SureSelect^{XT} RNA Target Enrichment for Illumina Multiplexed Sequencing

Strand-Specific RNA Library Prep and Target Enrichment Protocols

Protocol

Version D0, July 2015

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

Manual Part Number

G9691-90000

Edition

Version D0, July 2015

Printed in USA

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

Acknowledgement

Technical Support

For technical product support, contact your local Agilent Support Services representative.

For US and Canada, call (800) 227-9770 (option 3,4,4). For other countries, find your support center telephone numbers at www.agilent.com/chem/contactus.

Or send an e-mail to:
SureSelect.Support@agilent.com

Notice to Purchaser

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

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Notice to Purchaser: Limited License

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 6,258,569, 6,171,785, 6,127,155, 6,030,787, 5,994,056, 5,876,930, 5,804,375, 5,789,224, 5,773,258 (claims 1 and 6 only), 5,723,591, 5,677,152 (claims 1 to 23 only), 5,618,711, 5,538,848, and claims outside the US corresponding to expired US Patent No. 5,079,352. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{XT} RNA Target Enrichment system.

This protocol is specifically developed and optimized to enrich targeted regions of the transcriptome from repetitive sequences and sequences unrelated to the research focus prior to sample sequencing.

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 for target enrichment.

3 Hybridization

This chapter describes the steps to hybridize and capture the prepared cDNA for target enrichment.

4 Indexing and Sample Processing for Multiplexed Sequencing

This chapter describes the steps to index, purify, and assess quality and quantity of the target-enriched libraries. Samples are pooled by mass prior to sequencing.

5 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 60. For nucleotide sequences of the 8-bp indexes in this revised configuration, see Table 33 on page 64.

  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 1–96 provided in a clear plate (96 Reaction kits). For kit content details see page 65. For nucleotide sequences of the 8-bp indexes in this original configuration, see Table 40 on page 69 through Table 45 on page 74.

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 cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols or instruments to process samples for enrichment.

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^{XT} RNA Target Enrichment

Description	Vendor and part number
SureSelect RNA Capture Library	Select one library from Table 2
SureSelect^{XT} RNA Reagent Kit Illumina platforms (ILM), 16 Samples Illumina platforms (ILM), 96 Samples	Agilent p/n G9692A p/n G9692B
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
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Dynabeads MyOne Streptavidin T1 2 mL 10 mL 100 mL	Life Technologies Cat #65601 Cat #65602 Cat #65603
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 15 for additional information.

Table 2 SureSelect RNA Capture Libraries

Capture Library	16 Samples	96 Samples	480 Samples
SureSelect RNA Kinome	5190-4801	5190-4802	5190-4803
Custom RNA Capture 1 kb up to 499 kb (reorder)	5190-4934 (5190-4939)	5190-4935 (5190-4940)	5190-4937 (5190-4942)
Custom RNA Capture 0.5 Mb up to 2.9 Mb (reorder)	5190-4944 (5190-4949)	5190-4945 (5190-4950)	5190-4947 (5190-4952)
Custom RNA Capture 3 Mb up to 5.9 Mb (reorder)	5190-4954 (5190-4959)	5190-4955 (5190-4960)	5190-4957 (5190-4962)

Required Equipment

Table 3 Required Equipment for SureSelect^{XT} RNA Target Enrichment

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
Compression mats	Agilent p/n 410187
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Magnetic separator for 96-well plates	Phenix Research Products p/n RX-IMAG-96P or equivalent
Nutator plate mixer	BD Diagnostics p/n 421105 or equivalent
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vortex mixer	
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

Optional Reagents and Equipment

Table 4 Optional Reagents and Equipment

Description	Vendor and part number
2200 TapeStation	Agilent p/n G2964AA or G2965AA
2200 TapeStation consumables	
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Labnet MPS1000 Mini Plate Spinner	Labnet International p/n C1000

2 Sample Preparation

See Figure 1 for a summary of the SureSelect RNA sequencing target enrichment 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 21 for more information.)

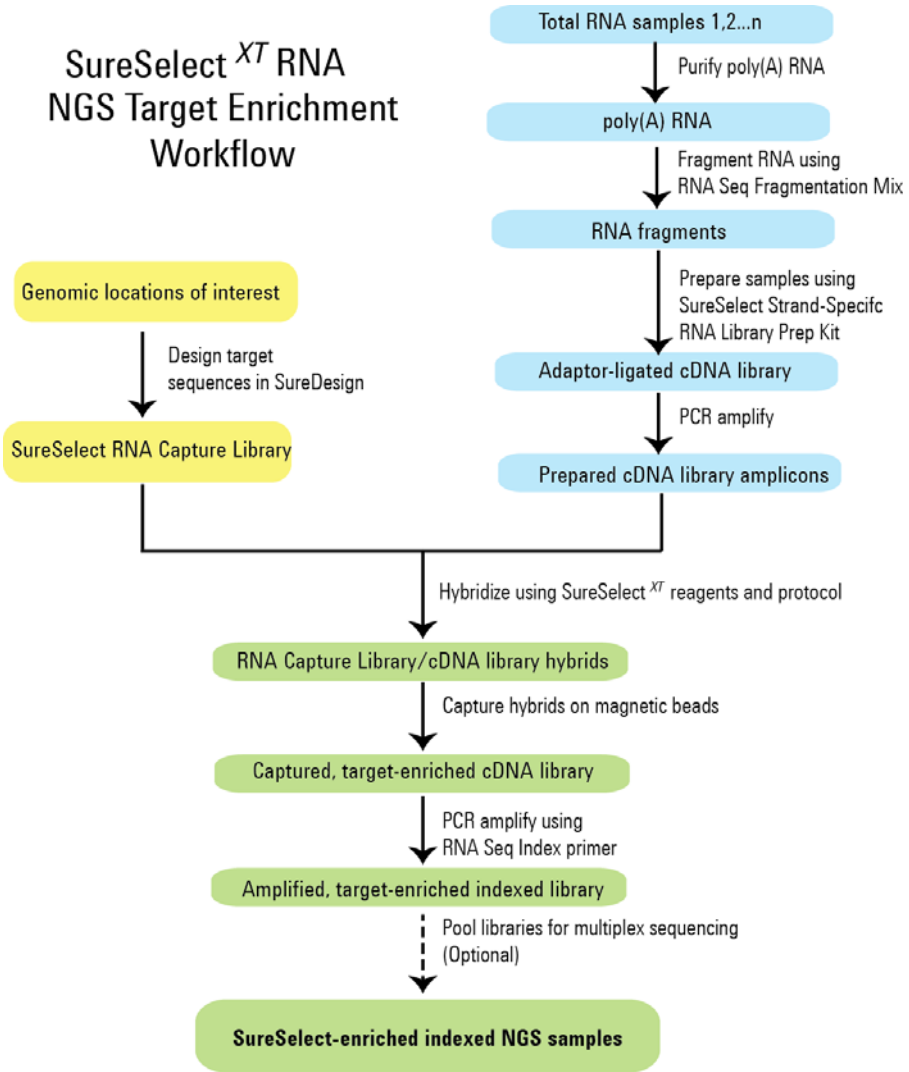

Figure 1 Overall RNA sequencing sample preparation workflow.

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 prior to target enrichment is 200 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 5 to room temperature before starting the protocol.

Table 5 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 17
RNA Seq Bead Washing Buffer	RNA Library Prep Kit Box 2, 4°C	page 18
RNA Seq Bead Elution Buffer	RNA Library Prep Kit Box 2, 4°C	page 18
RNA Seq Bead Binding Buffer	RNA Library Prep Kit Box 2, 4°C	page 19
RNA Seq Fragmentation Mix	RNA Library Prep Kit Box 1, –20°C	page 20

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 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 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 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

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 8.

Table 8 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 21 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 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**

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

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
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 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 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 and the Target Enrichment protocols require an additional 0.8 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 25.

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 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 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 13. **Do not use a heated lid**.

Table 13 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 14. **Do not use a heated lid**.

Table 14 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 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. Pipette up and down 10 times to mix.
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 23 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 22 µL of cleared supernatant to a fresh plate well. You can discard the beads at this time.

Step 10. Amplify the adaptor-ligated cDNA library

In this step, the adaptor ligated cDNA is PCR-amplified using a cycle number appropriate for the initial amount of RNA sample used for library preparation.

Use the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw and mix the reagents listed in Table 15 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 the appropriate volume of PCR reaction mix, as described in Table 15, on ice. Mix well on a vortex mixer.

.

Table 15 Preparation of pre-capture 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
RNA Seq ILM Reverse PCR Primer	1 µL	17 µL
Total	**28 µL**	**476 µL**

2 Add 28 µL of the pre-capture PCR reaction mix prepared in step 1 to each 22-µL purified, adaptor-ligated cDNA sample.

Mix by pipetting. Change pipette tips between samples.

3 Incubate the plate in the thermal cycler (with the heated lid ON) and run the program in Table 16.

Table 16 Thermal cycler program for pre-capture PCR

Segment	Number of Cycles	Temperature	Time
1	1	37°C	15 minutes
2	1	95°C	2 minutes
3	9–13 cycles (see Table 17)	95°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	1	4°C	Hold

Table 17 Pre-capture PCR cycle number recommendations

Amount of total RNA used for library prep	Cycle Number
200 ng–2 µg	11–13
2.1 µg–4 µg	9–11

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 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. Check that the electropherogram shows a distribution with a peak size approximately 180 to 550 bp. Measure the concentration of the library by integrating under the peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

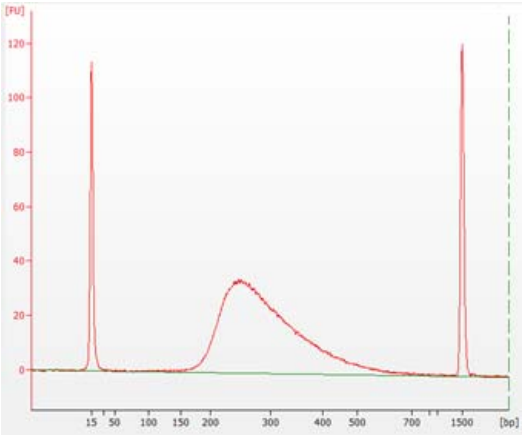

Figure 2 Analysis of amplified prepped library cDNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 180 to 550 bp.

3 Hybridization

This chapter describes the steps to hybridize the prepped cDNA library with the SureSelect capture library in combination with the hybridization reagents and blocking agents.

CAUTION The ratio of SureSelect capture library to prepped library is critical for successful capture.

Step 1: Hybridize the library

In this step, the prepared cDNA libraries are hybridized to a SureSelect RNA Capture Library.

Use reagents from SureSelect Target Enrichment Box 1 and Box 2 for this step.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 24 hour incubation.

If you want to use a different combination of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape), first test the conditions. Incubate 27 µL of water at 65°C for 24 hours as a test. Include water in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 3 to 4 µL.

The hybridization reaction requires 100 ng of prepared cDNA in a volume of 3.4 µL (initial concentration of 30 ng/µL).

1. For prepped libraries with cDNA concentrations above 30 ng/µL, prepare 3.4 µL of a 30 ng/µL dilution of each library.
2. For prepped libraries with cDNA concentrations below 30 ng/µL, use a vacuum concentrator to concentrate the samples at ≤ 45°C.
   a. Add the entire volume of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

      You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.
   b. Dehydrate using a vacuum concentrator on low heat (less than 45°C).
   c. Reconstitute with nuclease-free water to a final concentration of 30 ng/µL. Pipette up and down along the sides of the tube for optimal recovery.
   d. Mix well on a vortex mixer and spin in a centrifuge for 1 minute.

3 Mix the components in Table 18 at room temperature to prepare the hybridization buffer.

Keep the prepared hybridization buffer at 37°C until it is used in step 8.

Table 18 Hybridization Buffer

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect Hyb #1 (orange cap or bottle)	25 µL	350 µL
SureSelect Hyb #2 (red cap)	1 µL	14 µL
SureSelect Hyb #3 (yellow cap)	10 µL	140 µL
SureSelect Hyb #4 (black cap or bottle)	13 µL	182 µL
Total	**49 (40 µL needed)**	**686 (40 µL/sample)**

4 Mix the components in Table 19 to prepare the SureSelect block mix.

Table 19 SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect Indexing Block #1 (green cap)	2.5 µL	42.5 µL
SureSelect Block #2 (blue cap)	2.5 µL	42.5 µL
SureSelect Indexing Block #3 (brown cap)	0.6 µL	10.2 µL
Total	**5.6 µL**	**95.2 µL**

5 Prepare the required volume of a 1:4 dilution of SureSelect RNase Block (for a final concentration of 25%), as shown in Table 20. Keep on ice.

Table 20 Preparation of 25% RNase Block solution

Component	Volume for 1 reaction	Volume for 16 reactions (includes excess)
RNase Block (purple cap)	0.5 µL	8.5 µL
Nuclease-free water	1.5 µL	25.5 µL
Total	**2 µL**	**34 µL**

6 For each hybridization reaction well, combine 5 µL of the SureSelect RNA Capture Library and 2 µL of the 25% RNase Block solution.

Mix well by pipetting. Keep the mixture on ice.

Table 21 Preparation of Capture Library/RNase Block mixture

Component	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Dilute RNase Block	2 µL	34 µL
Capture Library	5 µL	85 µL
Total	**7 µL**	**119 µL**

7 In a PCR plate, prepare the prepped gDNA libraries for target enrichment.

a Add 3.4 µL of 30 ng/µL prepped library to the "B" row in the PCR plate. Put each sample into a separate well. The example in Figure 3 is for 12 captures.

b Add 5.6 µL of the SureSelect block mix (prepared in step 4) to each well in row B.

c Mix by pipetting up and down.

d Seal the wells of row "B" with caps and put the PCR plate in the thermal cycler. Do not heat the Hybridization Buffer or capture library yet; heat only the prepped library with block mix.

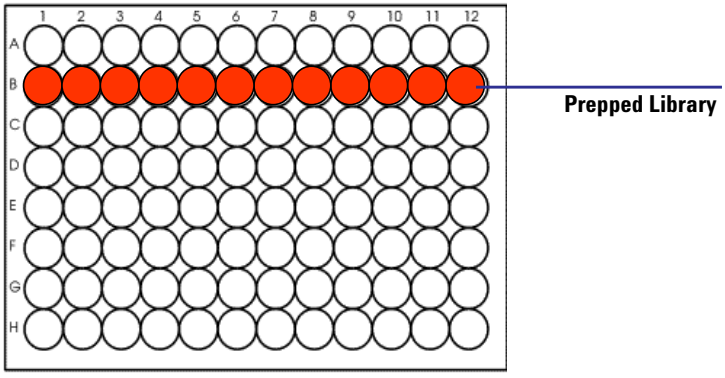

Figure 3 Configuration of Hybridization plate for 12 hybridization reactions; prepped libraries/block mixture shown in red

e Run the following thermal cycler program in Table 22.

Use a heated lid on the thermal cycler at 105°C to hold the temperature of the plate on the thermal cycler at 65°C.

CAUTION The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

Table 22 Thermal cycler program

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

8 Maintain the plate at 65°C while you load 40 µL of hybridization buffer (prepared in step 3) per well into the "A" row of the PCR plate.

Make sure that the plate is at 65°C for a minimum of 5 minutes before you go to step 9.

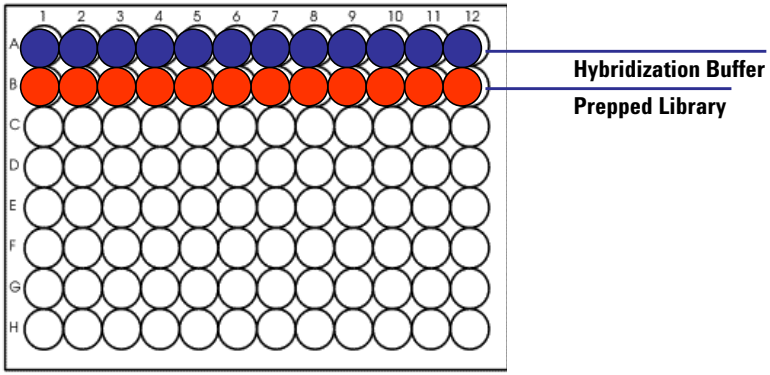

Figure 4 Configuration of Hybridization plate for 12 reactions; Hybridization buffer shown in blue

9 Add the Capture Library mix from step 6 to the PCR plate:

a Add 7 µL of capture library mix to "C" row wells in the PCR plate.

Keep the plate at 65°C during this step.

b Seal the wells with strip caps. Use a capping tool to make sure the fit is tight.

c Incubate the samples at 65°C for 2 minutes.

10 Maintain the plate at 65°C while you use a multi-channel pipette to take 13 µL of Hybridization Buffer from the "A" row and add it to the SureSelect capture library mix contained the "C" row of the PCR plate for each sample. (See Figure 5.)

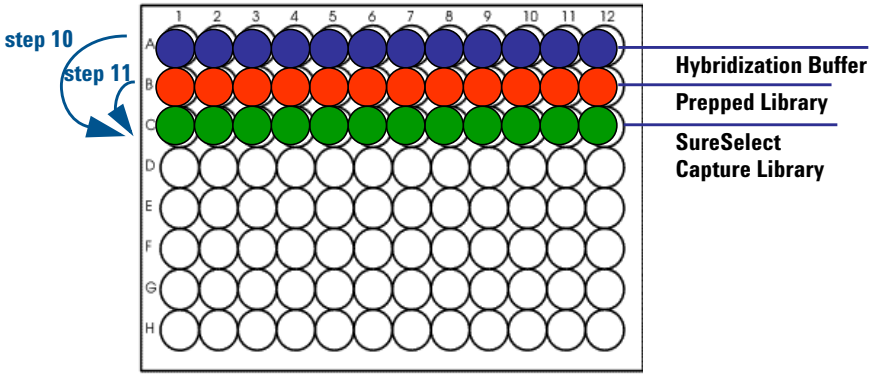

Figure 5 Configuration of Hybridization plate for 12 reactions; Capture Library mix shown in green

11 Maintain the plate at 65°C while you use a multi-channel pipette to transfer the entire contents (approximately 9 µL) of each prepped library mix in row "B" to the hybridization solution in row "C". (See Figure 5.) Mix well by slowly pipetting up and down 8 to 10 times.

The hybridization mixture is now 27 to 29 µL, depending on degree of evaporation during the preincubations.

12 Seal the wells with strip caps or two layers of adhesive film. Make sure all wells are completely sealed.

CAUTION Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted.

Use new adhesive seals or strip caps. The structural integrity of seals and caps can be compromised during the previous incubation steps.

If you use strip tubes, test for evaporation using mock hybridization conditions as described on page 36 before you do the first hybridization with your samples. Make sure that the tube/capping method is appropriate for the thermal cycler by checking that no more than 4 µL is lost to evaporation.

13 Incubate the hybridization mixture in the SureCycler thermal cycler, with compression mat, for 24 hours at 65°C with a heated lid at 105°C.

Step 2. Prepare streptavidin beads

In this step, Dynabeads MyOne Streptavidin T1 magnetic beads (see Table 1 on page 11 for ordering information) are prepared for use in capturing the cDNA library/Capture library hybrids.

Use reagents from SureSelect Target Enrichment Box 1 for this step.

1. Prewarm SureSelect Wash Buffer 2 at 65°C in a circulating water bath for use in "Step 3. Capture hybrids using streptavidin beads".
2. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. Magnetic beads settle during storage.
3. For each hybridization, add 50 µL of the magnetic bead suspension to wells of a PCR plate.
4. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix the beads on a vortex mixer for 5 seconds.
   c. Put the plate into a magnetic separator device and allow the solution to clear.
   d. Remove and discard the supernatant.
   e. Repeat step a through step d for a total of 3 washes.
5. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

Step 3. Capture hybrids using streptavidin beads

In this step, the cDNA library/Capture library hybrids are captured on the prepared streptavidin magnetic beads.

Use reagents from SureSelect Target Enrichment Box 1 for this step.

1. Estimate and record the volume of hybridization solution that remains after the 24 hour incubation.
2. Maintain the hybridization plate at 65°C on the thermal cycler while you use a multichannel pipette to transfer the entire volume (approximately 29 µL) of each hybridization mixture to the plate wells containing 200 µL of washed streptavidin beads.

   Mix well by slowly pipetting up and down at least 10 times.
3. Cap the wells, then incubate the capture plate on a Nutator mixer or equivalent for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells by inspecting the bottom of the plate for settled beads after 5 minutes.
4. Briefly spin the plate in a centrifuge or mini-plate spinner.
5. Put the plate in a magnetic separator to collect the beads from the suspension. Remove and discard the supernatant.
6. Resuspend the beads in 200 µL of SureSelect Wash Buffer 1 by mixing on a vortex mixer for 5 seconds.
7. Incubate the samples for 15 minutes at room temperature.
8. Separate the beads and buffer on a magnetic separator and remove the supernatant.

CAUTION

It is important to maintain bead suspensions at 65°C during the washing procedure below to ensure specificity of capture.

Make sure that the SureSelect Wash Buffer #2 is pre-warmed to 65°C before use.

Do not use a tissue incubator, or other devices with significant temperature fluctuations, for the incubation steps.

9 Wash the beads with SureSelect Wash Buffer 2:

   a Resuspend the beads in 200 µL of 65°C prewarmed SureSelect Wash Buffer 2. Cap the wells and mix on a vortex mixer for 5 seconds to resuspend the beads.

   b Incubate the sample plate for 10 minutes at 65°C on the thermal cycler.

      Invert the sealed plate to mix occasionally. The beads may settle.

   c Briefly spin the plate in a centrifuge or mini-plate spinner.

   d Put the plate in the magnetic separator.

   e Wait for the solution to clear, then remove and discard the supernatant.

   f Repeat step a through step e for a total of 3 washes.

      Make sure all of the wash buffer has been removed during the final wash.

10 Mix the beads in 50 µL of SureSelect Elution Buffer on a vortex mixer for 5 seconds to resuspend the beads.

11 Incubate the samples for 10 minutes at room temperature.

12 Separate the beads and buffer on the magnetic separator.

13 Transfer the supernatant to a fresh plate well.

   The supernatant contains the captured DNA. The beads can now be discarded.

14 Add 50 µL of SureSelect Neutralization Buffer to the captured cDNA library.

Step 4. Purify the captured 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 mL 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 180 µL of the homogeneous bead suspension to each sample well containing 100 µL of cDNA library. 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 in a magnetic separator at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
7. While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution. 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.

    Do not dry the bead pellets to the point that the pellets appear cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
12. Add 40 µL nuclease-free water to each sample well.
13. Seal the plate, mix well on a vortex mixer, and then 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 to 3 minutes or until the solution is clear.
16. Remove the supernatant (approximately 40 µL) 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.

4 Indexing and Sample Processing for Multiplexed Sequencing

This chapter describes the steps to add index tags by amplification, purify, and assess quality and quantity of the captured libraries. Sample pooling instructions are provided to prepare the indexed samples for multiplexed sequencing.

Step 1. Amplify the captured libraries to add index tags

In this step, the SureSelect-enriched indexed cDNA libraries are PCR amplified. The protocol uses half of the captured library for amplification. The remainder can be saved at –20°C for future use, if needed.

CAUTION To avoid cross-contaminating libraries, set up PCR master mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

Use reagents from the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Thaw the reagents listed in Table 23 below and keep on ice.

Prepare 1 indexing amplification reaction for each cDNA library.

1 Determine the appropriate index assignments for each sample.

CAUTION This guide contains two sets of index sequence information. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

See the Reference section for sequences of the index portion of the indexing primers used to amplify cDNA libraries in this step. For indexing primers A01 through H12 (provided in white capped tubes or blue plate) see Table 33 on page 64. For indexing primers 1 through 96 (provided in clear capped tubes or clear plate), see Table 40 on page 69 through Table 45 on page 74.

Use a different index primer for each sample to be sequenced in the same lane.

2 Prepare the appropriate volume of PCR reaction mixture, according to Table 23. Mix well using a vortex mixer and keep on ice.

Table 23 Preparation of Post-capture PCR Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
RNA Seq PCR Master Mix	25 µL	425 µL
RNA Seq ILM Post-Capture PCR Primer	1 µL	17 µL
Total Volume	**26 µL**	**442 µL**

3 For each sample to be amplified, place 26 µL of the post-capture PCR reaction mixture from step 2 in a PCR plate well.

4 Add 5 µL of the appropriate indexing primer to each PCR reaction mixture well.

5 Add 19 µL of purified cDNA library to each PCR reaction mixture well. Mix thoroughly by pipetting.

6 Place the PCR plate in a thermal cycler and run the amplification program shown in Table 24. Use a heated lid on the thermal cycler at 105°C.

Table 24 Post-Capture PCR indexing thermal cycler program

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	12	95°C	30 seconds
		57°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Step 2. Purify the amplified captured libraries 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 (approximately 3 to 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.

    Do not dry the bead pellets to the point that the pellets appear cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
12. Add 30 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 to 3 minutes or until the solution is clear.

16 Remove the cleared supernatant (approximately 30 µL) 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 plate and store at –20°C.

Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay

NOTE You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples at this step. For analysis of captured DNA, use the High-Sensitivity D1000 ScreenTape (p/n 5067-5584) and associated reagents. See the 2200 TapeStation and High-Sensitivity D1000 ScreenTape protocols for information on sample preparation and data analysis.

Use a High Sensitivity DNA Assay kit to assess sample quality and quantity using the 2100 Bioanalyzer.

The concentration of each sample loaded on the High Sensitivity DNA Assay chip must be within the linear range of the assay (5 pg to 500 pg) to accurately quantify the DNA. You may need to dilute your sample accordingly. Refer to the *Agilent High Sensitivity DNA Kit Guide* at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=59504.

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 required to run the High Sensitivity Kit), turn on the 2100 Bioanalyzer, and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
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 High Sensitivity DNA 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. Check that the electropherogram shows a distribution with a fragment size peak between approximately 200 to 700 bp. A sample electropherogram is shown in Figure 6.
8. Determine the concentration of each target-enriched library by integration under the peak in the electropherogram.

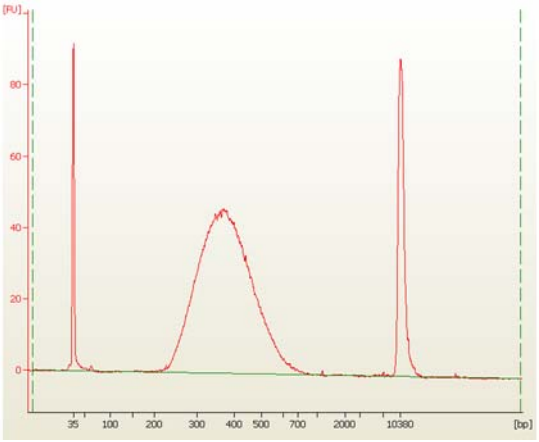

Figure 6 Analysis of amplified captured DNA using the High Sensitivity DNA Assay. The electropherogram shows a peak in the size range of approximately 200 to 700 bp.

Step 4. Pool 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 each sample based on your research objectives.

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 25 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 25 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 5. 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 RNA target-enriched libraries is approximately 8-10 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 26. 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 the Reference chapter starting on page 59.

Table 26 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.

5 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 73 for kit content and indexing primer information.

Kit Contents

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

Table 27 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
SureSelect Target Enrichment Box 1	Room Temperature	5190-4393	5190-4394
SureSelect Target Enrichment Box 2	–20°C	5190-6261	5190-6262

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 27 are described in the tables below.

Table 28 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*	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†

* See Table 33 on page 64 for index sequences.

† See Table 32 on page 63 for a plate map.

Table 29 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 30 SureSelect Target Enrichment Box 1 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 1	tube with orange cap	tube with orange cap
SureSelect Hyb 2	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle
SureSelect Elution Buffer	tube with blue cap	bottle
SureSelect Neutralization Buffer	tube with clear cap	bottle

Table 31 SureSelect Target Enrichment Box 2 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap
SureSelect Indexing Block 1	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap
SureSelect ILM Indexing Block 3	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap

Table 32 Plate map for SSEL 8bp Indexes A01 through H12 provided in blue plate in Library Prep kit p/n 5500-0135

	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 56 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 33 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 60 for kit content and indexing primer information.

Kit Contents

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

Table 34 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
SureSelect Target Enrichment Box 1	Room Temperature	5190-4393	5190-4394
SureSelect Target Enrichment Box 2	–20°C	5190-6261	5190-6262

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 34 are described in the tables below.

Table 35 SureSelect Strand Specific RNA Library Prep, ILM, Box 1 Content-Original Index Configuration

Kit Component	16 Reactions (p/n 5500-0116)	96 Reactions (p/n 5500-0117)
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†

* See Table 40 on page 69 through Table 45 on page 74 for index sequence information.

† See Table 39 on page 68 for a plate map.

Table 36 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 37 SureSelect Target Enrichment Box 1 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 1	tube with orange cap	tube with orange cap
SureSelect Hyb 2	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle
SureSelect Elution Buffer	tube with blue cap	bottle
SureSelect Neutralization Buffer	tube with clear cap	bottle

Table 38 SureSelect Target Enrichment Box 2 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap
SureSelect Indexing Block 1	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap
SureSelect ILM Indexing Block 3	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap

Table 39 Plate map for RNA Seq Indexes 1-96, 8 bp, provided in clear plate in Library Prep kit p/n 5500-0117

	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 56 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 40 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 41 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 42 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

Table 43 SureSelect RNA Seq Indexes 49-64

Index Number	Sequence
49	GATAGACA
50	GCCACATA
51	GCGAGTAA
52	GCTAACGA
53	GCTCGGTA
54	GGAGAACA
55	GGTGCGAA
56	GTACGCAA
57	GTCGTAGA
58	GTCTGTCA
59	GTGTTCTA
60	TAGGATGA
61	TATCAGCA
62	TCCGTCTA
63	TCTTCACA
64	TGAAGAGA

Table 44 SureSelect RNA Seq Indexes 65-80

Index Number	Sequence
65	TGGAACAA
66	TGGCTTCA
67	TGGTGGTA
68	TTCACGCA
69	AACTCACC
70	AAGAGATC
71	AAGGACAC
72	AATCCGTC
73	AATGTTGC
74	ACACGACC
75	ACAGATTC
76	AGATGTAC
77	AGCACCTC
78	AGCCATGC
79	AGGCTAAC
80	ATAGCGAC

Table 45 SureSelect RNA Seq Indexes 81-96

Index Number	Sequence
81	ATCATTCC
82	ATTGGCTC
83	CAAGGAGC
84	CACCTTAC
85	CCATCCTC
86	CCGACAAC
87	CCTAATCC
88	CCTCTATC
89	CGACACAC
90	CGGATTGC
91	CTAAGGTC
92	GAACAGGC
93	GACAGTGC
94	GAGTTAGC
95	GATGAATC
96	GCCAAGAC

www.agilent.com

In This Book

This guide contains information to run the SureSelect^{XT} RNA Target Enrichment for Illumina Multiplexed Sequencing protocol.

Version D0, July 2015

p/n G9691-90000

Agilent Technologies