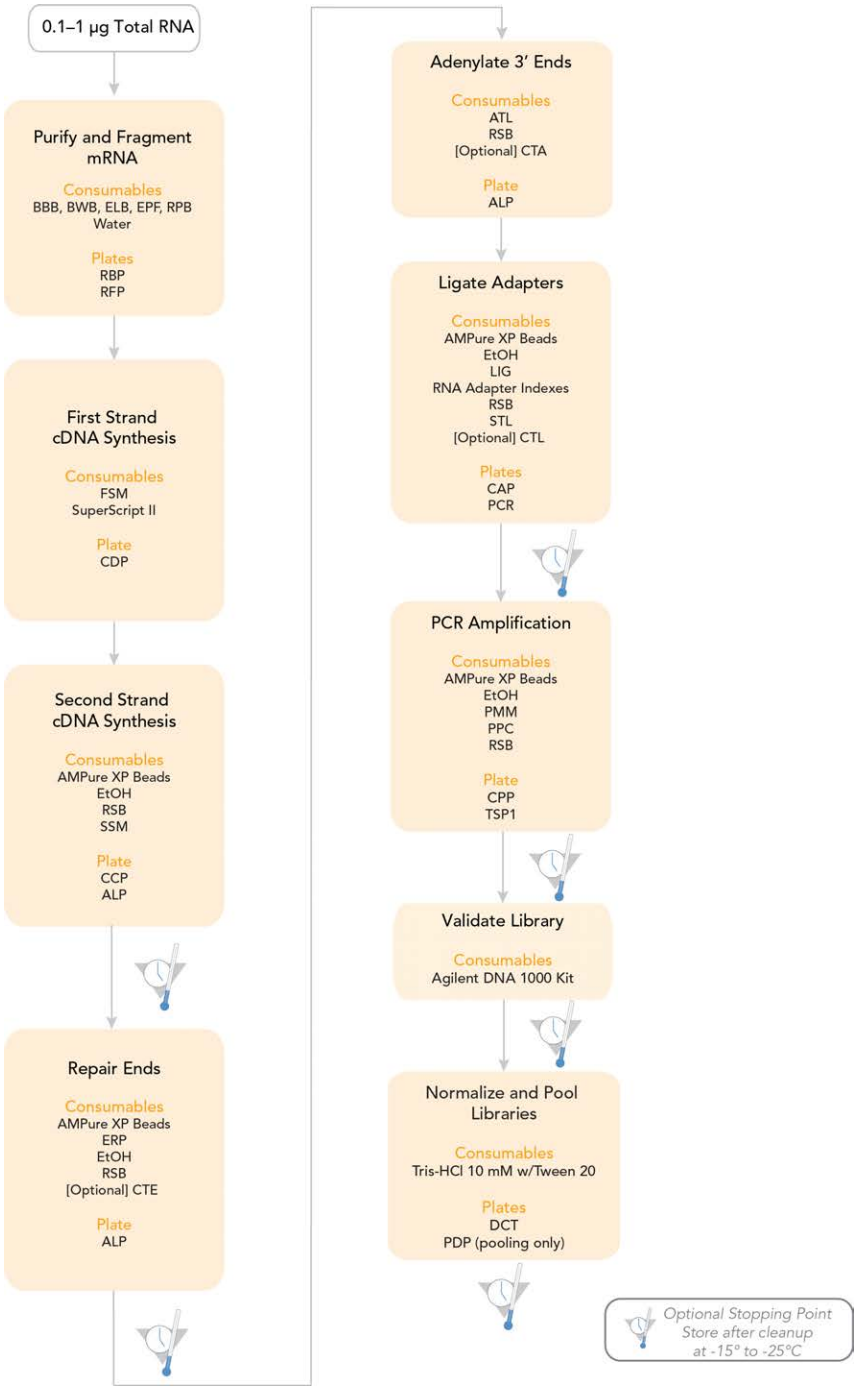
TruSeq RNA Sample Prep v2 HS Protocol

Experienced User Card and Lab Tracking Form

FOR RESEARCH USE ONLY

Date: _____ Description: _____

Illumina Kit Lot #: _____ _____



NOTE

Unless familiar with the HS protocol in the latest version of the *TruSeq RNA Sample Preparation v2 Kit Guide (part # 15026495)*, new or less experienced users are advised to follow the protocol in the guide before using this Experienced User Card and Lab Tracking Form.

Experienced User Card and Lab Tracking Form

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Consumables

Item	Lot Number
A-Tailing Control (CTA)	Lot #: _____
A-Tailing Mix (ATL)	Lot #: _____
Bead Binding Buffer (BBB)	Lot #: _____
Bead Washing Buffer (BWB)	Lot #: _____
Elute, Prime, Fragment Mix (EPF)	Lot #: _____
Elution Buffer (ELB)	Lot #: _____
End Repair Control (CTE)	Lot #: _____
End Repair Mix (ERP)	Lot #: _____
First Strand Master Mix (FSM)	Lot #: _____
Ligation Control (CTL)	Lot #: _____
Ligation Mix (LIG)	Lot #: _____
PCR Master Mix (PMM)	Lot #: _____
PCR Primer Cocktail (PPC)	Lot #: _____
Resuspension Buffer (RSB)	Lot #: _____
RNA Purification Beads (RPB)	Lot #: _____
Second Strand Master Mix (SSM)	Lot #: _____
Stop Ligation Buffer (STL)	Lot #: _____
80% Ethanol	Date Prepared: _____

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Consumables

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 #: _____

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Prepare Adapter Setup

If you are pooling, 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: _____

Comments

_____

_____

Date/Time: _____ Operator: _____

Purify and Fragment mRNA

This process purifies the polyA containing mRNA molecules using oligo-dT attached magnetic beads using two rounds of purification. During the second elution of the polyA RNA, the RNA is also fragmented and primed for cDNA synthesis.

Consumables

Item	Quantity	Storage	Supplied By
Bead Binding Buffer (BBB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Bead Washing Buffer (BWB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Elute, Prime, Fragment Mix (EPF)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C	Illumina
RNA Purification Beads (RPB)	1 tube per 48 reactions	2°C to 8°C	Illumina
Barcode labels for: • RBP (RNA Bead Plate) • RFP (RNA Fragmentation Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	6	15°C to 30°C	User

Make RBP

[_] 1 Dilute the total RNA with nuclease-free ultra pure water to a final volume of 50 µl in the new 96-well MIDI plate labeled with the RBP barcode.

[_] 2 Vortex the room temperature RNA Purification Beads tube vigorously to resuspend the oligo-dT beads.

[_] 3 Add 50 µl RNA Purification Beads to each well of the RBP plate to bind the polyA RNA to the oligo-dT beads. Mix thoroughly as follows:

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

[_] b Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

Incubate 1 RBP

[_] 1 Place the sealed RBP plate on the pre-heated microheating system. Close the lid and incubate at 65°C for 5 minutes to denature the RNA and facilitate binding of the polyA RNA to the beads.

Start time: _____ Stop time: _____

[_] 2 Remove the RBP plate from the microheating system and place on ice for 1 minute.

[_] 3 Place the RBP plate on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads.

Start time: _____ Stop time: _____

[_] 4 Pre-heat the microheating system to 80°C for the subsequent incubation.

Wash RBP

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

[_] 2 Place the RBP plate on the magnetic stand at room temperature for 5 minutes to separate the polyA RNA bound beads from the solution.

Start time: _____ Stop time: _____

[_] 3 Remove and discard all of the supernatant from each well of the RBP plate.

[_] 4 Remove the RBP plate from the magnetic stand.

[_] 5 Wash the beads by adding 200 µl Bead Washing Buffer in each well of the RBP plate to remove unbound RNA. Mix thoroughly as follows:

- [_] a Seal the RBP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

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

[_] 7 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 8 Centrifuge the thawed Elution Buffer at 600 × g for 5 seconds.

[_] 9 Remove and discard all of the supernatant from each well of the RBP plate.

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

[_] 11 Add 50 µl Elution Buffer in each well of the RBP plate. Mix thoroughly as follows:

- [_] a Seal the RBP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

[_] 12 Store the Elution Buffer tube at 4°C.

Incubate 2 RBP

[_] 1 Place the sealed RBP plate on the pre-heated microheating system. Close the lid and incubate at 80°C for 2 minutes to elute the mRNA from the beads.

Start time: _____ Stop time: _____

Date/Time: _____ Operator: _____

[_] 2 Remove the RBP plate from the microheating system and place on ice for 1 minute.

[_] 3 Place the RBP plate on the bench at room temperature.

[_] 4 Remove the adhesive seal from the RBP plate.

Make RFP

[_] 1 Centrifuge the thawed Bead Binding Buffer at 600 × g for 5 seconds.

[_] 2 Add 50 µl Bead Binding Buffer to each well of the RBP plate. Mix thoroughly as follows:

- [_] a Seal the RBP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

[_] 3 Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 2°C to 8°C.

Start time: _____ Stop time: _____

[_] 4 Remove the adhesive seal from the RBP plate.

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

Start time: _____ Stop time: _____

[_] 6 Remove and discard all of the supernatant from each well of the RBP plate.

[_] 7 Remove the RBP plate from the magnetic stand.

[_] 8 Wash the beads by adding 200 µl Bead Washing Buffer in each well of the RBP plate. Mix thoroughly as follows:

- [_] a Seal the RBP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

[_] 9 Store the Bead Washing Buffer tube at 2°C to 8°C.

[_] 10 Remove the adhesive seal from the RBP plate.

[_] 11 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 12 Remove and discard all of the supernatant from each well of the RBP plate.

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

[_] 14 Add 19.5 µl Elute, Prime, Fragment Mix to each well of the RBP plate. Mix thoroughly as follows:

- [_] a Seal the RBP plate with a Microseal 'B' adhesive seal.
- [_] b Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

[_] 15 Remove the adhesive seal from the RBP plate.

[_] 16 Transfer the entire contents from each well of the RBP plate to the corresponding well of the new HSP plate labeled with the RFP barcode.

[_] 17 Seal the RFP plate with a Microseal 'B' adhesive seal.

[_] 18 Store the Elute, Prime, Fragment Mix tube at -25°C to -15°C.

Incubate RFP

[_] 1 Place the sealed RFP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA.

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

[_] 3 Proceed immediately to *Synthesize First Strand cDNA* on page 11.

Comments

_____

_____

Date/Time: _____ Operator: _____

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase and random primers.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Master Mix (FSM)	1 tube	-25°C to -15°C	Illumina
CDP (cDNA Plate) barcode label	1 label per plate	15°C to 30°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 reagent reservoirs (if using multichannel pipettes)	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
SuperScript II Reverse Transcriptase	1 tube	-25°C to -15°C	User

Make CDP

[_] 1 Place the RFP plate on the magnetic stand at room temperature for 5 minutes. Do not remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

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

[_] 3 Transfer 17 µl of the supernatant (fragmented and primed mRNA) from each well of the RFP plate to the corresponding well of the new HSP plate labeled with the CDP barcode.

[_] 4 Centrifuge the thawed First Strand Master Mix tube at 600 × g for 5 seconds.

[_] 5 Add 50 µl SuperScript II to the First Strand Master Mix tube. Mix gently, but thoroughly and centrifuge briefly. If you are not using the entire contents of the First Strand Master Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Master Mix. Label the First Strand Master Mix tube to indicate that the SuperScript II has been added.

[_] 6 Add 8 µl First Strand Master Mix and SuperScript II mix to each well of the CDP plate. Mix thoroughly as follows:

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

[_] 7 Return the First Strand Master Mix tube to -25°C to -15°C storage immediately after use.

Date/Time: _____ Operator: _____

Incubate 1 CDP

[_] 1 Place the sealed CDP plate on the pre-programmed thermal cycler. Close the lid, and then select and run the **1st Strand** program.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 25°C for 10 minutes
- [_] c 42°C for 50 minutes
- [_] d 70°C for 15 minutes
- [_] e Hold at 4°C

[_] 2 When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 13.

Comments

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_____

Date/Time: _____ Operator: _____

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand to generate ds cDNA. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Master Mix (SSM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CCP (cDNA Clean Up Plate) • IMP (Insert Modification Plate)	1 label per plate	15°C to 30°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 reagent reservoirs (if using multichannel pipettes)	4	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4	15°C to 30°C	User

Add SSM

[_] 1 Centrifuge the thawed Second Strand Master Mix at 600 × g for 5 seconds.

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

[_] 3 Add 25 µl thawed Second Strand Master Mix to each well of the CDP plate. Mix thoroughly as follows:

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

[_] b Shake the CDP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.

Incubate 2 CDP

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

Start time: _____ Stop time: _____

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

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

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

Purify CDP

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

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

[_] 3 Transfer the entire contents from each well of the CDP 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 15 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 of 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 15 minutes to dry, and then remove the 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 52.5 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 to 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: _____

[_] 19 Transfer 50 µl supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled with the IMP barcode.

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Perform End Repair* on page 17, you can safely stop the protocol here. If you are stopping, seal the IMP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Comments

_____

_____

Synthesize Second Strand cDNA

Date/Time: _____ Operator: _____

Perform End Repair

This process converts the overhangs resulting from fragmentation into blunt ends using an End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix (ERP)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] End Repair Control (CTE)	1 tube per 48 reactions	-25°C to -15°C	Illumina
ALP (Adapter Ligation Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	160 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 'B' adhesive seals	4	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	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

Make IMP

[_] 1 Do one of the following:

- If using the in-line control reagent:
  - Centrifuge the thawed End Repair Control tube at 600 × g for 5 seconds.
  - Dilute the End Repair Control to 1/100 in Resuspension Buffer (1 µl End Repair Control + 99 µl Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
  - Add 10 µl diluted End Repair Control to each well of the IMP plate that contains 50 µl ds cDNA.
- If not using the in-line control reagent, add 10 µl Resuspension Buffer to each well of the IMP plate that contains 50 µl ds cDNA.

[_] 2 Add 40 µl End Repair Mix to each well of the IMP plate containing the ds cDNA. Mix thoroughly as follows:

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

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

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

Incubate IMP

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

Start time: _____ Stop time: _____

[_] 2 Remove the IMP plate from the microheating system and place the plate on ice until you are ready for the next step.

Clean Up IMP

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

[_] 2 Vortex the AMPure XP beads until they are well dispersed.

[_] 3 Add 160 µl well-mixed AMPure XP beads to each well of the IMP plate containing 100 µl End Repair Mix. Mix thoroughly as follows:

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

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

[_] 4 Incubate the IMP plate at room temperature for 15 minutes.

Start time: _____ Stop time: _____

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

Start time: _____ Stop time: _____

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

[_] 7 Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of supernatant from each well of the IMP plate.

[_] 8 Repeat step 7 one time.

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

[_] 10 Incubate the IMP 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 IMP plate stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 13 Resuspend the dried pellet in each well with 17.5 µl Resuspension Buffer. Mix thoroughly as follows:

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

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

[_] 14 Centrifuge the IMP plate at 280 × g for 1 minute.

[_] 15 Remove the adhesive seal from the IMP plate.

[_] 16 Incubate the IMP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 17 Place the IMP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 18 Transfer 15 µl of supernatant from each well of the IMP plate to the corresponding well of the new MIDI plate labeled with the ALP barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 21, 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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_____

Perform End Repair

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
[Optional] A-Tailing Control (CTA)	1 tube per 48 reactions	-25°C to -15°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 Do one of the following:
- If using the in-line control reagent:
  - Centrifuge the thawed A-Tailing Control tube at 600 × g for 5 seconds.
  - Dilute the A-Tailing Control to 1/100 in Resuspension Buffer (For example, 1 µl A-Tailing Control + 99 µl Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
  - Add 2.5 µl diluted A-Tailing Control to each well of the ALP plate.
- If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well of the ALP plate.

[_] 2 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.

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

Date/Time: _____ Operator: _____

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 23.

Comments

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_____

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
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
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
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
[Optional] Ligation Control (CTL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CAP (Clean Up ALP Plate) • PCR (Polymerase Chain Reaction)	1 label per plate	15°C to 30°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 reagent reservoirs (if using multichannel pipettes)	4–28	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

Add LIG

[_] 1 Centrifuge the thawed RNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer 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.

Date/Time: _____ Operator: _____

[_] 4 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl diluted Ligation Control to each well of the ALP plate.
- If not using the in-line control reagent, 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 back 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.

[_] 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.

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

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 15 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.

Date/Time: _____ Operator: _____

[_] 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 15 minutes.

Start time: _____ Stop time: _____

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

[_] 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 with the CAP barcode.

[_] 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 15 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 15 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 with the PCR barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 27, 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: _____

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. 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)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Barcode labels for: • CPP (Clean Up PCR Plate) • TSP1 (Target Sample Plate)	1 label per plate	15°C to 30°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.

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 10°C

Clean Up PCR

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

[_] 2 Vortex the AMPure XP Beads until they are well dispersed.

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

[_] 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 15 minutes.

Start time: _____ Stop time: _____

[_] 6 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

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

[_] 8 Remove and discard 95 µl of supernatant from each well of the CPP plate.

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

[_] 10 Incubate the CPP 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 While keeping the CPP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.

Start time: _____ Stop time: _____

[_] 13 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. 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: _____

[_] 14 Incubate the CPP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 15 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 16 Remove the adhesive seal from the CPP plate.

[_] 17 Transfer 30 µl of clear supernatant from each well of the CPP plate to the corresponding well of the new HSP plate labeled with the TSP1 barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 31, 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 seven days.

Comments

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_____

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 Libraries

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

Quality Control

[_] 1 Do one of the following:

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

[_] 2 Check the size and purity of the sample. Check the size and purity of the sample. The final product should be a band at approximately 260 bp (for single-read libraries).

Comments

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Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-indexed DNA libraries are normalized to 10 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate (for pooling only)	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15°C to 30°C	User

Make DCT

[_] 1 Transfer 10 µl of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode.

[_] 2 Normalize the concentration of sample library in each well of the DCT plate to 10 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

[_] 3 Mix the DCT plate as follows:

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

[_] b Shake the DCT plate on a microplate shaker at 1000 rpm for 2 minutes.

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

[_] 5 Remove the adhesive seal from the DCT plate.

[_] 6 Depending on the type of library you want to generate, do one of the following:

- For non-pooled libraries, the protocol stops here. Do one of the following:
  - Proceed to cluster generation.
  - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.
- For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)

[_] 1 Determine the number of samples to be combined together for each pool.

Date/Time: _____ Operator: _____

[_] 2 Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new HSP plate labeled with the PDP barcode.
The total volume in each well of the PDP plate is 10X the number of combined sample libraries and 20–240 µl (2–24 libraries).

[_] 3 Mix the PDP plate as follows:

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

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

[_] 4 Do one of the following:

- Proceed to cluster generation.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.

Comments

_____
_____

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

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

Table 2 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 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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