SureSelect Strand-Specific RNA Library Prep System

Poly-A Selection and Strand-Specific mRNA Library Preparation for the Illumina Platform

Protocol

Version F0, June 2020

SureSelect platform manufactured with Agilent SurePrint Technology

For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies

Notices

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Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

Acknowledgment

Technical Support

For US and Canada

Call (800) 227-9770 (option 3,4,4)

Or send an e-mail to:
ngs.support@agilent.com

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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 total RNA samples after selection of poly-A RNA.

3 Reference

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

What's New in Version F0

- Support for renamed Library Preparation Kit components. See Table 22 on page 46 and Table 23 on page 47 for a summary of component name changes. The new component names are utilized throughout the protocols in this document. No changes were made to the reagent formulations or methods of use.
- Support for revised Poly-A Selection Module subkit. See Table 22 on page 46 and Table 24 on page 48 for a summary of component name and configuration changes. The new component names are utilized throughout the protocols in this document. No changes were made to the reagent formulations or methods of use. Water is no longer provided in this module (see Table 24 on page 48) and has been added to the list of required reagents in Table 1 on page 11.
- Updates to thermal cycler recommendations and usage instructions (see Table 2 on page 12, and example usage instructions in step 5 on page 20).
- Updates to page 27 to clarify details of the second-strand synthesis/end repair step.
- Updates to sequencing support guidelines for RNA strandedness (see page 44).
- Updated recommendation for control reference RNA (see Table 3 on page 13 and page 19).
- Minor wording updates to poly-A RNA selection steps page 19 through page 22.
- Updates to Agilent 2100 Bioanalyzer system ordering information (see Table 2 on page 12)
- Updates to ordering information for 1X TE Buffer and AMPure XP Kits (see Table 1 on page 11).
- Addition of product guarantee and support statement (see *Note* on page 9)
- Updated *Notice to Purchaser* (see page 2).
- Updated Technical Support contact information (see page 2).

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.

NOTE Agilent guarantees performance and provides technical support for the SureSelect reagents required for this workflow only when used as directed in this Protocol.

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 thoroughly on a vortex mixer at high speed for 5 seconds, then briefly spin in a centrifuge 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 RNA Library Preparation

Description	Vendor and part number
SureSelect Strand Specific RNA Reagent Kit	Agilent
Illumina platforms (ILM), 16 Samples	p/n G9691A
Illumina platforms (ILM), 96 Samples	p/n G9691B
Actinomycin D*	Sigma p/n A1410
DMSO	Sigma p/n D8418
AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
DNA 1000 Kit	Agilent p/n 5067-1504
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Actinomycin D should be obtained as a solid and prepared at 4 μg/μl concentration in DMSO then stored in single-use aliquots at –20°C, protected from light. The aliquots may be stored for up to one year before use. See page 16 for additional information.

Required Equipment

Table 2 Required Equipment for SureSelect^{XT} mRNA Library Preparation

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent, and compatible plasticware (consult the thermal cycler manufacturer's recommendations for compatible plasticware)	Agilent p/n G8800A (SureCycler 8800 Thermal Cycler) p/n G8810A (96 well plate module) p/n 410088 (96 well plates) p/n 410092 (8-well strip tubes) p/n 410096 (Tube cap strips, domed) p/n 410187 (Compression mat)
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Low-Adhesion Tubes (RNase, DNase, and DNA-free) 1.5 mL 0.5 mL	USA Scientific p/n 1415-2600 p/n 1405-2600
Magnetic separator	Thermo Fisher Scientific p/n 12331D 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	

* Select a magnetic separator compatible with the 96-well plates or 8-well strip tubes used for sample processing and configured to collect magnetic particles on one side of each well. Do not use a magnetic separator configured to collect the particles in a ring formation.

Optional Reagents and Equipment

Table 3 Optional Reagents and Equipment

Description	Vendor and part number
Labnet MPS1000 Mini Plate Spinner	Labnet International p/n C1000
QPCR Human Reference Total RNA	Agilent p/n 750500

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.

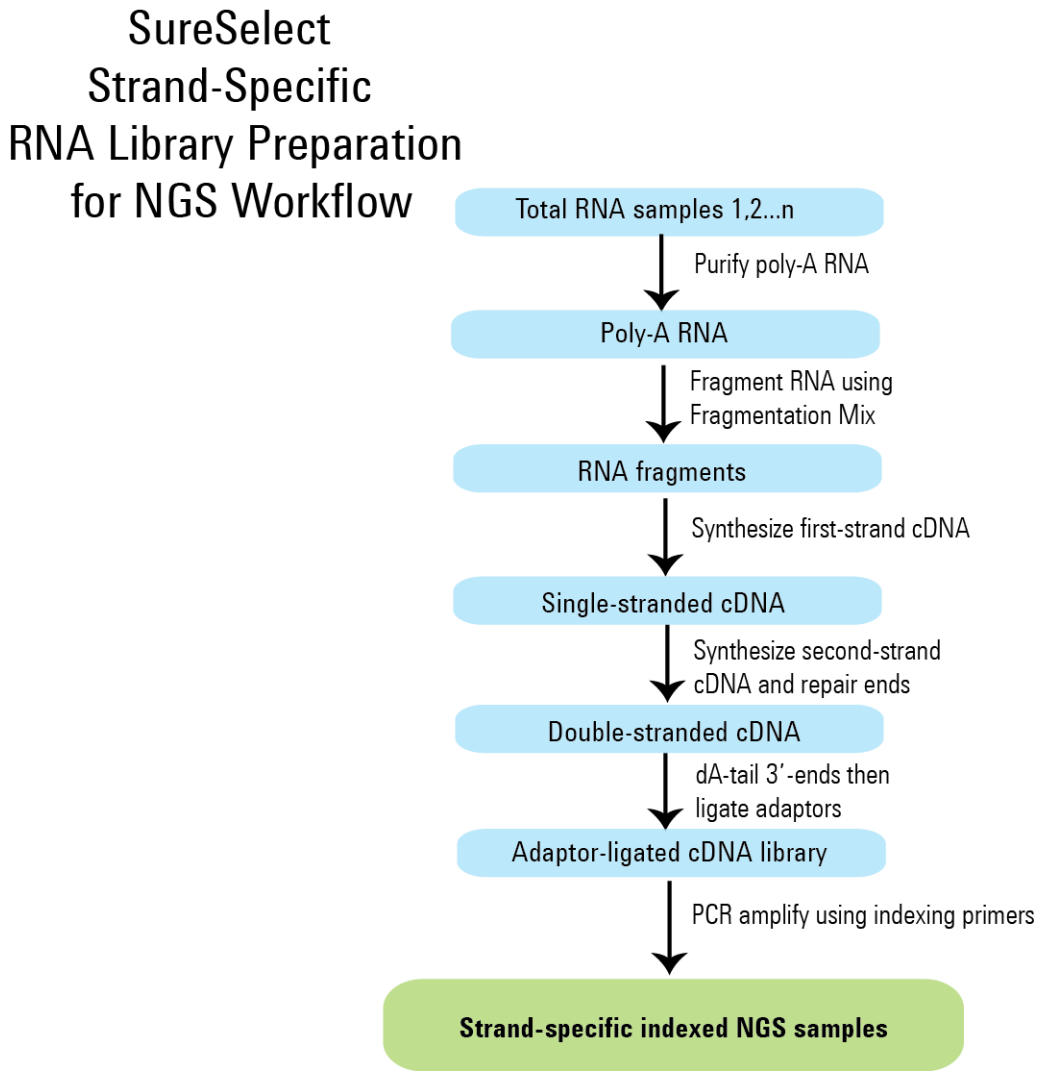

Figure 1 Overall mRNA sequencing sample preparation workflow.

Before you begin, prepare a stock solution of 4 µg/µL Actinomycin D in DMSO. Aliquot the stock solution into single-use volumes (typically 3 µL). Store the aliquots at –20°C, protected from light. Do not subject the aliquots to multiple freeze-thaw cycles. The aliquots may be stored for up to one year before use in the library preparation protocol. During the library preparation protocol, an aliquot of the DMSO stock is diluted with water, immediately before use, to a final Actinomycin D concentration of 120 ng/µL. (See page 23 for more information.)

Just before starting the protocol, bring the reagents listed in Table 4 to room temperature and thaw the reagents listed in Table 5 on ice.

Table 4 Reagents brought to room temperature before use in protocol

Storage Location	Kit Component	Where Used in Protocol
SureSelect Poly-A Selection Module*, 4°C	Oligo(dT) Microparticles (tube with brown cap or bottle)	page 19
	Bead Washing Buffer† (bottle)	page 20
	Bead Elution Buffer‡ (tube with green cap or bottle)	page 20
	Bead Binding Buffer** (tube with purple cap or bottle)	page 21

* May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 2.*

† May also be labeled as *RNA Seq Bead Washing Buffer.*

‡ May also be labeled as *RNA Seq Bead Elution Buffer.*

** May also be labeled as *RNA Seq Bead Binding Buffer.*

Table 5 Reagents thawed and held on ice before use in protocol

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR)*, –20°C	Fragmentation Mix† (red cap or bottle)	page 22
	First Strand Master Mix‡ (orange cap)	page 23
	Second Strand Enzyme Mix** (blue cap or bottle)	page 27
	Second Strand Oligo Mix†† (yellow cap)	page 27

* May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

† May also be labeled as *RNA Seq Fragmentation Mix*.

‡ May also be labeled as *RNA Seq First Strand Master Mix.*

** May also be labeled as *RNA Seq Second Strand + End Repair Enzyme Mix.*

†† May also be labeled as *RNA Seq Second Strand + End Repair Oligo Mix.*

Step 1. Select poly-A RNA from total RNA

In this step, poly-A RNA is selectively enriched 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.

Consider preparing an additional sequencing library in parallel, using a high-quality control RNA sample, such as Agilent's QPCR Human Reference Total RNA (p/n 750500). Use of this control is especially recommended during the first run of the protocol, to verify that all protocol steps are being successfully performed. Routine use of this control is helpful for any required troubleshooting, in order to differentiate any performance issues related to RNA input from other factors.

Make sure the reagents to be used in the protocol have been thawed and kept at the appropriate temperature, as indicated in Table 4 on page 17 and Table 5 on 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 or strip tube.
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 wells, then gently vortex for 5 seconds and briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.

5 Incubate the plate or strip tube in a thermal cycler (with the heated lid ON) and run the program in Table 6 to denature the RNA.

Table 6 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 or strip tube and incubate at room temperature for 5 minutes, to allow poly-A RNA binding to the oligo(dT) beads.

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

8 While keeping the plate or strip tube 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 or strip tube from the magnetic stand. Gently add 200 µL of Bead Washing Buffer to each well.

Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µL, without introducing bubbles.

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 or strip tube in a centrifuge before continuing.

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

11 While keeping the plate or strip tube 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.

12 Remove the plate or strip tube from the magnetic stand. Add 25 µL of Bead Elution Buffer to each sample well.

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

14 Incubate the plate or strip tube in a thermal cycler (with the heated lid ON) and run the program in Table 7.

Table 7 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

15 After the thermal cycler reaches the 4°C Hold step, remove the plate or strip tube and add 25 µL of Bead Binding Buffer to each sample well.

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

17 Incubate the samples at room temperature for 5 minutes, to allow poly-A RNA to re-bind the beads.

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

19 While keeping the plate or strip tube 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.

20 Remove the plate or strip tube from the magnetic stand. Gently add 200 µL of Bead Washing Buffer to each well.

Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µL, without introducing bubbles. If bubbles or foam are present, spin the plate or strip tube briefly before continuing.

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

22 While keeping the plate or strip tube 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.

23 Proceed immediately to Step 2. Fragment the poly-A RNA.

Step 2. Fragment the poly-A RNA

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

1 Remove the plate or strip tube, containing the collected poly-A RNA-bound beads, from the magnetic stand.

2 To each sample well add 19 µL of Fragmentation Mix.

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

4 Incubate the plate or strip tube in a thermal cycler (with the heated lid ON) and run the program in Table 8.

Table 8 Thermal cycler program for RNA fragmentation

Step	Temperature	Time
Step 1	94°C	8 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

5 During the 8-minute incubation at 94°C, complete the first-strand cDNA reagent preparation steps (step 1 through step 3) on page 23.

Step 3. Synthesize first-strand cDNA

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 year prior to use and stored in single-use aliquots at –20°C, protected from light.

1 Prepare a fresh 120 ng/µL Actinomycin D dilution in water, using an aliquot of the 4 µg/µL Actinomycin D stock solution in DMSO, according to Table 9.

Table 9 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**

CAUTION The First Strand Master Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions. Pipetting up and down is not sufficient to mix this reagent.

2 Vortex the thawed vial of First Strand Master Mix 5 seconds at high speed to ensure homogeneity.

3 Prepare the appropriate amount of First Strand Master Mix + Actinomycin D mixture according to Table 10 below. Mix by vortexing, then spin briefly and keep on ice.

Table 10 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
First Strand Master Mix (orange cap)	8.0 µL	136 µL
Total	**8.5 µL**	**144.5 µL**

4 Once the RNA fragmentation thermal cycler program has reached the 4°C Hold step (Step 3 in Table 8), transfer the sample plate or strip tube from the thermal cycler to the magnetic stand at room temperature. Leave the plate or strip tube on the magnetic stand for at least 2 minutes, until the solution is clear.

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

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

7 Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.

8 Spin the plate or strip tube briefly to collect the liquid.

9 Incubate the plate or strip tube in a thermal cycler (with the heated lid ON) and run the program in Table 11.

Table 11 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 or strip tube. Seal the wells, then vortex the plate or strip tube for 5 seconds. Briefly spin to collect the liquid.

5 Incubate samples for 5 minutes at room temperature.

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

7 While keeping the plate or strip tube 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 or strip tube 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 or strip tube briefly, return the samples to the magnetic stand, and then remove any remaining ethanol droplets with a pipette.

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

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

14 Seal the wells, then vortex the plate or strip tube for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. Briefly spin to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate or strip tube 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 well and keep on ice. You can discard the beads at this time.

18 Proceed immediately to "Step 5. Synthesize second-strand cDNA and repair the ends" on page 27.

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

In this step, the second strand of cDNA is synthesized using a dUTP second strand-marking method to allow strand-specific RNA sequencing. The ends of the cDNA fragments are also repaired in this step using end-repair reagents included in the Second Strand Enzyme Mix and Second Strand Oligo Mix.

CAUTION The Second Strand Enzyme Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions. Pipetting up and down is not sufficient to mix this reagent.

1. Vortex the thawed vials of Second Strand Enzyme Mix (blue capped tube or bottle) and Second Strand Oligo Mix (yellow capped tube) at high speed for 5 seconds to ensure homogeneity.
2. Add 25 µL of Second Strand Enzyme Mix to each 20-µL purified first-strand cDNA sample. Keep on ice.
3. Add 5 µL of Second Strand Oligo Mix to each sample well, for a total reaction volume of 50 µL. Keep on ice.
4. Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.
5. Spin the plate or strip tube briefly to collect the liquid.
6. Incubate the plate or strip tube in a thermal cycler and run the program in Table 12. **Do not use a heated lid**.

Table 12 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 or strip tube. Seal the wells, then vortex the plate or strip tube for 5 seconds. Briefly spin to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate or strip tube 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 or strip tube 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 beads on the thermal cycler (with lid open) at 37°C for up to 2 minutes. Do not overdry the beads.
12. Add 21 µL nuclease-free water to each sample well.
13. Seal the wells, then vortex the plate or strip tube for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. Briefly spin to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or strip tube 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 well and keep the samples on ice. You can discard the beads at this time.

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

Step 7. dA-Tail the cDNA 3' ends

Before continuing the protocol, thaw reagents listed in Table 13 (used for the remainder of library preparation steps) and keep on ice.

Table 13 Reagents thawed and held on ice before use in protocol

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR)*, –20°C	dA Tailing Master Mix† (green cap or bottle)	page 29
	SureSelect Ligation Master Mix (purple cap)	page 31
	SureSelect Oligo Adaptor Mix (blue cap)	page 31
	ILM Reverse PCR Primer‡ (black cap)	page 33
	PCR Master Mix** (red cap or bottle)	page 33
	Uracil DNA Glycosylase (UDG) (yellow cap)	page 34
	SureSelect Primer (brown cap)	page 34
	SureSelect^{XT} Indexes, 8 bp (tubes with white caps or blue 96-well plate)	page 34

* May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

† May also be labeled as *RNA Seq dA Tailing Master Mix.*

‡ May also be labeled as *RNA Seq ILM Reverse PCR Primer.*

** May also be labeled as *RNA Seq PCR Master Mix.*

CAUTION The dA Tailing Master Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions. Pipetting up and down is not sufficient to mix this reagent.

1 Vortex the thawed vial of dA Tailing Master Mix (green-capped tube or bottle) 5 seconds at high speed to ensure homogeneity.

2 Add 20 µL of dA Tailing Master Mix to each 20-µL purified, end-repaired cDNA sample.

Pipette the master mix slowly to ensure that the full volume is dispensed.

3 Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.

4 Spin the plate or strip tube briefly to collect the liquid.

5 Incubate the plate or strip tube in a thermal cycler and run the program in Table 14. **Do not use a heated lid**.

Table 14 Thermal cycler program for dA-tailing

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

Step 8. Ligate adaptors

CAUTION The SureSelect Ligation Master Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions. Pipetting up and down is not sufficient to mix this reagent.

1 Vortex the thawed vials of SureSelect Ligation Master Mix and SureSelect Oligo Adaptor Mix at high speed for 5 seconds to ensure homogeneity.

2 Transfer the cDNA samples to ice, then add 5 µL of SureSelect Ligation Master Mix to each A-tailed cDNA sample well.

   Pipette the master mix slowly to ensure that the full volume is dispensed.

3 Add 5 µL of SureSelect Oligo Adaptor Mix to each sample.

4 Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.

5 Spin the plate or strip tube briefly to collect the liquid.

6 Incubate the plate or strip tube in a thermal cycler and run the program in Table 15. **Do not use a heated lid**.

Table 15 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 or strip tube. Seal the wells, then vortex the plate or strip tube for 5 seconds. Briefly spin to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate or strip tube 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 or strip tube 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 beads on the thermal cycler (with lid open) at 37°C for up to 3 minutes. Do not overdry the beads.
12. Add 18 µL nuclease-free water to each sample well.
13. Seal the wells, then vortex the plate or strip tube for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. Briefly spin to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or strip tube in the magnetic stand and leave for 2 minutes or until the solution is clear.
16. Remove 17 µL of cleared supernatant to a fresh well and keep on ice. 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 (see Table 19 on page 35).

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.

1 Prepare a 1:20 dilution of the ILM Reverse PCR Primer, according to Table 16.

Table 16 Preparation of reverse primer dilution

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

CAUTION

The PCR Master Mix used at this step is highly viscous and thorough mixing is critical for optimal kit performance. Mix by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions in Table 17. Pipetting up and down is not sufficient to mix this reagent.

2 Vortex the thawed vial of PCR Master Mix 5 seconds at high speed to ensure homogeneity.

3 Prepare the appropriate volume of PCR reaction mix, as described in Table 17, on ice. Mix well by vortexing at high speed then spin briefly to collect the liquid.

Table 17 Preparation of PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
PCR Master Mix (tube with red cap or bottle)	25 µL	425 µL
Uracil DNA Glycosylase (UDG) (tube with yellow cap)	1 µL	17 µL
SureSelect Primer (forward primer; tube with brown cap)	1 µL	17 µL
1:20 dilution of ILM Reverse PCR Primer prepared in Table 16	1 µL	17 µL
Total	**28 µL**	**476 µL**

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

Mix by pipetting. Change pipette tips between samples.

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

CAUTION

For 96-reaction kits, only use the indexing primers provided in columns 1 through 6 (Indexes A01–H06) in step 5. Provided plates also contain indexing primers in columns 7 through 12, which are used in other SureSelect protocols.

See page 48 for a plate map.

6 Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.

7 Spin the plate or strip tube briefly to collect the liquid.

8 Incubate the plate or strip tube in a thermal cycler (with the heated lid ON) and run the program in Table 18.

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Table 18 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 19)	95°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	1	4°C	Hold

Table 19 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 18).

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 PCR plate or strip tube. Seal the wells, then vortex the plate or strip tube for 5 seconds. Briefly spin to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate or strip tube 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 or strip tube 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 beads on the thermal cycler (with lid open) at 37°C for up to 3 minutes. Do not overdry the beads.
12. Add 26 µL nuclease-free water to each sample well.
13. Seal the wells, then vortex the plate or strip tube for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. Briefly spin to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or strip tube 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 well and keep on ice. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells 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. Perform the assay according to the Agilent DNA 1000 Kit Guide.

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.02.02 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. Within the instrument context, choose the DNA 1000 assay from the drop down list. Start the run within five minutes after preparation.

5 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 39.

   If adaptor-dimers are not observed, proceed to "Step 14. Prepare samples for multiplexed sequencing" on page 41.

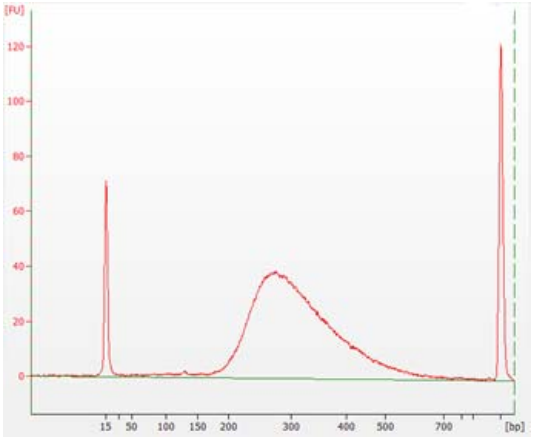

Figure 2 Analysis of amplified prepped library cDNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 200 to 600 bp.

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 PCR plate or strip tube. Seal the wells, then vortex the plate or strip tube for 5 seconds. Briefly spin to collect the liquid.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (at least 5 minutes).
7. While keeping the plate or strip tube 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 or strip tube 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 beads on the thermal cycler (with lid open) at 37°C for up to 3 minutes. Do not overdry the beads.
12. Add 26 µL nuclease-free water to each sample well.
13. Seal the wells, then vortex the plate or strip tube for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. Briefly spin to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or strip tube 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 well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells 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 20 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 20 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 21. 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 Table 26 on page 49.

Table 21 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 RNA sequencing library preparation method preserves RNA strandedness using dUTP second-strand marking. The sequence of read 1, which starts at the P5 end, matches the reverse complement of the poly-A RNA transcript strand. Read 2, which starts at the P7 end, matches 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 (https://broadinstitute.github.io/picard) 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.

Kit Contents

The SureSelect Strand Specific RNA Reagent Kits include the component kits listed in Table 22. The contents of each component kit are detailed in Table 23 through Table 24.

Table 22 Kit Content

Component Kits	Storage Condition	16 Samples	96 Samples
SureSelect RNA Library Prep, ILM (Pre PCR)*	–20°C	5500-0134	5500-0135
SureSelect Poly-A Selection Module (Pre PCR)†	4°C	5190-6410	5190-6411

* May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

† May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 2.*

Table 23 SureSelect RNA Library Prep, ILM (Pre PCR) Content

Equivalent RNA Library Preparation Kit Component Names*		Format	
Current name	**Retired name**	**16 Reactions (p/n 5500-0134)**	**96 Reactions (p/n 5500-0135)**
Fragmentation Mix	RNA Seq Fragmentation Mix	tube with red cap	bottle
First Strand Master Mix	RNA Seq First Strand Master Mix	tube with orange cap	tube with orange cap
Second Strand Enzyme Mix†	RNA Seq Second-Strand + End Repair Enzyme Mix	tube with blue cap	bottle
Second Strand Oligo Mix	RNA Seq Second-Strand + End Repair Oligo Mix	tube with yellow cap	tube with yellow cap
dA Tailing Master Mix	RNA Seq dA Tailing Master Mix	tube with green cap	bottle
SureSelect Ligation Master Mix	(no change)	tube with purple cap	tube with purple cap
SureSelect Oligo Adaptor Mix	(no change)	tube with blue cap	tube with blue cap
PCR Master Mix	RNA Seq PCR Master Mix	tube with red cap	bottle
Uracil DNA Glycosylase (UDG)	(no change)	tube with yellow cap	tube with yellow cap
SureSelect Primer	(no change)	tube with brown cap	tube with brown cap
ILM Reverse PCR Primer	RNA Seq ILM Reverse PCR Primer	tube with black cap	tube with black cap
ILM Post-Capture PCR Primer‡	RNA Seq ILM Post-Capture PCR Primer	tube with green cap	tube with green cap
SureSelect^{XT} Indexes, 8 bp**	(no change)	SureSelect 8 bp Indexes A01 through H02, provided in 16 tubes with white caps	SureSelect 8 bp Indexes A01 through H12, provided in blue 96-well plate††

* Some component names were updated in June, 2020. Formulations of the reagents supplied and protocols for use of the reagents are unchanged. All components are supported through the expiration date listed on the Certificate of Analysis.

† The *Second Strand Enzyme Mix* and *Second Strand Oligo Mix* also supply the reagents used for cDNA end repair; formulations are equivalent to vials labeled as *RNA Seq Second Strand + End Repair Enzyme Mix* and *Second Strand + End Repair Oligo Mix,* respectively.

‡ The provided ILM Post-capture PCR Primer is not used in the workflow described in this manual.

** See Table 26 on page 49 for index sequences.

†† See Table 25 on page 48 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 34.

Table 24 SureSelect Poly-A Selection Module (Pre PCR) Content

Kit Component	16 Reactions (p/n 5190-6410)*	96 Reactions (p/n 5190-6411)*
Oligo(dT) Microparticles	tube with brown cap	bottle
Bead Binding Buffer	tube with purple cap	bottle
Bead Washing Buffer	bottle	bottle
Bead Elution Buffer	tube with green cap	bottle

* Component kit part numbers 5190-6410 and 5190-6411 labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 2* also contain a vial of Nuclease Free Water and contain reagent tubes labeled as *RNA Seq Bead Binding Buffer, RNA Seq Bead Washing Buffer*, and *RNA Seq Bead Elution Buffer*. Formulations of the reagents supplied and protocols for use of these reagents are unchanged. Ordering information for nuclease-free water is provided in Table 1 on page 11.

Table 25 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 Indexes A01 to H12

Each index is 8 nt in length. See page 43 for sequencing run setup requirements for sequencing libraries using 8-bp indexes..

Table 26 SureSelect 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

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In This Book

This guide contains information to run the SureSelect Strand-Specific RNA Library Prep System protocol.

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