or [Tube].
  - Follow the instructions for the container that you are using.
  - Guidelines for using plates vs. tubes are as follows:

Table 1 Workflow Options

	Plates	Tubes
Workflow designator	[Plate]	[Tube]
Number of library prep samples processed at the same time	> 16	≤ 16
Number of enrichment libraries processed at the same time	> 8	≤ 8
Container	• 96-well Hard-Shell 0.3 ml PCR plates • 96-well midi plates	• 1.5 ml microcentrifuge tubes • 0.2 ml thin-wall PCR tubes • 8-tube strips
Mixing method	• Microplate shaker • Pipette	• Pipette
Incubation Equipment	• Microheating systems • 96-well thermal cycler	• Heat block • Thermal cycler

- Review best practices before proceeding. See *Additional Resources* on page 1 for information on how to access Nextera DNA Exome best practices on the Illumina website.
- Before proceeding, confirm kit contents and make sure that you have the required equipment and consumables. For more information, see *Supporting Information* on page 31.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate, and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.

Handling Beads

- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogenous.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the specified magnetic stand for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.

Library Prep Workflow

This diagram represents the Illumina-only workflow. If performing the Illumina-IDT Exome Enrichment Workflow, follow this protocol up to Hybridize Probes and then switch to the IDT xGen hybridization protocol. For more information, see the *Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents* protocol guide found on the Integrated DNA Technologies website.

Figure 1 Nextera DNA Exome Workflow

Prepare for Pooling

When pooling samples for sequencing, use IEM, LRM, or BaseSpace Prep Tab to record information about your samples before beginning library preparation.

- Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software.
- Use LRM and BaseSpace Prep Tab to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software.

Include a common index in each column. A common index facilitates pipetting operations when dispensing index adapters and pooling indexed libraries.

Nextera DNA Exome kits support the following reactions and plexity. For more information on the kit configurations, see *Product Contents* on page 31.

Samples	Enrichment Reactions	Plexity
24	8	3
96	8	12

Tagment Genomic DNA

In this step, the Nextera transposome tagments gDNA, which is a process that simultaneously fragments and tags gDNA with adapter sequences.

Consumables

- SPB (Sample Purification Beads)
- RSB (Resuspension Buffer)
- ST (Stop Tagment Buffer)
- TD (Tagment DNA Buffer)
- TDE2 (Tagment DNA Enzyme 2)
- gDNA (50 ng per sample)
- Tris-HCl 10 mM, pH 8.5
- Choose from the following containers:
  - [Plate] 96-well Hard-Shell 0.3 ml PCR plate
  - [Tube] 0.2 ml thin-wall PCR tube or 8-tube strips
- [Plate] Microseal 'B' adhesive seals

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
gDNA	-25°C to -15°C	Thaw on ice. Gently invert the thawed tubes 3–5 times, and then centrifuge briefly.
RSB	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C after the initial thaw for later use in the protocol.
TD	-25°C to -15°C	Thaw on ice. Gently invert the thawed tubes 3–5 times, and then centrifuge briefly.
TDE2	-25°C to -15°C	Thaw on ice. Gently invert the thawed tubes 3–5 times, and then centrifuge briefly. Set aside on ice.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Keep at room temperature for later use in the protocol.
ST	15°C to 30°C	Check for precipitates. If present, vortex until all particulates are resuspended.

2 Save the following TAG58 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 58°C for 10 minutes
- Hold at 10°C
- Each well or tube contains 50 µl.

3 Save the following TAG60 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 60°C for 5 minutes
- Hold at 10°C
- Each well or tube contains 65 µl.

Procedure

Quantify and Normalize gDNA

1 Quantify gDNA using a fluorometric method, such as QuantiFluor or Qubit.

2 Normalize gDNA in Tris-HCl 10 mM, pH 8.5 to 10 ng/µl.

3 Requantify the normalized gDNA using the same fluorometric quantification method.

4 Dilute the normalized gDNA in Tris-HCl 10 mM, pH 8.5 to a final volume of 10 µl at 5 ng/µl (50 ng total).

Tagment DNA

1 Add the following items in the order listed to each well of a new Hard-Shell PCR plate or to a new 0.2 ml thin-wall PCR tube or 8-tube strip.
- TD (25 µl)
- Normalized gDNA (10 µl)
- TDE2 (15 µl)

2 Mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

3 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

4 Place on the preprogrammed thermal cycler and run the tagmentation program. When the sample reaches 10°C, *immediately* proceed to step 5 because the transposome is still active.

5 Add 15 µl ST to each well or tube, and then pipette to mix

6 Place on the preprogrammed thermal cycler and run the TAG60 program.

Clean Up Tagmented DNA

This step uses SPB (Sample Purification Beads) to purify the tagmented DNA from the Nextera transposome. The cleanup step removes the Nextera transposome that can otherwise bind to DNA ends and interfere with downstream processes.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well midi plate and 96-well Hard-Shell 0.3 ml PCR plate
  - [Tube] 1.5 ml microcentrifuge tubes and 8-tube strips
- [Plate] Microseal 'B' adhesive seals

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.
- Minimize splashing and frothing while mixing to prevent carryover of ST buffer into PCR amplification. Carryover can reduce final yield before enrichment.

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.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Transfer total sample volume to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

2 Add 52 µl SPB to each well or tube, and then mix thoroughly as follows.
  - [Plate] Pipette up and down 10 times.
  - [Tube] Pipette up and down.

3 Incubate at room temperature for 5 minutes.

4 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

5 Transfer 98 µl supernatant to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

6 Add 137 µl SPB to each well or tube, and then mix thoroughly as follows.
- [Plate] Pipette up and down 10 times.
- [Tube] Pipette up and down.

7 Incubate at room temperature for 5 minutes.

8 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

9 Remove and discard all supernatant from each well or from the tube.

10 Wash 2 times as follows.

a Add 200 µl freshly prepared 80% EtOH to each well or to the tube.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well or from the tube.

11 Using a 20 µl pipette, remove residual 80% EtOH from each well or from the tube.

12 Air-dry on the magnetic stand for 5 minutes.

13 Remove from the magnetic stand.

14 Add 22.5 µl RSB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

15 Incubate at room temperature for 2 minutes.

16 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

17 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

18 Transfer 20 µl supernatant to the corresponding well of a new Hard-Shell PCR plate or to a new 8-tube strip.

19 [Optional] Run 1 µl undiluted DNA library on an Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit or an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit.

Figure 2 Example of Post-Tagmentation Library Run on Fragment Analyzer Using High Sensitivity NGS Kit

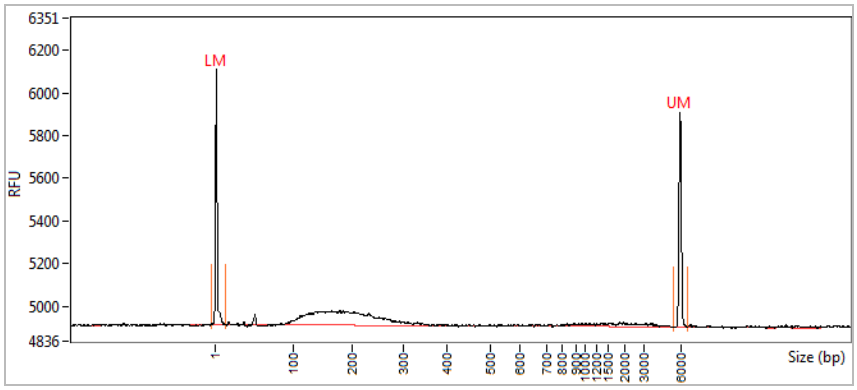
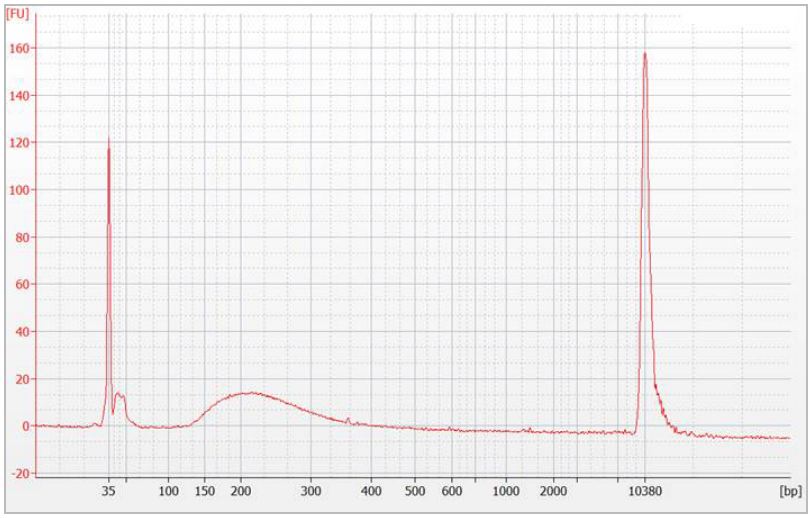


Figure 3 Example of Post-Tagmentation Library Run on a Bioanalyzer Using a High Sensitivity DNA Kit



Amplify Tagmented DNA

This step amplifies purified tagmented DNA and adds index adapters using a 10-cycle PCR program. This PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for cluster amplification.

Index adapters are included in the Nextera DNA Exome Kit for the Nextera DNA Exome workflow. If performing the Illumina-IDT Exome Enrichment workflow, index adapters must be ordered separately. For information on compatible index adapters, see *Supporting Information* on page 31.

Consumables

- DNA Adapters (tubes or index adapter plate)
- LAM (Library Amplification Mix)
- 1.7 ml microcentrifuge tubes (1 per index adapter tube)
- [Plate] Microseal 'A' film

- [Plate] Microseal 'B' adhesive seal
- [Optional] [Tube] TruSeq Index Plate Fixture Kit

NOTE

Use Microseal 'A' when sealing the plate before placing it on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
DNA Adapters (i5 and i7)	-25°C to -15°C	Only remove adapters being used. Thaw at room temperature for 20 minutes. Vortex each tube to mix. Centrifuge briefly using a 1.7 ml Eppendorf tube.
LAM	-25°C to -15°C	Thaw on ice.

2 Save the following LAM AMP program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Set volume to 50 µl
- 72°C for 3 minutes
- 98°C for 30 seconds
- 10 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

Procedure

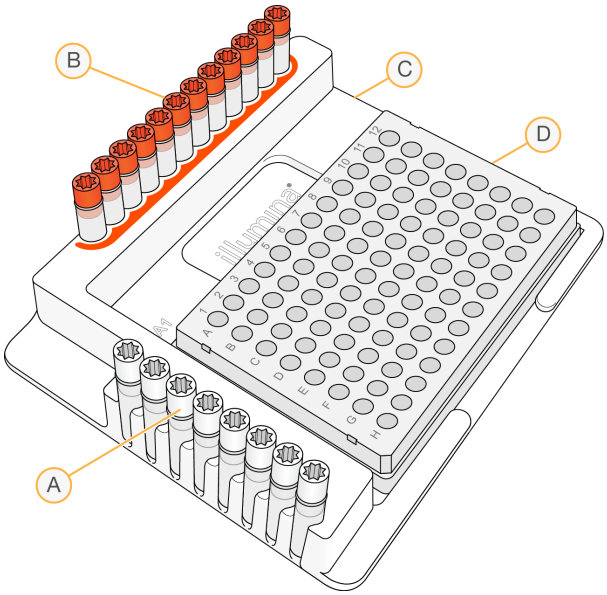
1 [Plate] Remove the Index Adapter Plate seal.

2 [Tube] Arrange Index 1 (i7) adapters in columns 1–12 of the TruSeq Index Plate Fixture.

3 [Tube] Arrange Index 2 (i5) adapters in rows A–H of the TruSeq Index Plate Fixture.

4 [Tube] Place the plate on the TruSeq Index Plate Fixture.

Figure 4 TruSeq Index Plate Fixture (24-Indexes)



NOTE

This image depicts eight i7 adapters and 12 i5 adapters. The Nextera DNA CD Indexes- 12 Indexes only includes six i7 adapters and four i5 adapters.

A Rows A–H: Index 2 (i5) adapters (white caps)
B Columns 1–12: Index 1 (i7) adapters (orange caps)
C TruSeq Index Plate Fixture
D 96-well plate

5 [Plate] Using a multichannel pipette, add 10µL to each sample well.

6 [Tube] Add 5 µl of each Index 1 (i7) adapter as follows.
- Add a different index to each sample well.

7 Replace the cap on each i7 adapter tube with a new orange cap.

8 [Tube] Add 5 µl of each Index 2 (i5) adapter as follows.
- Add a different index to each sample well.

9 Replace the cap on each i5 adapter tube with a new white cap.

10 Add 20 µl LAM to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 1 minute.
- [Tube] Pipette up and down.

11 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.

12 Place on the preprogrammed thermal cycler and run the LAM AMP program.

SAFE STOPPING POINT

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

Clean Up Amplified DNA

This step uses SPB (Sample Purification Beads) to purify the DNA library and remove unwanted products.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well midi plate and 96-well Hard-Shell 0.3 ml PCR plate
  - [Tube] 1.5 ml microcentrifuge tubes or 8-tube strips
- [Plate] Microseal 'B' adhesive seals

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

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.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Centrifuge at 280 × g for 1 minute.

2 Transfer 50 µl total volume to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

3 Add 90 µl SPB to each well or tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1800 rpm for 1 minute.
   - [Tube] Pipette up and down.

4 Incubate at room temperature for 5 minutes.

5 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.

6 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

7 Remove and discard all supernatant from each well or from the tube.

8 Wash 2 times as follows.

   a Add 200 µl freshly prepared 80% EtOH to each well or to the tube.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well or from the tube.

9 Using a 20 µl pipette, remove residual 80% EtOH from each well or from the tube.

10 Air-dry on the magnetic stand for 5 minutes.

11 Remove from the magnetic stand.

12 Add 17 µl RSB to each well or tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1800 rpm for 1 minute.
   - [Tube] Pipette up and down.

13 Incubate at room temperature for 2 minutes.

14 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.

15 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

16 Transfer 15 µl supernatant to the corresponding well of a new Hard-Shell PCR plate or to a new 1.5 ml microcentrifuge tube or 8-tube strip.

17 Quantify the library using a fluorometric method, such as QuantiFluor or Qubit. For an example protocol using the Promega QuantiFluor method, see *DNA Quantification* on page 37.

> **NOTE**
>
> Inaccurate quantification and pooling can result in a higher representation of some samples compared to others in the same pool.

18 [Optional] Run on an Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit or an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit as follows. You can expect to see a distribution of DNA fragments with a size range from 200–500 bp.
   - Dilute the DNA library 1:10 with water.
   - Run 1 µl diluted DNA library.

Figure 5 Example of Post-PCR, Pre-Enriched Library Run on Fragment Analyzer Using High Sensitivity NGS Kit

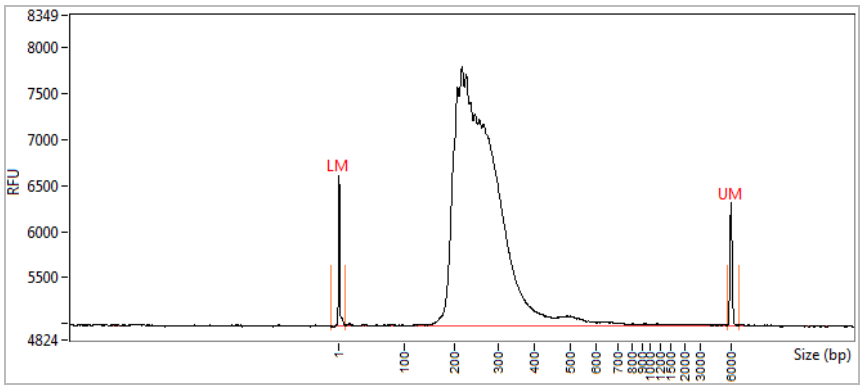
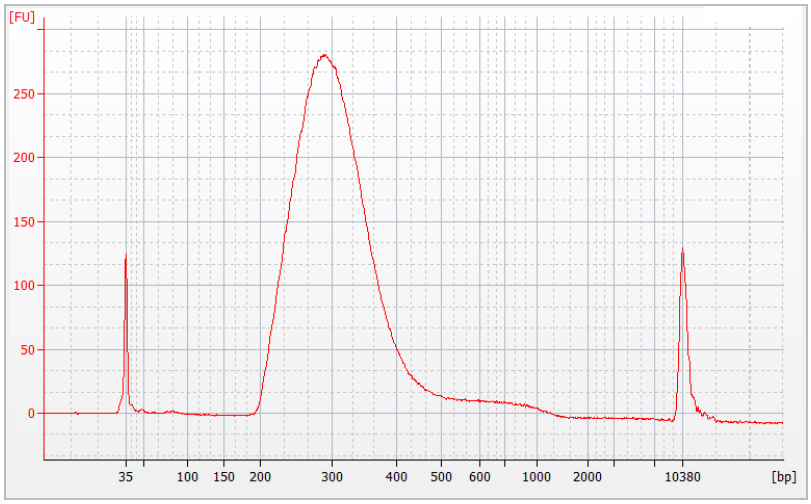


Figure 6 Example of Post-PCR, Pre-Enriched Library Run on Bioanalyzer Using High Sensitivty DNA Kit



SAFE STOPPING POINT

If you are stopping, seal the plate or cap the tube and store at -25°C to -15°C for up to 14 days.

Hybridize Probes

This step combines DNA libraries containing unique indexes into a single pool, and then binds targeted regions of the DNA with capture probes.

NOTE

If performing the Illumina-IDT Exome Enrichment workflow, do not proceed with the Illumina protocol as documented in the remainder of this guide, switch to the IDT xGen hybridization protocol. For more information, see the *Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents* protocol guide found on the Integrated DNA Technologies website.

If you are following the Nextera DNA Exome workflow using the Nextera DNA Exome Kit, continue with the sections that follow.

Consumables

- BLR (Blocker)
- CEX (Coding Exome Oligos)
- EHB1 (Enrichment Hybridization Buffer 1)
- EHB2 (Enrichment Hybridization Buffer 2)
- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well midi plate and 96-well Hard-Shell 0.3 ml PCR plate
  - [Tube] 1.5 ml microcentrifuge tubes and 0.2 ml thin-wall PCR tubes, and 8-tube strips
- [Plate] Microseal 'B' adhesive seal
- [Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) (1 per pooled sample)

About Reagents

- Before using BLR, vortex to resuspend the solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.
- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
BLR	-25°C to -15°C	Thaw at room temperature.
CEX	-25°C to -15°C	Thaw at room temperature.
EHB1	-25°C to -15°C	Thaw at room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
EHB2	2°C to 8°C	Remove from storage.

2 Save the TRE HYB program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 95°C for 10 minutes
- 58°C for 30 minutes
- Each well or tube contains 10 µl.

Pool Libraries

1 Combine 500 ng of each DNA library, making sure that each library has a unique index.

Library Pool Complexity	Total DNA Library Mass (ng)
1-plex	500
2-plex	1000
3-plex	1500
4-plex	2000
5-plex	2500
6-plex	3000
7-plex	3500
8-plex	4000
9-plex	4500
10-plex	5000
11-plex	5500
12-plex*	6000

* [Plate] For improved enrichment in the 12-plex pool, combine 300 ng of each DNA library, making sure that each library has a unique index. This method maximizes on-target reads, but reduces library size.

- If the total volume is > 30 µl, use a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) to concentrate the pooled sample to 30 µl.
  - If you are using a vacuum concentrator, use a no heat setting and a medium drying rate.
  - If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), it is not required to rinse the device before use. Most of the volume filters through in 5 minutes, but up to 30 minutes can be required depending on the starting volume.
- If the total volume is < 30 µl, increase the volume to 30 µl with RSB.

Procedure

1 Add the following reagents in the order listed to each well of a new midi plate or to a new 1.5 ml microcentrifuge tube.
- DNA library sample or pool (30 µl)
- BLR (10 µl)
- CEX (10 µl)

2 Mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 1 minute.
- [Tube] Pipette up and down.

3 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

4 Add 125 µl SPB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

5 Incubate at room temperature for 10 minutes.

6 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

7 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

8 Remove and discard all supernatant from each well or from the tube.

9 Wash 2 times as follows.

a Add 200 µl freshly prepared 80% EtOH to each well or to the tube.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well or from the tube.

10 Using a 20 µl pipette, remove residual 80% EtOH from each well or from the tube.

11 Air-dry on the magnetic stand for 10 minutes.

12 Remove from the magnetic stand.

13 Add 7.7 µl EHB1 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

14 Incubate at room temperature for 2 minutes.

15 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

16 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

17 Transfer 7.5 µl supernatant to the corresponding well of a new Hard-Shell PCR plate or to a new thin-wall PCR tube or 8-tube strip.

NOTE

To reduce evaporation, avoid transferring samples to wells near the edge of the plate.

18 Add 2.5 µl EHB2 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

19 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

20 Place on the preprogrammed thermal cycler and run the TRE HYB program.

Capture Hybridized Probes

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

- EE1 (Enrichment Elution Buffer 1)

- ET2 (Elute Target Buffer 2)
- EEW (Enhanced Enrichment Wash Solution)
- HP3 (2 N NaOH)
- RSB (Resuspension Buffer)
- SMB (Streptavidin Magnetic Beads)
- Choose from the following containers:
  - [Plate] 96-well midi plates (2)
  - [Tube] 1.5 ml microcentrifuge tubes
- 1.5 ml microcentrifuge tube
- [Plate] Microseal 'B' adhesive seals

About Reagents

- EEW can be cloudy after reaching room temperature.
- EEW must be at room temperature for use.
- Protect EEW from light.
- EEW can appear yellow.
- Make sure that you use SMB (2 ml tube) and not SPB (15 ml tube) for this procedure.
- Invert SMB to mix before use.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EEW	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 [Plate] Preheat a microheating system with midi plate insert to 50°C.

3 [Tube] Preheat a heat block to 50°C.

Procedure

First Bind

1 Centrifuge at 280 × g for 1 minute.

2 Transfer all (~10 µl) to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

3 Add 250 µl SMB to each well or tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1200 rpm for 5 minutes.
   - [Tube] Pipette up and down.
4 Incubate at room temperature for 25 minutes.
5 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.
6 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).
7 Remove and discard all supernatant from each well or from the tube.
8 Remove from the magnetic stand.

First Wash

1 Add 200 µl EEW to each well or tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1800 rpm for 4 minutes. Pipette to resuspend the bead pellet further.
   - [Tube] Pipette up and down.

NOTE

Proper resuspension is required to ensure efficient removal of nonspecific DNA from the reaction, which otherwise results in poor enrichment statistics.

2 Incubate as follows.
   - [Plate] Place on the 50°C microheating system with the lid closed for 30 minutes.
   - [Tube] Place on the 50°C heat block for 30 minutes.
3 Immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).
4 Remove and discard all supernatant from each well or from the tube.
5 Remove from the magnetic stand.
6 Repeat steps 1–5 for a total of 2 washes.

First Elution

1 Create elution premix in a 1.5 ml microcentrifuge tube, and then vortex.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)
2 Add 23 µl elution premix to each well or tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1800 rpm for 2 minutes.
   - [Tube] Pipette up and down.
3 Incubate at room temperature for 2 minutes.
4 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.
5 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
6 Transfer 21 µl supernatant to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

7 Add 4 µl ET2 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 1 minute.
- [Tube] Pipette up and down.

8 Add 5 µl RSB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 1 minute.
- [Tube] Pipette up and down.

9 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, seal the plate or cap the tube and store at -25°C to -15°C for up to 7 days.

Perform Second Hybridization

This step binds targeted regions of the enriched DNA with capture probes a second time. This second hybridization ensures high specificity of the captured regions.

Consumables

- BLR (Blocker)
- CEX (Coding Exome Oligos)
- EHB1 (Enrichment Hybridization Buffer 1)
- EHB2 (Enrichment Hybridization Buffer 2)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- [Plate] Microseal 'B' adhesive seals

About Reagents

- Before using BLR, vortex to resuspend the solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.
- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
BLR	-25°C to -15°C	Thaw at room temperature.
CEX	-25°C to -15°C	Thaw at room temperature.
EHB1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

Item	Storage	Instructions
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
EHB2	2°C to 8°C	Remove from storage.

Procedure

1 Add the following reagents in the order listed to each well or tube.
- BLR (10 µl)
- CEX (10 µl)

2 Mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 1 minute.
- [Tube] Pipette up and down.

3 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

4 Add 125 µl SPB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

5 Incubate at room temperature for 10 minutes.

6 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

7 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

8 Remove and discard all supernatant from each well or from the tube.

9 Wash 2 times as follows.

a Add 200 µl freshly prepared 80% EtOH to each well or to the tube.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well or from the tube.

10 Using a 20 µl pipette, remove residual 80% EtOH from each well or from the tube.

11 Air-dry on the magnetic stand for 10 minutes.

12 Remove from the magnetic stand.

13 Add 7.7 µl EHB1 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

14 Incubate at room temperature for 2 minutes.

15 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

16 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

17 Transfer 7.5 µl supernatant to the corresponding well of a new Hard-Shell PCR plate or to a new 8-tube strip.

18 Add 2.5 µl EHB2 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

19 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

20 Place on the preprogrammed thermal cycler and run the TRE HYB program.

Perform Second Capture

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- ET2 (Elute Target Buffer 2)
- EEW (Enhanced Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- Choose from the following containers:
  - [Plate] 96-well midi plates (2)
  - [Tube] 1.5 ml microcentrifuge tubes or 8-tube strips
- 1.5 ml microcentrifuge tube
- [Plate] Microseal 'B' adhesive seals

About Reagents

- EEW can be cloudy after reaching room temperature.
- For best results, EEW must be thawed before use.
- Protect EEW from light.
- EEW can appear yellow.
- Make sure that you use SMB (2 ml tube) and not SPB (15 ml tube) for this procedure.
- Invert SMB to mix before use.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.

Item	Storage	Instructions
EEW	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Return to storage after use.

2 [Plate] Preheat a microheating system with midi plate insert to 50°C.

3 [Tube] Preheat a heat block to 50°C.

Procedure

Second Bind

1 Centrifuge at 280 × g for 1 minute.

2 Transfer 10 µl supernatant to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

3 Add 250 µl SMB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 5 minutes.
- [Tube] Pipette up and down.

4 Incubate at room temperature for 25 minutes.

5 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

6 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

7 Remove and discard all supernatant from each well or from the tube.

8 Remove from the magnetic stand.

Second Wash

1 Add 200 µl EEW to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 4 minutes. Pipette to resuspend the bead pellet further.
- [Tube] Pipette up and down.

NOTE

Proper resuspension is required to ensure efficient removal of nonspecific DNA from the reaction, which otherwise results in poor enrichment statistics.

2 Incubate as follows.
- [Plate] Place on the 50°C microheating system with the lid closed for 30 minutes.
- [Tube] Place on the 50°C heat block for 30 minutes.

3 Immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).

4 Remove and discard all supernatant from each well or from the tube.

5 Remove from the magnetic stand.

6 Repeat steps 1–5 for a total of 2 washes.

Second Elution

1 Create elution premix in a 1.5 ml microcentrifuge tube, and then vortex.
- EE1 (28.5 µl)
- HP3 (1.5 µl)

2 Add 23 µl elution premix to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 2 minutes.
- [Tube] Pipette up and down.

3 Incubate at room temperature for 2 minutes.

4 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

5 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

6 Transfer 21 µl supernatant to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube or 8-tube strip.

7 Add 4 µl ET2 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

8 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

Clean Up Captured Library

This step uses SPB (Sample Purification Beads) to purify the captured library before PCR amplification.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well Hard-Shell 0.3 ml PCR plate
  - [Tube] 1.5 ml microcentrifuge tubes and 8-tube strips
- [Plate] Microseal 'B' adhesive seals

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

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.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Vortex SPB until well-dispersed.

2 Add 45 µl SPB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

3 Incubate at room temperature for 5 minutes.

4 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

5 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

6 Remove and discard all supernatant from each well or from the tube.

7 Wash 2 times as follows.

a Add 200 µl freshly prepared 80% EtOH to each well or to the tube.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well or from the tube.

8 Use a 20 µl pipette to remove residual EtOH from each well or from the tube.

9 Air-dry on the magnetic stand until dry (~5 minutes).

10 Remove from the magnetic stand.

11 Add 27.5 µl RSB to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1800 rpm for 1 minute.
- [Tube] Pipette up and down.

12 Incubate at room temperature for 2 minutes.

13 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

14 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

15 Transfer 25 µl supernatant to the corresponding well of a new Hard-Shell PCR plate or to a new 8-tube strip.

SAFE STOPPING POINT

If you are stopping, seal the plate or cap the tube and store at -25°C to -15°C for up to 7 days.

Amplify Enriched Library

This step uses a 10-cycle PCR program to amplify the enriched library.

Consumables

- EAM (Enrichment Amplification Mix)
- PPC (PCR Primer Cocktail)
- [Plate] Microseal 'A' film
- [Plate] Microseal 'B' adhesive seal

NOTE

Use Microseal 'A' when sealing the plate before placing it on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EAM	-25°C to -15°C	Thaw on ice.
PPC	-25°C to -15°C	Thaw on ice.

2 Save the following AMP10 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 98°C for 30 seconds
- 10 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C
- Each well or tube contains 50 µl.

Procedure

1 Add 5 µl PPC to each well or to the tube.

2 Add 20 µl EAM to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 1 minute.
- [Tube] Pipette up and down.

3 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

4 Place on the preprogrammed thermal cycler and run the AMP10 program.

SAFE STOPPING POINT

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

Clean Up Amplified Enriched Library

This step uses SPB (Sample Purification Beads) to purify the enriched library and remove unwanted products.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well midi plate and 96-well Hard-Shell 0.3 ml PCR plate
  - [Tube] 1.5 ml microcentrifuge tubes or 8-tube strips
- [Plate] Microseal 'B' adhesive seals

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

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.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

Procedure

1 Centrifuge as follows.
  - [Plate] Centrifuge at 280 × g for 1 minute.
  - [Tube] Centrifuge briefly.

2 Vortex SPB until well-dispersed.

3 Transfer 50 µl to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

4 Add 50 µl SPB to each well or tube, and then mix thoroughly as follows.
  - [Plate] Shake at 1800 rpm for 1 minute.
  - [Tube] Pipette up and down.

5 Incubate at room temperature for 5 minutes.

6 Centrifuge as follows.
  - [Plate] Centrifuge at 280 × g for 1 minute.
  - [Tube] Centrifuge briefly.

7 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

8 Remove and discard all supernatant from each well or from the tube.

9 Wash 2 times as follows.

   a Add 200 µl freshly prepared 80% EtOH to each well or to the tube.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well or from the tube.

10 Use a 20 µl pipette to remove residual EtOH from each well or from the tube.

11 Air-dry on the magnetic stand until dry (~5 minutes).

12 Remove from the magnetic stand.

13 Add 32 µl RSB to each well or tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1800 rpm for 1 minute.
   - [Tube] Pipette up and down.

14 Incubate at room temperature for 2 minutes.

15 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.

16 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

17 Transfer 30 µl supernatant to the corresponding well of a new Hard-Shell PCR plate or to a new 1.5 ml microcentrifuge tube or 8-tube strip.

NOTE

For best practice, perform normalization and pooling directly prior to sequencing. To minimize index hopping, do not store libraries in the pooled form. For more information, see *Minimize index hopping in multiplexed runs* on the Illumina website.

SAFE STOPPING POINT

If you are stopping, seal the plate or cap the tube and store at -25°C to -15°C for up to 7 days.

Check Enriched Libraries

Perform the following procedures to check the quality of the enriched library.

Quantify Libraries

Accurately quantify DNA libraries to ensure optimum cluster densities on the flow cell.

1 Quantify the postenriched library using the Qubit dsDNA BR Assay Kit.

2 Use the following formula to convert from ng/µl to nM. Assume a 400 bp library size or calculate based on the average size of the enriched library:

$$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol * average library size)}} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{\text{(15 ng/µl)}}{\text{(660 g/mol * 400)}} \times 10^6 = 57 \text{ nM}$$

Alternatively, you can quantify libraries using qPCR according to the *Sequencing Library qPCR Quantification Guide (document # 11322363)*.

Assess Quality

1 If the library concentration is higher than the supported quantitative range for the High Sensitivity DNA kit, dilute the library 1:10 with RSB.

2 Run 1 µl of either the pooled library, or the individual libraries, on an Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit or an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit.
Expect a distribution of DNA fragments with a size range from ~200 bp to ~400 bp. Depending on the level of indexing, insert size distribution can vary slightly. However, the sample peak must not be significantly shifted compared to the following example.

Figure 7 Fragment Analyzer Example Post Enrichment Library Distribution

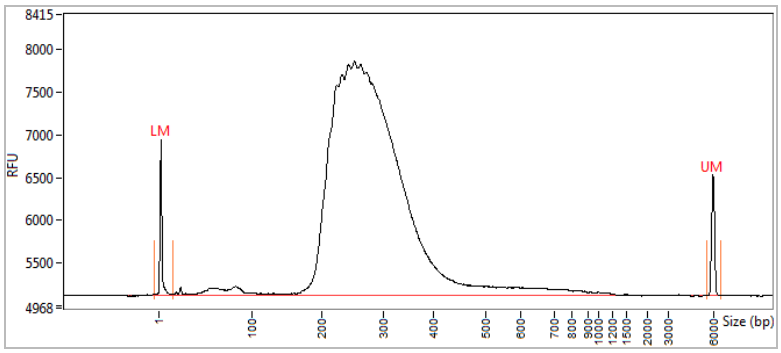
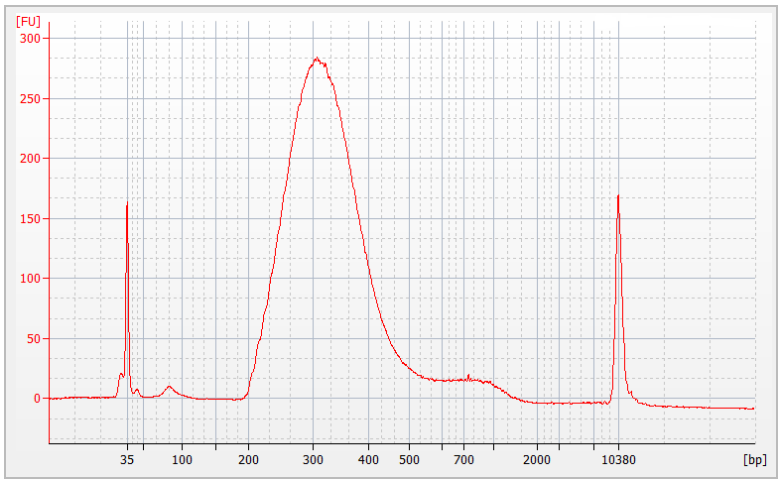


Figure 8 BioAnalyzer Example Post Enrichment (12-plex Enrichment) Library Distribution



Supporting Information

Introduction

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed workflow contents, and obtained all required consumables and equipment.

Product Contents

Make sure that you have all the reagents identified in this section before proceeding to the library preparation and enrichment procedures.

Nextera DNA Exome

The following kits are available for performing the Nextera DNA Exome workflow using Illumina reagents for library preparation and enrichment. These kits include index adapters and do not need to be ordered separately.

Kit Name	Catalog #
Nextera DNA Exome Kit (24 samples)	20020616
Nextera DNA Exome Kit (96 samples)	20020617

Illumina-IDT Exome Enrichment

The following library prep and index adapter components are available to order through Illumina to support the Illumina-IDT Exome Enrichment workflow.

From Illumina, order one catalog number for the library prep component and one catalog number for the index adapter component depending on the number of samples for your experiment.

Additional components provided by IDT are required for the IDT portion of the workflow. For more information, see the Integrated DNA Technologies website.

Library Prep Component	Catalog #
Nextera DNA Library Prep for Enrichment (24 samples)	20020187
Nextera DNA Library Prep for Enrichment (96 samples)	20020188

Index Adapter Component	Catalog #
Nextera DNA CD Indexes (24 indexes, 24 samples)	20018707
Nextera DNA CD Indexes (96 indexes, 96 samples)	20018708

Nextera DNA Exome Kit (24 Samples)

This Nextera DNA Exome kit is made up of four components of reagents, library preparation, enrichment reagents, indexed adapters, and coding exome oligos. Sufficient reagents are provided to support 24 samples in 8 × 3-plex enrichment reactions.

Nextera DNA Library Prep for Enrichment

This component contains two boxes, requiring different storage conditions.

Box 1, Store as specified

Quantity	Reagent	Description	Storage Temperature
1	SPB	Sample Purification Beads	2°C to 8°C
1	ST	Stop Tagment Buffer	15°C to 30°C

Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
1	RSB	Resuspension Buffer
1	TDE2	Tagment DNA Enzyme 2
1	LAM	Library Amplification Mix
1	TD	Tagment DNA Buffer

Nextera DNA for Enrichment

This component contains two boxes, requiring different storage conditions.

Box 1, Store at -25°C to -15°C

Quantity	Reagent	Description
1	COT	Blocker
1	EAM	Enrichment Amplification Mix
1	EE1	Enrichment Elution Buffer 1
2	EEW	Enhanced Enrichment Wash Solution
1	EHB1	Enrichment Hybridization Buffer 1
1	HP3	Finished Reagent
1	PPC	PCR Primer Cocktail

Box 2, Store at 2°C to 8°C

Quantity	Reagent	Description
1	EHB2	Enrichment Hybridization Buffer 2
1	ET2	Elute Target Buffer 2
4	SMB	Strepravidin Magnetic Beads

Nextera DNA CD Indexes (24 Samples), Store at -25°C to -15°C

Quantity	Index Name	Description
1	H503	DNA Adapter
1	H505	DNA Adapter
1	H506	DNA Adapter
1	H517	DNA Adapter
1	H705	DNA Adapter
1	H706	DNA Adapter
1	H707	DNA Adapter
1	H710	DNA Adapter
1	H711	DNA Adapter
1	H714	DNA Adapter

Index Adapter Replacement Caps, Store at 15°C to 30°C

Description
i7 Index Tube Caps, Orange
i5 Index Tube Caps, White

Exome Panel (45mb), Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

Nextera DNA Exome Kit (96 Samples)

This Nextera DNA Exome kit is made up of four components of reagents, library preparation, enrichment reagents, indexed adapters, and coding exome oligos. Sufficient reagents are provided to support 96 samples in 8 × 12-plex enrichment reactions.

Nextera DNA Library Prep for Enrichment

This component contains two boxes, requiring different storage conditions.

Box 1, Store as specified

Quantity	Reagent	Description	Storage Temperature
3	SPB	Sample Purification Beads	2°C to 8°C
1	ST	Stop Tagment Buffer	15°C to 30°C

Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
1	RSB	Resuspension Buffer
1	TDE2	Tagment DNA Enzyme 2
2	LAM	Library Amplification Mix
2	TD	Tagment DNA Buffer

Nextera DNA for Enrichment

This component contains two boxes, requiring different storage conditions.

Box 1, Store at -25°C to -15°C

Quantity	Reagent	Description
1	COT	Blocker
1	EAM	Enrichment Amplification Mix
1	EE1	Enrichment Elution Buffer 1
2	EEW	Enhanced Enrichment Wash Solution
1	EHB1	Enrichment Hybridization Buffer 1
1	HP3	Finished Reagent
1	PPC	PCR Primer Cocktail

Box 2, Store at 2°C to 8°C

Quantity	Reagent	Description
1	EHB2	Enrichment Hybridization Buffer 2
1	ET2	Elute Target Buffer 2
4	SMB	Strepravidin Magnetic Beads

Nextera DNA CD Indexes (96 samples), Store at -25°C to -15°C

Quantity	Description
1	96 Dual Adapter Index Plate

Exome Panel (45mb), Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

Consumables and Equipment

Some items required depend on the workflow performed (Plate or Tube) and these items are specified in separate tables.

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

If performing the Illumina-IDT Exome Enrichment Workflow, all user-supplied consumables and equipment for library prep listed in this section are required. Additional user-supplied consumables and equipment to complete the Illumina-IDT Exome Enrichment Workflow are listed in the IDT xGen hybridization capture protocol. Make sure that you have all the necessary user-supplied consumables and equipment from both the Illumina and IDT protocols before starting the workflow. For more information, see the Integrated DNA Technologies website.

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
1000 µl barrier pipette tips	General lab supplier
96-well flat clear bottom black microplates Note: Used when quantifying samples with a SpectraMax M5 spectrofluorometer.	Corning, part # 3904
Aluminum foil	General lab supplier
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Distilled water	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
Ultrapure water	General lab supplier
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) Note: Use to concentrate a pooled library. Otherwise, use a vacuum concentrator.	Millipore, part # UFC503008
[Optional] High Sensitivity NGS Fragment Analysis Kit	Advanced Analytical, catalog # DNF-474
[Optional] High Sensitivity DNA Kit	Agilent Technologies, part # 5067-4626

Consumables for Plate Workflow

Consumable	Supplier
96-well storage plates, round well, 0.8 ml (midi plate)	Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier
Hard-Shell 96-well PCR Plates	Bio-Rad, part # HSP-9601
Microseal 'A' film	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001

Consumables for Tube Workflow

Consumable	Supplier
0.2 ml thin-wall PCR tubes	General lab supplier
1.5 ml microcentrifuge tubes	General lab supplier

Equipment

Equipment	Supplier
DNA Engine Multi-Bay Thermal Cycler See *Equipment* on page 36.	Bio-Rad, part # PTC-0240G or PTC-0220G, with Alpha Unit, part # ALS-1296GC
Microcentrifuge	General lab supplier
QuantiFluor dsDNA System or similar fluorometric-based DNA quantification system	Promega, catalog # E2670
Fluorometric quantification with dsDNA binding dye reagents	General lab supplier
SpectraMax M5 spectrofluorometer or similar fluorometric-based DNA quantification system	Molecular Devices, part # 0112-0159
Vortexer	General lab supplier
[Optional] Fragment Analyzer™	Advanced Analytical
[Optional] 2100 Bioanalyzer Desktop System	Agilent Technologies, part # G2940CA
[Optional] Vacuum concentrator Note: Use to concentrate a pooled library. Otherwise, use Amicon Ultra-0.5 centrifugal filter units.	General lab supplier

Equipment for Plate Workflow

Equipment	Supplier
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V) or • 14216-214 (230 V)
Magnetic Stand-96	Life Technologies, part # AM10027
Microheating System-SciGene TruTemp Heating System	Illumina, catalog # • SC-60-503 (115 V) or • SC-60-504 (220 V)
Microplate centrifuge	General lab supplier
Midi plate insert for microheating system	Illumina, catalog # BD-60-601
[Optional] TruSeq Index Plate Fixture Kit Note: Recommended for setting up indexed adapters. This part is reusable.	Illumina, catalog # FC-130-1005

Equipment for Tube Workflow

Equipment	Supplier
DynaMag-2 Magnet	Life Technologies, catalog # 12321D

Thermal Cyclers

The following table lists the recommended settings for the thermal cycler. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

Index Adapter Sequences

For information on index adapter sequences, see *Illumina Adapter Sequences (document # 1000000002694)* which provides information regarding the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.

DNA Quantification

Perform the QuantiFluor dsDNA assay to quantify dsDNA samples. The assay can quantify small DNA volumes and measure DNA directly. Other techniques can pick up contaminates, such as RNA and proteins. Use a spectrofluorometer for DNA-specific quantification. Spectrophotometry can also measure RNA and yield values that are too high.

Consumables

- 1X TE
- 96-well flat clear bottom black microplates (2)
- 96-well midi plates (2)
- Aluminum foil
- Conical centrifuge tube (15 ml or 50 ml)
- Lambda DNA
- Microseal 'B' adhesive seals
- QuantiFluor dsDNA dye
- RNase/DNase-free Reagent Reservoir

About Reagents

- QuantiFluor dsDNA dye often crystallizes at room temperature. Make sure that the dye is thawed and liquid.

Preparation

1 Remove the QuantiFluor dsDNA dye from to 2°C to 8°C and let stand at room temperature for 60 minutes in a light-impermeable container.

Procedure

Make Lambda DNA Stock Plate

1 Dilute lambda DNA in well A1 of a new midi plate to 1 ng/µl in a final volume of 300 µl. Pipette to mix.

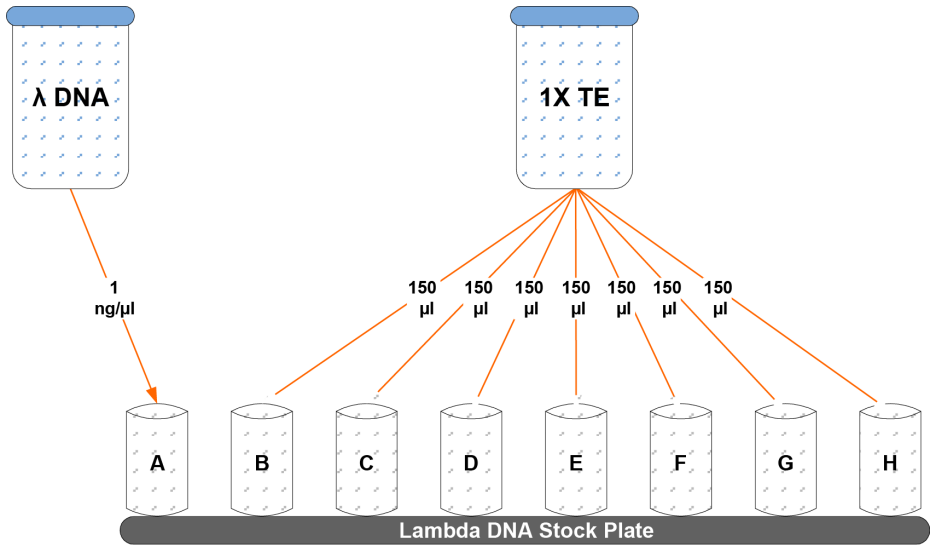
- Use the following formula to calculate the amount of lambda DNA to add to A1:

$$\frac{(300\ \mu l)\ X\ (1\ ng/\mu l)}{(\text{stock Lambda DNA concentration})} = \mu l \text{ of stock Lambda DNA to add to A1}$$

- Dilute DNA in well A1 using the following formula:
  (300 µl) - (µl of stock Lambda DNA in well A1) = µl of 1X TE to add to A1

2 Add 150 µl 1X TE to wells B, C, D, E, F, G, and H of column 1.

Figure 9 Dilution of Stock Lambda DNA Standard



3 Transfer 150 µl lambda DNA from well A1 to well B1. Pipette to mix.

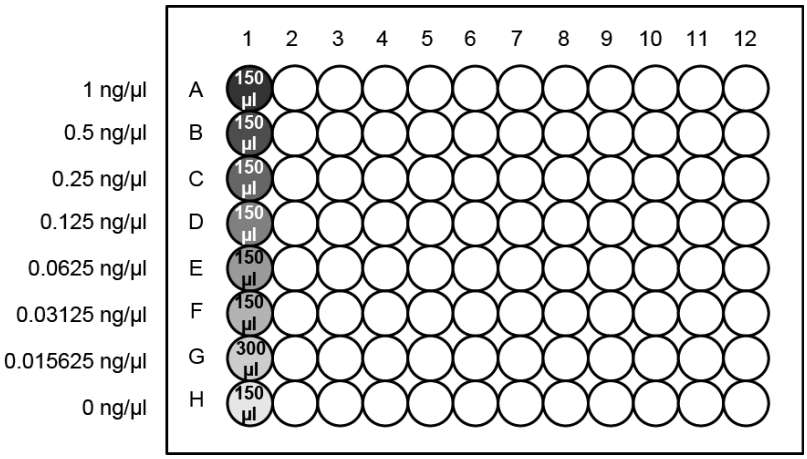
4 Transfer 150 µl from well B1 to well C1. Pipette to mix.

5 Repeat the transfer for wells D1, E1, F1, and G1, changing tips each time. Well H1 serves as the blank 0 ng/µl Lambda DNA.

Table 2 Concentrations of Lambda DNA

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
A1	1	150
B1	0.5	150
C1	0.25	150
D1	0.125	150
E1	0.0625	150
F1	0.03125	150
G1	0.015625	300
H1	0	150

Figure 10 Serial Dilutions of Lambda DNA



Make DNA Stock Plate

In a new midi plate, prepare the appropriate dilutions of your DNA samples using 1X TE. Measure each sample in triplicate. Make sure that at least 50 µl of diluted sample is prepared for quantification with the QuantiFluor dsDNA dye. Scale for replicate measurements.

1 Dilute using 1 of the following options, depending on the sample quality or library type:
   - **High-quality gDNA**—Dilute 1:1000. For example: 2 µl of gDNA + 1998 µl of 1X TE.
   - **Pre-enriched Nextera DNA Exome libraries**—Dilute 1:200. For example: 2 µl of library sample + 398 µl of 1X TE.
   - Post-enriched Nextera DNA Exome library dilution:
     - **1-plex, 3-plex, 6-plex, and 9-plex (8 reaction kits)**—Dilute 1:50. For example: 2 µl of postenriched library + 98 µl of 1X TE.
     - **12-plex**—Dilute 1:100. For example: 2 µl of postenriched library + 198 µl of 1X TE.
2 Shake at 1200 rpm for 1 minute.
3 Centrifuge at 280 × g for 1 minute

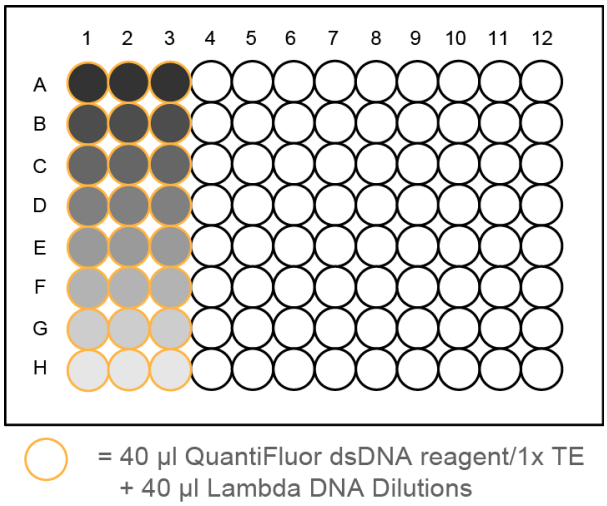
Dilute QuantiFluor dsDNA Dye

1 Prepare a 1:200 dilution of QuantiFluor dsDNA dye in 1X TE in a conical centrifuge tube wrapped in aluminum foil.
   Run each sample and standard in triplicate. For each measurement, 40 µl of diluted QuantiFluor dye is required. Scale as appropriate.
2 Vortex to mix.

Make Lambda DNA Quant Plate

1 Pour the diluted QuantiFluor dsDNA dye/1X TE into a new reagent reservoir.
2 Transfer 40 µl diluted QuantiFluor dsDNA dye/1X TE into each well of columns 1–3 of a new microplate.
3 Transfer 40 µl from each well of the lambda DNA stock plate to columns 1–3.

Figure 11 Lambda DNA Quant Plate with QuantiFluor dsDNA Dye/1X TE



4 Shake at 1200 rpm for 1 minute.

5 Centrifuge at 280 × g for 1 minute

6 Protect from light until read by the spectrofluorometer.

Make DNA Quant Plate

1 Transfer 40 µl QuantiFluor dsDNA reagent/1X TE dilution to each well of the microplate.

2 Transfer 40 µl DNA sample in the DNA stock plate to the microplate.

3 Shake at 1200 rpm for 1 minute.

4 Centrifuge at 280 × g for 1 minute

5 Protect from light until read by the spectrofluorometer.

Read Quant Plate

1 Measure fluorescence (485 nm Ex / 538 nm Em) of both the Lambda DNA quant and DNA quant plates according to the spectrofluorometer/software recommendations.

2 Calculate the DNA concentration of your unknown samples using the fluorescence values determined from step 1 as follows:

   a Calculate the average relative fluorescence units (RFU) of the Lambda DNA standards run in triplicate on the lambda DNA quant plate.
   b Calculate an Adjusted RFU by subtracting the RFU of the blank Lambda DNA standard (0 ng/µl) Row H from all unknown and standard samples.
   c Create a scatter plot of the lambda DNA standard curve values with the Adjusted RFU on the Y axis and DNA concentration (ng/µl) on the X axis.
   d Determine the equation of the line for the lambda DNA standard curve values, which is in the format of y = mx + b is equivalent to RFU = (slope*concentration) + y_int.
   e Calculate the concentration for each unknown sample by using the RFU for each sample for y in the equation and determining the value for x in ng/µl.
   f Multiply the resulting concentration by the appropriate dilution factor.

g Use the following formula to convert from ng/µl to nM.

$$\frac{(\text{concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{15\ \text{ng/µl}}{(660\ \text{g/mol} \times 400)} \times 10^6 = 57\ \text{nM}$$

Acronyms

Acronym	Definition
BLR	Blocker
CEX	Coding Exome Oligos
EAM	Enrichment Amplification Mix
EE1	Enrichment Elution Buffer 1
EEW	Enhanced Enrichment Wash Solution
EHB1	Enrichment Hybridization Buffer 1
EHB2	Enrichment Hybridization Buffer 2
ET2	Elute Target Buffer 2
HP3	2N NaOH
IEM	Illumina Experiment Manager
LAM	Library Amplification Mix
LRM	Local Run Manager
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SPB	Sample Purification Beads
ST	Stop Tagment Buffer
TD	Tagment DNA Buffer
TDE2	Tagment DNA Enzyme 2

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.635.9898	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

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