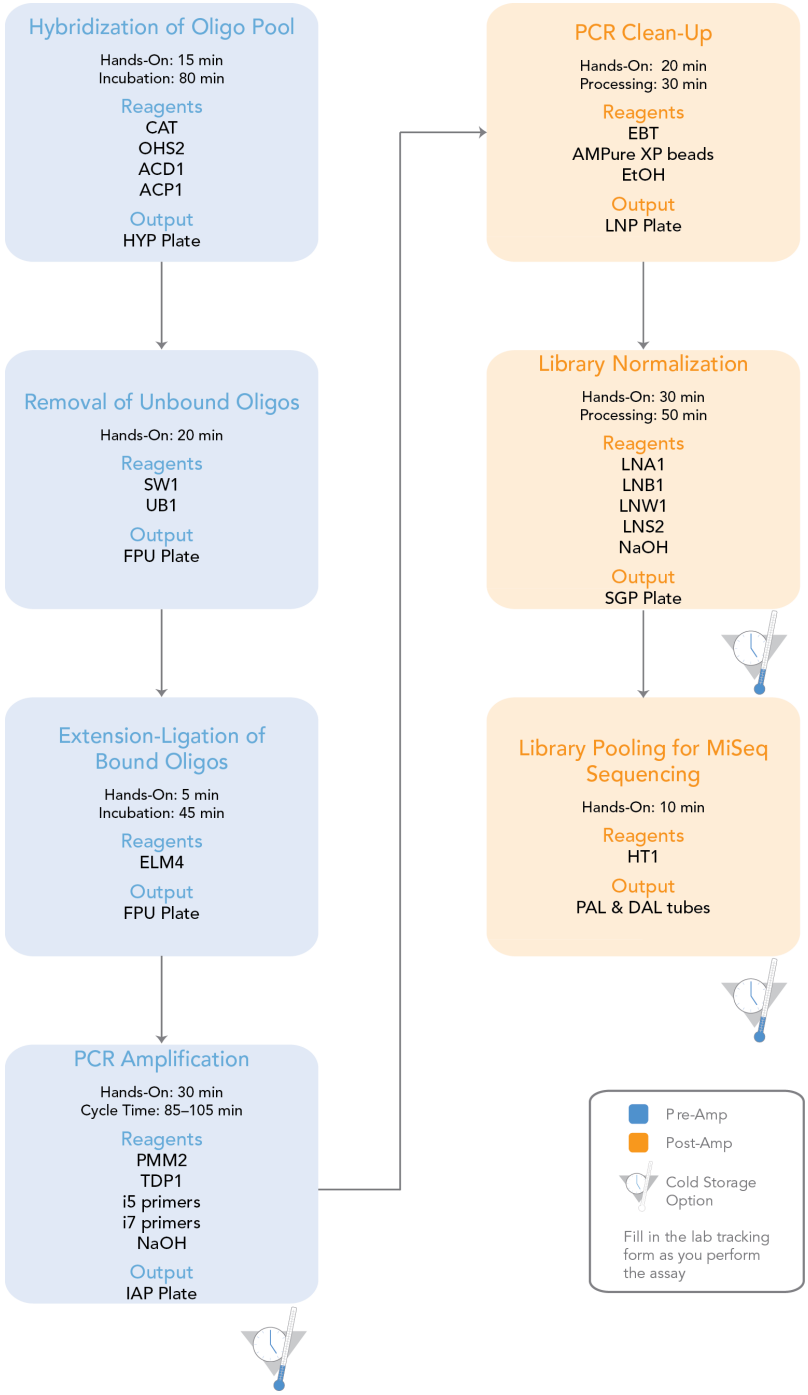
TruSeq Custom Amplicon Library Preparation

Experienced User Card

FOR RESEARCH USE ONLY

Date: _____ Description: _____

Illumina Kit Lot #: _____ _____



NOTE

Unless familiar with the protocol in the latest version of the *TruSeq Custom Amplicon Library Preparation Guide (part # 15027983)*, new or less experienced users are strongly advised to follow the protocol in the guide before using this Experienced User Card.

Experienced User Card

Date/Time: _____ Operator: _____

Consumables

Item	Lot Number
Amplicon Control DNA 1 (ACD1)	Lot #: _____
Amplicon Control Oligo Pool 1 (ACP1)	Lot #: _____
Oligo Hybridization for Sequencing Reagent 2 (OHS2)	Lot #: _____
Extension Ligation Mix 4 (ELM4)	Lot #: _____
PCR Master Mix 2 (PMM2)	Lot #: _____
TruSeq DNA Polymerase 1 (TDP1)	Lot #: _____
Stringent Wash 1 (SW1)	Lot #: _____
Universal Buffer 1 (UB1)	Lot #: _____
Library Normalization Wash 1 (LNW1)	Lot #: _____
Library Normalization Additives 1 (LNA1)	Lot #: _____
Library Normalization Storage Buffer 2 (LNS2)	Lot #: _____
Custom Amplicon oligo Tube (CAT)	Lot #: _____
Hybridization Buffer (HT1)	Lot #: _____
Elution Buffer with Tris (EBT)	Lot #: _____

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Index 1 Primers	Lot Number
Index Primer A701	Lot #: _____
Index Primer A702	Lot #: _____
Index Primer A703	Lot #: _____
Index Primer A704	Lot #: _____
Index Primer A705	Lot #: _____
Index Primer A706	Lot #: _____
Index Primer A707	Lot #: _____
Index Primer A708	Lot #: _____
Index Primer A709	Lot #: _____
Index Primer A710	Lot #: _____
Index Primer A711	Lot #: _____
Index Primer A712	Lot #: _____

Index 2 Primers	Lot Number
Index Primer A501	Lot #: _____
Index Primer A502	Lot #: _____
Index Primer A503	Lot #: _____
Index Primer A504	Lot #: _____
Index Primer A505	Lot #: _____
Index Primer A506	Lot #: _____
Index Primer A507	Lot #: _____
Index Primer A508	Lot #: _____

Consumables

Date/Time: _____ Operator: _____

Hybridization of Oligo Pool

During this step, a custom pool containing upstream and downstream oligos specific to your targeted regions of interest is hybridized to your genomic DNA samples.

Estimated Time

- Total duration: 1 hour 35 minutes
- Hands-on: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
CAT (Custom Amplicon Oligo Tube)	1 tube	-15°C to -25°C	Illumina
OHS2 (Oligo Hybridization for Sequencing 2)	1 tube	-15°C to -25°C	Illumina
ACD1	1 tube	-15°C to -25°C	Illumina
ACP1	1 tube	-15°C to -25°C	Illumina
Genomic DNA	As needed	-15°C to -25°C	User
96-well skirted PCR plate	1 plate		User
Adhesive aluminum foil seal	2 seals		User
Troughs	As needed		User

Preparation

[_] 1 Refer to the following table to qualify/quantitate DNA samples:
DNA Input Type (High Quality or FFPE): _____

Type of DNA	Supported Amplicon Size	Input (Up to 15 µl)	A260/A280	FFPE DNA QC
High-quality genomic DNA	150, 175, 250, 425 bp	50 ng (minimum) 250 ng (recommended)	1.8–2.0	Not required
FFPE genomic DNA	150 and 175 bp ONLY	250 ng (minimum)	1.8–2.0	Illumina FFPE QC Kit (WG-321-1001) Δ Cq ≤ 2.0

[_] 2 Record the CAT/DNA sample information and plate position as well as the plate position of ACP1/ACD1 control samples at the end of this document. Sample information and plate position is needed for the Illumina Experiment Manager.

Date/Time: _____ Operator: _____

[_] 3 Remove the CAT, OHS2, ACD1, ACP1, and genomic DNA from -15°C to -25°C storage and thaw at room temperature.

[_] 4 Set a 96-well heat block to 95°C.

[_] 5 Pre-heat an incubator to 37°C to prepare for the extension-ligation step.

[_] 6 Create your sample plate layout using the table on the last page or the Illumina Experiment Manager (Select TruSeq Amplicon as the Sample Prep Kit Type). Use the TruSeq Custom Amplicon Calculator for additional guidance. Record the amplicon size, number of amplicons, and the plate positions of each sample DNA/CAT, ACD1/ACP1 (TSCA_Control), and index primers. Illumina recommends processing samples in batches no smaller than 16.
Number of amplicons: _____
Amplicon Size: _____

Procedure

[_] 1 Apply the **HYP** (Hybridization Plate) barcode plate sticker to a new 96-well PCR plate.
Barcode: _____

[_] 2 Add 5 µl of control DNA ACD1 and 5 µl of TE or water to 1 well in the **HYP** plate for the assay control.

[_] 3 Add 10 µl of Genomic or FFPE DNA to each remaining well of the HYP plate to be used in the assay. For more dilute samples (that is, < 25 ng/µl) up to 15 µl of DNA can be used.
DNA stock concentration: _____
DNA volume used: _____

- Example Setup for High Quality Genomic DNA

Input	Volume	DNA Concentration
250 ng	10 µl	25 ng/µl
250 ng	up to 15 µl	≥ 16.7 ng/µl
50 ng	10 µl	5 ng/µl
50 ng	up to 15 µl	≥ 3.3 ng/µl

- Example Setup for FFPE Genomic DNA

Input	Volume	DNA Concentration
250 ng	10 µl	25 ng/µl
250 ng	up to 15 µl	≥ 16.7 ng/µl

[_] 4 Add 5 µl of control oligo pool ACP1 to the well containing control DNA ACD1.

[_] 5 Using a multichannel pipette, add 5 µl of CAT to the wells containing genomic DNA. Change tips after each column to avoid cross-contamination.

[_] 6 If samples are not sitting at the bottom of the well seal the **HYP** plate with adhesive aluminum foil and centrifuge to 1,000 × g at 20°C for 1 minute.

Experienced User Card

Date/Time: _____ Operator: _____

[_] 7 Using a multichannel pipette, add 35 µl of OHS2 to each sample in the **HYP** plate. When dispensing, gently pipette up and down 3–5 times to mix. Change tips after each column to avoid cross-contamination.

NOTE
Ensure any crystals or precipitate in OHS2 have dissolved.

[_] 8 Seal the **HYP** plate with adhesive aluminum foil and centrifuge to 1,000 × g at 20°C for 1 minute.

[_] 9 Place the **HYP** plate in the pre-heated block at 95°C and incubate for 1 minute.

Start time: _____ Stop time: _____

[_] 10 While the plate remains on the pre-heated block, set the temperature to 40°C and continue incubating for 80 minutes.

Start time: _____ Stop time: _____

Comments

_____

_____

Hybridization of Oligo Pool

Date/Time: _____ Operator: _____

Removal of Unbound Oligos

This process removes unbound oligos from genomic DNA using a filter capable of size selection. Two wash steps using SW1 ensure complete removal of unbound oligos. A third wash step using UB1 removes residual SW1 and prepares samples for the extension-ligation step.

Estimated Time

- Total duration: 20 minutes
- Hands-on: 20 minutes

Consumables

Item	Quantity	Storage	Supplied By
ELM4 (thawed in preparation for Extension-Ligation)	1 tube	-15°C to -25°C	Illumina
SW1 (Stringent Wash 1)	1 tube	2°C to 8°C	Illumina
UB1 (Universal Buffer 1)	1 tube	2°C to 8°C	Illumina
Filter plate with lid	1 plate		Illumina
Adapter collar (reusable)	1 plate		Illumina
MIDI plate	1 plate		User
Troughs	As needed		User

Preparation

[_] 1 Remove ELM4 from -15°C to -25°C storage and thaw at room temperature.

[_] 2 Remove SW1 and UB1 from 2°C to 8°C storage and set aside at room temperature.

[_] 3 Assemble the filter plate assembly unit in the order from top to bottom: Lid, Filter Plate, Adapter Collar, and MIDI plate. Apply the **FPU** (Filter Plate Unit) barcode plate sticker.

[_] 4 Pre-wash the **FPU** plate membrane as follows:

- [_] a Using a multichannel pipette, add 45 µl of SW1 to each well.
- [_] b Cover the **FPU** plate with the filter plate lid and keep it covered during each centrifugation step.
- [_] c Centrifuge the **FPU** at 2,400 × g at 20°C for 10 minutes.

Start time: _____ Stop time: _____

[_] 5 After the pre-wash step, if there is a significant amount (>15 µl/well) of residual buffer in multiple wells (≥10 wells/plate) switch to a fresh filter plate.

Procedure

[_] 1 After the 80 minute incubation, confirm that the heat block has cooled to 40°C. While the **HYP** plate is still in the heat block, reinforce the seal using a rubber roller or sealing wedge.

[_] 2 Remove the **HYP** plate from the heat block and centrifuge to 1,000 × g at 20°C for 1 minute to collect condensation.

[_] 3 Using a multichannel pipette set to 65 µl, transfer the entire volume of each sample onto the center of the corresponding pre-washed wells of the **FPU** plate. Change tips after each column to avoid cross-contamination.

[_] 4 Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 × g at 20°C for 2 minutes.

[_] 5 Wash the **FPU** plate as follows:

[_] a Using a multichannel pipette, add 45 µl of SW1 to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.

[_] b Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 × g for 2 minutes.

NOTE
If the SW1 does not drain completely after 2 minutes, the plate can be centrifuged again for up to 10 minutes. Significantly incomplete drainage of SW1 compromises target enrichment specificity.

[_] 6 Repeat the wash as follows:

[_] a Using a multichannel pipette, add 45 µl of SW1 to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.

[_] b Cover the **FPU** plate with the filter plate lid and centrifuge to 2,400 × g for 2 minutes.

NOTE
If the SW1 does not drain completely after 2 minutes, the plate can be centrifuged again for up to 10 minutes. Significantly incomplete drainage of SW1 compromises target enrichment specificity.

[_] 7 Discard all the flow-through (containing formamide waste and unbound oligos) collected up to this point in an appropriate hazardous waste container, then reassemble the **FPU**. The same MIDI plate can be reused for the rest of the pre-amplification process.

[_] 8 Using a multichannel pipette add 45 µl of UB1 to each sample well.

[_] 9 Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 × g for 2 minutes.

Comments

_____

_____

Date/Time: _____ Operator: _____

Extension-Ligation of Bound Oligos

This process connects the hybridized upstream and downstream oligos. A DNA polymerase extends from the upstream oligo through the targeted region, followed by ligation to the 5′ end of the downstream oligo using a DNA ligase. The extension-ligation results in the formation of products containing the targeted regions of interest flanked by sequences required for amplification.

Estimated Time

- Total duration: 50 minutes
- Hands-on: 5 minutes

Consumables

Item	Quantity	Storage	Supplied By
ELM4 (Extension-Ligation Mix 4)	1 tube	-15°C to -25°C	Illumina
Adhesive aluminum foil seal	1 seal		User
Troughs	As needed		User

Procedure

[_] 1 Using a multichannel pipette, add 45 µl of ELM4 to each sample well of the **FPU** plate.

[_] 2 Seal the **FPU** plate with adhesive aluminum foil, and then cover with the lid to secure the foil during incubation.

[_] 3 Incubate the entire **FPU** assembly in the pre-heated 37°C incubator for 45 minutes.

Start time: _____ Stop time: _____

[_] 4 While the **FPU** plate is incubating, prepare the **IAP** (Indexed Amplification Plate) as described in the following section.

Comments

_____

_____

Extension-Ligation of Bound Oligos

Date/Time: _____ Operator: _____

PCR Amplification

In this step, the extension-ligation products are amplified using primers that add sample multiplexing index sequences (i5 and i7) as well as common adapters required for cluster generation (P5 and P7).

Estimated Time

- Total duration: 85–105 minutes (depending on the number of PCR cycles used)
- Hands-on: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
PMM2 (PCR Master Mix 2)	1 tube	-15°C to -25°C	Illumina
i5 primers (A5XX)	1 tube per primer	-15°C to -25°C	Illumina
i7 primers (A7XX)	1 tube per primer	-15°C to -25°C	Illumina
TDP1 (TruSeq DNA Polymerase 1)	1 tube	-15°C to -25°C	Illumina
Microseal 'A' adhesive film	1		User
50 mM NaOH (less than one week old; prepared from 10 N NaOH) Date Prepared: _____	3.5 ml for 96 samples		User
96-well skirted PCR plate	1 plate		User
Troughs	As needed		User

Preparation

[_] 1 Record the amplicon size and number of amplicons in your CAT.
This information is used to determine PCR cycling conditions for your assay, and can be found either in DesignStudio or your manifest file.
Number of amplicons: _____
Amplicon Size: _____

[_] 2 Prepare fresh 50 mM NaOH.

[_] 3 Determine the index primers to be used in the assay using the Illumina Experiment Manager and the TruSeq Custom Amplicon Calculator. Record index primer positions on the last page.

[_] 4 Remove PMM2 and the index primers (i5 and i7) from -15°C to -25°C storage and thaw on a bench at room temperature.
Vortex each tube to mix and briefly centrifuge the tubes in a microcentrifuge.

Date/Time: _____ Operator: _____

[_] 5 Arrange i5 primer tubes (white caps, clear solution) vertically in a rack, aligned with rows A through H.

[_] 6 Arrange i7 primer tubes (orange caps, yellow solution) horizontally in a rack, aligned with columns 1 through 12.

[_] 7 Apply the **IAP** (Indexed Amplification Plate) barcode plate sticker to a new 96-well PCR plate.
Barcode: _____

[_] 8 Using a multichannel pipette, add 4 µl of i5 primers (clear solution) to each column of the **IAP** plate.

[_] 9 To avoid index cross-contamination, discard the original *white* caps and apply new *white* caps provided in the TruSeq Custom Amplicon Index Kit.

[_] 10 Using a multichannel pipette, add 4 µl of i7 primers (yellow solution) to each row of the **IAP** plate. ***Tips must be changed after each row to avoid index cross-contamination.***

[_] 11 To avoid index cross-contamination, discard the original *orange* caps and apply new *orange* caps provided in the TruSeq Custom Amplicon Index Kit.

[_] 12 For 96 samples, add 56 µl of TDP1 to 2.8 ml of PMM2 (1 full tube). Invert the PMM2/TDP1 PCR master mix 20 times to mix well. You will add this mix to the **IAP** plate in the next section.
Number of samples: _____
PMM volume: _____
TDP1 volume: _____

Procedure

[_] 1 When the 45 minute extension-ligation reaction is complete, remove the **FPU** from the incubator. Remove the aluminum foil seal and replace with the filter plate lid.

[_] 2 Centrifuge the **FPU** at 2,400 × g for 2 minutes.

[_] 3 Using a multichannel pipette, add 25 µl of 50 mM NaOH to each sample well on the **FPU** plate. Ensuring that pipette tips come in contact with the membrane, pipette the NaOH up and down 5–6 times. Tips must be changed after each column.

[_] 4 Incubate the **FPU** plate at room temperature for 5 minutes.
Start time: _____ Stop time: _____

[_] 5 While the **FPU** plate is incubating, use a multichannel pipette to transfer 22 µl of the PMM2/TDP1 PCR master mix to each well of the **IAP** plate containing index primers. Change tips between samples.

[_] 6 Transfer samples eluted from the **FPU** plate to the **IAP** plate as follows:

- [_] a Set a multichannel P20 pipette to 20 µl.
- [_] b Using fine tips, pipette the NaOH in the first column of the **FPU** plate up and down 5–6 times. Then transfer 20 µl from the **FPU** plate to the corresponding column of the **IAP** plate. Gently pipette up and down 5–6 times to combine the DNA with the PCR master mix.

NOTE
Slightly tilt the **FPU** plate to ensure complete aspiration and to avoid air bubbles.

[_] c Transfer the remaining columns from the **FPU** plate to the **IAP** plate in a similar manner. Tips must be changed after each column to avoid index and sample cross-contamination.

[_] d After all the samples have been transferred, the waste collection MIDI plate of the **FPU** can be discarded. Put the metal adapter collar away for future use. If only a partial **FPU** plate is used, clearly mark which wells have been used. Store the **FPU** plate and lid in a sealed plastic bag to avoid contamination of the filter membrane.

[_] 7 Cover the **IAP** plate with Microseal 'A' film and seal with a rubber roller.

[_] 8 Centrifuge to 1,000 × g at 20°C for 1 minute.

[_] 9 Transfer the **IAP** plate to the post-amplification area.

[_] 10 Perform PCR on a thermal cycler using the following program and the recommended number (X) of PCR cycles. The following tables contain amplicon size, number of amplicons in your CAT, type of DNA input, and DNA input quantity to help you calculate the number of PCR cycles required.

NOTE
The ACD1/ACP1 control can be processed using the same conditions as your CAT.

Table 1 100–250 ng

Amplicon Size	150/175 bp		250 bp	425 bp
DNA Input	High Quality	FFPE	High Quality	High Quality
	Number of PCR Cycles (X)			
<96 amplicons	29	31	30	30
97–384 amplicons	25	27	25	26
385–768 amplicons	23	25	24	25
769–1,536 amplicons	22	24	23	24

Table 2 50–99 ng

Amplicon Size	150/175 bp	250 bp	425 bp
DNA Input	High Quality	High Quality	High Quality
	Number of PCR Cycles (X)		
<96 amplicons	32	33	33
97–384 amplicons	28	28	29
385–768 amplicons	26	27	28
769–1,536 amplicons	25	26	27

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- 95°C for 3 minutes
- X cycles of:
  - 95°C for 30 seconds
  - 66°C for 30 seconds
  - 72°C for 60 seconds
- 72°C for 5 minutes
- Hold at 10°C

PCR machine: _____

Number of PCR cycles: _____

Start time: _____ Stop time: _____

SAFE STOPPING POINT

If you do not plan to proceed to *PCR Clean-Up on page 17* immediately, the plate can remain on the thermal cycler overnight. You can also store it at 2°C to 8°C up to two days. If storing at 2°C to 8°C, replace Microseal 'A' with Microseal 'B'

Comments

_____

_____

Date/Time: _____ Operator: _____

PCR Clean-Up

This process uses AMPure XP beads to purify the PCR products from the other reaction components.

Estimated Time

- Total duration: 50 minutes
- Hands-on: 20 minutes

Consumables

Item	Quantity	Storage	Supplied By
EBT (Elution Buffer with Tris)	1 tube	Room temperature	Illumina
AMPure XP beads	As needed	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH) Date Prepared: _____	40 ml per 96 samples	Room temperature	User
96-well MIDI plates	2		User
Microseal 'B' adhesive film	As needed		User
Troughs	As needed		User

Preparation

[_] 1 Bring the AMPure XP beads to room temperature.

[_] 2 Prepare fresh 80% ethanol from absolute ethanol.

Procedure

[_] 1 Centrifuge the **IAP** plate at 1,000 × g at 20°C for 1 minute to collect condensation.

[_] 2 To confirm that the library successfully amplified, run an aliquot of the control and selected test samples on a 4% agarose (5 µl) or on a Bioanalyzer (1 µl). The expected PCR product sizes for each amplicon length are indicated in the following table.

Amplicon Size	Expected PCR Product Size
150 bp	~280 bp
175 bp	~310 bp
250 bp	~350 bp
425 bp	~570 bp

[_] 3 Apply the **CLP** (Clean-up Plate) barcode plate sticker to a new MIDI plate.

Date/Time: _____ Operator: _____

Barcode: _____

[_] 4 Using a multichannel pipette, add the appropriate volume of AMPure XP beads indicated in the following table, corresponding to your amplicon size, to each well of the **CLP** plate.

NOTE
The ACD1/ACP1 control can be processed using the same conditions as your CAT.

Amplicon Size	µl AMPure XP beads
150 bp	60 µl
175 bp	60 µl
250 bp	45 µl
425 bp	35 µl

AMPure XP Beads volume: _____

[_] 5 Using a multichannel pipette set to 60 µl, transfer the entire PCR product from the **IAP** plate to the **CLP** plate. Change tips between samples.

[_] 6 Seal the **CLP** plate with a Microseal 'B' adhesive seal.

[_] 7 Shake the **CLP** plate on a microplate shaker at 1,800 rpm for 2 minutes.
Start time: _____ Stop time: _____

[_] 8 Incubate at room temperature without shaking for 10 minutes.
Start time: _____ Stop time: _____

[_] 9 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
Start time: _____ Stop time: _____

[_] 10 Using a multichannel pipette set to 100 µl and with the **CLP** plate on the magnetic stand, carefully remove and discard the supernatant. Change tips between samples.

[_] 11 With the **CLP** plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

- [_] a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well. Changing tips is not required if you use care to avoid cross-contamination. You do not need to resuspend the beads currently.
- [_] b Incubate the plate on the magnetic stand for 30 seconds or until the supernatant appears clear.
- [_] c Carefully remove and discard the supernatant.

[_] 12 Repeat the 80% ethanol wash described in the previous step.
Use a P20 multichannel pipette to remove excess ethanol.

[_] 13 Use a P20 multichannel pipette set to 20 µl to remove excess ethanol.

[_] 14 Remove the **CLP** plate from the magnetic stand and allow the beads to air-dry for 10 minutes.
Start time: _____ Stop time: _____

[_] 15 Using a multichannel pipette, add 30 µl of EBT to each well of the **CLP** plate.

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[_] 16 Seal the **CLP** plate with Microseal 'B' and shake on a microplate shaker at 1,800 rpm for 2 minutes. After shaking, if any samples are not resuspended, gently pipette up and down or lightly tap the plate on the bench to mix, then repeat this step.

Start time: _____ Stop time: _____

[_] 17 Incubate at room temperature without shaking for 2 minutes.

Start time: _____ Stop time: _____

[_] 18 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

Start time: _____ Stop time: _____

[_] 19 Apply the **LNP** (Library Normalization Plate) barcode plate sticker to a new MIDI plate.
Barcode: _____

[_] 20 Carefully transfer 20 µl of the supernatant from the **CLP** plate to the **LNP** plate. Change tips between samples.

[_] 21 Seal the **LNP** plate with Microseal 'B' and then centrifuge to 1,000 × g for 1 minute.

Comments

_____
_____

PCR Clean-Up

Date/Time: _____ Operator: _____

Library Normalization

This process normalizes the quantity of each library to ensure more equal library representation in your pooled sample.

Estimated Time

- Total duration: 1 hour 20 minutes
- Hands-on: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
LNA1 (Library Normalization Additives 1)	1 tube	-15°C to -25°C	Illumina
LNB1 (Library Normalization Beads 1)	1 tube	2°C to 8°C	Illumina
LNW1 (Library Normalization Wash 1)	2 tubes	2°C to 8°C	Illumina
LNS2 (Library Normalization Storage buffer 2)	1 tube	Room temperature	Illumina
0.1 N NaOH (less than one week old) Date Prepared: _____	3 ml per 96 samples		User
96-well skirted PCR plate	1 plate		User
15 ml conical tube	1 tube		User
Microseal 'B' adhesive film	As needed		User

Preparation

[_] 1 Prepare fresh 0.1N NaOH.

[_] 2 Remove LNA1 from -15°C to -25°C storage and bring to room temperature. Use a 20°C to 25°C water bath as needed. When at room temperature, vortex vigorously and make sure that all precipitates have dissolved.

[_] 3 Remove LNB1 and LNW1 from 2°C to 8°C storage and bring to room temperature.

[_] 4 Vigorously vortex LNB1 for at least 1 minute with intermittent inversion until the beads are well-resuspended and no pellet is found at the bottom of the tube when the tube is inverted.

Date/Time: _____ Operator: _____

Procedure

[_] 1 For 96 samples, add 4.4 ml of LNA1 to a fresh 15 ml conical tube.
Number of samples: _____
LNA1 volume: _____
LNB1 volume: _____

[_] 2 Use a P1000 pipette set to 1000 µl to resuspend LNB1 thoroughly by pipetting up and down 15–20 times, until the bead pellet at the bottom is resuspended.

NOTE
It is critical to completely resuspend the LNB1 bead pellet at the bottom of the tube. The use of a P1000 ensures that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. Resuspension is essential for achieving consistent cluster density on the flow cell.

[_] 3 Immediately after LNB1 is thoroughly resuspended, use a P1000 pipette to transfer 800 µl of LNB1 to the 15 ml conical tube containing LNA1. Mix well by inverting the tube 15–20 times. The resulting LNA1/LNB1 bead mix is enough for 96 samples. Pour the bead mix into a trough and use it immediately in the next step.

NOTE
If you do not plan to use full tubes for 96 samples, a P1000 set to 1000 µl is required to resuspend the beads completely in step 2. Mix only the required amounts of LNA1 and LNB1 for the current experiment. Never use a P200 pipette to handle LNB1. Store the remaining LNA1 and LNB1 separately at their respective recommended temperatures. To preserve stability, never freeze LNB1 beads or mix with LNA1 if not used immediately.

[_] 4 Add 45 µl of the combined LNA1/LNB1 to each well of the **LNP** plate containing libraries.

[_] 5 Seal the **LNP** plate with a Microseal 'B' adhesive seal and shake on a microplate shaker at 1,800 rpm for 30 minutes.
Start time: _____ Stop time: _____

[_] 6 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
Start time: _____ Stop time: _____

[_] 7 With the **LNP** plate on the magnetic stand, use a multichannel pipette set to 80 µl to remove the supernatant and then discard in an appropriate hazardous waste container.

[_] 8 Remove the **LNP** plate from the magnetic stand and wash the beads with LNW1, as follows:

- [_] a Using a multichannel pipette, add 45 µl of LNW1 to each sample well.
- [_] b Seal the **LNP** plate with a Microseal 'B' adhesive seal.
- [_] c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
  Start time: _____ Stop time: _____
- [_] d Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
  Start time: _____ Stop time: _____
- [_] e Carefully remove and discard the supernatant in an appropriate hazardous waste container.

[_] 9 Repeat the LNW1 wash described in the previous step. Use a P20 multichannel pipette to remove excess LNW1.

Date/Time: _____ Operator: _____

NOTE
Using a P20 multichannel to remove residual LNW1 is important to avoid reagent carryover into the storage buffer, and to reduce volume variability, which would affect library normalization.

[_] 10 Remove the **LNP** plate from the magnetic stand and add 30 µl of 0.1 N NaOH (less than a week old) to each well to elute the sample.

[_] 11 Seal the **LNP** plate with a Microseal 'B' adhesive seal and shake on a microplate shaker at 1,800 rpm for 5 minutes.

Start time: _____ Stop time: _____

[_] 12 During the 5 minute elution, apply the **SGP** (Storage Plate) barcode plate sticker to a new 96-well PCR plate.

Barcode: _____

[_] 13 Add 30 µl LNS2 to each well to be used in the **SGP** plate.

[_] 14 After the 5 minute elution, make sure that all samples in the **LNP** plate are resuspended completely. If the samples are not resuspended, gently pipette up and down or lightly tap the plate on the bench to resuspend the beads. Then shake for another 5 minutes.

[_] 15 Place the **LNP** plate on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 16 Using a multichannel pipette set to 30 µl, transfer the supernatant from the **LNP** plate to the **SGP** plate. Change tips between samples to avoid cross-contamination.

[_] 17 Seal the **SGP** plate with Microseal 'B' and then centrifuge to 1,000 × g for 1 minute.

SAFE STOPPING POINT
If you do not plan to proceed to *Library Pooling and MiSeq Sample Loading* and subsequent sequencing on the MiSeq, store the sealed **SGP** plate at -15°C to -25°C.

Comments

_____

_____

Date/Time: _____ Operator: _____

Library Pooling and MiSeq Sample Loading

In preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer, and heat denatured before sequencing on the MiSeq.

Estimated Time

- Total duration: 10 minutes
- Hands-on: 10 minutes

Consumables

Item	Quantity	Storage	Supplied By
HT1 (Hybridization buffer)	1 tube	-15°C to -25°C	Illumina
Eppendorf tubes (screw-cap recommended)	2 tubes		User
PCR eight-tube strip	1		User
2.5 L Ice bucket	1		User

Preparation

[_] 1 Set a heat block suitable for 1.5 ml centrifuge tubes to 96°C.

[_] 2 Remove a MiSeq reagent cartridge from -15°C to -25°C storage and thaw at room temperature.

[_] 3 In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

Procedure

[_] 1 If the **SGP** plate was stored frozen, thaw the **SGP** plate at room temperature.

[_] 2 Centrifuge the **SGP** plate at 1,000 × g at 20°C for 1 minute to collect condensation.

[_] 3 Apply the **PAL** (Pooled Amplicon Library) barcode sticker to a fresh Eppendorf tube.
PAL Barcode: _____

[_] 4 Determine the samples to be pooled for sequencing. Calculate your supported sample multiplexing level, based on the number of targeted regions and desired coverage. Use the following table or the TruSeq Custom Amplicon Calculator.

Date/Time: _____ Operator: _____

Amplicons per CAT	Desired Mean Coverage*	Suggested Maximum Samples per MiSeq Run*	
		MiSeq v2	MiSeq v3
16	150x	96	96
	500x	96	96
48	150x	96	96
	500x	96	96
96	150x	96	96
	500x	96	96
384	150x	96	96
	500x	48	80
768	150x	72	96
	500x	24	40
1536	150x	36	60
	500x	12	20

* Actual performance varies depending on the genes being targeted. If unexpectedly low coverage is found with certain genes in a newly designed CAT, deeper sequencing might be required.

[_] 5 If the **SGP** plate was stored frozen, mix each library to be sequenced by pipetting up and down 3–5 times using a P200 multichannel pipette. Change tips between samples.

[_] 6 Using a P20 multichannel pipette, transfer 5 µl of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip. Change tips after each column to avoid cross-contamination. Seal **SGP** with Microseal 'B' and set aside.

NOTE
After use, store the sealed **SGP** plate at -15°C to -25°C.

[_] 7 Combine and transfer the contents of the PCR eight-tube strip into the **PAL** tube. Mix **PAL** well.

[_] 8 Apply the **DAL** (Diluted Amplicon Library) barcode sticker to a fresh Eppendorf tube.
DAL Barcode: _____

[_] 9 Create **DAL** by combining the volumes of HT1 and **PAL** indicated in Table 3 based on your MiSeq Reagent Kit version. Upon transferring PAL, using the same tip, pipette up and down 3–5 time to rinse the tip and ensure complete transfer.

NOTE
Volumes for diluting PAL with HT1 were established using recommended equipment (e.g., plate shaker calibrated for shaking speed). Typical laboratory conditions (e.g., 20°C to 25°C) were strictly followed during the normalization procedure. If cluster density is too high or too low, adjust the dilution ratio to better suit the equipment, temperature, and handling in your laboratory after validation.

Experienced User Card

Date/Time: _____ Operator: _____

Table 3 Pooling Dilution

	Volume of HT1	Volume of PAL
MiSeq v2	594 µl	6 µl
MiSeq v3	580 µl	20 µl

[_] 10 Mix **DAL** by vortexing the tube at top speed.

NOTE
If you would like to save the remaining **PAL** for future use, store the **PAL** tube at -15°C to -25°C.

Make sure that the diluted library **DAL** is freshly prepared and used immediately for MiSeq loading. Storage of the **DAL** results in a significant reduction of cluster density.

[_] 11 Using a heat block, incubate the **DAL** tube at 96°C for 2 minutes.

Start time: _____ Stop time: _____

[_] 12 After the incubation, invert **DAL** 1–2 times to mix and immediately place in the ice-water bath.

[_] 13 Keep the **DAL** tube in the ice-water bath for 5 minutes.

[_] 14 Load **DAL** into a thawed MiSeq reagent cartridge into the **Load Samples** reservoir.
MiSeq flow cell: _____
MiSeq reagent cartridge: _____

PAL description:				
Number of samples:				
Sample wells used:				

[_] 15 Sequence your library as indicated in the *MiSeq System User Guide*.

NOTE
Illumina recommends choosing a read length that does not exceed the CAT amplicon size.

Comments

_____
_____

For each well, record: 1) DNA sample name (i.e. ACD1), 2) ACP or CAT (Controls or Custom Pool), and 3) Index primer pairs (i.e. A701/A501).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
B	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
C	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
D	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
E	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
F	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
G	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____
H	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____	1. _____ 2. _____ 3. _____/_____

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 4 Illumina General Contact Information

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

Table 5 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
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

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

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