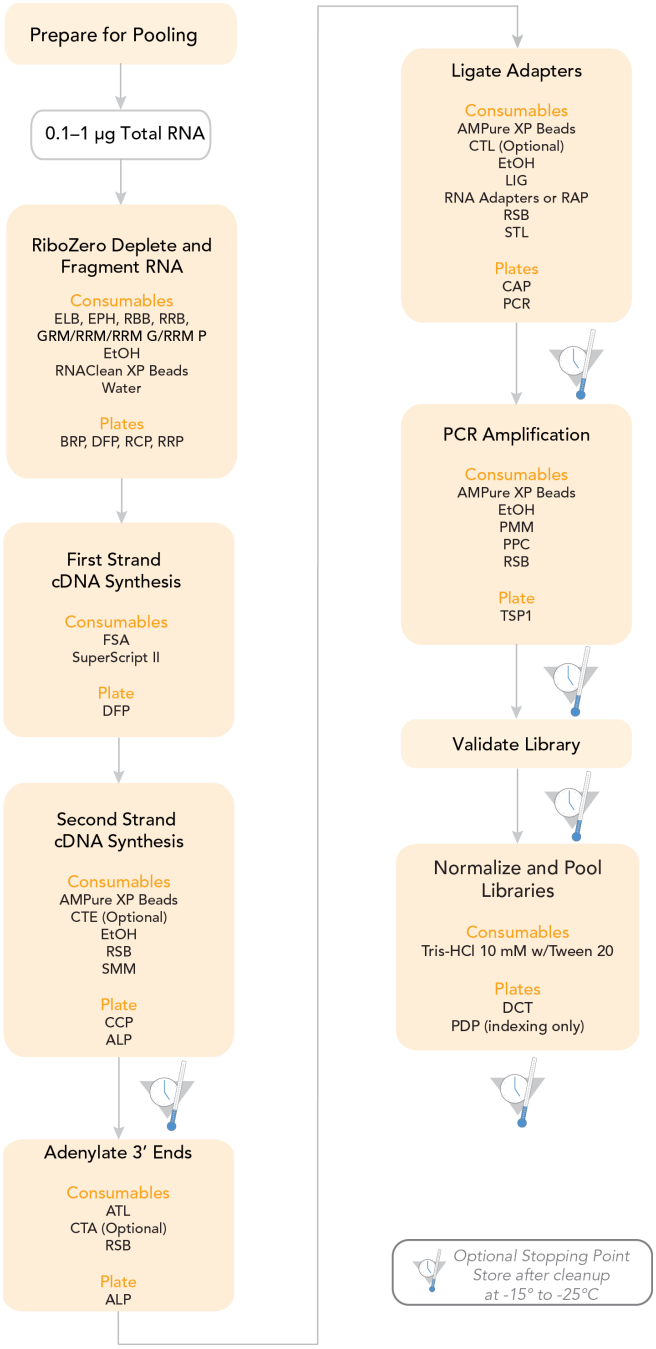
TruSeq Stranded Total RNA Sample Prep LS Protocol

Experienced User Card and Lab Tracking Form

FOR RESEARCH USE ONLY

Date: _____ Description: _____

Illumina Kit Lot #: _____ _____



NOTE

Unless familiar with the LS protocol in the latest version of the *TruSeq Stranded Total RNA Sample Preparation Guide (part # 15031048)*, 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 #: _____
Elute, Prime, Fragment High Mix (EPH)	Lot #: _____
Elution Buffer (ELB)	Lot #: _____
End Repair Control (CTE)	Lot #: _____
First Strand Synthesis Act D Mix (FSA)	Lot #: _____
Globin Removal Mix (GRM)	Lot #: _____
Ligation Control (CTL)	Lot #: _____
Ligation Mix (LIG)	Lot #: _____
PCR Master Mix (PMM)	Lot #: _____
PCR Primer Cocktail (PPC)	Lot #: _____
Resuspension Buffer (RSB)	Lot #: _____
rRNA Binding Buffer (RBB)	Lot #: _____
rRNA Removal Beads (RRB)	Lot #: _____
rRNA Removal Mix (RRM)	Lot #: _____
rRNA Removal Mix Gold (RRM G)	Lot #: _____
rRNA Removal Mix Plant (RRM P)	Lot #: _____
Second Strand Marking Master Mix (SMM)	Lot #: _____
Stop Ligation Buffer (STL)	Lot #: _____
80% Ethanol	Date Prepared: _____

Adapter Indices or RAP	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 #: _____

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Consumables

Adapter Indices or RAP	Lot Number
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 #: _____
RNA Adapter Plate, 96plex (RAP)	Lot #: _____

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Date/Time: _____ Operator: _____

Ribo-Zero™ Deplete and Fragment RNA

This process depletes rRNA from total RNA. After the rRNA is depleted, the remaining RNA is purified, fragmented, and primed for cDNA synthesis.

Consumables

Item	Quantity	Storage	Supplied By
Elute, Prime, Fragment High Mix (EPH)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	2°C to 8°C	Illumina
One of the following, depending on the kit you are using: • Globin Removal Mix (GRM) • rRNA Removal Mix (RRM) • rRNA Removal Mix - Gold (RRM G) • rRNA Removal Mix - Plant (RRM P)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	-15°C to -25°C	Illumina
rRNA Binding Buffer (RBB)	1 tube per 48 reactions	-15°C to -25°C	Illumina
rRNA Removal Beads (RRB)	1 tube per 48 reactions	2°C to 8°C	Illumina
Barcode labels for: • BRP (Bind rRNA Plate) • DFP (Depleted RNA Fragmentation Plate) • RCP (RNA Clean Up Plate) • RRP (rRNA Removal Plate)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR Plates	4	15°C to 30°C	User
Freshly Prepared 70% Ethanol (EtOH)	200 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	15°C to 30°C	User
RNAClean XP Beads	99 µl per sample	2°C to 8°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User
Ultra Pure Water	Enough to dilute each total RNA sample to a final volume of 10 µl	15°C to 30°C	User

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Make BRP

[_] 1 Dilute the total RNA with nuclease-free ultra pure water to a final volume of 10 µl in the new 96-well 0.3 ml PCR plate labeled with the BRP barcode.

[_] 2 Add 5 µl of rRNA Binding Buffer to each well of the BRP plate.

[_] 3 Add 5 µl of one of the following reagents to each well of the BRP plate, depending on the kit you are using:

- Globin Removal Mix
- rRNA Removal Mix
- rRNA Removal Mix - Gold
- rRNA Removal Mix - Plant

[_] 4 Gently pipette the entire volume of each well of the BRP plate up and down 6 times to mix thoroughly.

[_] 5 Seal the BRP plate with a Microseal 'B' adhesive seal.

[_] 6 Return the following to -15°C to -25°C storage:

- rRNA Binding Buffer
- One of the following, depending on the kit you are using:
  - Globin Removal Mix
  - rRNA Removal Mix
  - rRNA Removal Mix - Gold
  - rRNA Removal Mix - Plant

Incubate 1 BRP

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

[_] a Choose the pre-heat lid option and set to 100°C

[_] b 68°C for 5 minutes

[_] 2 After the 5 minute incubation, place the BRP plate on the bench and incubate at room temperature for 1 minute.

Make RRP

[_] 1 Vortex the room temperature rRNA Removal Bead tube vigorously to resuspend the beads.

[_] 2 Add 35 µl of rRNA Removal Beads to each well of the new 96-well 0.3 ml PCR plate labeled with the RRP barcode.

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

[_] 4 Tansfer the entire contents from each well of the BRP plate to the corresponding well of the RRP plate containing rRNA Removal Beads.

[_] 5 Adjust the pipette to 45 µl, then with the tip of the pipette at the bottom of the well, pipette quickly up and down 20 times to mix thoroughly.

[_] 6 Incubate the RRP plate at room temperature for 1 minute.

Date/Time: _____ Operator: _____

[_] 7 Place the RRP plate on the magnetic stand at room temperature for 1 minute.

[_] 8 Transfer all of the supernatant from each well of the RRP plate to the corresponding well of the new 96-well 0.3 ml PCR plate labeled with the RCP barcode.

[_] 9 Place the RCP plate on the magnetic stand at room temperature for 1 minute.

[_] 10 Return the rRNA Removal Beads to 2°C to 8°C storage.

Clean Up RCP

[_] 1 Vortex the RNAClean XP beads until they are well dispersed, then add 99 µl of well-mixed RNAClean XP beads to each well of the RCP plate containing ribosomal depleted RNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
If starting with degraded total RNA, add 193 µl of well-mixed RNAClean XP beads to each well of the RCP plate containing ribosomal depleted RNA.

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

Start time: _____ Stop time: _____

[_] 3 Place the RCP 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: _____

[_] 4 Remove and discard all of the supernatant from each well of the RCP plate.

[_] 5 With the RCP plate on the magnetic stand, add 200 µl freshly prepared 70% EtOH to each well without disturbing the beads.

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

[_] 7 Let the RCP plate stand at room temperature for 15 minutes to dry, and then remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 8 Centrifuge the thawed, room temperature Elution Buffer to 600 × g for 5 seconds.

[_] 9 Add 11 µl Elution Buffer to each well of the RCP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

Start time: _____ Stop time: _____

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

Start time: _____ Stop time: _____

[_] 12 Return the Elution Buffer to 2°C to 8°C storage.

[_] 13 Transfer 8.5 µl supernatant from the RCP plate to the new 96-well 0.3 ml PCR plate labeled with the DFP barcode.

[_] 14 Add 8.5 µl Elute, Prime, Fragment High Mix to each well of the DFP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 15 Seal the DFP plate with a Microseal 'B' adhesive seal.

[_] 16 Return the Elute, Prime, Fragment High Mix to -15°C to -25°C storage and the RNAClean XP Beads tube to 2°C to 8°C storage.

Incubate 1 DFP

[_] 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **Elution 2 - Frag - Prime** program.

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

[_] 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

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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 and random primers. 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 per 48 reactions	-15°C to -25°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	-15°C to -25°C	User

Add FSA

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

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

[_] 3 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 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. Gently pipette the entire volume up and down 6 times to mix thoroughly.

[_] 5 Seal the DFP plate with a Microseal 'B' adhesive seal and centrifuge briefly.

[_] 6 Return the First Strand Synthesis Mix Act D tube to -15°C to -25°C storage immediately after use.

Incubate 2 DFP

[_] 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **Synthesize 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 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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_____

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
(Optional) End Repair Control (CTE)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-15°C to -25°C	Illumina
ALP (Adapter Ligation Plate) Barcode Label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR 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	2	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 Do one of the following:

- If using the in-line control reagent:
  - Centrifuge the thawed End Repair Control tube to 600 × g 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 DFP plate.
- If not using the in-line control reagent, add 5 µl of Resuspension Buffer to each well of the DFP plate.

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

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

[_] 5 Seal the DFP plate with a Microseal 'B' adhesive seal.

[_] 6 Return the Second Strand Marking Master Mix tube to -15°C to -25°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: _____

[_] 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 DFP plate containing 50 µl of ds cDNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

Start time: _____ Stop time: _____

[_] 4 Place the DFP 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: _____

[_] 5 Remove and discard 135 µl supernatant from each well of the DFP plate.

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

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

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

[_] 9 Let the DFP plate stand at room temperature for 15 minutes to dry, and then remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 10 Centrifuge the thawed, room temperature Resuspension Buffer to 600 × g for 5 seconds.

[_] 11 Add 17.5 µl Resuspension Buffer to each well of the DFP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 12 Incubate the DFP plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 13 Place the DFP plate on the magnetic stand at room temperature for 5 minutes.

Start time: _____ Stop time: _____

Date/Time: _____ Operator: _____

[_] 14 Transfer 15 µl supernatant (ds cDNA) from the DFP 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 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 -15°C to -25°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
(Optional) A-Tailing Control (CTA)	1 tube per 48 reactions	-15°C to -25°C	Illumina
A-Tailing Mix (ATL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°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)	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 to 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 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.

Date/Time: _____ Operator: _____

Incubate 1 ALP

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

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 37°C for 30 minutes
- [_] c 70°C for 5 minutes
- [_] d Hold at 4°C

[_] 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 17.

Comments

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Date/Time: _____ Operator: _____

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°C to -25°C	Illumina

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

Ligation Mix (LIG)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Barcode labels for: • CAP (Clean Up ALP Plate) • PCR (Polymerase Chain Reaction Plate) • RAP (RNA Adapter Plate) (if using the HT kit)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR Plates	2	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	2	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

Date/Time: _____ Operator: _____

Add LIG

[_] 1 Do one of the following:

- If using RNA Adapter tubes, centrifuge the thawed tubes to 600 × g for 5 seconds.
- If using a RAP:
  - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.

    Start time: _____ Stop time: _____
  - Remove the adapter plate tape seal.
  - Centrifuge the plate to 280 × g for 1 minute to collect all of the adapter to the bottom of the well.
  - Remove the plastic cover. Save the cover if you are not processing the entire plate at one time.
  - If it 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 × g for 5 seconds.

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

[_] 8 Do one of the following:

- If using RNA Adapter tubes, add 2.5 µl of the 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 on the lower left.

Date/Time: _____ Operator: _____

- Do one of the following to pierce the foil seal:
  - If using the entire plate at one time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press 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 seals of the wells that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.
- Using an eight-tip multichannel pipette, transfer 2.5 µl of the 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 × g 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.

Start time: _____ Stop time: _____

[_] 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 for at least 1 minute or until they are well dispersed.

[_] 2 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.

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

Start time: _____ Stop time: _____

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

Start time: _____ Stop time: _____

[_] 5 Remove and discard 79.5 µl supernatant from each well of the ALP plate.

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

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

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

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Date/Time: _____ Operator: _____

[_] 9 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.
Start time: _____ Stop time: _____

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

[_] 11 Add 52.5 µl Resuspension Buffer to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.

[_] 12 Incubate the ALP plate at room temperature for 2 minutes.
Start time: _____ Stop time: _____

[_] 13 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
Start time: _____ Stop time: _____

[_] 14 Transfer 50 µl 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.

[_] 15 Vortex the AMPure XP Beads until they are well dispersed.

[_] 16 Add 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second cleanup. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 17 Incubate the CAP plate at room temperature for 15 minutes.
Start time: _____ Stop time: _____

[_] 18 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
Start time: _____ Stop time: _____

[_] 19 Remove and discard 95 µl supernatant from each well of the CAP plate.

[_] 20 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well.

[_] 21 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 22 Repeat steps 20 and 21 one time for a total of two 80% EtOH washes.

[_] 23 With 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.
Start time: _____ Stop time: _____

[_] 24 Add 22.5 µl Resuspension Buffer to each well of the CAP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.

[_] 25 Incubate the CAP plate at room temperature for 2 minutes.
Start time: _____ Stop time: _____

[_] 26 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
Start time: _____ Stop time: _____

[_] 27 Transfer 20 µl 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.

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 23, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to seven days.

Comments

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Ligate Adapters

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	-15°C to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
TSP1 (Target Sample Plate) Barcode Label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR 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
Microseal 'B' Adhesive Seals	2	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 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.

Experienced User Card and Lab Tracking Form

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 until they are well dispersed.

[_] 3 Do one of the following, depending on the adapter type used:

- 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.

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

Start time: _____ Stop time: _____

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

Start time: _____ Stop time: _____

[_] 6 Remove and discard 95 µl supernatant from each well of the PCR plate.

[_] 7 With the PCR plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 8 Incubate the PCR plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 9 Repeat steps 7 and 8 one time for a total of two 80% EtOH washes.

[_] 10 With 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.

Start time: _____ Stop time: _____

[_] 11 Add 32.5 µl Resuspension Buffer to each well of the PCR plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 12 Incubate the PCR plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

Experienced User Card and Lab Tracking Form

Date/Time: _____ Operator: _____

[_] 13 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 14 Transfer 30 µl 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 immediately to *Validate Library* on page 27, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 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

To achieve the highest quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide (part # 11322363).*

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.

Comments

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_____

Date/Time: _____ Operator: _____

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. DNA libraries not intended for pooling 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 MIDI plates	2 (second plate for pooling only, if pooling > 40 samples)	15°C to 30°C	User
96-well 0.3 ml PCR plate (for pooling only, if pooling ≤ 40 samples)	1	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	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 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-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 -15°C to -25°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.

[_] 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 is 10 X the number of combined sample libraries and 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 of the DCT plate to column 1 of the new 0.3 ml PCR or MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 of 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 is 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°C to -25°C.

Comments

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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

MSDSs

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

Product Documentation

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

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