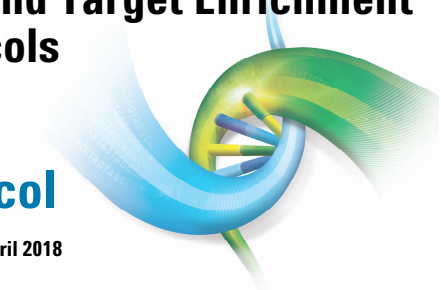
SureSelect^{XT} RNA Target Enrichment for Illumina Multiplexed Sequencing

Strand-Specific RNA Library Prep and Target Enrichment Protocols



Protocol

Version F0, April 2018

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 F0, April 2018

Printed in USA

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

For Europe, Middle East, Africa, and India
Call 00800 345 600 (toll free) or
+49 69 8679 7730

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

For all other regions
Agilent's world-wide sales and support center telephone numbers can be obtained at www.agilent.com/genomics under Contact Us.

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

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 and minimize 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 F0

- Support for new on-bead post-capture PCR protocol. Make sure to use the updated streptavidin bead supplier information in Table 1 on page 11. See revised instructions for DNA capture through on-bead post-capture PCR and clean-up on page 43 through page 51. See revised kit configuration details in Table 30 on page 60 and Table 33 on page 62.
- Correction to storage location for post-capture hybridization reagents (see Table 25 on page 43)
- Updates to sequencing support guidelines (see page 57 to page 58)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see Table 3 on page 13)
- Addition of Agilent 4200 TapeStation system-compatible plasticware ordering information (see page 19)
- Updates to product guarantee and support statement (see *Note* on page 9)
- Clarification to AMPure XP Bead drying steps after ethanol washes (for example, see step 12 on page 24)
- Clarification to mixing instructions for step 3 on page 23 to include vortexing duration and speed
- Updates to reagent descriptions to include vial format and cap color (see page 26, page 28, page 30)
- Update to plate sealing method prior to hybridization (see step 11 on page 42)
- Clarification to instructions for initiation of hybridization (see step 12 on page 42)
- Updates to library description (to RNA Capture Library) on page 41 to page 44
- Updates to 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^{XT} RNA Target Enrichment

Description	Vendor and part number
SureSelect or ClearSeq 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 M-270 Streptavidin Beads† 2 mL 10 mL	Thermo Fisher Scientific p/n 65305 p/n 65306
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090015, 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 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 17 for additional information.

† It is important to use the specified Dynabeads M-270 Streptavidin Beads in the on-bead post-capture PCR protocol detailed in this user manual. Other sources of streptavidin-coated beads, including those listed in previous versions of this user manual, are not supported for use in the current protocol.

:

Table 2 Compatible SureSelect and ClearSeq Capture Libraries

Capture Library	16 Samples	96 Samples	480 Samples
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)
ClearSeq RNA Kinome XT	5190-4801	5190-4802	5190-4803

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 plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
Compression mats	Agilent p/n 410187
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
Low-Adhesion Tubes (RNase, DNase, and DNA-free) 1.5 mL 0.5 mL	USA Scientific p/n 1415-2600 p/n 1405-2600
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent*
96-well plate mixer	Eppendorf ThermoMixer C, p/n 5382 000.015 and Eppendorf SmartBlock 96 PCR, p/n 5306 000.006, 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	

* 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 4 Optional Reagents and Equipment

Description	Vendor and part number
Agilent 4200 TapeStation*	Agilent p/n G2991AA
TapeStation consumables	
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
Labnet MPS1000 Mini Plate Spinner	Labnet International p/n C1000
Universal Human Reference RNA	Agilent p/n 740000

* DNA samples may also be analyzed using the Agilent 2200 TapeStation, p/n G2964AA or G2965AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.

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.

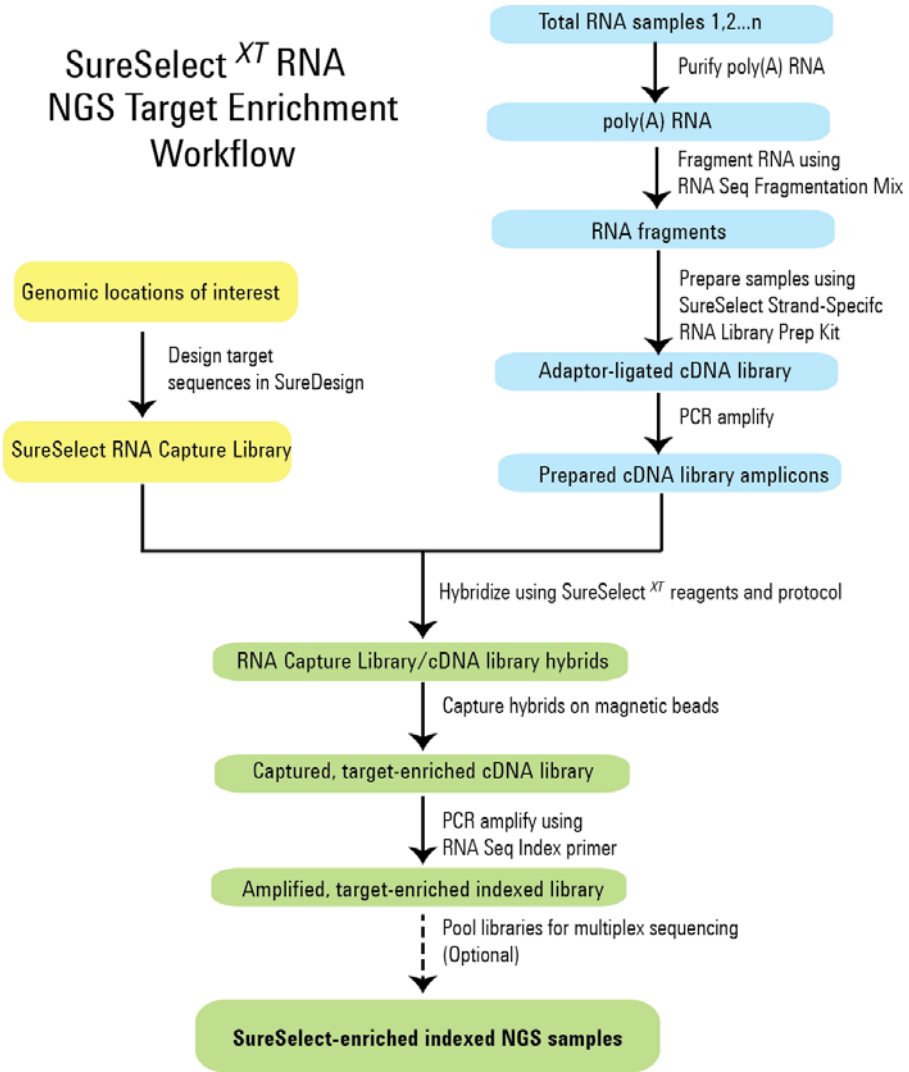


Figure 1 Overall RNA sequencing sample preparation workflow.

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 22 for more information.)

Just before starting the protocol, bring the reagents listed in Table 5 to room temperature and thaw the reagents listed in Table 6 on ice.

Table 5 Reagents brought to room temperature before use in protocol

Kit Component	Storage Location	Where Used in Protocol
Nuclease Free Water (bottle)	RNA Library Prep Kit Box 2, 4°C	page 18
Oligo(dT) Microparticles (tube with brown cap or bottle)	RNA Library Prep Kit Box 2, 4°C	page 18
RNA Seq Bead Washing Buffer (bottle)	RNA Library Prep Kit Box 2, 4°C	page 19
RNA Seq Bead Elution Buffer (tube with green cap or bottle)	RNA Library Prep Kit Box 2, 4°C	page 20
RNA Seq Bead Binding Buffer (tube with purple cap or bottle)	RNA Library Prep Kit Box 2, 4°C	page 20

Table 6 Reagents thawed and held on ice before use in protocol

Kit Component	Storage Location	Where Used in Protocol
RNA Seq Fragmentation Mix (tube with red cap or bottle)	RNA Library Prep Kit Box 1, –20°C	page 21
RNA Seq First Strand Master Mix (tube with orange cap)	RNA Library Prep Kit Box 1, –20°C	page 22
RNA Seq Second-Strand + End-Repair Enzyme Mix (tube with blue cap or bottle)	RNA Library Prep Kit Box 1, –20°C	page 26
RNA Seq Second-Strand + End-Repair Oligo Mix (tube with yellow cap)	RNA Library Prep Kit Box 1, –20°C	page 26

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.

Consider preparing an additional sequencing library in parallel, using a high-quality control RNA sample, such as Agilent's Universal Human Reference RNA (p/n 740000). 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 5 and Table 6 on page 17.

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 the SureCycler thermal cycler (with the heated lid ON) and run the program in Table 7 to denature the RNA.

Table 7 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 RNA Seq 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 RNA Seq 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 the thermal cycler (with the heated lid ON) and run the program in Table 8.

Table 8 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 RNA Seq 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 RNA Seq 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 poly(A) RNA.

Step 2. Fragment poly(A) RNA

In this step, the purified 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 RNA Seq Fragmentation Mix.

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

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

Table 9 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 Complete step 1 and step 3 on page 22 during the 8-minute incubation at 94°C.

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 10.

Table 10 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 RNA Seq 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 RNA Seq First Strand Master Mix 5 seconds at high speed to ensure homogeneity.

3 Prepare the appropriate amount of RNA Seq First Strand Master Mix + Actinomycin D mixture, on ice, according to Table 11. Mix by vortexing at high speed for 5 seconds, then spin briefly and keep on ice.

Table 11 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 (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 9), 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 3 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 the thermal cycler (with the heated lid ON) and run the program in Table 12.

Table 12 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 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 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 samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (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 sample 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 26.

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

CAUTION The RNA Seq Second Strand + End Repair 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 RNA Seq Second Strand + End Repair Enzyme Mix (blue capped tube or bottle) and RNA Seq Second Strand + End Repair Oligo Mix (yellow capped tube) at high speed for 5 seconds to ensure homogeneity.
2. Add 25 µL of RNA Seq Second Strand + End Repair Enzyme Mix to each 20-µL purified first-strand cDNA sample. Keep on ice.
3. Add 5 µL of RNA Seq Second Strand + End Repair 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 at high speed 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 the thermal cycler and run the program in Table 13. **Do not use a heated lid**.

Table 13 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 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 once for a total of two washes.
11. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (up to 3 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 sample 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 7. dA-Tail the cDNA 3' ends

Before continuing the protocol, thaw the reagents listed in Table 14 (used for the remainder of library preparation steps) and keep on ice.

Table 14 Reagents thawed and held on ice before use in protocol

Kit Component	Storage Location	Where Used in Protocol
RNA Seq dA Tailing Master Mix (tube with green cap or bottle)	RNA Library Prep Kit Box 1, –20°C	page 28
SureSelect Ligation Master Mix (tube with purple cap)	RNA Library Prep Kit Box 1, –20°C	page 30
SureSelect Oligo Adaptor Mix (tube with blue cap)	RNA Library Prep Kit Box 1, –20°C	page 30
RNA Seq ILM Reverse PCR Primer (tube with black cap)	RNA Library Prep Kit Box 1, –20°C	page 32
RNA Seq PCR Master Mix (tube with red cap or bottle)	RNA Library Prep Kit Box 1, –20°C	page 32
Uracil DNA Glycosylase (UDG) (tube with yellow cap)	RNA Library Prep Kit Box 1, –20°C	page 32
SureSelect Primer (tube with brown cap)	RNA Library Prep Kit Box 1, –20°C	page 32

CAUTION The RNA Seq 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 RNA Seq dA Tailing Master Mix (green-capped tube or bottle) 5 seconds at high speed to ensure homogeneity.

2 Add 20 µL of RNA Seq 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 the thermal cycler and run the program in Table 15. **Do not use a heated lid**.

Table 15 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 (purple-capped tube) and SureSelect Oligo Adaptor Mix (blue-capped tube) 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 the thermal cycler and run the program in Table 16. **Do not use a heated lid**.

Table 16 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 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 once for a total of two washes.
11. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (up to 3 minutes). Do not overdry the beads.
12. Add 23 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 22 µL of cleared supernatant to a fresh well and keep on ice. 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.

CAUTION The RNA Seq 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.

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

2 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 pre-capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
RNA Seq 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
RNA Seq ILM Reverse PCR Primer (tube with black cap)	1 µL	17 µL
Total	**28 µL**	**476 µL**

3 Add 28 µL of the pre-capture PCR reaction mix prepared in step 2 to each 22-µL purified, adaptor-ligated cDNA 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 the thermal cycler (with the heated lid ON) and run the program in Table 18.

Table 18 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 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 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 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 once for a total of two washes.
11. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (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. See the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

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. 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.

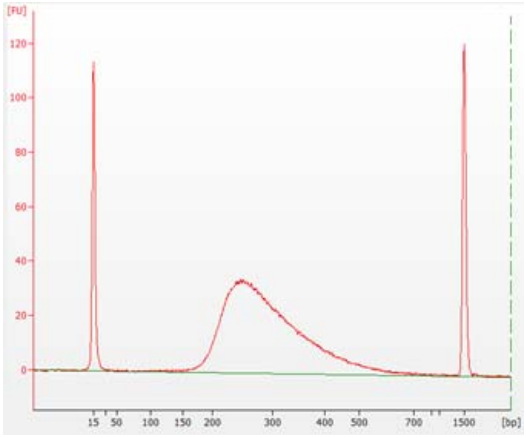


Figure 2 Analysis of amplified prepped library cDNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 180 to 550 bp.

3 Hybridization

This chapter describes the steps to hybridize the prepped cDNA library with the SureSelect or ClearSeq RNA Capture Library in combination with the hybridization reagents and blocking agents.

After hybridization, libraries are captured using Dynabeads M-270 Streptavidin magnetic beads in preparation for on-bead PCR.

NOTE

Previous versions of the RNA-Seq target enrichment kits and protocols included elution of DNA libraries from Dynabeads MyOne T1 streptavidin beads using SureSelect Elution Buffer prior to post-capture PCR. For support with using kits containing SureSelect Elution Buffer and the associated protocols, please contact ngs.support@agilent.com.

CAUTION

The ratio of RNA Capture Library to prepped cDNA library is critical for successful capture.

Step 1: Hybridize the library

In this step, the prepared cDNA libraries are hybridized to a SureSelect or ClearSeq 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. Transfer each 3.4-µL DNA library sample (100 ng) to a separate well of a 96-well plate or strip tube. Seal the wells and keep on ice.

4 Prepare the Hybridization Buffer by mixing the components in Table 20 at room temperature.

If a precipitate forms, warm the Hybridization Buffer at 65°C for 5 minutes.

Keep the prepared Hybridization Buffer at room temperature until it is used in step 9.

Table 20 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction*	Volume for 16 reactions (includes excess)
SureSelect Hyb 1 (tube with orange cap or bottle)	6.63 µL	116 µL
SureSelect Hyb 2 (tube with red cap)	0.27 µL	4.7 µL
SureSelect Hyb 3 (tube with yellow cap or bottle)	2.65 µL	46.4 µL
SureSelect Hyb 4 (tube with black cap or bottle)	3.45 µL	60.4 µL
Total	**13 µL**	**227.5**

* Prepare Hybridization Buffer for at least 5 reaction equivalents per run to allow accurate pipetting volumes.

5 Prepare the SureSelect Block Mix by mixing the components in Table 21. Keep the mixture on ice until it is used in step 6.

Table 21 Preparation of SureSelect Block Mix

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

6 To each cDNA library sample well prepared in step 3 on page 38, add 5.6 µL of the SureSelect Block Mix prepared in Table 21. Pipette up and down to mix.

7 Cap the wells, then transfer the sealed plate or strip tube to the thermal cycler and run the following program shown in Table 22.

Use a heated lid, set at 105°C, to hold the temperature at 65°C.

Make sure that the DNA + Block Mix samples are held at 65°C for at least 5 minutes before adding the remaining hybridization reaction components in step 10 below.

Table 22 Thermal cycler program for DNA + Block Mix prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold (at least 5 minutes)

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

8 Prepare the required volume of a 1:4 dilution of SureSelect RNase Block (for a final concentration of 25%), as shown in Table 23. Keep on ice.

Table 23 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**

NOTE

Prepare the RNA Capture Library mixture described in step 9, below, near the end of the 65°C hold step of >5 minute duration) described in Table 22. Keep the mixture at room temperature briefly, until adding the mixture to sample wells in step 10. Do not keep solutions containing the RNA Capture Library at room temperature for extended periods.

9 Prepare the RNA Capture Library Hybridization Mix by combining the solutions listed in Table 24, in the order listed.

Mix well by vortexing at high speed for 5 seconds then spin down briefly. Keep the mixture at room temperature briefly, until use in step 10.

Table 24 Preparation of RNA Capture Library Hybridization Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 4	13 µL	221 µL
25% RNase Block solution from step 8	2 µL	34 µL
RNA Capture Library	5 µL	85 µL
Total	**20 µL**	**340 µL**

10 Maintain the DNA library + Block Mix plate or strip tube at 65°C while you add 20 µL of the RNA Capture Library Hybridization Mix from step 9 to each sample well. Mix well by pipetting up and down 8 to 10 times.

The hybridization reaction wells now contain approximately 27 to 29 µL, depending on the degree of evaporation during the thermal cycler incubation.

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

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

When using the SureCycler 8800 thermal cycler and sealing with strip caps, make sure to use domed strip caps and to place a compression mat over the PCR plate or strip tubes in the thermal cycler.

12 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 M-270 Streptavidin magnetic beads (see Table 1 on page 11 for ordering information) are prepared for use in capturing the cDNA library/RNA Capture Library hybrids.

The reagents listed in Table 25 are used to prepare the Dynabeads M-270 Streptavidin magnetic beads and for subsequent steps in the capture protocol.

Table 25 Reagents for post-hybridization capture using streptavidin beads

Kit Component	Storage Location	Where Used in Protocol
SureSelect Binding Buffer (bottle)	SureSelect Target Enrichment Box 1, RT	page 43
SureSelect Wash Buffer 1 (bottle)	SureSelect Target Enrichment Box 1, RT	page 44
SureSelect Wash Buffer 2 (bottle)	SureSelect Target Enrichment Box 1, RT	page 44

1. Vigorously resuspend the Dynabeads M-270 Streptavidin magnetic beads on a vortex mixer. Magnetic beads settle during storage.
2. For each hybridization, add 50 µL of the magnetic bead suspension to wells of a PCR plate or strip tube.
3. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix the beads by pipetting up and down until the beads are fully resuspended.
   c. Put the plate or strip tube into a magnetic separator device and allow the solution to clear (approximately 5 minutes).
   d. Remove and discard the supernatant.
   e. Repeat step a through step d for a total of 3 washes.
4. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

Step 3. Capture hybrids using streptavidin beads

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

1. After the 24-hour hybridization, remove samples from the thermal cycler and spin the plate or strip tube at room temperature to collect the liquid.

   Estimate and record the volume of hybridization solution that remains after the 24 hour incubation in each well.

2. Use a multichannel pipette to transfer the entire volume (approximately 29 µL) of each hybridization mixture to the plate or strip tube wells containing 200 µL of washed streptavidin beads.

   Mix well by slowly pipetting up and down at least 10 times, until beads are fully resuspended.

3. Cap the wells, then incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (1400–1800 rpm) for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells by inspecting the bottom of the wells for settled beads after 5 minutes.

4. During the 30-minute incubation for capture, prewarm Wash Buffer 2 at 65°C as described below.

   a. Place 200-µl aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 3 wells of buffer for each DNA sample in the run.

   b. Cap the wells then incubate in the thermal cycler, with heated lid ON, held at 65°C until used in step 10.

5. At the end of the 30-minute incubation for capture, briefly spin the sample plate or strip tube in a centrifuge or mini-plate spinner.

6. Put the plate or strip tube on the magnetic stand at room temperature for approximately five minutes. Remove and discard the supernatant.

7. Resuspend the beads in 200 µL of SureSelect Wash Buffer 1. Pipette up and down until beads are resuspended. Cap the wells then mix on a vortex mixer for 5 seconds.

8. Incubate the samples for 15 minutes at room temperature.

9 Put the plate or strip tube on the magnetic stand at room temperature for approximately five minutes. Remove and discard 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.

10 Wash the beads with SureSelect Wash Buffer 2:

- **a** Resuspend the beads in 200 µL of 65°C prewarmed SureSelect Wash Buffer 2. Pipette up and down until beads are resuspended.
- **b** Seal the wells with fresh caps and then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid without pelleting the beads.

  Make sure the beads are in suspension before proceeding.
- **c** Incubate the samples for 10 minutes at 65°C on the thermal cycler (with the lid closed and heated lid ON).
- **d** Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner.
- **e** Put the plate or strip tube in the magnetic separator.
- **f** Wait for the solution to clear, then remove and discard the supernatant.
- **g** Repeat step a through step f for a total of 3 washes.

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

11 Add 40 µL of nuclease-free water to each sample well. Pipette up and down to resuspend the beads.

Keep the samples on ice until they are used on page 49.

NOTE Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

Stopping Point If you do not continue to the next step, seal the wells and store at 4°C for same-day processing or store at –20°C for up to one month.

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 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. Prepare 1 indexing amplification reaction for each cDNA library.

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

See Table 36 on page 64 for sequences of the index portion of the indexing primers used to amplify cDNA libraries in this step.

Thaw the appropriate indexing primer vials (white-capped tubes) for 16-reaction kits or the blue indexing primer plate for 96-reaction kits and keep on ice.

CAUTION The RNA Seq 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 as directed in Table 26. Pipetting up and down is not sufficient to mix this reagent.

2 Thaw the reagents listed in Table 26 below and keep on ice. Vortex the master mix vial at high speed for 5 seconds just before use to ensure homogeneity.

3 Prepare the appropriate volume of PCR reaction mixture, according to Table 26. Mix well using a vortex mixer and keep on ice.

Table 26 Preparation of Post-capture PCR Reaction Mix

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

4 For each sample to be amplified, place 26 µL of the Post-capture PCR Reaction Mix prepared in step 3 into wells of a PCR plate or strip tube.

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

6 Add the cDNA library samples to the PCR reactions:

a Obtain the PCR plate containing 40 µL of bead-bound target-enriched cDNA library samples from ice (from page 45).

b Mix thoroughly by pipetting up and down. Make sure the bead suspension is homogeneous before removing liquid, then transfer 19 µL of the sample to the appropriate well of the PCR plate or strip tube containing PCR reaction mix and indexing primer.

c Mix the PCR reactions well by pipetting.

d Store the remaining bead-bound library samples at –20°C for future use, if needed.

7 Place the PCR plate in a thermal cycler and run the amplification program shown in Table 27. Use a heated lid on the thermal cycler at 105°C.

Table 27 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 9.
3. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 90 µL of the homogeneous AMPure XP bead suspension to each 50-µL amplified DNA sample bead suspension in the PCR plate.
5. Mix thoroughly by pipetting up and down.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
6. Incubate samples for 5 minutes at room temperature.
7. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 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 the beads while removing the solution.
9. 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.
10. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
11. Repeat step 9 and step 10 once for a total of two washes.
12. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (up to 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.
13. Add 30 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 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 wells and store at –20°C.

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

NOTE You can use Agilent's 4200 TapeStation or 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. For more information, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information.

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 3.
8. Determine the concentration of each target-enriched library by integration under the peak in the electropherogram.

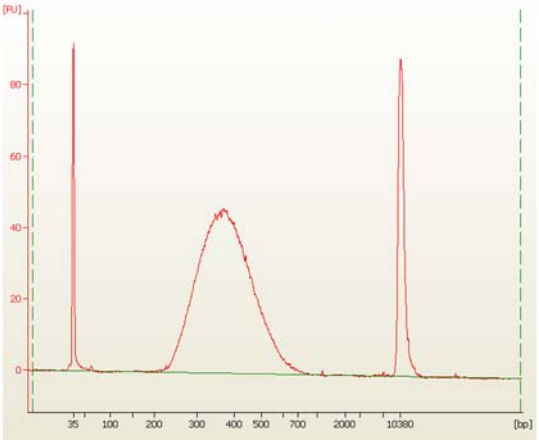


Figure 3 Analysis of amplified captured DNA using the High Sensitivity DNA Assay. The electropherogram shows a peak in the size range of approximately 200 to 700 bp.

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 28 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 28 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 appropriate Illumina Paired-End Cluster Generation Kit. See Table 29 for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect^{XT} target-enriched RNA sequencing libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 29 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the fragment size range for the library and on the desired output and data quality.

Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 29 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	10–13 pM
HiSeq 2500	High Output	2 × 100 bp	200 Cycle Kit	v3	10–13 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	13–16 pM
HiSeq 2000	All Runs	2 × 100 bp	200 Cycle Kit	v3	7–11 pM
HiSeq 2000	All Runs	2 × 100 bp	250 Cycle Kit	v4	10–14 pM
MiSeq	All Runs	2 × 100 bp	300 Cycle Kit	v2	10–13 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	14–19 pM
NextSeq 500/550	All Runs	2 × 100 bp	300 Cycle Kit	v2	1.7–2.0 pM
HiSeq 3000/4000	All Runs	2 × 100 bp	300 Cycle Kit	v1	200 pM

Sequencing run setup guidelines for 8-bp indexes

Sequencing runs must be set up to perform an 8-bp index read. See the Reference chapter for complete index sequence information.

For the HiSeq and NextSeq 500 (v1) platforms, use the *Cycles* settings shown in Table 30. 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.

Table 30 Cycle Number settings for HiSeq and NextSeq platforms

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	0
Read 2	100

For the MiSeq platform, use the Illumina Experiment Manager (IEM) software to generate a Sample Sheet that includes the run parameters specified in Table 31.

Table 31 Run parameters for MiSeq platform Sample Sheet

Parameter	Entry
Workflow	GenerateFASTQ
Cycles for Read 1	100 for v2 chemistry 75 for v3 chemistry
Cycles for Read 2	100 for v2 chemistry 75 for v3 chemistry
Index 1 (i7) Sequence (enter in Data Section for each sample)	Type the 8-nt index sequence for each individual sample (see Table 37 on page 67).

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.

Kit Contents

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

Table 30 SureSelect RNA-Seq Kit Content

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	5190-8645 or 5190-4393*	5190-8646 or 5190-4394*
SureSelect Target Enrichment Box 2	–20°C	5190-6261	5190-6262

* Part numbers 5190-4393 and 5190-4394 were replaced with part numbers 5190-8645 and 5190-8646 in May, 2018. The retired kits are used for previous versions of the RNA-Seq target enrichment protocol that include elution of DNA libraries from streptavidin beads prior to post-capture PCR. For support with using the retired kits and protocols, please contact ngs.support@agilent.com.

The contents of each of the component kits listed in Table 30 are described in Table 31 through Table 34 below.

Table 31 SureSelect Strand Specific RNA Library Prep, ILM, Box 1 Content

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 36 on page 64 for index sequences.

† See Table 35 on page 63 for a plate map.

Table 32 SureSelect Strand Specific RNA Library Prep, ILM, Box 2 Content

Kit Component	16 Reactions (p/n 5190-6410)	96 Reactions (p/n 5190-6411)
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 33 SureSelect Target Enrichment Box 1 Content

Kit Component	16 Reactions (p/n 5190-8645)	96 Reactions (p/n 5190-8646)	16 Reactions (p/n 5190-4393)*	96 Reactions (p/n 5190-4394)*
SureSelect Hyb 1	tube with orange cap	bottle	tube with orange cap	bottle
SureSelect Hyb 2	tube with red cap	tube with red cap	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle	bottle	bottle
SureSelect Elution Buffer	Not supplied (not required for updated protocol with on-bead PCR)	Not supplied (not required for updated protocol with on-bead PCR)	tube with blue cap†	bottle†
SureSelect Neutralization Buffer	Not supplied (not required for updated protocol with on-bead PCR)	Not supplied (not required for updated protocol with on-bead PCR)	tube with clear cap†	bottle†

* Part numbers 5190-4393 and 5190-4394 were replaced with part numbers 5190-8645 and 5190-8646 in May, 2018.

† This component is not used in the protocol supported by this publication, which includes on-bead post-capture PCR. For support with using the retired kit components and protocol including elution of DNA libraries from streptavidin beads prior to post-capture PCR, please contact ngs.support@agilent.com.

Table 34 SureSelect Target Enrichment Box 2 Content

Kit Component	16 Reactions (p/n 5190-6261)	96 Reactions (p/n 5190-6262)
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 Indexing Block 3	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap

Table 35 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 61 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 36 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

www.agilent.com

In This Book

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

Version F0, April 2018

p/n G9691-90000

Agilent Technologies