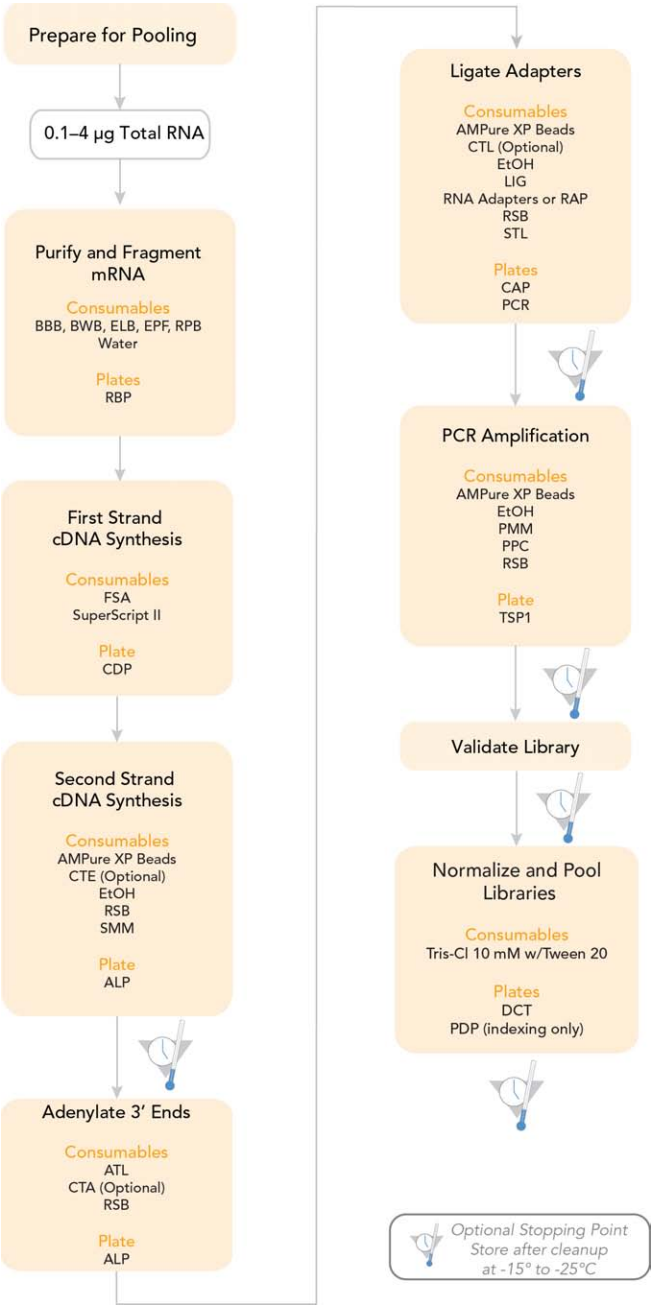
TruSeq Stranded mRNA Sample Prep LS Protocol

Experienced User Card

FOR RESEARCH USE ONLY



NOTE

- Unless familiar with the protocol in the latest version of the TruSeq Stranded mRNA Sample Preparation Guide, new or less experienced users are strongly advised to follow the protocol in the guide before using this Experienced User Card.
- For optimal sample tracking and quality control, fill out the TruSeq Stranded mRNA Sample Preparation LS Lab Tracking Form as you perform TruSeq Stranded mRNA Sample Preparation.

Purify and Fragment mRNA

This process purifies the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads using two rounds of purification. During the second elution of the poly-A 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	-15° to -25°C	Illumina
Bead Washing Buffer (BWB)	1 tube per 48 reactions	-15° to -25°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	-15° to -25°C	Illumina
Fragment, Prime, Finish Mix (FPF)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	-15° to -25°C	Illumina
RNA Purification Beads (RPB)	1 tube per 48 reactions	2° to 8°C	Illumina
RBP (RNA Bead Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	3	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	6	15° 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 0.3 ml PCR plate labeled with the RBP barcode.

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

[_] 3 Add 50 µl of RNA Purification Beads to each well of the RBP to bind the poly-A RNA to the oligo dT magnetic beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 4 Seal the RBP plate with a Microseal 'B' Adhesive seal.

Incubate 1 RBP

[_] 1 Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **mRNA Denaturation** to denature the RNA and facilitate binding of the poly-A RNA to the beads.

[_] 2 Remove the RBP plate from the thermal cycler when it reaches 4°C.

[_] 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.

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 poly-A RNA bound beads from the solution.

[_] 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 of Bead Washing Buffer in each well of the RBP plate to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 6 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

[_] 7 Centrifuge the thawed Elution Buffer to 600 xg for 5 seconds.

[_] 8 Remove and discard all of the supernatant from each well of the RBP plate. The supernatant contains the majority of the ribosomal and other non-messenger RNA.

[_] 9 Remove the RBP plate from the magnetic stand.

[_] 10 Add 50 µl of Elution Buffer in each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 11 Seal the RBP plate with a Microseal 'B' Adhesive seal.

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

Incubate 2 RBP

[_] 1 Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **mRNA Elution 1** to elute the mRNA from the beads.

[_] 2 Remove the RBP plate from the thermal cycler when it reaches 25°C.

[_] 3 Place the RBP plate on the bench at room temperature and remove the adhesive seal from the plate.

Make RFP

[_] 1 Centrifuge the thawed Bead Binding Buffer to 600 xg for 5 seconds.

[_] 2 Add 50 µl of Bead Binding Buffer to each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

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

[_] 4 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

[_] 5 Remove and discard all of the supernatant from each well of the RBP plate.

[_] 6 Remove the RBP plate from the magnetic stand.

[_] 7 Wash the beads by adding 200 µl of Bead Washing Buffer in each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

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

[_] 9 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

[_] 10 Remove and discard all of the supernatant from each well of the RBP plate.

[_] 11 Remove the RBP plate from the magnetic stand.

[_] 12 Add 19.5 µl of Fragment, Prime, Finish Mix to each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 13 Seal the RBP plate with a Microseal 'B' Adhesive seal.

[_] 14 Store the Fragment, Prime, Finish Mix tube at -15° to -25°C.

Incubate RFP

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

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

[_] 3 Proceed immediately to *Synthesize First Strand cDNA* on page 7.

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 and random primers.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Synthesis Act D Mix (FSA)	1 tube per 48 reactions	-15° to -25°C	Illumina
CDP (cDNA Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seal	1	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	1	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	1	15° to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-15° to -25°C	User

Make CDP

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

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

[_] 3 Transfer 17 µl of the supernatant (fragmented and primed mRNA) from each well of the RBP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CDP barcode.

[_] 4 Centrifuge the thawed First Strand Synthesis Act D Mix tube to 600 xg for 5 seconds.

[_] 5 Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. 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. Mix gently, but thoroughly, and centrifuge briefly.
Label the First Strand Synthesis Act D Mix tube to indicate that the SuperScript II has been added.

[_] 6 Add 8 µl of First Strand Synthesis Act D Mix and SuperScript II mix to each well of the CDP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 7 Seal the CDP plate with a Microseal 'B' Adhesive seal and centrifuge briefly.

[_] 8 Return the First Strand Synthesis Act D Mix tube to -15° to -25°C storage immediately after use.

Incubate 1 CDP

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

[_] 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 9.

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. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you will have blunt-ended cDNA.

Consumables

Item	Quantity	Storage	Supplied By
(Optional) End Repair Control (CTE)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-15° to -25°C	Illumina
ALP (Adapter Ligation Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
AMPure XP beads	90 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	5	15° to 30°C	User

Add SMM

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

[_] 2 Do one of the following:

- If using the in-line control reagent:
  - Centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds.
  - Dilute the End Repair Control to 1/50 in Resuspension Buffer (For example, 2 µl End Repair Control + 98 µl Resuspension Buffer) before use.
  - Add 5 µl of diluted End Repair Control to each well of the CDP plate.
- If not using the in-line control reagent, add 5 µl of Resuspension Buffer to each well of the CDP plate.

[_] 3 Centrifuge the thawed Second Strand Marking Master Mix to 600 xg for 5 seconds.

[_] 4 Add 20 µl of thawed Second Strand Marking Master Mix to each well of the CDP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 5 Seal the CDP plate with a Microseal 'B' Adhesive seal.

[_] 6 Return the Second Strand Marking Master Mix tube to -15° to -25°C storage after use.

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.

[_] 2 Remove the CDP plate from the thermal cycler, remove the adhesive seal, and let stand to bring the plate to room temperature.

Purify CDP

[_] 1 Vortex the AMPure XP beads until they are well dispersed, then add 90 µl of well-mixed AMPure XP beads to each well of the CDP plate containing 50 µl of ds cDNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

[_] 3 Place the CDP 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.

[_] 4 Remove and discard 135 µl of the supernatant from each well of the CDP plate.

[_] 5 With the CDP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 6 Incubate the CDP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

[_] 7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.

[_] 8 Let the plate stand at room temperature for 15 minutes to dry and then remove the CDP plate from the magnetic stand.

[_] 9 Centrifuge the thawed, room temperature Resuspension Buffer to 600 xg for 5 seconds.

[_] 10 Add 17.5 µl Resuspension Buffer to each well of the CDP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 11 Incubate the CDP plate at room temperature for 2 minutes.

[_] 12 Place the CDP plate on the magnetic stand at room temperature for 5 minutes.

[_] 13 Transfer 15 µl of the supernatant (ds cDNA) from the CDP plate to the new 96-well 0.3 ml PCR plate labeled with the ALP barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* on page 11 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

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
(Optional) A-Tailing Control (CTA)	1 tube per 48 reactions	-15° to -25°C	Illumina
A-Tailing Mix (ATL)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Microseal 'B' Adhesive Seal	1	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	3	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	3	15° 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 to 600 xg 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 of diluted A-Tailing Control to each well of the ALP plate.
- If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.

[_] 2 Add 12.5 µl of thawed A-Tailing Mix to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 3 Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

[_] 1 Place the sealed ALP plate on the pre-programmed thermal cycler. Close the lid and select **ATAIL70**.

[_] 2 When the thermal cycler temperature is 4°C, remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 13.

Ligate Adapters

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

Consumables

Item	Quantity	Storage	Supplied By
(Optional) Ligation Control (CTL)	1 tube per 48 reactions	-15° to -25°C	Illumina

Choose from the following depending on the kit you are using:
• TruSeq Stranded mRNA LT Sample Prep Kit contents:
  • RNA Adapter Indices (AR001–AR016, AR018–AR023, AR025, AR027)
• TruSeq Stranded mRNA HT Sample Prep Kit contents:
  • RAP (RNA Adapter Plate)
	1 tube per column of 8 reactions, of each indices being used
	or
	1 RAP
		-15° to -25°C	Illumina

Ligation Mix (LIG)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-15° to -25°C	Illumina
CAP (Clean Up ALP Plate) barcode label	1 label per plate	15° to 30°C	Illumina
PCR (Polymerase Chain Reaction) barcode label	1 label per plate	15° to 30°C	Illumina
RAP (RNA Adapter Plate) barcode label (if using the HT kit)	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plates	2	15° to 30°C	User
AMPure XP beads	92 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	800 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	4–28	15° to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	4–28	15° to 30°C	User

Add LIG

[_] 1 Do one of the following:
- If using RNA Adapter tubes, centrifuge the appropriate/desired thawed tubes to 600 xg for 5 seconds.
- If using a RAP:
  - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
  - Remove the adapter plate tape seal.
  - Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
  - Remove the plastic cover and save the cover if you are not processing the entire plate at once.
  - If this is the first time using this RAP, apply the RAP barcode label to the plate.

NOTE
- The RAP is single-use for each well.
- Illumina recommends that the RAP does not undergo more than 4 freeze-thaw cycles.

[_] 2 Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to 600 xg for 5 seconds.

[_] 3 Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.

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

[_] 5 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, 1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl of diluted Ligation Control to each well of the ALP plate.
- If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.

[_] 6 Add 2.5 µl of Ligation Mix to each well of the ALP plate.

[_] 7 Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.

[_] 8 Do one of the following:
- If using RNA Adapter tubes, add 2.5 µl of the appropriate/desired thawed RNA Adapter Index to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- If using a RAP:

- Place the RAP on the benchtop so that the part number barcode on the long side of the plate is facing you and the clipped corner is located on the lower left.
- Do one of the following to pierce the foil seal:
  - If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing the clean plate over the foil seal.
  - If using only part of the plate, use the bottom of a clean eight-tube strip, with caps attached, to pierce holes in the desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
- Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired thawed RNA Adapter from the RAP well to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 9 Seal the ALP plate with a Microseal 'B' adhesive seal.

[_] 10 Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 2 ALP

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

[_] 2 Remove the ALP plate from the thermal cycler.

Add STL

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

[_] 2 Add 5 µl of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

[_] 1 Vortex the AMPure XP Beads until they are well dispersed, then add 42 µl of mixed AMPure XP Beads to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

[_] 3 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 4 Remove and discard 79.5 µl of the supernatant from each well of the ALP plate.

[_] 5 With the ALP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 6 Incubate the ALP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

[_] 7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.

[_] 8 While keeping the ALP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

[_] 9 Resuspend the dried pellet in each well with 52.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 10 Incubate the ALP plate at room temperature for 2 minutes.

[_] 11 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 12 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode.

[_] 13 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 14 Incubate the CAP plate at room temperature for 15 minutes.

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

[_] 16 Remove and discard 95 µl of the supernatant from each well of the CAP plate.

[_] 17 With the CAP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 18 Incubate the CAP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

[_] 19 Repeat steps 17 and 18 once for a total of two 80% EtOH washes.

[_] 20 While keeping the CAP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

[_] 21 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 22 Incubate the CAP plate at room temperature for 2 minutes.

[_] 23 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 24 Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the PCR barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 17 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR primer cocktail that anneals to the ends of the adapters. The number of PCR cycles should be minimized to avoid skewing the representation of the library.

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-15° to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
TSP1 (Target Sample Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
AMPure XP beads	50 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	5	15° to 30°C	User

Make PCR

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

[_] 2 Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 3 Seal the PCR plate with a Microseal 'B' adhesive seal.

Amp PCR

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

Clean Up PCR

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

[_] 2 Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:
- If using the RNA Adapter tubes, add 50 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- If using the RAP, add 47.5 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 3 Incubate the PCR plate at room temperature for 15 minutes.

[_] 4 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 5 Remove and discard 95 µl of the supernatant from each well of the PCR plate.

[_] 6 With the PCR plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 7 Incubate the PCR plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

[_] 8 Repeat steps 6 and 7 once for a total of two 80% EtOH washes.

[_] 9 While keeping the PCR plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

[_] 10 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 11 Incubate the PCR plate at room temperature for 2 minutes.

[_] 12 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 13 Transfer 30 µl of the clear supernatant from each well of the PCR plate to the corresponding well of the new 0.3 ml PCR plate labeled with the TSP1 barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 19 immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

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

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide*.

Quality Control

[_] 1 Load 1 µl of the 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.

Validate Library

Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for indexing are normalized to 10 nM in the DCT plate without pooling.

Consumables

Item	Quantity	Storage	Supplied By
DCT (Diluted Cluster Template) barcode label	1 label per plate	15° to 30°C	Illumina
PDP (Pooled DCT Plate) barcode label (for indexing only)	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate (for indexing only)	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15° 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 DCT plate to 10 nM using Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.

[_] 3 Gently pipette the entire normalized sample library volume up and down 10 times to mix thoroughly.

[_] 4 Depending on the type of library you want to generate, do one of the following:
- For non-indexed 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 -15° to -25°C.
- For indexed libraries, proceed to Make PDP.

Make PDP (for indexing only)

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

[_] 2 Do one of the following:
- If pooling 2–24 samples:

- Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new 0.3 ml PCR plate labeled with the PDP barcode.

The total volume in each well of the PDP plate should be 10X the number of combined sample libraries and will be 20–240 µl (2–24 libraries).

- If pooling 25–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 from the DCT plate to column 1 of the new MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 from the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result will be a PDP plate with pooled samples in column 1. Gently pipette the entire volume of each well of column 1 up and down 10 times to mix thoroughly.
  - Combine the contents of each well of column 1 into well A2 of the PDP plate, for the final pool.

[_] 3 Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 4 Do one of the following:

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