SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Sequencing

mRNA Library Preparation Protocol

Protocol

Version D0, July 2015

SureSelect platform manufactured with Agilent SurePrint Technology

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Agilent Technologies

Notices

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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect Strand Specific RNA Library Prep system.

This protocol is specifically developed and optimized to prepare mRNA sequencing libraries from total RNA samples.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps to prepare cDNA sequencing libraries from mRNA isolated from total RNA samples.

3 Reference

This chapter contains reference information, including component kit contents and index sequences.

What's New in Version D.0

- Updated product labeling statement.

What's New in Version C.0

- Support for kits supplied with either of two indexing primer configurations.

  Kits with revised index configuration (typically received December, 2014 or later) include indexing primers A01 through H02 provided in white-capped tubes (16 Reaction kits) or indexing primers A01 through H12 provided in a blue plate (96 Reaction kits). For kit content details see page 40. For nucleotide sequences of the 8-bp indexes in this revised configuration, see Table 23 on page 43.

  Kits with original index configuration (typically received before December, 2014), include indexing primers 1–16 provided in clear-capped tubes (16 Reaction kits) or indexing primers 1–48 provided in a clear plate (96 Reaction kits). For kit content details see page 44. For nucleotide sequences of the 8-bp indexes in this original configuration, see Table 28 on page 47 through Table 30 on page 49.

Content

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Avoid repeated freeze-thaw cycles of stock and diluted RNA and cDNA solutions. Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.
- When preparing master mix reagent stock solutions for use:
  1. Thaw the reagent vial as rapidly as possible without heating above room temperature.
  2. Mix on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store vials used during an experiment on ice or in a cold block.
  4. Library Preparation Master Mixes should not be frozen and thawed more than five times. If you plan to use the reagents in more than five experiments, aliquot to multiple vials to minimize freeze/thaw cycles for each vial.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for SureSelect Strand Specific RNA Library Prep

Description	Vendor and part number
SureSelect Strand Specific RNA Reagent Kit Illumina platforms (ILM), 16 Samples Illumina platforms (ILM), 96 Samples	Agilent p/n G9691A p/n G9691B
Actinomycin D*	Sigma p/n A1410
DMSO	Sigma p/n D8418
Agencourt AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
DNA 1000 Kit	Agilent p/n 5067-1504
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

* Actinomycin D should be obtained as a solid and prepared at 4 μg/μl concentration in DMSO no more than one month before use. See page 13 for additional information.

Required Equipment

Table 2 Required Equipment for SureSelect Strand Specific RNA Library Prep

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible 96-well plates	Agilent p/n 410088
Tube cap strips, domed	Agilent p/n 410096
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Magnetic separator for 96-well plates	Phenix Research Products p/n RX-IMAG-96P or equivalent
Labnet MPS1000 Mini Plate Spinner (optional)	Labnet International p/n C1000 or equivalent
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

2 Sample Preparation

See Figure 1 for a summary of the SureSelect mRNA sequencing workflow.

This section contains instructions for strand-specific RNA sequencing cDNA library preparation for the Illumina platform.

Before you begin, prepare a stock solution of 4 µg/µL Actinomycin D in DMSO. Aliquots of the Actinomycin D DMSO stock solution may be stored at –20°C, protected from light, for up to one month for use in subsequent runs. During the library preparation protocol, the DMSO stock is diluted with water, immediately before use, to a final Actinomycin D concentration of 120 ng/µL. (See page 19 for more information.)

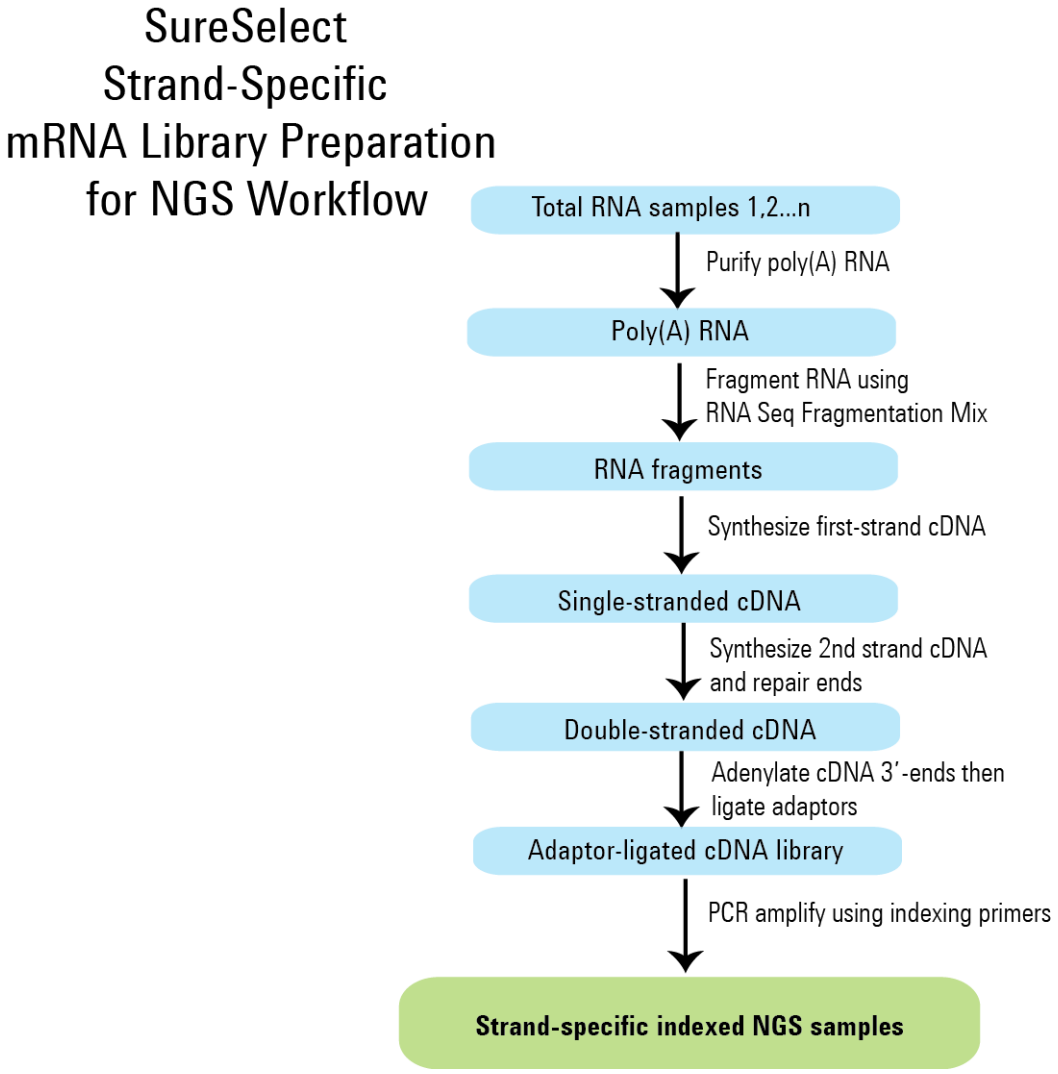

Figure 1 Overall mRNA sequencing sample preparation workflow.

Step 1. Purify poly(A) RNA from total RNA

In this step, poly(A) RNA is purified from total RNA using two serial rounds of binding to oligo(dT) magnetic particles.

Before you begin, prepare total RNA for each sample in the run. The amount of total RNA needed for the library preparation protocol is 50 ng to 4 µg.

NOTE For optimal performance, total RNA samples should have an RNA Integrity Number (RIN) of 8 or more, based on analysis using Agilent's 2100 Bioanalyzer.

Bring the reagents listed in Table 3 to room temperature before starting the protocol.

Table 3 Reagents for poly(A) RNA purification and RNA fragmentation

Kit Component	Storage Location	Where Used in Protocol
Oligo(dT) Microparticles	RNA Library Prep Kit Box 2, 4°C	page 15
RNA Seq Bead Washing Buffer	RNA Library Prep Kit Box 2, 4°C	page 16
RNA Seq Bead Elution Buffer	RNA Library Prep Kit Box 2, 4°C	page 17
RNA Seq Bead Binding Buffer	RNA Library Prep Kit Box 2, 4°C	page 17
RNA Seq Fragmentation Mix	RNA Library Prep Kit Box 1, –20°C	page 18

1 Prepare each total RNA sample in a final volume of 25 µL of nuclease-free water and place the samples in separate wells of a 96-well plate.

2 Vortex the Oligo(dT) Microparticles until the suspension appears homogeneous and consistent in color. If bead aggregates are still present after vortexing, mix thoroughly by pipetting up and down until the suspension appears homogeneous.

3 Add 25 µL of the homogeneous Oligo(dT) bead suspension to each total RNA sample well.

4 Seal the plate wells, then gently vortex the plate for 5 seconds and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.

5 Incubate the plate in the SureCycler thermal cycler (with the heated lid ON) and run the program in Table 4 to denature the RNA.

Table 4 Thermal cycler program for RNA denaturation

Step	Temperature	Time
Step 1	65°C	5 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

6 After the thermal cycler reaches the 4°C Hold step, remove the plate and incubate at room temperature for 5 minutes, to allow poly(A) RNA binding to the oligo(dT) beads.

7 Move the plate to a magnetic separation device at room temperature. Wait for the solution to clear (approximately 2 to 5 minutes).

8 While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

9 Remove the plate from the magnetic stand. Gently add 200 µL of RNA Seq Bead Washing Buffer to each well.

10 Seal the plate, then gently vortex the plate for 5 seconds. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.

CAUTION The Bead Washing Buffer contains detergent. It is important to process mixtures of the beads with the wash buffer without introducing bubbles or foam. If bubbles or foam are present during the wash steps, briefly spin the plate in a centrifuge before continuing.

11 Place the plate in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).

12 While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

13 Remove the plate from the magnetic stand. Add 25 µL of RNA Seq Bead Elution Buffer to each sample well.

14 Seal the plate, then gently vortex the plate for 5 seconds. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.

15 Incubate the plate in the thermal cycler (with the heated lid ON) and run the program in Table 5.

Table 5 Thermal cycler program for RNA elution

Step	Temperature	Time
Step 1	80°C	2 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

16 After the thermal cycler reaches the 4°C Hold step, remove the plate and add 25 µL of RNA Seq Bead Binding Buffer to each sample well.

17 Seal the plate, then gently vortex the plate for 5 seconds. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.

18 Incubate the plate at room temperature for 5 minutes, to allow poly(A) RNA to re-bind the beads,

19 Place the plate in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).

20 While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

21 Remove the plate from the magnetic stand. Gently add 200 µL of RNA Seq Bead Washing Buffer to each well.

22 Seal the plate, then gently vortex the plate for 5 seconds. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.

23 Place the plate in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).

24 While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

25 Proceed immediately to Step 2. Fragment poly(A) RNA.

Step 2. Fragment poly(A) RNA

In this step, the purificd poly(A) RNA is chemically-fragmented to a size appropriate for RNA sequencing library preparation.

1 Remove the plate, containing the collected poly(A) RNA-bound beads, from the magnetic stand. Add 19 µL of RNA Seq Fragmentation Mix to each sample well.

2 Seal the plate, then gently vortex the plate for 5 seconds. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.

3 Incubate the plate in the thermal cycler (with the heated lid ON) and run the program in Table 6.

Table 6 Thermal cycler program for RNA fragmentation

Step	Temperature	Time
Step 1	94°C	8 minutes
Step 2	4°C	Hold

4 Complete step 1 and step 2 on page 19 during the 8-minute incubation at 94°C.

Step 3. Synthesize first-strand cDNA

Use SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw and mix the RNA Seq First Strand Master Mix, and keep on ice.

Hold samples and enzyme mixtures on ice during the following setup steps.

CAUTION To ensure strand-specificity, you must prepare the 120 ng/µL Actinomycin D solution in step 1, below, immediately before use. The stock solution of 4 µg/µL Actinomycin D in DMSO must be prepared less than one month prior to use and stored in aliquots at –20°C, protected from light.

CAUTION SureSelect Strand Specific RNA Library Prep master mixes are viscous. Mix thoroughly by vortexing before removing an aliquot for use and after combining the master mixes with other solutions.

1 Prepare a fresh 120 ng/µL Actinomycin D dilution in water from a stock solution of 4 µg/µL Actinomycin D in DMSO, according to Table 7.

Table 7 Preparation of 120 ng/µl Actinomycin D

Reagent	Volume for up to 96-reaction run (includes excess)
Actinomycin D (4 µg/µl in DMSO)	3 µL
Nuclease-free water	97 µL
Total	**100 µL**

2 Prepare the appropriate amount of RNA Seq First Strand Master Mix + Actinomycin D mixture, on ice, according to the table below.

Table 8 Preparation of First Strand Master Mix/Actinomycin D mixture

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Actinomycin D (120 ng/µl in H_2O)	0.5 µL	8.5 µL
RNA Seq First Strand Master Mix	8.0 µL	136 µL
Total	**8.5 µL**	**144.5 µL**

3 Transfer the sample plate, containing fragmented RNA and beads, from the thermal cycler to the magnetic stand at room temperature. Leave the plate on the magnetic stand for at least 5 minutes.

4 Keep the RNA sample plate in the magnetic stand at room temperature while you carefully transfer 17 µL of each fragmented RNA-containing supernatant to a fresh plate well. Do not touch or disturb the beads while removing the RNA solution. Once all of the samples are transferred, place the plate on ice or in a cold block.

5 Add 8.5 µL of First Strand Master Mix/Actinomycin D mixture prepared in step 2 to each RNA sample well.

6 Seal the plate wells, then gently vortex the plate for 5 seconds.

7 Spin the plate at 1500 x *g* for 1 minute.

8 Incubate the plate in the thermal cycler (with the heated lid ON) and run the program in Table 9.

Table 9 Thermal cycler program for first-strand cDNA synthesis

Step	Temperature	Time
Step 1	25°C	10 minutes
Step 2	37°C	40 minutes
Step 3	4°C	Hold

Step 4. Purify first strand cDNA using AMPure XP beads

1 Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*

2 Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete SureSelect RNA Library Preparation protocol requires 1.6 mL of fresh 70% ethanol per sample.

3 Mix the bead suspension well so that the suspension appears homogeneous and consistent in color.

4 Add 46 µL of the homogeneous bead suspension to each 25.5-µL sample in the PCR plate. Seal the plate wells, then vortex the plate for 5 seconds. Briefly spin the plate to collect the liquid.

5 Incubate samples for 5 minutes at room temperature.

6 Put the plate on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).

7 While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

8 Continue to keep the plate in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.

9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

10 Repeat step 8 and step 9 step once for a total of two washes.

11 After removing the 200 µL ethanol supernatant from the second wash, spin the plate briefly, return the plate to the magnetic stand, and then remove any remaining ethanol droplets with a pipette.

12 Dry the samples on the thermal cycler (with lid open) at 37°C for 1 minute. Do not overdry the samples.

13 Add 21 µL nuclease-free water to each sample well.

14 Cover the sample wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate in the magnetic stand and leave for 2 minutes or until the solution is clear.

17 Remove 20 µL of cleared supernatant to a fresh plate well. You can discard the beads at this time.

18 Proceed immediately to "Step 5. Synthesize second-strand cDNA and repair the ends" on page 23.

Step 5. Synthesize second-strand cDNA and repair the ends

Use reagents from the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw the vial of RNA Seq Second Strand + End Repair Enzyme Mix and the vial of RNA Seq Second Strand + End Repair Oligo Mix and keep on ice. Vortex each vial for 5 seconds to mix before use.

Hold samples and reagent mixtures on ice during the following setup steps.

CAUTION SureSelect Strand Specific RNA Library Prep reagents are viscous. Mix thoroughly by vortexing before removing an aliquot for use and after combining the reagents with other solutions.

1. Add 25 µL of RNA Seq Second Strand + End Repair Enzyme Mix to each 20-µL purified first-strand cDNA sample.
2. Add 5 µL of RNA Seq Second Strand + End Repair Oligo Mix to each sample well, for a total reaction volume of 50 µL.
3. Seal the plate wells, then vortex the plate for 5 seconds.
4. Spin the plate at 1500 x *g* for 1 minute.
5. Incubate the plate in the thermal cycler and run the program in Table 10. **Do not use a heated lid**.

Table 10 Thermal cycler program for second-strand synthesis and end repair

Step	Temperature	Time
Step 1	16°C	30 minutes
Step 2	4°C	Hold

Step 6. Purify cDNA using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 90 µL of the homogeneous bead suspension to each 50-µL sample in the PCR plate. Seal the plate wells, then vortex the plate for 5 seconds. Briefly spin the plate to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 step once for a total of two washes.
11. Dry the samples on the thermal cycler (with lid open) at 37°C for 3 minutes.
12. Add 21 µL nuclease-free water to each sample well.
13. Cover the sample wells with strip caps, then vortex the plate for 5 seconds and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 minutes or until the solution is clear.
16. Remove 20 µL of cleared supernatant to a fresh plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 7. Adenylate cDNA 3' ends

Use the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw and mix the RNA Seq dA Tailing Master Mix and keep on ice.

CAUTION SureSelect Strand Specific RNA Library Prep master mixes are viscous. Mix thoroughly by vortexing before removing an aliquot for use and after combining the master mixes with other solutions.

1. Add 20 µL of RNA Seq dA Tailing Master Mix to each 20-µL purified, end-repaired cDNA sample.
2. Seal the plate wells, then vortex the plate for 5 seconds.
3. Spin the plate at 1500 x *g* for 1 minute.
4. Incubate the plate in the thermal cycler and run the program in Table 11. **Do not use a heated lid**.

Table 11 Thermal cycler program for dA-tailing

Step	Temperature	Time
Step 1	37°C	15 minutes
Step 2	4°C	Hold

Step 8. Ligate adaptors

Use the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw and mix the SureSelect Ligation Master Mix and the SureSelect Oligo Adaptor Mix and keep on ice.

CAUTION SureSelect Strand Specific RNA Library Prep master mixes are viscous. Mix thoroughly by vortexing before removing an aliquot for use and after combining the master mixes with other solutions.

1. Transfer the cDNA sample plate to ice, then add 5 µL of SureSelect Ligation Master Mix to each A-tailed cDNA sample well. Mix by pipetting.
2. Add 5 µL of SureSelect Oligo Adaptor Mix to each sample.
3. Seal the plate wells, then vortex the plate for 5 seconds and briefly spin the plate to collect the liquid.
4. Incubate the plate in the thermal cycler and run the program in Table 12. **Do not use a heated lid**.

Table 12 Thermal cycler program for adaptor ligation

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Step 9. Purify adaptor-ligated DNA using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 60 µL of the homogeneous bead suspension to each 50-µL sample in the PCR plate. Seal the plate wells, then vortex the plate for 5 seconds. Briefly spin the plate to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 step once for a total of two washes.
11. Dry the samples on the thermal cycler (with lid open) at 37°C for 3 minutes.
12. Add 18 µL nuclease-free water to each sample well.
13. Cover the sample wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 3 minutes or until the solution is clear.
16. Remove 17 µL of cleared supernatant to a fresh plate well. You can discard the beads at this time.

Step 10. Amplify and index the adaptor-ligated cDNA library

In this step, the adaptor ligated cDNA is amplified in a three-primer PCR that includes the appropriate indexing primer. Amplification cycle number is based on the initial amount of RNA sample used for library preparation.

NOTE

The PCR protocol detailed below is appropriate for mRNA library amplification and PCR indexing without target enrichment. If samples will be target-enriched after library preparation, see publication part number G9691-90000 at www.genomics.agilent.com for the appropriate pre-capture amplification and post-capture indexing protocols.

Use the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw and mix the reagents listed in Table 13 and Table 14 below and keep on ice.

CAUTION

SureSelect Strand Specific RNA Library Prep master mixes are viscous. Mix thoroughly by vortexing before removing an aliquot for use and after combining the master mixes with other solutions.

1 Prepare a 1:20 dilution of the RNA Seq ILM Reverse PCR Primer, according to Table 13.

Table 13 Preparation of reverse primer dilution

Reagent	Volume for up to 16 reactions (includes excess)
Nuclease-free water	19 µL
RNA Seq ILM Reverse PCR Primer	1 µL
Total	**20 µL**

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 14, on ice. Mix well on a vortex mixer.

Table 14 Preparation of PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
RNA Seq PCR Master Mix	25 µL	425 µL
Uracil DNA Glycosylase (UDG)	1 µL	17 µL
SureSelect Primer (forward primer)	1 µL	17 µL
1:20 dilution of RNA Seq ILM Reverse PCR Primer prepared in Table 13	1 µL	17 µL
Total	**28 µL**	**476 µL**

3 Add 28 µL of the PCR reaction mix prepared in step 2 to each sample well containing 17 µL of purified, adaptor-ligated cDNA.

Mix by pipetting. Change pipette tips between samples.

4 Add 5 µL of the appropriate indexing primer **(Index A01–H06 OR Index 1–48)** to each PCR reaction mixture well.

CAUTION

Only use the indexing primers provided in columns 1 through 6 of the plate provided with 96 reaction kits. Provided plates also contain indexing primers in columns 7 through 12, which are used in other SureSelect protocols.

For 96 reaction kits with indexes provided in a blue plate (revised configuration), use only Indexes A01–H06 in step 4. See page 42 for a plate map.

For 96 reaction kits with indexes provided in a clear plate (original configuration), use only RNA Seq Indexes 1–48 in step 4. See page 46 for a plate map.

5 Incubate the plate in the thermal cycler (with the heated lid ON) and run the program in Table 15

.

Table 15 Thermal cycler program for mRNA Library PCR indexing

Segment	Number of Cycles	Temperature	Time
1	1	37°C	15 minutes
2	1	95°C	2 minutes
3	10–16 cycles (see Table 16)	95°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	1	4°C	Hold

Table 16 mRNA Library PCR indexing cycle number recommendations

Amount of total RNA used for library prep	Cycle Number
50 ng–200 ng	14–16
201 ng–2 µg	12–14
2.1 µg–4 µg	10–12

NOTE If you started with the minimum total RNA input amount of 50 ng, use 16 amplification cycles in the PCR indexing amplification program (Table 15).

Step 11. Purify the amplified library with AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 60 µL of the homogeneous bead suspension to each 50-µL PCR reaction in the plate. Seal the plate wells, then vortex the plate for 5 seconds. Briefly spin the plate to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 step once for a total of two washes.
11. Dry the samples on the thermal cycler (with lid open) at 37°C for 3 minutes.
12. Add 26 µL nuclease-free water to each sample well.
13. Cover the sample wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 minutes or until the solution is clear.
16. Remove 25 µL of cleared supernatant to a fresh plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 12. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit. See the *DNA 1000 Kit Guide*, at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=46764.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results. A sample electropherogram is shown in Figure 2.
   a. Measure the concentration of the library by integrating under the peak at approximately 200 to 600 bp. For accurate quantification, make sure that the concentration falls within the linear range of the assay.
   b. Analyze the 100 to 150-bp range of the electropherogram. A peak at this position indicates the presence of adaptor-dimers in the cDNA sample, which need to be removed for optimal sequencing results.

      If adaptor-dimers are observed, dilute the prepared library sample to a final volume of 50 µL using nuclease-free water, then proceed to "Step 13. Remove adaptor-dimers with AMPure XP beads (optional)" on page 34.

      If adaptor-dimers are not observed, proceed to "Step 14. Prepare samples for multiplexed sequencing" on page 35.

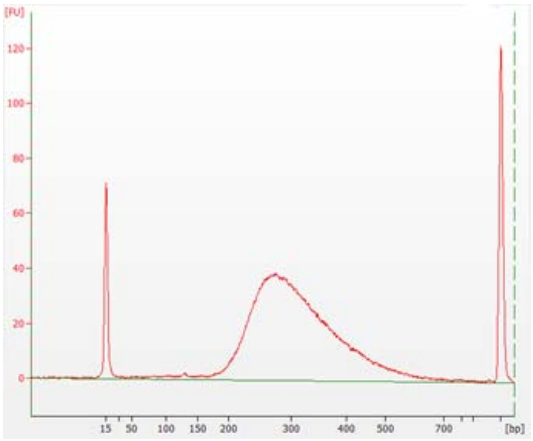

Figure 2 Analysis of amplified prepped library cDNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 200 to 600 bp.

Step 13. Remove adaptor-dimers with AMPure XP beads (optional)

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 60 µL of the homogeneous bead suspension to each 50-µL library sample in the plate. Seal the plate wells, then vortex the plate for 5 seconds. Briefly spin the plate to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 step once for a total of two washes.
11. Dry the samples on the thermal cycler (with lid open) at 37°C for 3 minutes.
12. Add 26 µL nuclease-free water to each sample well.
13. Cover the sample wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 minutes or until the solution is clear.
16. Remove 25 µL of cleared supernatant to a fresh plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 14. Prepare samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of indexed sample to use.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where *V(f)* is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool

is the number of indexes, and

C(i) is the initial concentration of each indexed sample.

Table 17 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 17 Example of indexed sample volume calculation for total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

Step 15. Prepare and analyze sequencing samples

Proceed to cluster amplification using the Illumina Paired-End Cluster Generation Kit; refer to the manufacturer's instructions for this step. The optimal seeding concentration for cluster amplification from SureSelect mRNA libraries is approximately 10-12 pM.

NOTE The optimal seeding concentration may vary, depending on the method used for library quantification and fragment size distribution.

This protocol has been validated with 2 x 100-base paired-end reads. However, read length can be adjusted to achieve the desired research goals.

Sequencing run setup guidelines for 8-bp indexes

Sequencing runs must be set up to perform an 8-nt index read. For the HiSeq platform, use the *Cycles* settings shown in Table 18. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

For complete index sequence information, see tables starting on page 47.

Table 18 HiSeq platform Run Configuration screen Cycle Number settings*

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	9
Index 2 (i5)	0
Read 2	100

* Settings apply to v3.0 SBS chemistry.

Sequence analysis guidelines

The SureSelect^{XT} RNA sequencing library preparation method preserves RNA strandedness as described here. The first strand of cDNA is the reverse complement of the poly(A) RNA transcript strand. Since the second strand of cDNA is eliminated before PCR, the sequence of read 1, which starts at the P5 end, matches only the first strand of cDNA. Read 2, which starts at the P7 end, matches the second strand of cDNA (the poly(A) RNA transcript strand). When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (http://picard.sourceforge.net/) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY*= *SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

3 Reference

This chapter contains reference information, including component kit contents and index sequences.

CAUTION This chapter contains two sets of index sequence and kit content information. The first section covers kits with indexing primers supplied in Library Prep Kit p/n 5500-0134 or 5500-0135 (typically received December, 2014 or later). The second section covers kits with indexing primers supplied in Library Prep Kit p/n 5500-0116 or 5500-0117 (typically received before December, 2014). **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Reference Information for Kits with Revised Index Configuration (indexing primers in white-capped tubes or blue plate)

Use the reference information in this section if your kit includes Library Prep Kit p/n 5500-0134 or 5500-0135. If your kit does not include one of these component kits, see page 44 for kit content and indexing primer information.

Kit Contents

The SureSelect^{XT} RNA Reagent Kits contain the following component kits:

Table 19 SureSelect RNA-Seq Kit Content-Revised Index Configuration

Component Kits	Storage Condition	16 Samples	96 Samples
SureSelect Strand Specific RNA Library Prep, ILM, Box 1	–20°C	5500-0134	5500-0135
SureSelect Strand Specific RNA Library Prep, ILM, Box 2	4°C	5190-6410	5190-6411

NOTE SureSelect capture libraries and reagents must be used within one year of receipt.

The contents of each of the component kits listed in Table 19 are described in the tables below.

Table 20 SureSelect Strand Specific RNA Library Prep, ILM, Box 1 Content-Revised Index Configuration

Kit Component	16 Reactions (p/n 5500-0134)	96 Reactions (p/n 5500-0135)
RNA Seq Fragmentation Mix	tube with red cap	bottle
RNA Seq First Strand Master Mix	tube with orange cap	tube with orange cap
RNA Seq Second-Strand + End-Repair Enzyme Mix	tube with blue cap	bottle
RNA Seq Second-Strand + End-Repair Oligo Mix	tube with yellow cap	tube with yellow cap
RNA Seq dA Tailing Master Mix	tube with green cap	bottle
SureSelect Ligation Master Mix	tube with purple cap	tube with purple cap
SureSelect Oligo Adaptor Mix	tube with blue cap	tube with blue cap
RNA Seq PCR Master Mix	tube with red cap	bottle
Uracil DNA Glycosylase (UDG)	tube with yellow cap	tube with yellow cap
SureSelect Primer	tube with brown cap	tube with brown cap
RNA Seq ILM Reverse PCR Primer	tube with black cap	tube with black cap
RNA Seq ILM Post-capture PCR Primer*	tube with green cap	tube with green cap
SureSelect^{XT} Indexes, 8 bp†	SSEL 8bp Indexes A01 through H02, provided in 16 tubes with white caps	SureSelect 8 bp Indexes A01 through H12, provided in blue 96-well plate‡

* The provided SureSelect ILM Post-capture PCR Primer is not used in the workflow described in this manual. This primer is used in the SureSelect RNA Sequencing Target Enrichment workflow detailed in publication part number G9691-90000.

† See Table 23 on page 43 for index sequences.

‡ See Table 22 on page 42 for a plate map. Although the provided plate contains 96 indexing primers, only indexes A01–H06 should be used for the mRNA library preparation workflow. Wells contain enough volume for two mRNA library preparation reactions per index, using the protocol on page 29.

Table 21 SureSelect Strand Specific RNA Library Prep, ILM, Box 2 Content

Kit Component	16 Reactions	96 Reactions
Oligo(dT) Microparticles	tube with brown cap	bottle
RNA Seq Bead Binding Buffer	tube with purple cap	bottle
RNA Seq Bead Washing Buffer	bottle	bottle
RNA Seq Bead Elution Buffer	tube with green cap	bottle
Nuclease Free Water	bottle	bottle

Table 22 Plate map for SSEL 8bp Indexes A01 through H12 provided in blue plate in Library Prep kit p/n 5500-0135. Use only indexes A01–H06 (Columns 1 to 6) for the mRNA library preparation workflow.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**A01**	**A02**	**A03**	**A04**	**A05**	**A06**	A07	A08	A09	A10	A11	A12
B	**B01**	**B02**	**B03**	**B04**	**B05**	**B06**	B07	B08	B09	B10	B11	B12
C	**C01**	**C02**	**C03**	**C04**	**C05**	**C06**	C07	C08	C09	C10	C11	C12
D	**D01**	**D02**	**D03**	**D04**	**D05**	**D06**	D07	D08	D09	D10	D11	D12
E	**E01**	**E02**	**E03**	**E04**	**E05**	**E06**	E07	E08	E09	E10	E11	E12
F	**F01**	**F02**	**F03**	**F04**	**F05**	**F06**	F07	F08	F09	F10	F11	F12
G	**G01**	**G02**	**G03**	**G04**	**G05**	**G06**	G07	G08	G09	G10	G11	G12
H	**H01**	**H02**	**H03**	**H04**	**H05**	**H06**	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of SureSelect^{XT} Indexes A01 to H12

Each index is 8 nt in length. See page 37 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 23 SureSelect RNA Seq Indexes, for indexing primers in white-capped tubes or blue 96-well plates

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10	AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10	TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10	AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10	AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10	CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10	TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10	CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10	ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

Reference Information for Kits with Original Index Configuration (indexing primers in clear-capped tubes or clear plate)

Use the reference information in this section if your kit includes Library Prep Kit p/n 5500-0116 or 5500-0117. If your kit does not include one of these component kits, see page 40 for kit content and indexing primer information.

Kit Contents

The SureSelect^{XT} RNA Reagent Kits contain the following component kits:

Table 24 SureSelect RNA-Seq Kit Contents-Original Index Configuration

Component Kits	Storage Condition	16 Samples	96 Samples
SureSelect Strand Specific RNA Library Prep, ILM, Box 1	–20°C	5500-0116	5500-0117
SureSelect Strand Specific RNA Library Prep, ILM, Box 2	4°C	5190-6410	5190-6411

NOTE SureSelect capture libraries and reagents must be used within one year of receipt.

The contents of each of the component kits listed in Table 24 are described in the tables below.

Table 25 SureSelect Strand Specific RNA Library Prep, ILM, Box 1 Content-Original Index Configuration

Kit Component	16 Samples	96 Samples
RNA Seq Fragmentation Mix	tube with red cap	bottle
RNA Seq First Strand Master Mix	tube with orange cap	tube with orange cap
RNA Seq Second-Strand + End-Repair Enzyme Mix	tube with blue cap	bottle
RNA Seq Second-Strand + End-Repair Oligo Mix	tube with yellow cap	tube with yellow cap
RNA Seq dA Tailing Master Mix	tube with green cap	bottle
SureSelect Ligation Master Mix	tube with purple cap	tube with purple cap
SureSelect Oligo Adaptor Mix	tube with blue cap	tube with blue cap
RNA Seq PCR Master Mix	tube with red cap	bottle
Uracil DNA Glycosylase (UDG)	tube with yellow cap	tube with yellow cap
SureSelect Primer	tube with brown cap	tube with brown cap
RNA Seq ILM Reverse PCR Primer	tube with black cap	tube with black cap
RNA Seq ILM Post-capture PCR Primer*	tube with green cap	tube with green cap
RNA Seq Indexes, 8 bp	RNA Seq Indexes 1-16, 8 bp provided in 16 tubes with clear caps	RNA Seq Indexes 1-96, 8 bp provided in clear 96-well plate†

* The provided SureSelect ILM Post-capture PCR Primer is not used in the workflow described in this manual. This primer is used in the SureSelect RNA Sequencing Target Enrichment workflow detailed in publication part number G9691-90000.

† See Table 27 on page 46 for a plate map. Although the provided plate contains 96 RNA Seq Indexes, only Indexes 1–48 should be used for the mRNA library preparation workflow. Wells contain enough volume for two mRNA library preparation reactions per index, using the protocol on page 29.

Table 26 SureSelect Strand Specific RNA Library Prep, ILM, Box 2 Content

Kit Component	16 Samples	96 Samples
Oligo(dT) Microparticles	tube with brown cap	bottle
RNA Seq Bead Binding Buffer	tube with purple cap	bottle
RNA Seq Bead Washing Buffer	bottle	bottle
RNA Seq Bead Elution Buffer	tube with green cap	bottle
Nuclease Free Water	bottle	bottle

Table 27 Plate map for RNA Seq Indexes 1-48, 8 bp, provided in clear plate in Library Prep kit p/n 5500-0117. Use only indexes 1–48 (Columns 1 to 6) for the mRNA library preparation workflow.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Index 1	Index 9	Index 17	Index 25	Index 33	Index 41	Index 49	Index 57	Index 65	Index 73	Index 81	Index 89
B	Index 2	Index 10	Index 18	Index 26	Index 34	Index 42	Index 50	Index 58	Index 66	Index 74	Index 82	Index 90
C	Index 3	Index 11	Index 19	Index 27	Index 35	Index 43	Index 51	Index 59	Index 67	Index 75	Index 83	Index 91
D	Index 4	Index 12	Index 20	Index 28	Index 36	Index 44	Index 52	Index 60	Index 68	Index 76	Index 84	Index 92
E	Index 5	Index 13	Index 21	Index 29	Index 37	Index 45	Index 53	Index 61	Index 69	Index 77	Index 85	Index 93
F	Index 6	Index 14	Index 22	Index 30	Index 38	Index 46	Index 54	Index 62	Index 70	Index 78	Index 86	Index 94
G	Index 7	Index 15	Index 23	Index 31	Index 39	Index 47	Index 55	Index 63	Index 71	Index 79	Index 87	Index 95
H	Index 8	Index 16	Index 24	Index 32	Index 40	Index 48	Index 56	Index 64	Index 72	Index 80	Index 88	Index 96

Nucleotide Sequences of SureSelect RNA Seq Indexes-Original Kit Configuration

The nucleotide sequence of each SureSelect RNA Seq Index provided in the original kit configuration is provided in the tables below.

Refer to the sequence information below only if your kit includes Library Prep kit p/n 5500-0116, with indexing primers provided in clear-capped tubes or includes Library Prep kit p/n 5500-0117, with indexing primers provided in a clear 96-well plate.

Each index is 8 nt in length. See page 37 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 28 SureSelect RNA Seq Indexes 1-16

Index Number	Sequence
1	AACGTGAT
2	AAACATCG
3	ATGCCTAA
4	AGTGGTCA
5	ACCACTGT
6	ACATTGGC
7	CAGATCTG
8	CATCAAGT
9	CGCTGATC
10	ACAAGCTA
11	CTGTAGCC
12	AGTACAAG
13	AACAACCA
14	AACCGAGA
15	AACGCTTA
16	AAGACGGA

Table 29 SureSelect RNA Seq Indexes 17-32

Index Number	Sequence
17	AAGGTACA
18	ACACAGAA
19	ACAGCAGA
20	ACCTCCAA
21	ACGCTCGA
22	ACGTATCA
23	ACTATGCA
24	AGAGTCAA
25	AGATCGCA
26	AGCAGGAA
27	AGTCACTA
28	ATCCTGTA
29	ATTGAGGA
30	CAACCACA
31	CAAGACTA
32	CAATGGAA

Table 30 SureSelect RNA Seq Indexes 33-48

Index Number	Sequence
33	CACTTCGA
34	CAGCGTTA
35	CATACCAA
36	CCAGTTCA
37	CCGAAGTA
38	CCGTGAGA
39	CCTCCTGA
40	CGAACTTA
41	CGACTGGA
42	CGCATACA
43	CTCAATGA
44	CTGAGCCA
45	CTGGCATA
46	GAATCTGA
47	GACTAGTA
48	GAGCTGAA

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In This Book

This guide contains information to run the SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Sequencing protocol.

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