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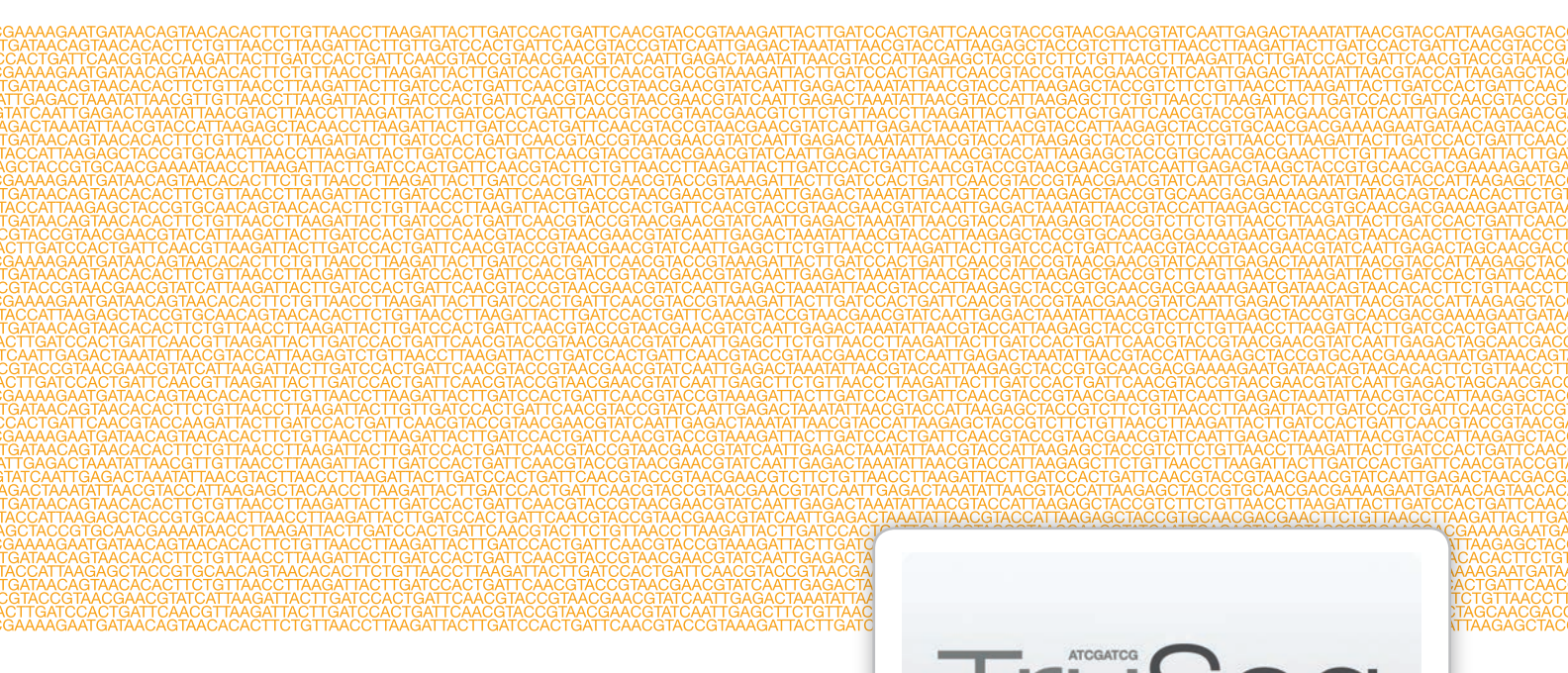
TruSeq Exome Library Prep Reference Guide



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Material # 20000408
Document # 15059911 v01
November 2015

Revision History

Document	Date	Description of Change

Material # 20000408
Document # 15059911 v01
	November 2015	• Renamed documentation to TruSeq Exome Library Prep
		• Removed content for TruSeq Expanded Exome Library Prep kits and oligos
		• Modified document hyperlinks from 'enrichment' to 'exome' in Additional Resources
		• Removed recommendation on number of enrichment libraries to process in a plate or tube
		• Change all shaking instructions to shake the plate at 1200 rpm
		• Added tube volume to thermal cycler program and removed volume from protocol
		• Add separate and additional instructions to centrifuge plate or tube throughout protocol
		• Add step to incubate on magnetic stand after wash and centrifuge throughout protocol
		• Add Microseal 'A' film to list of consumables for processes that require it
		• Remove step to centrifuge ERP3 before use
		• Added step to vortex SPB before use consistently throughout protocol
		• Change centrifuge ATL2 and STL, to centrifuge briefly before use
		• Separate plate and tube-specific steps in PCRNano thermal cycler program
		• Corrected RSB volume to 5 ml in shearing buffer premix to Normalize DNA
		• Amplify DNA Fragments:
		  • Changed volume on thermal cycler to 50 µl
		  • Changed to centrifuge briefly
		• Changed Quantify Libraries to measure in duplicate and use the average
		• Hybridize Probes:
		  • Removed Plate option
		  • Added step to the TE HYB thermal cycler program
		  • Removed single sample library option
		• Capture Hybridized Probes:
		  • Removed Plate option
		  • Removed initial First Bind centrifuge step
		  • Immediately transfer samples to tube containing SMB
		  • Removed 1.5 ml microcentrifuge tube as an option for the final transfer of supernatant in First Elution
		  • Removed safe stopping point

Document	Date	Description of Change

Material # 20000408
Document # 15059911 v01
	November 2015 (continued)	• Perform Second Hybridization:
		  • Removed Plate option
		  • Removed single sample library option
		  • Added step to the TE HYB program on the thermal cycler
		• Perform Second Capture:
		  • Removed Plate option until the end of Second Elution
		  • Removed initial First Bind centrifuge step
		  • Immediately transfer samples to tube containing SMB
		• Changed shaking the Plate to pipetting to Clean Up Captured Library
		• Changed shaking the Plate to pipetting to Amplify Enriched Library
		• Changed hold to 4°C in AMP8 thermal cycler program
		• Ethanol is not required to be 200 proof (absolute) and can be from a general lab supplier
		• DynaMag-2 Magnet required for both Plate and Tube workflows
		• Added minicentrifuge to equipment requirements
		• Added heat block to Tube equipment requirements
		• Changed magnetic stand-96 supplier to Thermo Fisher Scientific
		• Added Bio-Rad C1000 to thermal cyclers

Part # 15059911 Rev. A	June 2015	Initial release.

Table of Contents

Material # 20000408
Document # 15059911 v01

Overview

Chapter 1

Introduction

This TruSeq Exome Library Prep protocol explains how to prepare up to 96 indexed, paired-end libraries, followed by enrichment using reagents provided in an Illumina® TruSeq® Exome Library Prep 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 TruSeq Exome Library Prep protocol offers:

- Master-mixed reagents to reduce reagent containers and pipetting
- Optimized shearing for whole-genome resequencing with a 150 bp insert size
- Bead-based size selection
- Indexed adapters
  - The 8 × 3, 8 × 6, and 8 × 9 reaction kits contain adapter index tubes
  - The 8 × 12 kits contain a 96-well plate with 96 uniquely indexed adapter combinations designed for simultaneous manual or automated preparation of 96 dual-indexed DNA samples
- Advanced troubleshooting with process control checks built-in for quality control
- Compatible with single sample sequencing or lower indexing pooling levels

DNA Input Recommendations

For best results, follow the input recommendations. Use 100 ng input gDNA. Quantify the input gDNA and assess the gDNA quality before beginning library preparation.

Quantify Input DNA

Use the following recommendations to quantify input DNA:

- Successful library preparation depends on accurate quantification of input DNA. To verify results, use multiple methods.
- Use fluorometric-based methods for quantification, such as Qubit or PicoGreen.
- DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to the presence of excess nucleic acids.
- Do not use spectrophotometric-based methods, such as NanoDrop, which measure the presence of nucleotides and can result in an inaccurate measurement of gDNA.
- Quantification methods depend on accurate pipetting methods. Do not use pipettes at the extremes of volume specifications. Make sure that pipettes are calibrated.

Assess DNA Quality

Absorbance measurements at 260 nm are commonly used to assess DNA quality:

- The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. Values of 1.8–2.0 indicate relatively pure DNA.
- The presence of RNA or small nucleic acid fragments, such as nucleotides, can compromise both absorbance measurements.
- Make sure that samples are free of contaminants.

Positive Control

Use Coriell Institute gDNA (NA12878) as a positive control sample for this protocol.

Additional Resources

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

Resource	Description
TruSeq Exome Library Prep Protocol Guide (document # 15059912)	Provides only protocol instructions. The protocol guide is intended for experienced users.
TruSeq Exome Library Prep Checklist (document # 15059914)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
TruSeq Library Prep Pooling Guide (document # 15042173)	Provides TruSeq pooling guidelines for preparing libraries for Illumina sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.
Sequencing Library qPCR Quantification Guide (document # 11322363)	Describes a qPCR method for quantifying sequencing by synthesis (SBS) libraries generated using the Illumina library prep protocols.
Illumina Experiment Manager Guide (document # 15031335) and *IEM TruSeq DNA, RNA, or ChIP Quick Reference Card (document # 15037152)*	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
BaseSpace help (help.basespace.illumina.com)	Information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.

Visit the TruSeq Exome Library Prep Kits support page on the Illumina website for access to requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Material # 20000408
Document # 15059911 v01

Protocol

Chapter 2

Introduction

This chapter describes the TruSeq Exome Library Prep 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	> 24	≤ 24
Container	• 96-well Hard-Shell 0.3 ml PCR plates • 96-well midi plates • 1.5 ml microcentrifuge tubes • 8-tube strips	• 1.5 ml microcentrifuge tubes • 8-tube strips
Mixing method	• Microplate shaker • Pipette	• Pipette
Incubation Equipment	• Microheating systems • 96-well thermal cycler • Heat block	• Heat block • Thermal cycler

- Review best practices before proceeding. See *Additional Resources* on page 5 for information on how to access TruSeq Exome Library Prep 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 43.

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 effective for thermal cycling and easy to cut when using fewer than 96 wells.

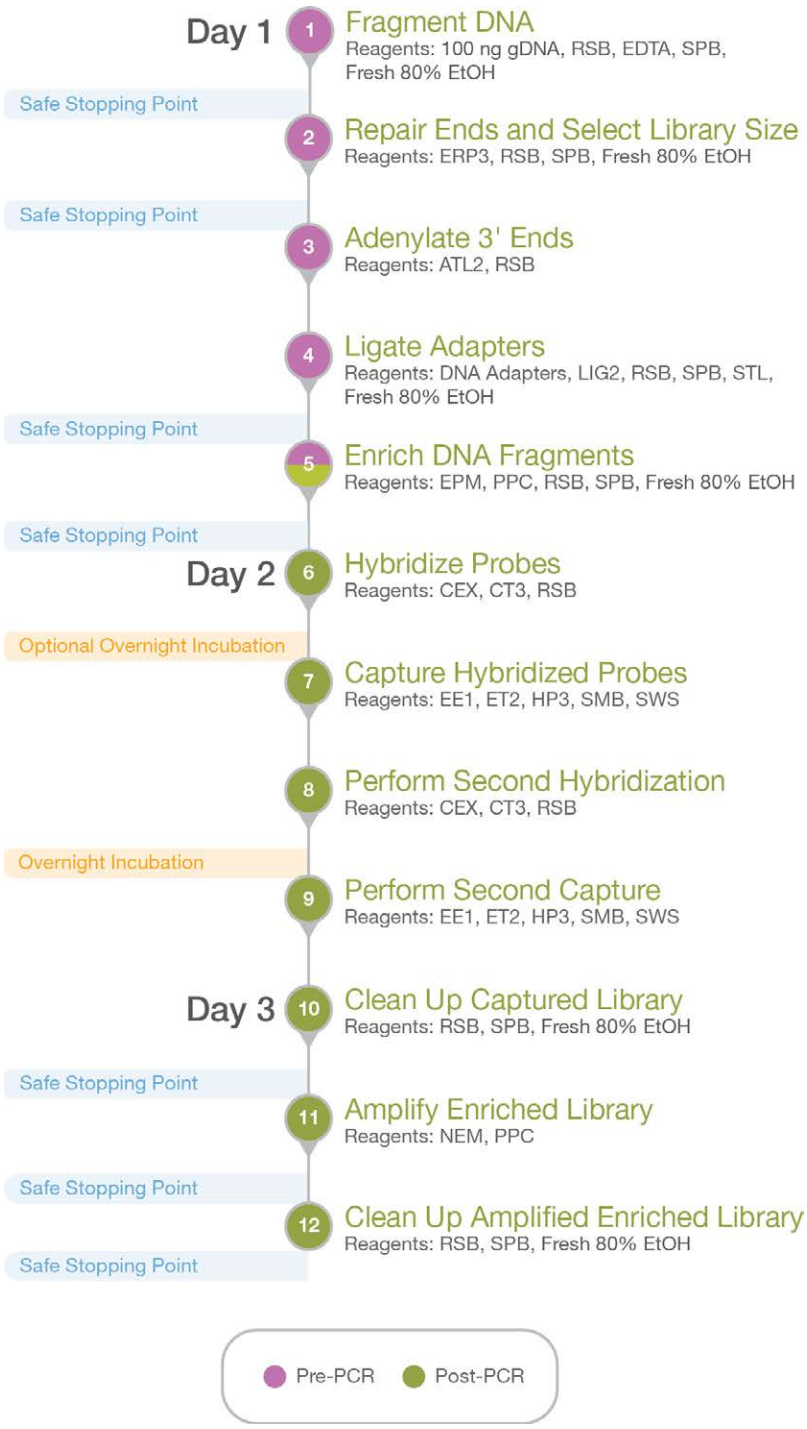
Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

Centrifugation

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

Library Prep Workflow



Material # 20000408
Document # 15059911 v01

Prepare for Pooling

If you are pooling, use IEM or BaseSpace to record information about your samples before beginning library prep.

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

Review the planning steps in the *TruSeq Library Prep Pooling Guide (document # 15042173)* when preparing libraries for Illumina sequencing systems that require balanced index combinations.

TruSeq Exome Library Prep kits support the following reactions and plexity. For more information on the kit configurations, see *Kit Contents* on page 45.

Samples	Enrichment Reactions	Plexity
24	8	3
48	8	6
72	8	9
96	8	12

Fragment DNA

This process describes how to optimally fragment gDNA to a 150 bp insert size. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs.

Consumables

- gDNA samples (100 ng per sample)
- EDTA
- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well midi plates (3)
  - [Tube] 1.5 ml microcentrifuge tubes and 8-tube strips
- Covaris tubes (1 per sample) or plate
- 15 ml conical tube
- [Plate] Microseal 'B' adhesive seal

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	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C after the initial thaw.
EDTA	-25°C to -15°C	Thaw at room temperature.
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.

2 Prepare fresh 80% ethanol.

3 Turn on and set up the Covaris instrument according to manufacturer guidelines.

4 [Plate] Calibrate the microplate shaker with a stroboscope and set it to 1200 rpm.

Procedure

Normalize gDNA

1 Quantify gDNA using a fluorometric-based method.

2 Create shearing buffer premix in a 15 ml conical tube.
- RSB (5 ml)
- EDTA (10 µl)

If the starting DNA sample concentration is below < 20 ng/µl, add more EDTA to make sure that the final concentration of EDTA is 1 mM in 50 µl of shearing buffer.

3 Normalize 100 ng gDNA samples with shearing buffer premix to a final volume of 50 µl, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

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

Fragment DNA

1 Transfer 50 µl DNA samples to separate Covaris tubes or plate wells.

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

3 Fragment the DNA using the following Covaris settings.

Covaris Setting	M220	S2	S220	E220	LE220
Duty Factor (%)	20	10	10	10	30
Intensity	—	5	—	—	—
Peak Power (W)	50	—	175	175	450
Cycles/Burst	200	200	200	200	200
Duration (seconds)	375	280	280	280	360/rack; 420/tube
Temperature (°C)	20	7	7	7	7
Water Level	—	12	12	6	6
Intensifier	—	—	—	Yes	—

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

5 Transfer 50 µl supernatant from each Covaris tube or plate well to a new midi plate or to a new 1.5 ml microcentrifuge tube or 8-tube strip.

Clean Up Fragmented DNA

1 Vortex SPB until well-dispersed.

2 Add 100 µl SPB to each well or to the tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [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 (~8 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 Centrifuge as follows.
   - [Plate] Centrifuge at 280 × g for 1 minute.
   - [Tube] Centrifuge briefly.

9 Incubate on the magnetic stand for 30 seconds.

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 Add 62.5 µl RSB to each well or to the tube.

13 Remove from the magnetic stand, and then mix thoroughly as follows.
   - [Plate] Shake at 1200 rpm for 2 minutes.
   - [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 60 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.

Repair Ends and Select Library Size

This process converts the overhangs resulting from fragmentation into blunt ends using ERP3 (End Repair Mix). The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. Following end repair, the library size is selected using SPB (Sample Purification Beads).

Consumables

- ERP3 (End Repair Mix)
- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [Plate] 96-well midi plates (2)
  - [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
ERP3	-25°C to -15°C	Thaw at room temperature, and then place on ice. Return to storage after use.
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% ethanol.

3 [Plate] Preheat the microheating system to 30°C.

4 [Tube] Save the following ERP program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 30 minutes
- Hold at 4°C
- Each tube contains 100 µl.

Procedure

Convert Overhangs

1 Add 40 µl ERP3 to each well or to the tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

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

3 Incubate as follows.
- [Plate] Place on the 30°C microheating system with the heated lid closed for 30 minutes, and then place on ice.
- [Tube] Place on the thermal cycler and run the ERP program.

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

Optimize Fragment Length

1 Vortex SPB until well-dispersed.

2 Add 90 µl SPB to each well or to the tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [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 Transfer 185 µl supernatant to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube.

7 Vortex SPB until well-dispersed.

8 Add 125 µl SPB to each well or to the tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

9 Incubate at room temperature for 5 minutes.

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

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

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

13 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.

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

15 Incubate on the magnetic stand for 30 seconds.

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

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

18 Add 20 µl RSB to each well or to the tube.

19 Remove from the magnetic stand, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

20 Incubate at room temperature for 2 minutes.

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

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

23 Transfer 17.5 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.

Adenylate 3′ Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to each other during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

- ATL2 (A Tailing Mix)
- RSB (Resuspension Buffer)
- [Plate] Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ATL2	-25°C to -15°C	Thaw at 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 2 microheating systems, one to 37°C and another to 70°C.

3 [Tube] Save the following ATAIL70 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 37°C for 30 minutes
- 70°C for 5 minutes
- 4°C for 5 minutes
- Hold at 4°C
- Each tube contains 30 µl.

Procedure

1 Centrifuge ATL2 briefly.

2 Add 12.5 µl ATL2 to each well or tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

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

4 [Plate] Incubate as follows.

a Place on the 37°C microheating system with the lid closed for 30 minutes.
b Move to the 70°C microheating system with the lid closed for 5 minutes.
c Place on ice for 5 minutes.

5 [Tube] Place on the thermal cycler and run the ATAIL70 program.

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

Ligate Adapters

This process ligates multiple indexing adapters to the ends of the DNA fragments, which prepares them for hybridization onto a flow cell.

Consumables

- DNA Adapters (tubes or DAP)
- LIG2 (Ligation Mix 2)
- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- STL (Stop Ligation Buffer)
- 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

- Do not remove LIG2 from storage until instructed to do so in the procedure.
- Return LIG2 to storage immediately after use.
- 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
DNA Adapters	-25°C to -15°C	Thaw at room temperature for 10 minutes. Return to storage after use. The DAP can undergo up to 4 freeze-thaw cycles.
STL	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
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% ethanol.

3 [Plate] Preheat a microheating system to 30°C.

4 [Tube] Save the following LIG program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 10 minutes
- Hold at 4°C
- Each tube contains 37.5 µl.

Procedure

Add Index Adapters

1 [96-sample kit only] Remove the tape seal from the DAP.

2 Centrifuge the DNA adapters as follows.

Reagent	Speed	Duration
Adapter tubes	N/A	5 seconds
DAP	280 × g	1 minute

3 [96-sample kit only] Remove the plastic cover from the DAP. Save the cover if you are not processing the entire plate.

4 Remove LIG2 from -25°C to -15°C storage.

5 Add the following reagents in the order listed to each well or to the tube.
- RSB (2.5 µl)
- LIG2 (2.5 µl)
- DNA adapters (2.5 µl)

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

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

8 Incubate as follows.
- [Plate] Place on the 30°C microheating system with the lid closed for 10 minutes, and then place on ice.
- [Tube] Place on the thermal cycler and run the LIG program.

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

10 Centrifuge STL briefly.

11 Add 5 µl STL to each well or to the tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

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

Clean Up Ligated Fragments

1 Vortex SPB until well-dispersed.

2 Perform steps 3 through 18 using the **Round 1** volumes.

3 Add SPB to each well or to the tube.

	Round 1	Round 2
SPB	42.5 µl	50 µl

4 Mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [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 Centrifuge as follows.
- [Plate] Centrifuge at 280 × g for 1 minute.
- [Tube] Centrifuge briefly.

11 Incubate on the magnetic stand for 30 seconds.

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

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

14 Add RSB to each well or to the tube.

	Round 1	Round 2
RSB	52.5 µl	27.5 µl

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

16 Incubate at room temperature for 2 minutes.

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

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

19 Transfer 50 µl supernatant to the corresponding well of a new midi plate or to a new 1.5 ml microcentrifuge tube or 8-tube strip.

20 Repeat steps 3 through 18 with the new plate or tube using the **Round 2** volumes.

21 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.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. PCR is performed with PPC (PCR Primer Cocktail) that anneals to the ends of the adapters. Minimize the number of PCR cycles to avoid skewing the representation of the library.

NOTE
Fragments with no adapters cannot hybridize to surface-bound primers in the flow cell. Fragments with an adapter on 1 end can hybridize to surface bound primers, but cannot form clusters.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- 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 or 8-tube strips
- [Plate] Microseal 'A' film
- [Plate] Microseal 'B' adhesive seals

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

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
PPC	-25°C to -15°C	Thaw at room temperature. Invert to mix, then centrifuge at 600 × g for 1 minute. Do not vortex. Return to storage after use.
EPM	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge at 600 × g for 1 minute. Do not vortex. Return to storage after use.
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% ethanol.

3 Save the following PCRNano program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 95°C for 3 minutes
   - 8 cycles of:
     - 98°C for 20 seconds
     - [Plate] 60°C for 20 seconds
     - [Tube] 60°C for 15 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 4°C
   - Each well or tube contains 50 µl.

Procedure

Amplify DNA Fragments

1 Place the plate or tube on ice and add 5 µl PPC to each well or to the tube.

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

3 Centrifuge briefly.

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

Clean Up Amplified DNA

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

2 Vortex SPB until well-dispersed

3 Add 35 µl SPB to each well or to the tube.

4 Mix thoroughly, as follows.
   - [Plate] Shake at 1200 rpm for 2 minutes.
   - [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 Transfer 82 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.

9 Vortex SPB until well-dispersed.

10 Add 82 µl SPB to each well or to the tube, and then mix thoroughly as follows.
   - [Plate] Shake at 1200 rpm for 2 minutes.
   - [Tube] Pipette up and down.

11 Incubate at room temperature for 5 minutes.

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

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

14 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.

15 Centrifuge briefly.

16 Incubate on the magnetic stand for 30 seconds.

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

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

19 Add 17.5 µl RSB to each well or to the tube, and then mix thoroughly as follows.
- [Plate] Shake at 1200 rpm for 2 minutes.
- [Tube] Pipette up and down.

20 Incubate at room temperature for 2 minutes.

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

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

23 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.

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.

Validate Libraries

Quantify Libraries

1 Quantify the libraries using the Qubit dsDNA HS Assay Kit.

   a Use 1 µl as the loading volume.
   b Use the dsDNA and high sensitivity settings.
   c Record STD1 and STD2 readings.
   d Measure the library concentration in duplicate and use the average of the 2 measurements.

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

Check Library Quality

1 Check the library size distribution on an Agilent Technologies 2100 Bioanalyzer:
   - If using a High Sensitivity DNA chip:
     - Dilute the DNA library 1:10 with RSB.
     - Run 1 µl diluted DNA library.
   - If using a DNA 1000 chip, run 1 µl undiluted DNA library.

It is normal to see some remaining adapter dimer (~150 bp) and a secondary peak, as a concatemer of the library (~550 bp–1000 bp).

Figure 1 Example 150 bp Insert Library Distribution (pre-enrichment) on a High Sensitivity DNA Chip at 1:10 Dilution

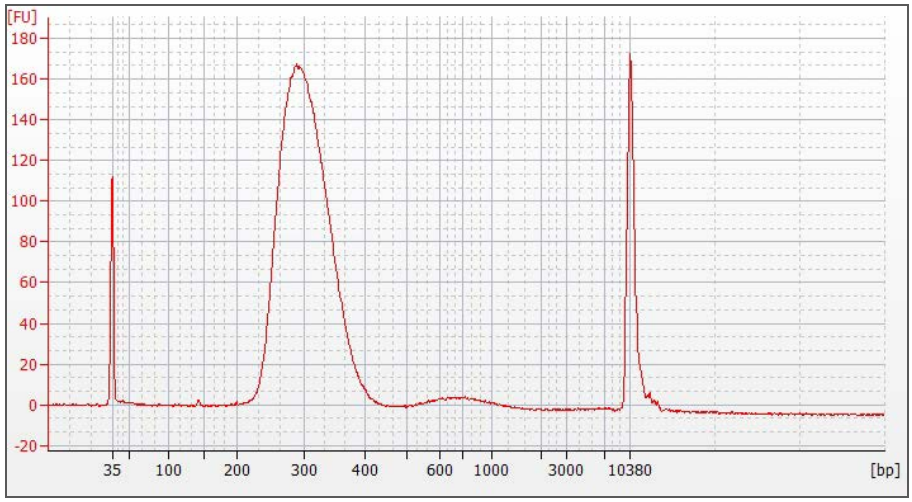
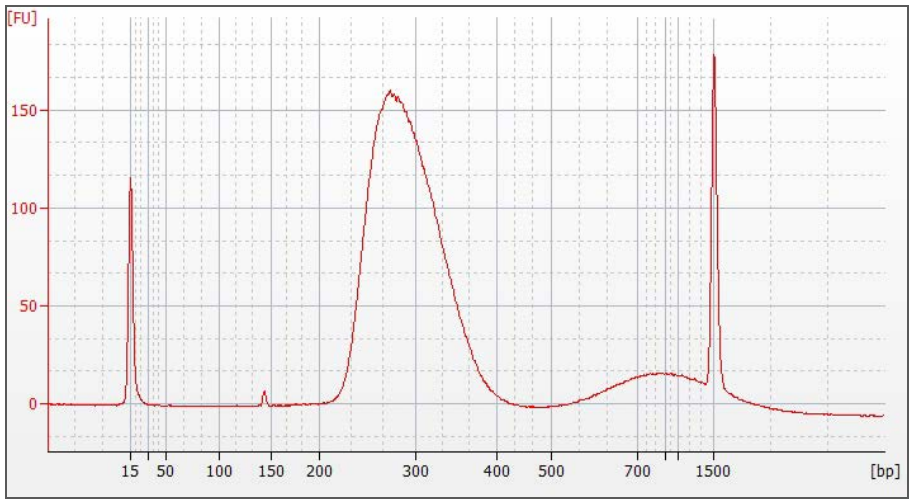


Figure 2 Example 150 bp Insert Library Distribution (pre-enrichment) on a DNA 1000 Chip Undiluted



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.

Consumables

- CEX (Coding Exome Oligos)
- CT3 (Capture Target Buffer 3)
- RSB (Resuspension Buffer)
- 1.5 ml microcentrifuge tubes and 8-tube strips
- [Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) (1 per pooled sample)

About Reagents

- Before using CT3, vortex to resuspend the thawed solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CEX	-25°C to -15°C	Thaw at room temperature.
CT3	-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.

2 Save the TE HYB program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 95°C for 10 minutes
- 18 cycles of 1 minute each, starting at 94°C, then decreasing 2°C per cycle
- 58°C for 90 minutes
- Hold at 58°C
- Each tube contains 100 µl.

NOTE
Incubate at the 58°C holding temperature for at least 90 minutes and up to a maximum of 24 hours.

Pool Libraries

1 Combine the following amount of each DNA library, making sure that each library has a unique index.

Plexity	Each Library	Total Pool
3-plex	250 ng	750 ng
6-plex	200 ng	1200 ng
9-plex	150 ng	1350 ng
12-plex	100 ng	1200 ng

- If the total volume is > 40 µl, use a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) to concentrate the pooled sample to 40 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 < 40 µl, increase the volume to 40 µl with RSB.

Procedure

1. Add the following reagents in the order listed to a new 8-tube strip. Pipette to mix.
   - DNA library pool (40 µl)
   - CT3 (50 µl)
   - CEX (10 µl)
2. Centrifuge briefly.
3. Place on the preprogrammed thermal cycler and run the TE HYB program.
4. Keep at the 58°C holding temperature for at least 90 minutes and up to 24 hours.

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)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- SWS (Streptavidin Wash Solution)
- 1.5 ml microcentrifuge tubes or 8-tube strips

About Reagents

- SWS can be cloudy after reaching room temperature.
- 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.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
SWS	-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 Preheat a heat block to 50°C.

Procedure

First Bind

1 Add 250 µl SMB to a new 1.5 ml microcentrifuge tube.

2 Immediately transfer the total sample volume (~100 µl) from the thermal cycler to the 1.5 ml microcentrifuge tube containing SMB. Pipette to mix.

NOTE
If you see a greater than 15% sample loss, do not proceed with the protocol. Poor sealing or insufficient heating of the lid can cause sample loss.

3 Incubate at room temperature for 25 minutes.

4 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 the tube.

7 Remove from the magnetic stand.

First Wash

1 Add 200 µl SWS to the tube. Pipette to mix.

2 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 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 the tube that contains the beads. Pipette to mix.

3 Incubate at room temperature for 2 minutes.

4 Centrifuge briefly.

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

6 Transfer 21 µl supernatant to a new 8-tube strip.

7 Add 4 µl ET2 to the tube. Pipette to mix.

8 Centrifuge briefly.

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

- CEX (Coding Exome Oligos)
- CT3 (Capture Target Buffer 3)
- RSB (Resuspension Buffer)

About Reagents

Before using CT3, vortex to resuspend the thawed solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CEX	-25°C to -15°C	Thaw at room temperature.
CT3	-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.

Procedure

1 Add the following reagents in the order listed to the 8-tube strip. Pipette to mix.
   - DNA library pool (25 µl)
   - RSB (15 µl)
   - CT3 (50 µl)
   - CEX (10 µl)

2 Centrifuge briefly.

3 Place on the preprogrammed thermal cycler and run the TE HYB program.

4 Keep at the 58°C holding temperature for at least 14.5 hours and up to 24 hours.

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)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- SWS (Streptavidin Wash Solution)
- 1.5 ml microcentrifuge tubes
- [Plate] 96-well Hard-Shell 0.3 ml PCR plate
- [Plate] Microseal 'B' adhesive seal

About Reagents

- SWS can be cloudy after reaching room temperature.
- 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.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
SWS	-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 Preheat a heat block to 50°C.

Procedure

Second Bind

1 Add 250 µl SMB to a new 1.5 ml microcentrifuge tube.

2 Immediately transfer the total sample volume (~100 µl) from the thermal cycler to the 1.5 ml microcentrifuge tube containing SMB. Pipette to mix.

NOTE
If you see a greater than 15% sample loss, do not proceed with the protocol. Poor sealing or insufficient heating of the lid can cause sample loss.

3 Incubate at room temperature for 25 minutes.

4 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 the tube.

7 Remove from the magnetic stand.

Second Wash

1 Add 200 µl SWS to the tube. Pipette to mix.

2 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 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 the tube that contains the beads. Pipette to mix.

3 Incubate at room temperature for 2 minutes.

4 Centrifuge briefly.

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

6 Transfer 21 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.

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 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% ethanol.

Procedure

1 Vortex SPB until well-dispersed.

2 Add 45 µl SPB to each well or tube. Pipette to mix.

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

9 Incubate on the magnetic stand for 30 seconds.

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 Add 27.5 µl RSB to each well or tube. Pipette to mix.

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 (2–5 minutes).

16 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 an 8-cycle PCR program to amplify the enriched library.

Consumables

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

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

Preparation

1 Prepare the following consumables.

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

2 Save the following AMP8 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 98°C for 30 seconds
- 8 cycles of:
  - 98°C for 10 seconds
  - [Plate] 60°C for 35 seconds
  - [Tube] 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°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 NEM to each well or tube. Pipette to mix.

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 AMP8 program.

SAFE STOPPING POINT

If you are stopping, seal the plate or cap 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 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% ethanol.

Procedure

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

2 Vortex SPB until well-dispersed.

3 Add 45 µl SPB to each well or tube. Pipette to mix.

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

10 Incubate on the magnetic stand for 30 seconds.

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

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

13 Add 22 µl RSB to each well or tube. Pipette to mix.

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 20 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.

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.

Validate Enriched Libraries

Perform the following procedures to quantify enriched libraries and check enriched library quality.

Quantify Libraries

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

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

   a Use 1 µl as the loading volume.
   b Use the dsDNA and high sensitivity settings.
   c Record STD1 and STD2 readings.
   d Measure the library concentration in duplicate and use the average of the 2 measurements.

2 Use the following formula to convert from ng/µl to nM. Assume a 300 bp library size or calculate based on the average size of the enriched library:

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

   For example:

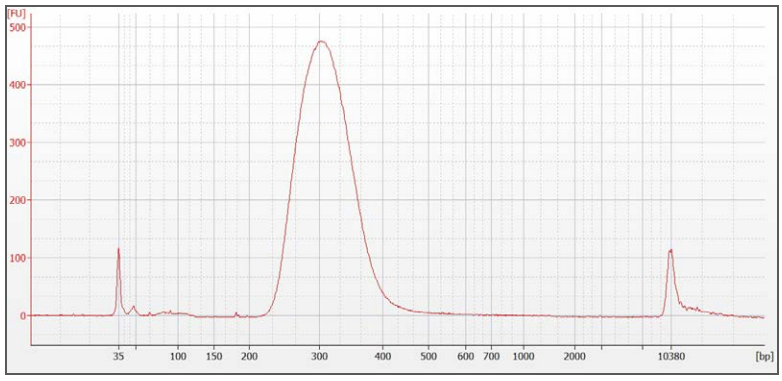
$$\frac{(4\ \text{ng/µl})}{(660\ \text{g/mol} * 300)} \times 10\text{^}6 = 20\ \text{nM}$$

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

Assess Quality

1 Run 1 µl of post enriched library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.
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 3 Example Post Enrichment (12-plex Enrichment) Library Distribution



Material # 20000408
Document # 15059911 v01

Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all the required consumables and equipment.

Material # 20000408
Document # 15059911 v01

Kit Contents

Make sure that you have all the reagents identified in this section before starting the protocol. The following kits are available.

Kit Name	Catalog #
TruSeq Exome Library Prep Kit (8 rxn × 3 plex)	FC-150-1001
TruSeq Exome Library Prep Kit (8 rxn × 6 plex)	FC-150-1002
TruSeq Exome Library Prep Kit (8 rxn × 9 plex)	FC-150-1003
TruSeq Exome Library Prep Kit (8 rxn × 12 plex)	FC-150-1004

24 Samples (8 rxn × 3 plex)

This kit contains 3 boxes of reagents to support 24 samples in 8 × 3-plex enrichment reactions.

-20°C Box, Store at -25°C to -15°C

Quantity	Reagent	Description
1	RSB	Resuspension Buffer
1	SWS	Streptavidin Wash Solution
1	ATL2	A Tailing Mix
1	LIG2	Ligation Mix 2
1	STL	Stop Ligation Buffer
1	PPC	PCR Primer Cocktail
1	EPM	Enhanced PCR Mix
1	ERP3	End Repair Mix
1	EDTA	EDTA 0.5 M
1	AD002	DNA Adapter Index 2
1	AD007	DNA Adapter Index 7
1	AD019	DNA Adapter Index 19
1	CT3	Capture Target Buffer 3
1	EE1	Enrichment Elution Buffer
1	HP3	HP3
3	NEM	Enrichment Amplification Mix

4°C Box, Store at 2°C to 8°C

Quantity	Reagent	Description
1	ET2	Elute Target Buffer 2
1	SPB	Sample Purification Beads
2	SMB	Streptavidin Magnetic Beads

Coding Exome Oligos (8 rxn), Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

48 Samples (8 rxn × 6 plex)

These kits contain 3 boxes of reagents to support 48 samples in 8 × 6-plex enrichment reactions.

-20°C Box, Store at -25°C to -15°C

Quantity	Reagent	Description
1	ATL2	A Tailing Mix
1	LIG2	Ligation Mix 2
1	STL	Stop Ligation Buffer
1	PPC	PCR Primer Cocktail
1	EPM	Enhanced PCR Mix
1	EDTA	EDTA 0.5 M
1	AD002	DNA Adapter Index 2
1	AD007	DNA Adapter Index 7
1	AD019	DNA Adapter Index 19
1	AD005	DNA Adapter Index 5
1	AD006	DNA Adapter Index 6
1	AD012	DNA Adapter Index 12
1	RSB	Resuspension Buffer
1	CT3	Capture Target Buffer 3
1	EE1	Enrichment Elution Buffer
1	HP3	HP3
3	NEM	Enrichment Amplification Mix
1	RSB	Resuspension Buffer
1	SWS	Streptavidin Wash Solution
1	ERP3	End Repair Mix

4°C Box, Store at 2°C to 8°C

Quantity	Reagent	Description
1	ET2	Elute Target Buffer 2
2	SPB	Sample Purification Beads
2	SMB	Streptavidin Magnetic Beads

Oligos (8 rxn), Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

72 Samples (8 rxn × 9 plex)

The kit contains 3 boxes of reagents to support 72 samples in 8 × 9-plex enrichment reactions.

-20°C Box, Store at -25°C to -15°C

Quantity	Reagent	Description
2	ATL2	A Tailing Mix
1	LIG2	Ligation Mix 2
1	STL	Stop Ligation Buffer
2	PPC	PCR Primer Cocktail
2	EPM	Enhanced PCR Mix
1	AD002	DNA Adapter Index 2
1	AD004	DNA Adapter Index 4
1	AD005	DNA Adapter Index 5
1	AD006	DNA Adapter Index 6
1	AD007	DNA Adapter Index 7
1	AD012	DNA Adapter Index 12
1	AD013	DNA Adapter Index 13
1	AD014	DNA Adapter Index 14
1	AD015	DNA Adapter Index 15
1	CT3	Capture Target Buffer 3
1	EE1	Enrichment Elution Buffer
1	HP3	HP3
3	NEM	Enrichment Amplification Mix
1	RSB	Resuspension Buffer
1	SWS	Streptavidin Wash Solution
2	ERP3	End Repair Mix
1	EDTA	EDTA 0.5 M

4°C Box, Store at 2°C to 8°C

Quantity	Reagent	Description
1	ET2	Elute Target Buffer 2
3	SPB	Sample Purification Beads
2	SMB	Streptavidin Magnetic Beads

Coding Exome Oligos (8 rxn), Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

96 Samples (8 rxn × 12 plex)

These kits contain 4 boxes of reagents to support 96 samples in 8 × 12-plex enrichment reactions.

-20°C Box, Store at -25°C to -15°C

Quantity	Reagent	Description
3	RSB	Resuspension Buffer
2	ERP3	End Repair Mix
2	ATL2	A Tailing Mix
1	LIG2	Ligation Mix 2

Quantity	Reagent	Description
1	STL	Stop Ligation Buffer
2	PPC	PCR Primer Cocktail
1	RSB	Resuspension Buffer
1	SWS	Streptavidin Wash Solution
2	EPM	Enhanced PCR Mix
1	EDTA	EDTA 0.5 M
1	CT3	Capture Target Buffer 3
1	EE1	Enrichment Elution Buffer
1	HP3	HP3
3	NEM	Enrichment Amplification Mix

4°C Box, Store at 2°C to 8°C

Quantity	Reagent	Description
1	ET2	Elute Target Buffer 2
4	SPB	Sample Purification Beads
2	SMB	Streptavidin Magnetic Beads

Oligos (8 rxn), Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

TruSeq Enrichment Adapters (96 adapters), Store at -25°C to -15°C

Quantity	Reagent	Description
1	DAP	DNA Adapter Plate, 96plex

Consumables and Equipment

Make sure that you have all necessary user-supplied consumables and equipment before starting the protocol. 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.

Consumables

Consumable	Supplier
1.5 ml microcentrifuge tubes	General lab supplier
15 ml conical tubes	General lab supplier
10 µl barrier pipette tips	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
One of the following: • High Sensitivity DNA Kit • DNA 1000 Kit	Agilent Technologies, part #: • 5067-4626 • 5067-1504
Ethanol for molecular biology (500 ml)	General lab supplier
One of the following types, depending on your Covaris system and sample number: • microTUBE AFA Fiber Snap-Cap • 8 microTUBE strip • 96 microTUBE plate • microTUBE AFA Fiber Screw-Cap • microTUBE AFA Fiber Crimp-Cap	Covaris, part # • 520045 • 520053 • 520078 • 520096 • 520052
PCR grade water	General lab supplier
Qubit dsDNA HS Assay Kit	Life Technologies, catalog # Q32851
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) Note: Used to concentrate a pooled library. Another option is to use a vacuum concentrator.	Millipore, part # UFC503008
[Optional] Fluorometric quantification with dsDNA binding dye reagents	General lab supplier

Consumables for Plate Workflow

Consumable	Supplier
96-well storage plates, round well, 0.8 ml ('midi' plate)	Fisher Scientific, part # AB-0859
96-well Hard-Shell 0.3 ml PCR plate	Bio-Rad, part # HSP-9601

Equipment

Equipment	Supplier/Description
2100 Bioanalyzer Desktop System	Agilent Technologies, part # G2940CA
One of the following Covaris systems: • S2 • S220 • E210 • E220 • M220 • LE220	Covaris M220, part # 500295 For all other models, contact Covaris
DynaMag-2 Magnet	Life Technologies, catalog # 12321D
Minicentrifuge	General lab supplier
Qubit 2.0 Fluorometer	Life Technologies, catalog # Q32866
SpectraMax M5 spectrofluorometer or similar fluorometric-based DNA quantification system	Molecular Devices, part # 0112-0159
Thermal cycler (with heated lid) See *Thermal Cyclers* on page 51.	General lab supplier
Vortexer	General lab supplier
[Optional] Fluorometer for quantification with dsDNA binding dyes	General lab supplier
[Optional] Vacuum concentrator Note: Used to concentrate a pooled library. Another option is to 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)

Equipment	Supplier
Magnetic stand-96	Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
One of the following: • SciGene TruTemp Heating System Note: Two systems are recommended to support successive heating procedures. • Heat block	• Illumina, catalog # • SC-60-503 (110 V) or • SC-60-504 (220 V) • General lab supplier
Midi plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier
SpectraMax M5 spectrofluorometer or similar fluorometric-based DNA quantification system	Molecular Devices, part # 0112-0159

Equipment for Tube Workflow

Equipment	Supplier
Heat Block	General lab supplier

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	Plate
MJ Research PTC-225 DNA Engine Tetrad	Calculated	Heated, constant at 100°C	Plate
Bio-Rad C1000	N/A	Heated, constant at 100°C	Plate
Bio-Rad S1000	N/A	Heated, constant at 100°C	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

Index Adapter Sequences

Index Adapter Tube Sequences

TruSeq Exome Library Prep 8 rxn × 3 plex, 8 rxn × 6 plex, and 8 rxn × 9 plex kits contain adapters in tubes with the following indexed adapter sequences.

- The sequence contains 7 bases. The seventh base, shown in parenthesis (), is not included in the Index Read. Record only the first 6 bases in a sample sheet. For indexes 13 and above, the seventh base (in parentheses) might not be A, which is seen in the cycle 7 of the Index Read.
- For more information on the number of cycles used to sequence the Index Read, see the system guide for your Illumina sequencing platform.

Table 2 TruSeq Exome Library Prep Index Adapter Sequences

Adapter	Sequence	8 × 3 Reaction Kit	8 × 6 Reaction Kits	8 × 9 Reaction Kits
AD002	CGATGT(A)	•	•	•
AD004	TGACCA(A)			•
AD005	ACAGTG(A)		•	•
AD006	GCCAAT(A)		•	•
AD007	CAGATC(A)	•	•	•
AD012	CTTGTA(A)		•	•
AD013	AGTCAA(C)			•
AD014	AGTTCC(G)			•
AD015	ATGTCA(G)			•
AD019	GTGAAA(C)	•	•	

Index Adapter Plate Sequences

The adapter plate contains the following index adapter sequences. The index adapter sequence recorded in the sample sheet contains 8 bases, and all 8 bases are sequenced during the Index Read.

Table 3 Index Adapter 1 Sequences

Adapter	Sequence	Adapter	Sequence
D701	ATTACTCG	D707	CTGAAGCT
D702	TCCGGAGA	D708	TAATGCGC
D703	CGCTCATT	D709	CGGCTATG
D704	GAGATTCC	D710	TCCGCGAA
D705	ATTCAGAA	D711	TCTCGCGC
D706	GAATTCGT	D712	AGCGATAG

Table 4 Index Adapter 2 Sequences

Adapter	Sequence	Adapter	Sequence
D501	TATAGCCT	D505	AGGCGAAG
D502	ATAGAGGC	D506	TAATCTTA
D503	CCTATCCT	D507	CAGGACGT
D504	GGCTCTGA	D508	GTACTGAC

Figure 4 Adapter Plate Dual-Index Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-D503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

Acronyms

Acronym	Definition
ATL2	A Tailing Mix
CEX	Coding Exome Oligos
CT3	Capture Target Buffer 3
DAP	DNA Adapter Plate
EE1	Enrichment Elution Buffer 1
EPM	Enhanced PCR Mix
ERP	End Repair Mix
ET2	Elute Target Buffer 2
HP3	2N NaOH
IEM	Illumina Experiment Manager
LIG	Ligation Mix
NEM	Enrichment Amplification Mix
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SPB	Sample Purification Beads
STL	Stop Ligation Buffer
SWS	Streptavidin Wash Solution

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 5 Illumina General Contact Information

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

Table 6 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	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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