

SureSelect^{XT} RNA Target Enrichment System

**Poly-A Selection, Strand-Specific
mRNA Library Preparation and Target
Enrichment for the Illumina Platform**

Protocol

Version G0, June 2020

**SureSelect platform manufactured with Agilent
SurePrint Technology**

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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 G0

- Support for renamed Library Preparation Kit components. See [Table 33](#) on page 62 and [Table 34](#) on page 63 for a summary of component name changes. The new component names are utilized throughout the protocols in this document. No changes were made to the reagent formulations or methods of use.
- Support for revised Poly-A Selection Module subkit. See [Table 33](#) on page 62 and [Table 35](#) on page 64 for a summary of component name and configuration changes. The new component names are utilized throughout the protocols in this document. No changes were made to the reagent formulations or methods of use. Water is no longer provided in this module (see [Table 35](#) on page 64) and has been added to the list of required reagents in [Table 1](#) on page 11.
- Updates to ordering information for custom RNA target enrichment probes (see [Table 2](#) on page 12) and updates to probe nomenclature in the Hybridization protocol steps (see [page 38](#) to [page 42](#)).
- Updates to thermal cycler recommendations and usage instructions (see [Table 3](#) on page 13, and example usage instructions in [step 5](#) on [page 20](#)).
- Updates to [page 27](#) to clarify details of the second-strand synthesis/end repair step.
- Updates to sequencing support guidelines for RNA strandedness (see [page 59](#)).
- Updated recommendation for control reference RNA, to Agilent QPCR Human Reference Total RNA (see [Table 4](#) on page 14 and [page 19](#)).
- Update to *Caution* on [page 38](#).
- Updates to [page 48](#) to include post-capture PCR reagent table ([Table 26](#)).
- Minor wording updates to poly-A RNA selection steps [page 19](#) through [page 22](#).
- Updates to ordering information for 1X TE Buffer and AMPure XP Kits (see [Table 1](#) on page 11).

- Updated Technical Support contact information (see [page 2](#)).
- Updated *Notice to Purchaser* (see [page 2](#)).
- End of support for component kits 5190-4393 and 5190-4394 used for target enrichment protocols that included a bead elution step prior to post-capture PCR. These kits were replaced with component kits 5190-8645 and 5190-8646, respectively, in May 2018 for use in the current protocol that includes on-bead post-capture PCR. See [Table 33](#) on page 62 and [Table 36](#) on page 64 for current kit contents.

Content

1	Before You Begin	9
	Procedural Notes	10
	Safety Notes	10
	Required Reagents	11
	Required Equipment	13
	Optional Reagents and Equipment	14
2	Sample Preparation	15
	Step 1. Select poly-A RNA from total RNA	19
	Step 2. Fragment the poly-A RNA	22
	Step 3. Synthesize first-strand cDNA	23
	Step 4. Purify first strand cDNA using AMPure XP beads	25
	Step 5. Synthesize second-strand cDNA and repair the ends	27
	Step 6. Purify cDNA using AMPure XP beads	28
	Step 7. dA-Tail the cDNA 3' ends	29
	Step 8. Ligate adaptors	31
	Step 9. Purify adaptor-ligated DNA using AMPure XP beads	32
	Step 10. Amplify the adaptor-ligated cDNA library	33
	Step 11. Purify the amplified library with AMPure XP beads	35
	Step 12. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay	36
3	Hybridization	37
	Step 1: Hybridize the library	38
	Step 2. Prepare streptavidin beads	43
	Step 3. Capture hybrids using streptavidin beads	44

Contents

4	Indexing and Sample Processing for Multiplexed Sequencing	47
	Step 1. Amplify the captured libraries to add index tags	48
	Step 2. Purify the amplified captured libraries using AMPure XP beads	51
	Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay	53
	Step 4. Pool samples for multiplexed sequencing	55
	Step 5. Prepare and analyze sequencing samples	57
5	Reference	61
	Kit Contents	62
	Nucleotide Sequences of SureSelect ^{XT} Indexes A01 to H12	66



1 Before You Begin

Procedural Notes	10
Safety Notes	10
Required Reagents	11
Required Equipment	13
Optional Reagents and Equipment	14

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 Library Preparation and Target Enrichment

Description	Vendor and part number
SureSelect or ClearSeq RNA Target Enrichment Probe	Select one probe from Table 2
SureSelect ^{XT} RNA Reagent Kit	Agilent
Illumina platforms (ILM), 16 Samples	p/n G9692A
Illumina platforms (ILM), 96 Samples	p/n G9692B
Actinomycin D*	Sigma p/n A1410
DMSO	Sigma p/n D8418
AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Dynabeads M-270 Streptavidin Beads [†]	Thermo Fisher Scientific
2 mL	p/n 65305
10 mL	p/n 65306
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

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

1 Before You Begin

Required Reagents

Table 2 Compatible SureSelect and ClearSeq Target Enrichment Probes

Target Enrichment Probe Library	16 Samples	96 Samples
Custom RNA Target Enrichment Probes 1–499 kb	Please contact Agilent’s SureSelect support team or your local representative for assistance with custom RNA probe design and ordering.	
Custom RNA Target Enrichment Probes 0.5–2.9 Mb		
Custom RNA Target Enrichment Probes 3– 5.9 Mb		
ClearSeq RNA Kinome XT	5190-4801	5190-4802

Required Equipment

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

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent, and compatible plasticware (consult the thermal cycler manufacturer's recommendations for compatible plasticware)	Agilent p/n G8800A (SureCycler 8800 Thermal Cycler) p/n G8810A (96 well plate module) p/n 410088 (96 well plates) p/n 410092 (8-well strip tubes) p/n 410096 (Tube cap strips, domed) p/n 410187 (Compression mat)
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
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
QPCR Human Reference Total RNA	Agilent p/n 750500

^{*} DNA samples may also be analyzed using the Agilent 4150 TapeStation, p/n G2992AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.



2 Sample Preparation

- Step 1. Select poly-A RNA from total RNA 19
- Step 2. Fragment the poly-A RNA 22
- Step 3. Synthesize first-strand cDNA 23
- Step 4. Purify first strand cDNA using AMPure XP beads 25
- Step 5. Synthesize second-strand cDNA and repair the ends 27
- Step 6. Purify cDNA using AMPure XP beads 28
- Step 7. dA-Tail the cDNA 3' ends 29
- Step 8. Ligate adaptors 31
- Step 9. Purify adaptor-ligated DNA using AMPure XP beads 32
- Step 10. Amplify the adaptor-ligated cDNA library 33
- Step 11. Purify the amplified library with AMPure XP beads 35
- Step 12. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay 36

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.



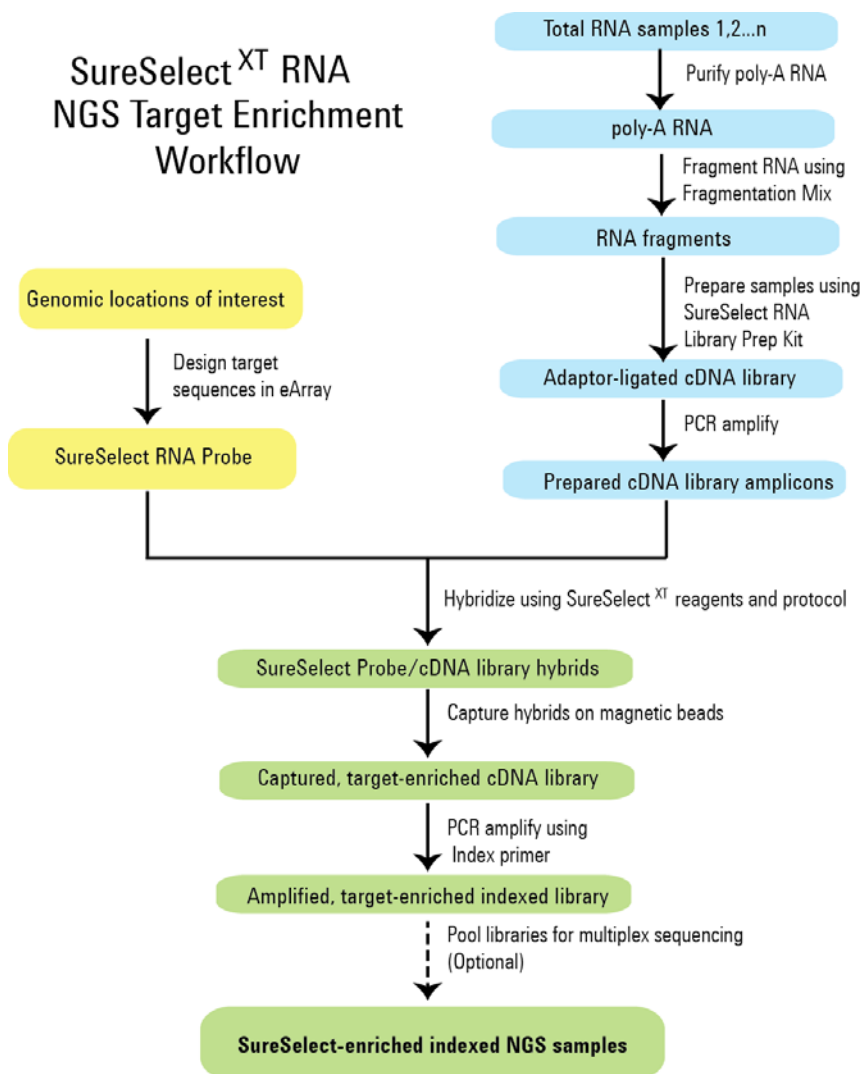


Figure 1 Overall RNA sequencing sample preparation workflow.

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 23](#) 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

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

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

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

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

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

2 Sample Preparation

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

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

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

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

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

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

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

Step 1. Select poly-A RNA from total RNA

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

Before you begin, prepare total RNA for each sample in the run. The amount of total RNA needed for the library preparation protocol 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 QPCR Human Reference Total RNA (p/n 750500). Use of this control is especially recommended during the first run of the protocol, to verify that all protocol steps are being successfully performed. Routine use of this control is helpful for any required troubleshooting, in order to differentiate any performance issues related to RNA input from other factors.

Make sure the reagents to be used in the protocol have been thawed and kept at the appropriate temperature, as indicated in [Table 5](#) and [Table 6](#) on page 18.

- 1 Prepare each total RNA sample in a final volume of 25 µL of nuclease-free water and place the samples in separate wells of a 96-well plate or strip tube.
- 2 Vortex the Oligo(dT) Microparticles until the suspension appears homogeneous and consistent in color. If bead aggregates are still present after vortexing, mix thoroughly by pipetting up and down until the suspension appears homogeneous.
- 3 Add 25 µL of the homogeneous Oligo(dT) bead suspension to each total RNA sample well.
- 4 Seal the wells, then gently vortex for 5 seconds and briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.

2 Sample Preparation

Step 1. Select poly-A RNA from total RNA

- 5 Incubate the plate or strip tube in a 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 Bead Washing Buffer to each well.
Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µL, without introducing bubbles.

CAUTION

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

- 10 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).
- 11 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.
- 12 Remove the plate or strip tube from the magnetic stand. Add 25 µL of Bead Elution Buffer to each sample well.

Step 1. Select poly-A RNA from total RNA

- 13** Seal the wells, then gently vortex for 5 seconds. Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid.
- 14** Incubate the plate or strip tube in a thermal cycler (with the heated lid ON) and run the program in [Table 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 Bead Binding Buffer to each sample well.
- 16** Seal the wells, then gently vortex for 5 seconds. Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid.
- 17** Incubate the samples at room temperature for 5 minutes, to allow poly-A RNA to re-bind the beads.
- 18** Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).
- 19** While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.
- 20** Remove the plate or strip tube from the magnetic stand. Gently add 200 µL of Bead Washing Buffer to each well.
Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µL, without introducing bubbles. If bubbles or foam are present, spin the plate or strip tube briefly before continuing.
- 21** Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).
- 22** While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.
- 23** Proceed immediately to [Step 2. Fragment the poly-A RNA](#).

2 Sample Preparation

Step 2. Fragment the poly-A RNA

Step 2. Fragment the poly-A RNA

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

- 1 Remove the plate or strip tube, containing the collected poly-A RNA-bound beads, from the magnetic stand.
- 2 To each sample well add 19 μ L of 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 a 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 23](#) during the 8-minute incubation at 94°C.

Step 3. Synthesize first-strand cDNA

In this step, first-strand cDNA is synthesized from the RNA fragments.

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

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

2 Sample Preparation

Step 3. Synthesize first-strand cDNA

- 3 Prepare the appropriate amount of 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/ μ L in H ₂ O)	0.5 μ L	8.5 μ L
First Strand Master Mix (orange cap)	8.0 μ L	136 μ L
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 μ L 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 μ L 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 a 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 in the thermal cycler block, 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.

2 Sample Preparation

Step 4. Purify first strand cDNA using AMPure XP beads

- 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 μ L 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 27.

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

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

CAUTION

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

- 1 Vortex the thawed vials of Second Strand Enzyme Mix (blue capped tube or bottle) and Second Strand Oligo Mix (yellow capped tube) at high speed for 5 seconds to ensure homogeneity.
- 2 Add 25 μL of Second Strand Enzyme Mix to each 20- μL purified first-strand cDNA sample. Keep on ice.
- 3 Add 5 μL of Second Strand Oligo Mix to each sample well, for a total reaction volume of 50 μL . Keep on ice.
- 4 Seal the wells. Mix thoroughly by vortexing the plate or strip tube 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 a 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

2 Sample Preparation

Step 6. Purify cDNA using AMPure XP beads

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 in 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

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR) [*] , -20°C	dA Tailing Master Mix [†] (green cap or bottle)	page 29
	SureSelect Ligation Master Mix (purple cap)	page 31
	SureSelect Oligo Adaptor Mix (blue cap)	page 31
	ILM Reverse PCR Primer [‡] (black cap)	page 33
	PCR Master Mix ^{**} (red cap or bottle)	page 33
	Uracil DNA Glycosylase (UDG) (yellow cap)	page 33
	SureSelect Primer (brown cap)	page 33

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

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

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

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

CAUTION

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

- 1 Vortex the thawed vial of dA Tailing Master Mix (green-capped tube or bottle) 5 seconds at high speed to ensure homogeneity.
- 2 Add 20 µL of dA Tailing Master Mix to each 20-µL purified, end-repaired cDNA sample.
Pipette the master mix slowly to ensure that the full volume is dispensed.

2 Sample Preparation

Step 7. dA-Tail the cDNA 3' ends

- 3 Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.
- 4 Spin the plate or strip tube briefly to collect the liquid.
- 5 Incubate the plate or strip tube in a thermal cycler and run the program in [Table 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 μ L 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 μ L of SureSelect Oligo Adaptor Mix to each sample.
- 4 Seal the wells. Mix thoroughly by vortexing the plate or strip tube for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.
- 5 Spin the plate or strip tube briefly to collect the liquid.
- 6 Incubate the plate or strip tube in a thermal cycler and run the program in [Table 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

2 Sample Preparation

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

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 in 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 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 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)
PCR Master Mix (tube with red cap or bottle)	25 μ L	425 μ L
Uracil DNA Glycosylase (UDG) (tube with yellow cap)	1 μ L	17 μ L
SureSelect Primer (forward primer; tube with brown cap)	1 μ L	17 μ L
ILM Reverse PCR Primer (tube with black cap)	1 μ L	17 μ L
Total	28 μL	476 μL

- 3 Add 28 μ L of the pre-capture PCR reaction mix prepared in [step 2](#) to each 22- μ L 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.

2 Sample Preparation

Step 10. Amplify the adaptor-ligated cDNA library

- 6 Incubate the plate or strip tube in a 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 in 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 .

2 Sample Preparation

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

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 μ L 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.

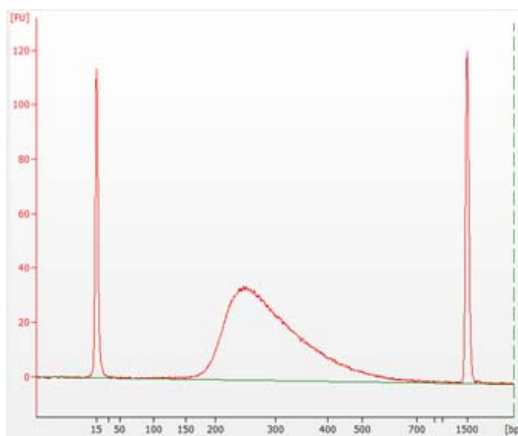


Figure 2 Analysis of amplified prepped library cDNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 180 to 550 bp.



3 Hybridization

- Step 1: Hybridize the library 38
- Step 2: Prepare streptavidin beads 43
- Step 3: Capture hybrids using streptavidin beads 44

This chapter describes the steps to hybridize the prepped cDNA library with the SureSelect or ClearSeq RNA Target Enrichment Probe 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.

CAUTION

The ratio of RNA Target Enrichment Probe to prepped cDNA library is critical for successful capture.



3 Hybridization

Step 1: Hybridize the library

Step 1: Hybridize the library

In this step, the prepared cDNA libraries are hybridized to a SureSelect or ClearSeq RNA Target Enrichment Probe.

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.

Before processing the first set of samples, first test the combination of thermal cycler, plates or strip tubes, and sealing method (strip caps or sealing tape) to be used in the protocol. 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 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 $\leq 45^\circ\text{C}$.
 - 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.

3 Hybridization

Step 1: Hybridize the library

- 7 Cap the wells, then transfer the sealed plate or strip tube to a 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 Probe Hybridization Mix 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 probe at room temperature for extended periods.

- 9** Prepare the Probe 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 Probe 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 Target Enrichment Probe	5 µL	85 µL
Total	20 µL	340 µL

3 Hybridization

Step 1: Hybridize the library

10 Maintain the DNA library + Block Mix plate or strip tube at 65°C while you add 20 µL of the Probe 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 thermal cycler, 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 probe 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

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

Step 3. Capture hybrids using streptavidin beads

- 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 μ L 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 μ L 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.

3 Hybridization

Step 3. Capture hybrids using streptavidin beads



4 Indexing and Sample Processing for Multiplexed Sequencing

- Step 1. Amplify the captured libraries to add index tags 48
- Step 2. Purify the amplified captured libraries using AMPure XP beads 51
- Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay 53
- Step 4. Pool samples for multiplexed sequencing 55
- Step 5. Prepare and analyze sequencing samples 57

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.



4 Indexing and Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured libraries to add index tags

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.

Before you begin, thaw the reagents listed in [Table 26](#) and keep on ice.

Table 26 Reagents for post-capture PCR

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR) [*] , -20°C	SureSelect ^{XT} Indexes, 8 bp (see step 1 below for index assignment and format information)	page 48
	PCR Master Mix [†] (red cap or bottle)	page 49
	ILM Post-Capture PCR Primer [‡] (green cap)	page 49

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

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

‡ May also be labeled as *RNA Seq ILM Post-Capture PCR Primer*.

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.

- 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 39](#) on page 66 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 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 27](#). Pipetting up and down is not sufficient to mix this reagent.

- 2 Thaw the reagents listed in [Table 27](#) 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 27](#). Mix well using a vortex mixer and keep on ice.

Table 27 Preparation of Post-capture PCR Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
PCR Master Mix (tube with red cap or bottle)	25 µL	425 µL
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.

4 Indexing and Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured libraries to add index tags

- 7 Place the PCR plate in a thermal cycler and run the amplification program shown in [Table 28](#). Use a heated lid on the thermal cycler at 105°C.

Table 28 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 in 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.

4 Indexing and Sample Processing for Multiplexed Sequencing

Step 2. Purify the amplified captured libraries using AMPure XP beads

- 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 μ L) 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 4150 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.

4 Indexing and Sample Processing for Multiplexed Sequencing

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

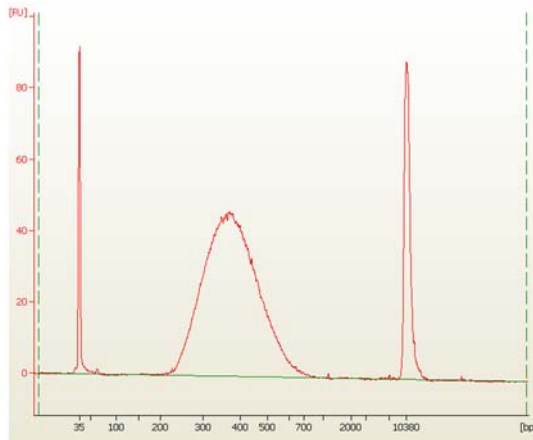


Figure 3 Analysis of amplified captured DNA using the High Sensitivity DNA Assay. The electropherogram shows a peak in the size range of approximately 200 to 700 bp.

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 29 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 29 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.

4 Indexing and Sample Processing for Multiplexed Sequencing

Step 4. Pool samples for multiplexed sequencing

- 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 30](#) 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 30](#) 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 30 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

4 Indexing and Sample Processing for Multiplexed Sequencing

Step 5. Prepare and analyze sequencing samples

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 31](#). 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 31 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 32](#).

Table 32 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 using dUTP second-strand marking. The sequence of read 1, which starts at the P5 end, matches the reverse complement of the poly-A RNA transcript strand. Read 2, which starts at the P7 end, matches the poly-A RNA transcript strand. When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (<https://broadinstitute.github.io/picard>) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY=SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

4 Indexing and Sample Processing for Multiplexed Sequencing
Step 5. Prepare and analyze sequencing samples



5 Reference

Kit Contents 62

Nucleotide Sequences of SureSelect^{XT} Indexes A01 to H12 66

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



Kit Contents

The SureSelect^{XT} RNA Reagent Kits include the component kits listed in [Table 33](#). The contents of each component kit are detailed in [Table 34](#) through [Table 37](#).

Table 33 SureSelect^{XT} RNA Reagent Kit Content

Component Kits	Storage Condition	16 Reactions	96 Reactions
SureSelect RNA Library Prep, ILM (Pre PCR) [*]	–20°C	5500-0134	5500-0135
SureSelect Poly-A Selection Module (Pre PCR) [†]	4°C	5190-6410	5190-6411
SureSelect Target Enrichment Box 1	Room Temperature	5190-8645	5190-8646
SureSelect Target Enrichment Box 2	–20°C	5190-6261	5190-6262

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

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

Table 34 SureSelect RNA Library Prep, ILM (Pre PCR) Content

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

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

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

‡ See [Table 39](#) on page 66 for index sequences.

** See [Table 38](#) on page 65 for a plate map.

5 Reference

Kit Contents

Table 35 SureSelect Poly-A Selection Module (Pre PCR) Content

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

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

Table 36 SureSelect Target Enrichment Box 1 Content

Kit Component	16 Reactions (p/n 5190-8645)	96 Reactions (p/n 5190-8646)
SureSelect Hyb 1	tube with orange cap	bottle
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

Table 37 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 38 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

5 Reference

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

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

Each index is 8 nt in length. See [page 58](#) for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 39 SureSelect Indexes, for indexing primers in white-capped tubes or blue 96-well plates

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

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In This Book

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

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