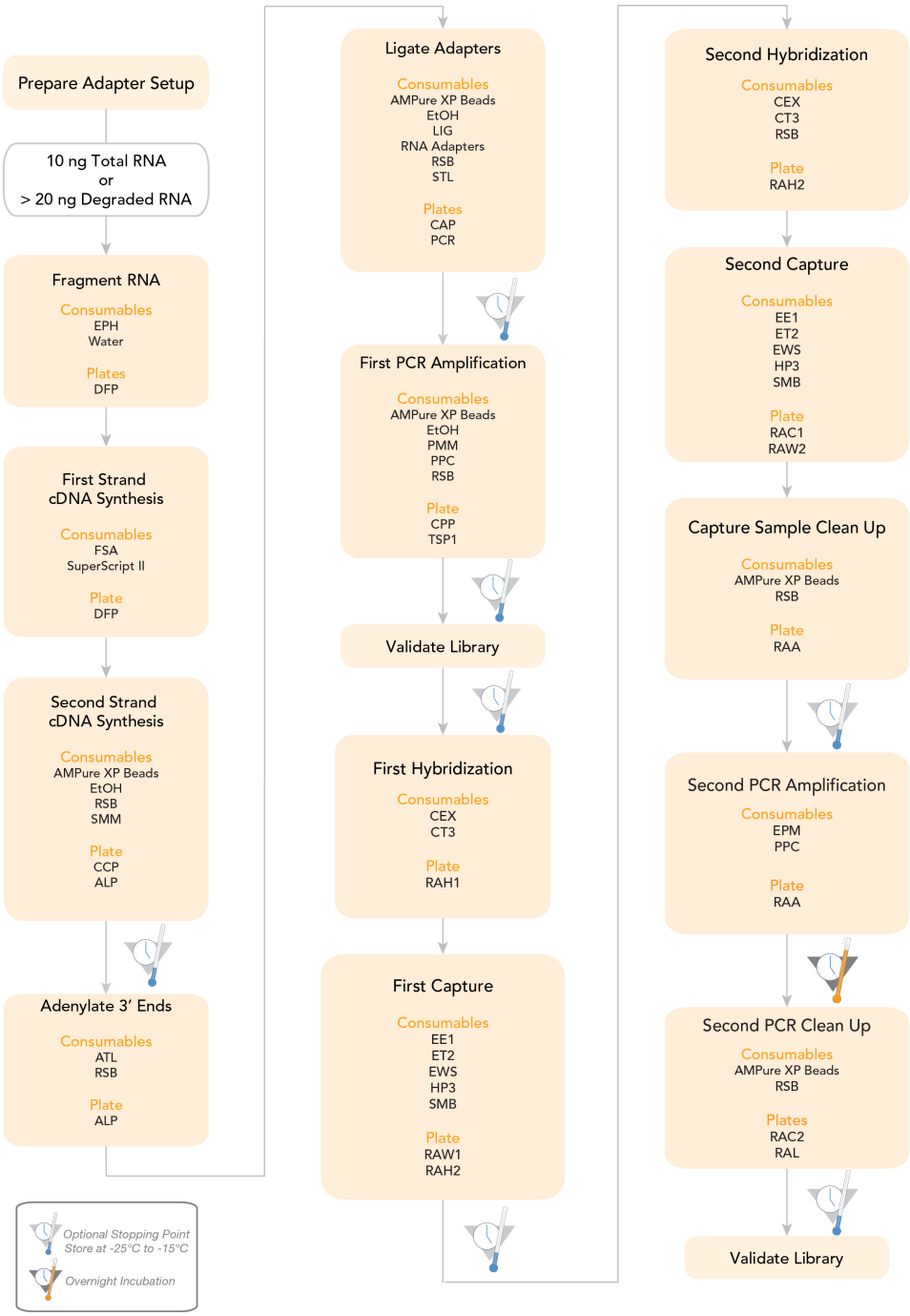
TruSeq RNA Access Library Prep

Experienced User Card and Lab Tracking Form

FOR RESEARCH USE ONLY

Date: _____ Description: _____

Illumina Kit Lot #: _____ _____



NOTE

New or less experienced users are advised to follow the protocol in the *TruSeq RNA Access Library Prep Guide (part # 15049525)* before using this document to perform the protocol.

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Consumables

Item	Lot Number
2N NaOH (HP3)	Lot #: _____
A-Tailing Mix (ATL)	Lot #: _____
Capture Target Buffer 3 (CT3)	Lot #: _____
Coding Exome Oligos (CEX)	Lot #: _____
Elute Target Buffer 2 (ET2)	Lot #: _____
Elute, Prime, Fragment High Mix (EPH)	Lot #: _____
Enhanced PCR Mix (EPM)	Lot #: _____
Enrichment Elution Buffer 1 (EE1)	Lot #: _____
Enrichment Wash Solution (EWS)	Lot #: _____
First Strand Synthesis Act D Mix (FSA)	Lot #: _____
Ligation Mix (LIG)	Lot #: _____
PCR Master Mix (PMM)	Lot #: _____
PCR Primer Cocktail (PPC)	Lot #: _____
Resuspension Buffer (RSB)	Lot #: _____
Second Strand Marking Master Mix (SMM)	Lot #: _____
Streptavidin Magnetic Beads (SMB)	Lot #: _____
Stop Ligation Buffer (STL)	Lot #: _____
80% Ethanol	Date Prepared: _____

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Adapter Indexes	Lot Number
RNA Adapter Index 1 (AR001)	Lot #: _____
RNA Adapter Index 2 (AR002)	Lot #: _____
RNA Adapter Index 3 (AR003)	Lot #: _____
RNA Adapter Index 4 (AR004)	Lot #: _____
RNA Adapter Index 5 (AR005)	Lot #: _____
RNA Adapter Index 6 (AR006)	Lot #: _____
RNA Adapter Index 7 (AR007)	Lot #: _____
RNA Adapter Index 8 (AR008)	Lot #: _____
RNA Adapter Index 9 (AR009)	Lot #: _____
RNA Adapter Index 10 (AR010)	Lot #: _____
RNA Adapter Index 11 (AR011)	Lot #: _____
RNA Adapter Index 12 (AR012)	Lot #: _____
RNA Adapter Index 13 (AR013)	Lot #: _____
RNA Adapter Index 14 (AR014)	Lot #: _____
RNA Adapter Index 15 (AR015)	Lot #: _____
RNA Adapter Index 16 (AR016)	Lot #: _____
RNA Adapter Index 18 (AR018)	Lot #: _____
RNA Adapter Index 19 (AR019)	Lot #: _____
RNA Adapter Index 20 (AR020)	Lot #: _____
RNA Adapter Index 21 (AR021)	Lot #: _____
RNA Adapter Index 22 (AR022)	Lot #: _____
RNA Adapter Index 23 (AR023)	Lot #: _____
RNA Adapter Index 24 (AR024)	Lot #: _____
RNA Adapter Index 25 (AR025)	Lot #: _____
RNA Adapter Index 27 (AR027)	Lot #: _____

Date/Time: _____ Operator: _____

Fragment RNA

This process fragments and primes RNA for cDNA synthesis.

NOTE
If starting with FFPE RNA, the sample input amount is based on sample quality. Illumina recommends using the percentage of RNA fragments > 200 nt DV_{200} as a reliable determinant of FFPE RNA quality.

Table 1 FFPE RNA Input Recommendations

Quality	DV_{200}	Input Requirement Per Reaction
High	> 70%	20 ng
Medium	50–70%	20–40 ng
Low	30–50%	40–100 ng
Too Degraded	< 30%	Not recommended

Use IEM or BaseSpace to record information about your samples before beginning library preparation. Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column.

Sample Sheet Name: _____

Date/Time: _____ Operator: _____

Consumables

Item	Quantity	Storage	Supplied By
Elute, Prime, Fragment High Mix (EPH)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
Total RNA	10 ng fresh/frozen RNA per reaction or 20–100 ng FFPE RNA per reaction (see Table 1)	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
Nuclease-free ultra pure water	Enough to dilute each total RNA sample to a final volume of 8.5 µl	15°C to 30°C	User

Make DFP

[_] 1 Dilute total RNA with nuclease-free ultra pure water to a final volume of 8.5 µl in each well of the new 96-well HSP plate labeled **DFP**.

[_] 2 Add 8.5 µl Elute, Prime, Fragment High Mix to each well of the DFP plate. Mix thoroughly as follows:

- [_] a Seal the DFP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.

[_] 3 Return the Elute, Prime, Fragment High Mix to -25°C to -15°C storage.

Incubate 1 DFP

WARNING
If starting with FFPE RNA, do not perform this incubation procedure. Proceed immediately to *Synthesize First Strand cDNA* on page 9.

[_] 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** to fragment and prime the RNA.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 94°C for 8 minutes
- [_] c Hold at 4°C

Date/Time: _____ Operator: _____

[_] 2 Remove the DFP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.

[_] 3 Proceed immediately to *Synthesize First Strand cDNA* on page 9.

Comments

_____

_____

Fragment RNA

Date/Time: _____ Operator: _____

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments that were primed with random hexamers into first strand cDNA using reverse transcriptase. The addition of actinomycin D to the First Stand Synthesis Act D mix (FSA) prevents spurious DNA-dependent synthesis, while allowing RNA-dependent synthesis, improving strand specificity.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Synthesis Act D Mix (FSA)	1 tube	-25°C to -15°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-25°C to -15°C	User

Add FSA

[_] 1 Remove the adhesive seal from the DFP plate.

[_] 2 Centrifuge the thawed First Strand Synthesis Mix Act D tube at 600 × g for 5 seconds.

[_] 3 Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. Mix gently, but thoroughly and centrifuge briefly. If you are not using the entire contents of the First Strand Synthesis Act D Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Synthesis Act D Mix.
Label the First Strand Synthesis Mix Act D tube to indicate that the SuperScript II has been added.

[_] 4 Add 8 µl of First Strand Synthesis Mix Act D and SuperScript II mix to each well of the DFP plate. Mix thoroughly as follows:

- [_] a Seal the DFP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.

[_] 5 Return the First Strand Synthesis Mix Act D tube to -25°C to -15°C storage immediately after use.

Date/Time: _____ Operator: _____

Incubate 2 DFP

[_] 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid and select **Synthesize 1st Strand**.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 25°C for 10 minutes
- [_] c 42°C for 15 minutes
- [_] d 70°C for 15 minutes
- [_] e Hold at 4°C

[_] 2 When the thermal cycler reaches 4°C, remove the DFP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 11.

Comments

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_____

Date/Time: _____ Operator: _____

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you have blunt-ended cDNA.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
96-well MIDI plates	2	15°C to 30°C	User
AMPure XP beads	90 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Add SMM

[_] 1 Remove the adhesive seal from the DFP plate.

[_] 2 Add 5 µl of Resuspension Buffer to each well of the DFP plate.

[_] 3 Centrifuge the thawed Second Strand Marking Master Mix at 600 × g for 5 seconds.

[_] 4 Add 20 µl of thawed Second Strand Marking Master Mix to each well of the DFP plate. Mix thoroughly as follows:

- [_] a Seal the DFP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.

[_] 5 Return the Second Strand Marking Master Mix tube to -25°C to -15°C storage after use.

Incubate 3 DFP

[_] 1 Place the sealed DFP plate on the pre-heated thermal cycler. Close the lid and incubate at 16°C for 1 hour.

Start time: _____ Stop time: _____

Date/Time: _____ Operator: _____

[_] 2 Remove the DFP plate from the thermal cycler and place it on the bench.

[_] 3 Remove the adhesive seal from the DFP plate.

[_] 4 Let the DFP plate stand to bring it to room temperature.

Clean Up DFP

[_] 1 Vortex the AMPure XP beads until they are well dispersed.

[_] 2 Add 90 µl of well-mixed AMPure XP beads to each well of the new MIDI plate labeled **CCP**.

[_] 3 Transfer the entire contents from each well of the DFP plate to the corresponding well of the CCP plate containing AMPure XP beads. Mix thoroughly as follows:

- [_] a Seal the CCP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 4 Incubate the CCP plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 5 Centrifuge the CCP plate at 280 × g for 1 minute.

[_] 6 Remove the adhesive seal from the CCP plate.

[_] 7 Place the CCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.

Start time: _____ Stop time: _____

[_] 8 Remove and discard 135 µl supernatant from each well of the CCP plate.

[_] 9 With the CCP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 10 Incubate the CCP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

[_] 12 Let the CCP plate stand at room temperature for 5 minutes to dry, and then remove the CCP plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 13 Centrifuge the thawed, room temperature Resuspension Buffer at 600 × g for 5 seconds.

[_] 14 Add 17.5 µl Resuspension Buffer to each well of the CCP plate. Mix thoroughly as follows:

- [_] a Seal the CCP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 15 Incubate the CCP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 16 Centrifuge the CCP plate at 280 × g for 1 minute.

[_] 17 Remove the adhesive seal from the CCP plate.

[_] 18 Place the CCP plate on the magnetic stand at room temperature for 5 minutes.

Start time: _____ Stop time: _____

Date/Time: _____ Operator: _____

[_] 19 Transfer 15 µl supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled **ALP**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 15, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Comments

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_____

Synthesize Second Strand cDNA

Date/Time: _____ Operator: _____

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another 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

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Add ATL

[_] 1 Centrifuge the thawed A-Tailing Mix tube at 600 × g for 5 seconds.

[_] 2 Add 2.5 µl Resuspension Buffer to each well of the ALP plate.

[_] 3 Add 12.5 µl thawed A-Tailing Mix to each well of the ALP plate. Mix thoroughly as follows:

- [_] a Seal the ALP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 4 Centrifuge the ALP plate at 280 × g for 1 minute.

[_] 5 Return the A-Tailing Mix tube to -25°C to -15°C storage.

Incubate 1 ALP

[_] 1 Place the sealed ALP plate on the pre-heated microheating system 1. Close the lid and incubate at 37°C for 30 minutes.

Start time: _____ Stop time: _____

[_] 2 Immediately after the 37°C incubation, remove the ALP plate from system 1 and place the plate on the pre-heated microheating system 2. Close the lid and incubate at 70°C for 5 minutes.

Start time: _____ Stop time: _____

[_] 3 Set the microheating system 1 to 30°C in preparation for *Ligate Adapters*.

[_] 4 Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 1 minute.

[_] 5 Proceed immediately to *Ligate Adapters* on page 17.

Comments

_____

_____

Date/Time: _____ Operator: _____

Ligate Adapters

This process ligates indexing adapters to the ends of the ds cDNA, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By
RNA Adapter Indexes (AR001–AR016, AR018–AR023, AR025, AR027)	1 tube of each index being used, per column of 8 reactions	-25°C to -15°C	Illumina
Ligation Mix (LIG)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	92 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4–28	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	4–28	15°C to 30°C	User

Add LIG

[_] 1 Centrifuge the thawed RNA Adapter tubes at 600 × g for 5 seconds.

[_] 2 Immediately before use, remove the Ligation Mix tube from -25°C to -15°C storage.

[_] 3 Remove the adhesive seal from the ALP plate.

[_] 4 Add 2.5 µl Resuspension Buffer to each well of the ALP plate.

[_] 5 Add 2.5 µl Ligation Mix to each well of the ALP plate.

[_] 6 Return the Ligation Mix tube to -25°C to -15°C storage immediately after use.

[_] 7 Add 2.5 µl thawed RNA Adapter Index to each well of the ALP plate.

[_] 8 Mix thoroughly as follows:

- [_] a Seal the ALP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 9 Centrifuge the ALP plate at 280 × g for 1 minute.

Date/Time: _____ Operator: _____

Incubate 2 ALP

[_] 1 Place the sealed ALP plate on the pre-heated microheating system. Close the lid and incubate at 30°C for 10 minutes.

Start time: _____ Stop time: _____

[_] 2 Remove the ALP plate from the microheating system.

Add STL

[_] 1 Remove the adhesive seal from the ALP plate.

[_] 2 Add 5 µl Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation mix. Mix thoroughly as follows:

- [_] a Seal the ALP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 3 Centrifuge the ALP plate at 280 × g for 1 minute.

Clean Up ALP

[_] 1 Remove the adhesive seal from the ALP plate.

[_] 2 Vortex the AMPure XP beads for at least 1 minute or until they are well dispersed.

[_] 3 Add 42 µl mixed AMPure XP beads to each well of the ALP plate. Mix thoroughly as follows:

- [_] a Seal the ALP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 4 Incubate the ALP plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 5 Centrifuge the ALP plate at 280 × g for 1 minute.

[_] 6 Remove the adhesive seal from the ALP plate.

[_] 7 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 8 Remove and discard 79.5 µl of supernatant from each well of the ALP plate.

[_] 9 With the ALP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 10 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

[_] 12 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 13 Remove the ALP plate from the magnetic stand.

Date/Time: _____ Operator: _____

[_] 14 Add 52.5 µl Resuspension Buffer to each well of the ALP plate. Mix thoroughly as follows:

[_] a Seal the ALP plate with a Microseal 'B' adhesive seal.

[_] b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 15 Incubate the ALP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 16 Centrifuge the ALP plate at 280 × g for 1 minute.

[_] 17 Remove the adhesive seal from the ALP plate.

[_] 18 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 19 Transfer 50 µl of supernatant from each well of the ALP plate to the corresponding well of the new MIDI plate labeled **CAP**.

[_] 20 Vortex the AMPure XP beads until they are well dispersed.

[_] 21 Add 50 µl mixed AMPure XP beads to each well of the CAP plate for a second cleanup. Mix thoroughly as follows:

[_] a Seal the CAP plate with a Microseal 'B' adhesive seal.

[_] b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 22 Incubate the CAP plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 23 Centrifuge the CAP plate at 280 × g for 1 minute.

[_] 24 Remove the adhesive seal from the CAP plate.

[_] 25 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 26 Remove and discard 95 µl of supernatant from each well of the CAP plate.

[_] 27 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well.

[_] 28 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 29 Repeat steps 27 and 28 one time for a total of two 80% EtOH washes.

[_] 30 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 31 Remove the CAP plate from the magnetic stand.

[_] 32 Add 22.5 µl Resuspension Buffer to each well of the CAP plate. Mix thoroughly as follows:

[_] a Seal the CAP plate with a Microseal 'B' adhesive seal.

[_] b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 33 Incubate the CAP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 34 Centrifuge the CAP plate at 280 × g for 1 minute.

[_] 35 Remove the adhesive seal from the CAP plate.

[_] 36 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 37 Transfer 20 µl of supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled **PCR**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *First PCR Amplification* on page 21, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Comments

_____

_____

Date/Time: _____ Operator: _____

First PCR Amplification

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. The PCR is performed with a 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.

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
PCR Primer Cocktail (PPC)		-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	50 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'A' film	1	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Make PCR

[_] 1 Add 5 µl thawed PCR Primer Cocktail to each well of the PCR plate.

[_] 2 Add 25 µl thawed PCR Master Mix to each well of the PCR plate.

- [_] a Seal the PCR plate with a Microseal 'A' film.
- [_] b Shake the PCR plate on a microplate shaker at 1600 rpm for 20 seconds.

[_] 3 Centrifuge the PCR plate at 280 × g for 1 minute.

[_] 4 Return the PCR Primer Cocktail and Enhanced PCR Mix tubes to -25°C to -15°C storage.

Date/Time: _____ Operator: _____

Amp PCR

[_] 1 Place the sealed PCR plate on the pre-programmed thermal cycler. Close the lid, then select and run **PCR** to amplify the plate.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 98°C for 30 seconds
- [_] c 15 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- [_] d 72°C for 5 minutes
- [_] e Hold at 4°C

Clean Up PCR

[_] 1 Remove the adhesive seal from the PCR plate.

[_] 2 Vortex the AMPure XP beads for at least 1 minute or until they are well dispersed.

[_] 3 Add 50 µl mixed AMPure XP beads to each well of the new MIDI plate labeled **CPP**.

[_] 4 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl mixed AMPure XP beads. Mix thoroughly as follows:

- [_] a Seal the CPP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 5 Incubate the CPP plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 6 Centrifuge the CPP plate at 280 × g for 1 minute.

[_] 7 Remove the adhesive seal from the CPP plate.

[_] 8 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 9 Remove and discard 95 µl of the supernatant from each well of the CPP plate.

[_] 10 With the CPP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 11 Incubate the CPP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 12 Repeat steps 10 and 11 one time for a total of two 80% EtOH washes.

[_] 13 With the CPP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes, and then remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 14 Add 17.5 µl Resuspension Buffer to each well of the CPP plate. Mix thoroughly as follows:

- [_] a Seal the CPP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.

Date/Time: _____ Operator: _____

[_] 15 Incubate the CPP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 16 Centrifuge the CPP plate at 280 × g for 1 minute.

[_] 17 Remove the adhesive seal from the CPP plate.

[_] 18 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 19 Transfer 15 µl of the clear supernatant from each well of the CPP plate to the corresponding well of the new HSP plate labeled **TSP1**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 25, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Comments

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_____

First PCR Amplification

Date/Time: _____ Operator: _____

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Quantify Library

Quantify your library using an Advanced Analytical Fragment Analyzer or Agilent Technologies 2100 Bioanalyzer. As an alternative, quantify using PicoGreen.

Quality Control

[_] 1 Do one of the following:

- Dilute 1 µl of resuspended construct with 1 µl Resuspension Buffer and load on an Advanced Analytical Fragment Analyzer using Standard Sensitivity NGS Fragment Analysis Kit.
- Load 1 µl of resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the Agilent DNA 1000.

[_] 2 Check the size and purity of the sample. The final product should be a band at approximately 260 bp.

Comments

_____

_____

Date/Time: _____ Operator: _____

First Hybridization

This process mixes the DNA library with capture probes to targeted regions of interest. The recommended hybridization time makes sure that targeted regions bind to the capture probes thoroughly. This process also describes how to combine multiple libraries with different indexes into a single pool before enrichment.

Consumables

Item	Quantity	Storage	Supplied By
Capture Target Buffer 3 (CT3)	1 tube	-25°C to -15°C	Illumina
Coding Exome Oligos (CEX)	1 tube	-25°C to -15°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (for multi-sample processing)	2	15°C to 30°C	User
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa)	1 per pooled sample	15°C to 30°C	User

Pool Libraries

Combine 200 ng of each DNA library for pooling.

- If the total volume is greater than 45 µl, concentrate the pooled sample. Use either a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) according to manufacturer instructions.
  - If you are using a vacuum concentrator, Illumina recommends concentrating samples with a no heat and medium drying rate setting.
  - If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), it is not required to pre-rinse the device before use.
- If the pooled sample volume after concentrating is less than 45 µl, bring the volume up to 45 µl with Resuspension Buffer.

Procedure

[_] 1 Thoroughly vortex the Capture Target Buffer 3 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

Date/Time: _____ Operator: _____

[_] 2 Add the following reagents in the order listed to each well of the new 96-well HSP plate labeled **RAH1**:

Reagent	Volume (µl)
DNA library sample or library pool from TSP1 plate	45
Capture Target Buffer 3	50
Coding Exome Oligos	5
Total Volume per Sample	**100**

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAH1 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAH1 plate on a microplate shaker at 1200 rpm for 1 minute.

[_] 4 Centrifuge the RAH1 plate at 280 × g for 1 minute.

[_] 5 Place the sealed RAH1 plate on the pre-programmed thermal cycler. Close the lid, then select and run the **RNA HYB** program.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 95°C for 10 minutes
- [_] c 18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle
- [_] d 58°C for forever

NOTE
Run the 58°C hybridization for 90 minutes.

Start time: _____ Stop time: _____

[_] 6 Immediately remove the plate from the thermal cycler after 90 minutes of 58°C hybridization and proceed immediately to *First Capture* on page 29.

Comments

_____

_____

Date/Time: _____ Operator: _____

First Capture

This process uses streptavidin beads to capture probes hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-25°C to -15°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2°C to 8°C	Illumina
Enrichment Elution Buffer 1 (EE1)	1 tube	-25°C to -15°C	Illumina
Enrichment Wash Solution (EWS)	1 tube	-25°C to -15°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2°C to 8°C	Illumina
1.7 ml microcentrifuge tube	1	15°C to 30°C	User
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Microseal 'B' adhesive seals	6	15°C to 30°C	User

First Bind

[_] 1 Remove the RAH1 plate from the thermal cycler.

[_] 2 Centrifuge the RAH1 plate at 280 × g for 1 minute.

[_] 3 Remove the adhesive seal from the RAH1 plate.

[_] 4 Transfer the entire contents (~100 µl) from each well of the RAH1 plate to the corresponding well of the new 96-well MIDI plate labeled **RAW1**.

[_] 5 Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl well-mixed Streptavidin Magnetic Beads to the wells of the RAW1 plate.

[_] 6 Mix thoroughly as follows:

- [_] a Seal the RAW1 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAW1 plate on a microplate shaker at 1200 rpm for 5 minutes.

[_] 7 Let the RAW1 plate stand at room temperature for 25 minutes.

Start time: _____ Stop time: _____

[_] 8 Centrifuge the RAW1 plate at 280 × g for 1 minute.

[_] 9 Remove the adhesive seal from the RAW1 plate.

[_] 10 Place the RAW1 plate on the magnetic stand for 2 minutes at room temperature or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 11 Carefully remove and discard all of the supernatant from each well of the RAW1 plate without disturbing the beads.

[_] 12 Remove the RAW1 plate from the magnetic stand.

First Wash

[_] 1 Make sure that the Enrichment Wash Solution tube is at room temperature, then thoroughly vortex the tube.

[_] 2 Add 200 µl Enrichment Wash Solution to each well of the RAW1 plate.

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAW1 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAW1 plate on a microplate shaker at 1800 rpm for 4 minutes.
- [_] c Remove the adhesive seal from the RAW1 plate.
- [_] d Gently pipette the entire volume of each well up and down to ensure complete resuspension of the sample.

[_] 4 Seal the RAW1 plate with a Microseal 'B' adhesive seal.

[_] 5 Place the sealed RAW1 plate on the **pre-heated** microheating system. Close the lid and incubate at 50°C for 20 minutes.

Start time: _____ Stop time: _____

Start time: _____ Stop time: _____

[_] 6 Place the magnetic stand next to the microheating system for immediate access.

[_] 7 Remove the RAW1 plate from the microheating system and ***immediately*** place it on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

Start time: _____ Stop time: _____

[_] 8 Remove the adhesive seal from the RAW1 plate.

[_] 9 Immediately remove and discard all of the supernatant from each well of the RAW1 plate.

[_] 10 Remove the RAW1 plate from the magnetic stand.

[_] 11 Repeat steps 2–10 one time for a total of two Enrichment Wash Solution washes.

Date/Time: _____ Operator: _____

First Elution

[_] 1 Add the following reagents in the order listed in a new 1.7 ml microcentrifuge tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared. The volumes include an excess amount for processing multiple samples.

Reagent	Volume (µl)
Enrichment Elution Buffer 1	28.5
2N NaOH	1.5
Total volume per enrichment	**30**

[_] 2 Vortex the elution pre-mix tube, then add **23** µl of the mix to each well of the RAW1 plate.

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAW1 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAW1 plate on a microplate shaker at 1800 rpm for 2 minutes.

[_] 4 Let the RAW1 plate stand at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 5 Centrifuge the RAW1 plate at 280 × g for 1 minute.

[_] 6 Carefully remove the adhesive seal from the RAW1 plate to avoid spilling the contents of the wells.

[_] 7 Place the RAW1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 8 Transfer 21 µl of clear supernatant from each well of the RAW1 plate to the corresponding well of the new HSP plate labeled **RAH2**.

[_] 9 Add 4 µl Elute Target Buffer 2 to each well of the RAH2 plate containing samples to neutralize the elution.

[_] 10 Mix thoroughly as follows:

- [_] a Seal the RAH2 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAH2 plate on a microplate shaker at 1200 rpm for 1 minute.

[_] 11 Centrifuge the RAH2 plate at 280 × g for 1 minute.

[_] 12 Store the remaining reagents as follows:

- [_] a Place the Elute Target Buffer 2 and Streptavidin Magnetic Beads tubes in 2°C to 8°C storage.
- [_] b Place the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution tubes in -25°C to -15°C storage.
- [_] c Discard any remaining elution pre-mix.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Second Hybridization* on page 33, you can safely stop the protocol here. If you are stopping, seal the RAH2 plate with a Microseal 'B' adhesive seal and store it at -25°C to -15°C for up to 7 days.

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

First Capture

Comments

_____

_____

Date/Time: _____ Operator: _____

Second Hybridization

This process combines the eluted DNA library from the first enrichment round with additional capture probes to targeted regions of interest. This second hybridization is required to ensure high specificity of the captured regions.

Consumables

Item	Quantity	Storage	Supplied By
Capture Target Buffer 3 (CT3)	1 tube	-25°C to -15°C	Illumina
Coding Exome Oligos (CEX)	1 tube	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Procedure

[_] 1 Thoroughly vortex the Capture Target Buffer 3 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

[_] 2 Remove the adhesive seal from the RAH2 plate.

[_] 3 Add the following reagents in the order listed to each well of the RAH2 plate:

Reagent	Volume (µl)
Resuspension Buffer	20
Capture Target Buffer 3	50
Coding Exome Oligos	5

[_] 4 Mix thoroughly as follows:

- [_] a Seal the RAH2 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAH2 plate on a microplate shaker at 1200 rpm for 1 minute

[_] 5 Centrifuge the RAH2 plate at 280 × g for 1 minute.

[_] 6 Place the sealed RAH2 plate on the pre-programmed thermal cycler. Close the lid, then select and run the **RNA HYB** program.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 95°C for 10 minutes
- [_] c 18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle
- [_] d 58°C for forever

NOTE
Run the 58°C hybridization for 90 minutes.

Start time: _____ Stop time: _____

[_] 7 Immediately remove the plate from the thermal cycler after 90 minutes of 58°C hybridization and proceed immediately to *Second Capture* on page 35.

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Comments

_____

_____

Date/Time: _____ Operator: _____

Second Capture

This process uses streptavidin beads to capture probes hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-25°C to -15°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2°C to 8°C	Illumina
Enrichment Elution Buffer 1 (EE1)	1 tube	-25°C to -15°C	Illumina
Enrichment Wash Solution (EWS)	1 tube	-25°C to -15°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2°C to 8°C	Illumina
1.7 ml microcentrifuge tube	1	15°C to 30°C	User
96-well MIDI plates	2	15°C to 30°C	User
Microseal 'B' adhesive seals	6	15°C to 30°C	User

Second Bind

[_] 1 Remove the RAH2 plate from the thermal cycler.

[_] 2 Centrifuge the room temperature RAH2 plate at 280 × g for 1 minute.

[_] 3 Remove the adhesive seal from the RAH2 plate.

[_] 4 Transfer the entire contents (~100 µl) from each well of the RAH2 plate to the corresponding well of the new 96-well MIDI plate labeled **RAW2**.

[_] 5 Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl well-mixed Streptavidin Magnetic Beads to the wells of the RAW2 plate.

[_] 6 Mix thoroughly as follows:

- [_] a Seal the RAW2 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAW2 plate on a microplate shaker at 1200 rpm for 5 minutes

[_] 7 Let the RAW2 plate stand at room temperature for 25 minutes.

Start time: _____ Stop time: _____

[_] 8 Centrifuge the RAW2 plate at 280 × g for 1 minute.

[_] 9 Remove the adhesive seal from the RAW2 plate.

[_] 10 Place the RAW2 plate on the magnetic stand for 2 minutes at room temperature or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 11 Carefully remove and discard all of the supernatant from each well of the RAW2 plate without disturbing the beads.

[_] 12 Remove the RAW2 plate from the magnetic stand.

Second Wash

[_] 1 Make sure that the Enrichment Wash Solution tube is at room temperature, then thoroughly vortex the tube.

[_] 2 Add 200 µl Enrichment Wash Solution to each well of the RAW2 plate.

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAW2 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAW2 plate on a microplate shaker at 1800 rpm for 4 minutes
- [_] c Remove the adhesive seal from the RAW2 plate.
- [_] d Gently pipette the entire volume of each well up and down to ensure complete resuspension of the sample.

[_] 4 Seal the RAW2 plate with a Microseal 'B' adhesive seal.

[_] 5 Incubate the RAW2 plate on the **pre-heated** microheating system, with the lid closed, at 50°C for 20 minutes.

Start time: _____ Stop time: _____

Start time: _____ Stop time: _____

[_] 6 Place the magnetic stand next to the microheating system for immediate access.

[_] 7 Remove the RAW2 plate from the microheating system and ***immediately*** place it on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

Start time: _____ Stop time: _____

[_] 8 Remove the adhesive seal from the RAW2 plate.

[_] 9 Immediately remove and discard all of the supernatant from each well of the RAW2 plate.

[_] 10 Remove the RAW2 plate from the magnetic stand.

[_] 11 Repeat steps 2–10 one time for a total of two Enrichment Wash Solution washes.

Second Elution

[_] 1 Add the following reagents in the order listed in a new 1.7 ml microcentrifuge tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared. The volumes include an excess amount for processing multiple samples.

Reagent	Volume (µl)
Enrichment Elution Buffer 1	28.5
2N NaOH	1.5
Total volume per enrichment	**30**

[_] 2 Vortex the elution pre-mix tube, then add **23** µl of the mix to each well of the RAW2 plate.

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAW2 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAW2 plate on a microplate shaker at 1800 rpm for 2 minutes.

Date/Time: _____ Operator: _____

[_] 4 Let the RAW2 plate stand at room temperature for 2 minutes.
Start time: _____ Stop time: _____

[_] 5 Centrifuge the RAW2 plate at 280 × g for 1 minute.

[_] 6 Carefully remove the adhesive seal from the RAW2 plate to avoid spilling the contents of the wells.

[_] 7 Place the RAW2 plate on the magnetic stand for 2 minutes or until the liquid is clear.
Start time: _____ Stop time: _____

[_] 8 Transfer 21 µl of clear supernatant from each well of the RAW2 plate to the corresponding well of the new MIDI plate labeled **RAC1**.

[_] 9 Add 4 µl Elute Target Buffer 2 to each well of the RAC1 plate containing samples to neutralize the elution.

[_] 10 Mix thoroughly as follows:
- [_] a Seal the RAC1 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.

[_] 11 Centrifuge the RAC1 plate at 280 × g for 1 minute.

[_] 12 Store the remaining reagents as follows:
- [_] a Place the Elute Target Buffer 2 and Streptavidin Magnetic Beads tubes in 2°C to 8°C storage.
- [_] b Place the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution tubes in -25°C to -15°C storage.
- [_] c Discard any remaining elution pre-mix.

Comments

_____
_____

Second Capture

Date/Time: _____ Operator: _____

Capture Sample Clean Up

This process uses AMPure XP beads to purify the captured library before PCR amplification.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
AMPure XP beads	45 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Procedure

[_] 1 Remove the adhesive seal from the RAC1 plate.

[_] 2 Vortex the AMPure XP beads tube until the beads are well dispersed, then add 45 µl well-mixed AMPure XP beads to each well of the RAC1 plate.

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAC1 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.

[_] 4 Incubate the RAC1 plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 5 Centrifuge the RAC1 plate at 280 × g for 1 minute.

[_] 6 Remove the adhesive seal from the RAC1 plate.

[_] 7 Place the RAC1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 8 Remove and discard all of the supernatant from each well of the RAC1 plate.

[_] 9 With the RAC1 plate on the magnetic stand, slowly add 200 µl freshly made 80% EtOH to each well without disturbing the beads.

[_] 10 Let the RAC1 plate stand at room temperature for 30 seconds.

[_] 11 Remove and discard the 80% EtOH from each well of the RAC1 plate.

[_] 12 Repeat steps 9–11 one time for a total of two 80% EtOH washes.

[_] 13 Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the RAC1 plate without disturbing the beads.

[_] 14 Let the RAC1 plate stand at room temperature for 5 minutes to dry on the magnetic stand.

Start time: _____ Stop time: _____

[_] 15 Remove the RAC1 plate from the magnetic stand.

[_] 16 Add 27.5 µl Resuspension Buffer to each well of the RAC1 plate.

[_] 17 Mix thoroughly as follows:

[_] a Seal the RAC1 plate with a Microseal 'B' adhesive seal.

[_] b Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.

[_] 18 Incubate the RAC1 plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 19 Centrifuge the RAC1 plate at 280 × g for 1 minute.

[_] 20 Remove the adhesive seal from the RAC1 plate.

[_] 21 Place the RAC1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 22 Transfer 25 µl of clear supernatant from each well of the RAC1 plate to the corresponding well of the new HSP plate labeled **RAA**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Second PCR Amplification* on page 41, you can safely stop the protocol here. If you are stopping, seal the RAA plate with a Microseal 'B' adhesive seal and store it at -25°C to -15°C for up to 7 days.

Comments

_____

_____

Date/Time: _____ Operator: _____

Second PCR Amplification

This process uses PCR to amplify the enriched DNA library for sequencing.

Consumables

Item	Quantity	Storage	Supplied By
Enhanced PCR Mix (EPM)	1 tube	-25°C to -15°C	Illumina
PCR Primer Cocktail (PPC)	1 tube	-25°C to -15°C	Illumina
Microseal 'A' film	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Procedure

[_] 1 Add 5 µl PCR Primer Cocktail to each well of the RAA plate.

[_] 2 Add 20 µl Enhanced PCR Mix to each well of the RAA plate.

[_] 3 Mix thoroughly as follows:

- [_] a Seal the RAA plate with a Microseal 'A' film.
- [_] b Shake the RAA plate on a microplate shaker at 1200 rpm for 1 minute

[_] 4 Centrifuge the RAA plate at 280 × g for 1 minute.

[_] 5 Place the sealed RAA plate on the pre-programmed thermal cycler. Close the lid, then select and run the **EPM AMP** program.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 98°C for 30 seconds
- [_] c **10 cycles** of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 5 minutes
- [_] d 72°C for 5 minutes
- [_] e Hold at 10°C

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Second PCR Clean Up* on page 43, the RAA plate can remain on the thermal cycler overnight. If you are stopping, replace the Microseal 'A' film with a Microseal 'B' adhesive seal and store the RAA plate at 2°C to 8°C for up to two days.

Comments

_____

_____

Second PCR Amplification

Date/Time: _____ Operator: _____

Second PCR Clean Up

This process uses AMPure XP beads to purify the enriched library and remove unwanted products.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	90 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Procedure

[_] 1 Centrifuge the RAA plate at 280 × g for 1 minute.

[_] 2 Remove the adhesive seal from the RAA plate.

[_] 3 Transfer the entire contents from each well of the RAA plate to the corresponding well of the new 96-well MIDI plate labeled **RAC2**.

[_] 4 Vortex the AMPure XP beads until the beads are well dispersed.

[_] 5 Add 90 µl well-mixed AMPure XP beads to each well of the RAC2 plate containing 50 µl of PCR amplified library.

[_] 6 Mix thoroughly as follows:

- [_] a Seal the RAC2 plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RAC2 plate on a microplate shaker at 1800 rpm for 1 minute.

[_] 7 Incubate the RAC2 plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 8 Centrifuge the RAC2 plate at 280 × g for 1 minute.

[_] 9 Remove the adhesive seal from the RAC2 plate.

[_] 10 Place the RAC2 plate on the magnetic stand at room temperature for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 11 Carefully remove and discard all of the supernatant from each well of the RAC2 plate.

[_] 12 With the RAC2 plate on the magnetic stand, slowly add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 13 Let the RAC2 plate stand at room temperature for 30 seconds.

Date/Time: _____ Operator: _____

[_] 14 Remove and discard the 80% EtOH from each well of the RAC2 plate.

[_] 15 Repeat steps 12–14 one time for a total of two 80% EtOH washes.

[_] 16 Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the RAC2 plate without disturbing the beads.

[_] 17 Let the RAC2 plate stand at room temperature for 5 minutes to dry on the magnetic stand.

Start time: _____ Stop time: _____

[_] 18 Remove the RAC2 plate from the magnetic stand.

[_] 19 Add 32 µl Resuspension Buffer to each well of the RAC2 plate.

[_] 20 Mix thoroughly as follows:

[_] a Seal the RAC2 plate with a Microseal 'B' adhesive seal.

[_] b Shake the RAC2 plate on a microplate shaker at 1800 rpm for 1 minute.

[_] 21 Incubate the RAC2 plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 22 Centrifuge the RAC2 plate at 280 × g for 1 minute.

[_] 23 Remove the adhesive seal from the RAC2 plate.

[_] 24 Place the RAC2 plate on the magnetic stand for 2 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 25 Transfer 30 µl of clear supernatant from each well of the RAC2 plate to the corresponding well of the new HSP plate labeled **RAL**.

[_] 26 Seal the RAL plate with a Microseal 'B' adhesive seal.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 45, store the sealed RAL plate at -25°C to -15°C for up to 7 days. If the plate is stored for more than 7 days, requantify your library to guarantee the accuracy of your enrichment results.

Comments

_____

_____

Date/Time: _____ Operator: _____

Validate Library

Illumina recommends performing the following procedures for quality control analysis and quantification of your enriched library.

Quantify Libraries

Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide (part # 11322363)*.

When quantitation is complete, proceed to cluster generation.

[Optional] Assess Quality

Do the following to assess library quality:

[_] 1 Load 1 µl of the post-enriched library on one of the following:
- Advanced Analytical Technologies Standard Sensitivity NGS Fragment Analysis Kit
- Agilent High Sensitivity DNA Chip

[_] 2 Check the size of the library for a distribution of DNA fragments with a size range from approximately 200 bp–1 kb.
Depending on the level of indexing, insert size distribution can vary slightly.

Comments

_____
_____

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 2 Illumina General Contact Information

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

Table 3 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

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.ilmn.

Product Documentation

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

Part # 15049526 Rev. B

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