SureSelect^{XT} RNA Direct System

Strand-Specific RNA Library Preparation and Target Enrichment for the Illumina Platform

Protocol

Version B0, June 2020

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

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Acknowledgment

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In this Guide...

This guide describes an optimized protocol for preparation of target-enriched, strand-specific RNA sequencing libraries from either high-quality RNA samples or formalin-fixed paraffin-embedded (FFPE) total RNA samples, without initial mRNA purification, using the SureSelect^{XT} RNA Direct Reagent Kit.

This protocol is specifically optimized to enrich targeted regions of the transcriptome from repetitive sequences and sequences unrelated to the research focus prior to Illumina paired-end multiplexed 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 target enrichment steps including hybridization and capture of the targeted regions of prepared cDNA.

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 B0

- Support for renamed Library Preparation Kit components. See Table 30 on page 68 and Table 31 on page 69 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.
- Updates to page 27 to clarify details of the second-strand synthesis/end repair step.
- Updates to page 52 to include post-capture PCR reagent table (Table 24)
- Updates to ordering information for custom RNA target enrichment probes (see Table 2 on page 11) and updates to probe nomenclature in the Hybridization protocol steps (see page 42 to page 46)
- Updates to thermal cycler recommendations and usage instructions (see Table 3 on page 12, and example usage instructions in step 1 on page 21)
- Updates to sequencing support guidelines for RNA strandedness (see page 65)
- Updates to organization of "Required Equipment" tables (see new Table 4 on page 13)
- Update to name of AMPure XP Kits (see Table 1 on page 11)
- Updates to Technical Support contact information (see page 2)

What's New in Version A1

- Correction to supplier information for Dynabeads M-270 Streptavidin Beads and Nuclease-free water (see Table 1 on page 11)
- Updates to sequencing support guidelines (see page 63 to page 64)

- Minor updates to 2100 Bioanalyzer and 4200 TapeStation use instructions and reference documents (see page 36, page 38, page 57 and page 59)
- Addition of *Note* on use of RNA Seq Fragmentation Mix with highly degraded FFPE samples (see page 22)
- Updates to RNA Target Enrichment Probe Library details in Table 2 on page 11.
- Updates to terminology for RNA libraries used for hybridization-based enrichment, e.g. from *Capture Library* to *Target Enrichment Probe Library* (see for example page 41)
- Updates to Figure 1 on page 16.
- Updates to Technical Support contact information (see page 2
- Updated *Notice to Purchaser* (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 on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store vials used during an experiment on ice or in a cold block.
  4. Library Preparation Master Mixes should not be frozen and thawed more than five times. If you plan to use the reagents in more than five experiments, aliquot to multiple vials to minimize freeze/thaw cycles for each vial.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

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

Required Reagents

Table 1 Reagents for RNA Direct 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 Direct Reagent Kit	Agilent
Illumina platforms (ILM), 16 Samples	p/n G7564A
Illumina platforms (ILM), 96 Samples	p/n G7564B
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
Dynabeads M-270 Streptavidin Beads	Thermo Fisher Scientific
2 mL	p/n 65305
10 mL	p/n 65306
Buffer EB (10 mM Tris-Cl, pH 8.5)	Qiagen p/n 19086
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 (see page 17). The aliquots may be stored for up to one year before use.

CAUTION Use only the recommended Dynabeads M-270 Streptavidin Beads for this protocol. Use of other streptavidin bead preparations may adversely affect performance and is not supported by Agilent.

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		
SureSelectXT Human All Exon V6 + UTRs	5190-8881	5190-8882
ClearSeq RNA Kinome XT	5190-4801	5190-4802

Required Equipment

Table 3 Equipment for RNA Direct 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)
Nucleic acid analysis system	See Table 4 on page 13
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
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
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent*
Labnet MPS1000 Mini Plate Spinner (**Optional**)	Labnet International p/n C1000
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

* Select a magnetic separator 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.

Table 4 Nucleic Acid Analysis Platform Options--Select One

Analysis system	Vendor and part number
Agilent 4200/4150 TapeStation Instrument	Agilent p/n G2991AA/G2992AA
Consumables:	
96-well sample plates	p/n 5042-8502
96-well plate foil seals	p/n 5067-5154
8-well tube strips	p/n 401428
8-well tube strip caps	p/n 401425
High Sensitivity RNA ScreenTape	p/n 5067-5579
High Sensitivity RNA ScreenTape Sample Buffer	p/n 5067-5580
High Sensitivity RNA ScreenTape Ladder	p/n 5067-5581
D1000 ScreenTape	p/n 5067-5582
D1000 Reagents	p/n 5067-5583
High Sensitivity D1000 ScreenTape	p/n 5067-5584
High Sensitivity D1000 Reagents	p/n 5067-5585
Agilent 2100 Bioanalyzer Instrument	p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	p/n G2953CA
Consumables:	
RNA 6000 Pico Kit	p/n 5067-1513
DNA 1000 Kit	p/n 5067-1504
High Sensitivity DNA Kit	p/n 5067-4626

2 Sample Preparation

See Figure 1 for a summary of the SureSelect^{XT} RNA Direct target enrichment workflow.

This section contains instructions for strand-specific RNA sequencing cDNA library preparation, without initial mRNA purification. This protocol differs from Agilent's protocol for RNA sequencing library preparation that includes initial mRNA purification (User Manual p/n G9691-90000) at several steps.

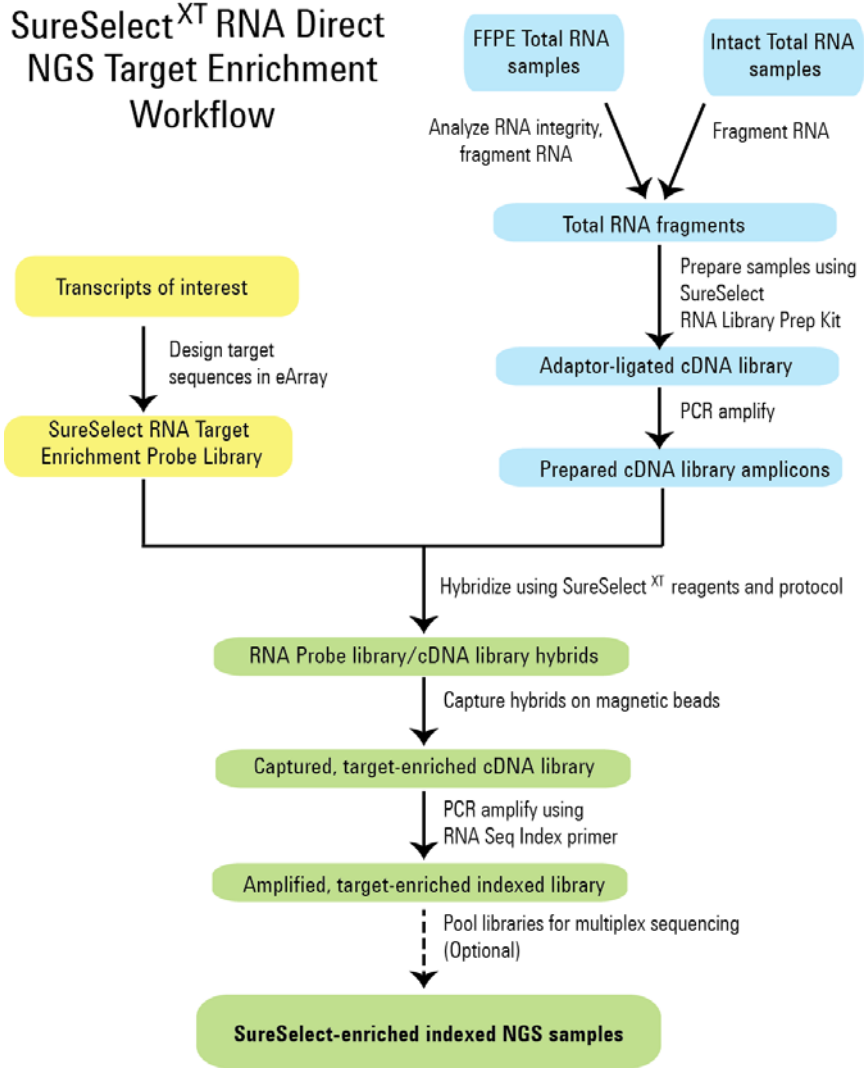

Figure 1 Overall RNA sequencing library preparation and target enrichment workflow.

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, thaw the reagents listed in Table 5 on ice.

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

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

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

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

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

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

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

Step 1. Prepare and assess quality of total RNA

Before you begin, prepare total RNA from each sample in the run in nuclease-free water. The library preparation protocol requires 200 ng FFPE total RNA or 100 ng of intact (non-FFPE) RNA.

For FFPE-derived RNA, you must determine the initial quality of each sample in order to determine the appropriate reaction conditions at several steps in the workflow. Use the steps below to qualify each FFPE total RNA sample. For intact (non-FFPE) RNA samples, proceed to "Step 2. Fragment total RNA and anneal primers" on page 20.

1 Use a small-volume spectrophotometer, such as the NanoDrop instrument, to determine sample absorbance at 260 nm, 280 nm, and 230 nm. Determine the 260/280 and 260/230 absorbance ratio values for the sample.

   High-quality RNA samples are indicated by a value close to 2.0 for both ratios. Ratios with significant deviation from 2.0 indicate the presence of organic or inorganic contaminants, which may require further purification or may indicate that the sample is not suitable for use in RNA target enrichment applications.

2 Examine the starting size distribution of RNA in the sample using the 2100 Bioanalyzer instrument or an Agilent TapeStation instrument and the appropriate assay system described in Table 6.

   Using either platform, determine the percentage of RNA in the sample that is >200 nt using the analysis mode described in Table 6. RNA molecules must be >200 nt for efficient conversion to cDNA library, and the percent convertible RNA value is used to determine the appropriate conditions at several protocol steps in the workflow.

Table 6 RNA qualification platforms

Analysis Instrument	RNA Qualification Assay	Analysis to Perform
2100 Bioanalyzer	RNA 6000 Pico Chip	Smear/Region analysis using 2100 Expert Software
4200 TapeStation OR 4150 TapeStation	High Sensitivity RNA ScreenTape	Region analysis using TapeStation Analysis Software

NOTE Grading of FFPE RNA quality by RNA Integrity Number (RIN) is not recommended for this application.

3 Grade each RNA sample based on the percentage of RNA in the sample >200 nucleotides, according to Table 7.

Table 7 Classification of RNA samples based on starting RNA size

Grade	% RNA >200 nt	Recommended input amount
Good FFPE RNA	>50%	200 ng
Poor FFPE RNA	20% to 50%	200 ng
Inapplicable FFPE RNA	<20%	Not recommended for further processing
Intact (non-FFPE) RNA	>70%	100 ng

Step 2. Fragment total RNA and anneal primers

In this step, total RNA is chemically-fragmented to a size appropriate for RNA sequencing library preparation, and the RNA fragments are bound to random primers included in the Fragmentation Mix.

Fragmentation is achieved by treatment with metal ions at elevated temperature. The recommended fragmentation conditions vary, depending on the initial quality (grade) of the RNA sample, with lower-quality samples subjected to fragmentation at lower temperature and for a shorter duration. **Since the fragmentation reaction also allows for binding of random primers supplied in the Fragmentation Mix, this step is required, even for highly degraded FFPE samples.**

1 Preprogram a thermal cycler (with the heated lid ON) with the appropriate program in Table 8, based on the RNA integrity grade for the samples. Immediately pause the program, allowing the heated lid to reach temperature while you prepare the RNA samples, and keep paused until samples are loaded in step 6.

Table 8 Thermal cycler program for RNA fragmentation and random primer binding

Step	Temperature	Time
Good FFPE RNA Samples*		
Step 1	94°C	3 minutes
Step 2	65°C	2 minutes
Step 3	4°C	1 minute
Step 4	4°C	Hold
Poor FFPE RNA Samples*		
Step 1	65°C	5 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold
Intact (non-FFPE) RNA Samples		
Step 1	94°C	8 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

* Due to the variability of FFPE-derived material, optimization of the duration of incubation at each temperature may be required for optimal performance. Use the conditions provided in the table as a starting point for optimization. For example, for certain Good FFPE RNA Samples, performance may be optimized by reducing the incubation time at 94°C and increasing the incubation time at 65°C (retaining a total elevated temperature incubation time of 5 minutes).

2 Transfer the appropriate amount of input RNA, in RNase-free water, to wells of a thermal cycler-compatible plate or strip tube.

For FFPE-derived RNA samples, use 200 ng RNA, and for intact RNA samples, use 100 ng RNA.

3 Completely lyophilize the total RNA samples using a plate or strip tube-compatible vacuum concentrator at ≤45°C. To preserve sample integrity, use the minimum amount of time required to dry each sample.

4 Add 19 µL of Fragmentation Mix (from red capped tube or bottle) to each lyophilized RNA sample well.

NOTE

All samples, including highly degraded FFPE samples, must be combined with Fragmentation Mix, which supplies the random primers for cDNA synthesis. Specific conditions for the fragmentation/primer binding reaction vary based on the initial quality of the RNA sample (see Table 8 on page 21) but all samples must undergo this reaction step.

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

6 Immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 8.

During the thermal cycler incubation steps in Table 8, complete step 1 through step 3 on page 23.

Step 3. Synthesize first-strand cDNA

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

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

Table 9 Preparation of 120 ng/µl Actinomycin D

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

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

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

3 Prepare the appropriate amount of First Strand Master Mix + Actinomycin D mixture, according to Table 10. Mix by vortexing at high speed for 5 seconds, then spin briefly and keep on ice.

Table 10 Preparation of First Strand Master Mix/Actinomycin D mixture

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

4 Once the thermal cycler program in Table 8 reaches the 4°C Hold step, transfer the fragmented RNA sample plate or strip tube from the thermal cycler to ice or a cold block.

5 Preprogram a thermal cycler with the program in Table 11. Immediately pause the program, and keep paused until samples are loaded in step 9.

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 Place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 11 (with heated lid ON).

Table 11 Thermal cycler program for first-strand cDNA synthesis

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

Step 4. Purify first strand cDNA using AMPure XP beads

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

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

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete SureSelect RNA Direct 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 48 µL of the homogeneous bead suspension to each 27.5-µL sample in the PCR plate or strip tube. Seal the wells, then vortex 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 samples on 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 samples briefly, return the plate or strip tube 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 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 the thermal cycler and run the program in Table 12. **Do not use a heated lid**.

Table 12 Thermal cycler program for second-strand synthesis and end repair

Step	Temperature	Time
Step 1	16°C	1 hour
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 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. 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 13 (used for the remainder of library preparation steps) and keep on ice.

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

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR)*, –20°C	dA Tailing Master Mix† (green cap or bottle)	page 29
	SureSelect Ligation Master Mix (purple cap)	page 31
	SureSelect Oligo Adaptor Mix (blue cap)	page 31
	ILM Reverse PCR Primer‡ (black cap)	page 34
	PCR Master Mix** (red cap or bottle)	page 34
	Uracil DNA Glycosylase (UDG) (yellow cap)	page 34
	SureSelect Primer (brown cap)	page 34

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

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

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

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

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

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

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

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

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

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

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

Table 14 Thermal cycler program for dA-tailing

Step	Temperature	Time
Step 1	37°C	30 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 15. **Do not use a heated lid**.

Table 15 Thermal cycler program for adaptor ligation

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

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

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 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 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 sample 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 Preprogram a thermal cycler with the program in Table 16. Immediately pause the program, and keep paused until samples are loaded in step 7.

Table 16 Thermal cycler program for pre-capture PCR

Segment	Number of Cycles	Temperature	Time
1	1	37°C	15 minutes
2	1	95°C	2 minutes
3	14 cycles*	95°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	1	4°C	Hold

* Cycle number may require optimization. The optimal cycle number depends on the yield of adapter-ligated library fragments and the desired pre-capture library yield (200 ng of the pre-capture library is required to perform target enrichment). If yield is insufficient, repeat library preparation for the sample using up to 16 cycles. Optimization of cycle number may also be required based on duplication rate in the sequencing data generated after enrichment. If sequencing results indicate excessive duplicates, repeat library preparation for the sample using 12 or 13 cycles.

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

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

Table 17 Preparation of pre-capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
PCR Master Mix (red cap or bottle)	25 µL	425 µL
Uracil DNA Glycosylase (UDG) (yellow cap)	1 µL	17 µL
SureSelect Primer (forward primer; brown cap)	1 µL	17 µL
ILM Reverse PCR Primer (black cap)	1 µL	17 µL
Total	**28 µL**	**476 µL**

4 Add 28 µL of the pre-capture PCR reaction mix prepared in step 3 to each 22-µL purified, adaptor-ligated cDNA sample.

5 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.

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

7 Immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 16.

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 90 µL of the homogeneous bead suspension to each 50-µL PCR reaction in the plate or strip tube. Seal the wells, then vortex 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 and quantity

Sample analysis can be done with the Agilent 2100 Bioanalyzer instrument, the Agilent 4200 TapeStation instrument or the Agilent 4150 TapeStation instrument.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit and perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer. Within the instrument context, choose the DNA 1000 assay from the drop down list. Start the run within five minutes after preparation.
5. Verify that the electropherogram shows the peak of DNA fragment size positioned between 150 to 300 bp. Measure the concentration of the library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay. Sample electropherograms are shown in Figure 2 (library prepared from high-quality RNA) and Figure 3 (library prepared from FFPE RNA).

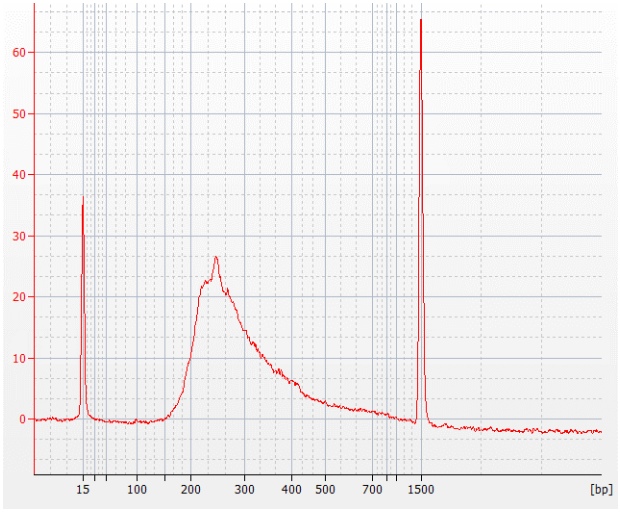

Figure 2 Amplified library DNA prepared from a high-quality RNA sample analyzed using a D1000 Assay.

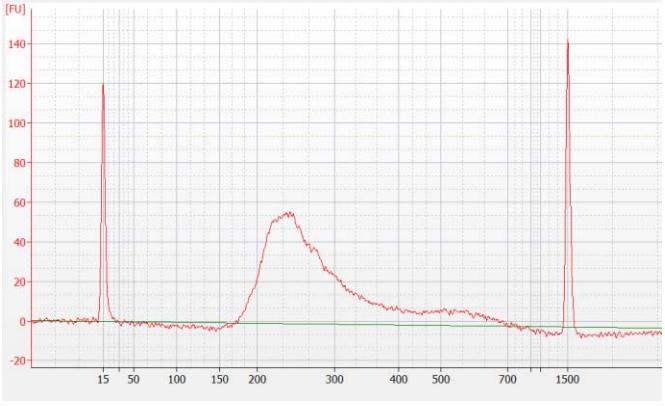

Figure 3 Amplified library DNA prepared from an FFPE RNA sample analyzed using a D1000 Assay.

Option 2: Analysis using an Agilent 4200/4150 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit. For more information to do this step, see the Agilent D1000 Assay Quick Guide.

1 Prepare the TapeStation samples as instructed as instructed in the reagent kit guide. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 150 to 300 bp. Determine the concentration of the library DNA by integrating under the entire peak. Sample electropherograms are shown in Figure 4 (library prepared from high-quality RNA) and Figure 5 (library prepared from FFPE RNA).

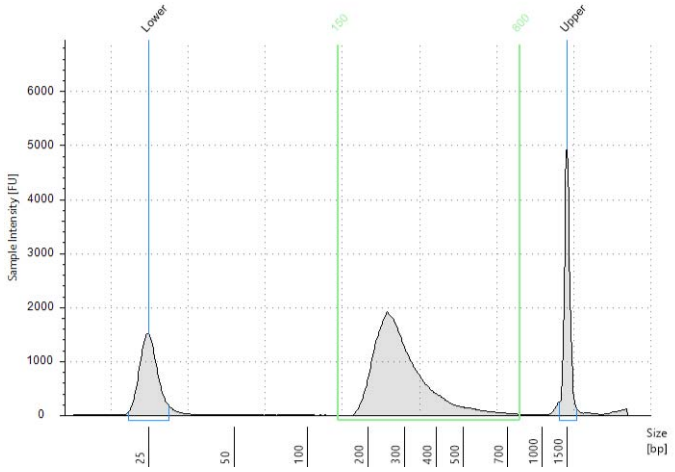

Figure 4 Amplified library DNA prepared from a high-quality RNA sample analyzed using a D1000 ScreenTape.

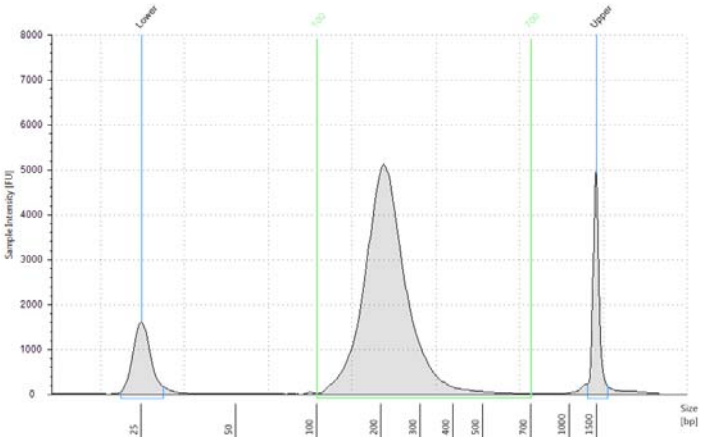

Figure 5 Amplified library DNA prepared from an FFPE RNA sample analyzed using a D1000 ScreenTape.

3 Hybridization

This chapter describes the steps to hybridize the prepped cDNA library with the SureSelect or ClearSeq Target Enrichment Probe in combination with the hybridization reagents and blocking agents.

CAUTION The ratio of RNA Target Enrichment Probe 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 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 200 ng of prepared cDNA in a volume of 3.4 µL (initial concentration of 58.8 ng/µL).

1. For prepped libraries with cDNA concentrations above 58.8 ng/µL, prepare 3.4 µL of a 58.8 ng/µL dilution of each library.
2. For prepped libraries with cDNA concentrations below 58.8 ng/µL, use a vacuum concentrator to concentrate the samples at ≤45°C.
   a. Add 200 ng 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 3.4 µL nuclease-free water. 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 cDNA library sample (200 ng) to a separate well of a 96-well plate or strip tube. Seal the wells and keep on ice.

4 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 18. Immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions, and keep paused until samples are loaded in step 8.

Table 18 Thermal cycler program for DNA + Block Mix prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	5 minutes
Step 3	65°C	Hold

5 Prepare the Hybridization Buffer by mixing the components in Table 19 at room temperature.

If a precipitate forms, warm the Hybridization Buffer at 65°C for 3 minutes.

Keep the prepared Hybridization Buffer at room temperature until it is used in step 10.

Table 19 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction*	Volume for 16 reactions (includes excess)
SureSelect Hyb 1 (orange cap or bottle)	6.63 µL	116 µL
SureSelect Hyb 2 (red cap)	0.27 µL	4.7 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	46.4 µL
SureSelect Hyb 4 (black cap)	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.

6 Prepare the SureSelect Block Mix by mixing the components in Table 20. Keep the mixture on ice until it is used in step 7.

Table 20 Preparation of SureSelect Block Mix

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

7 To each cDNA library sample well prepared in step 3 on page 42, add 5.6 µL of the SureSelect Block Mix prepared in Table 20. Pipette up and down to mix.

8 Cap the wells, then transfer the sealed sample plate or strip tube to the thermal cycler and resume the thermal cycling program in Table 18. Ensure that the heated lid is in use (set at 105°C), to hold the temperature at 65°C.

Complete step 9 and step 10 below while running the thermal cycling program in Table 18. Make sure that the thermal cycler has entered the 65°C Hold step (Segment 3) before adding the remaining hybridization reaction components in step 11.

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

9 Prepare the required volume of a 1:4 dilution of SureSelect RNase Block (for a final concentration of 25%), as shown in Table 21. Keep on ice.

Table 21 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 10, below, near the end of the Segment 2 (5 minutes at 65°C) in Table 18. Keep the mixture at room temperature only briefly, until adding the mixture to sample wells in step 11. Do not keep solutions containing the probe at room temperature for extended periods.

10 Prepare the Probe Hybridization Mix by combining the solutions listed in Table 22, 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 11.

Table 22 Preparation of Probe Hybridization Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 5	13 µL	221 µL
25% RNase Block solution from step 9	2 µL	34 µL
RNA Target Enrichment Probe*	5 µL	85 µL
Total	**20 µL**	**340 µL**

* These conditions are optimal for all probes listed in Table 2 on page 11, including SureSelect^{XT} Human All Exon V6 + UTRs, which was originally developed for DNA target enrichment applications. Use of other libraries designed for DNA target enrichment requires optimization of probe volume. For probes ≥3 Mb, begin optimization using 5 µl of probe in the hybridization as shown in Table 22. For probes <3 Mb, begin optimization by adding 2 µl of probe to the hybridization, and compensate for the volume difference by adding dilute RNase Block as 5 µl of a 10% solution.

11 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 10 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.

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

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

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.

13 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 23 are used to prepare the Dynabeads M-270 Streptavidin magnetic beads and for subsequent steps in the capture protocol.

Table 23 Reagents for post-hybridization capture using streptavidin beads

Storage Location	Kit Component	Where Used in Protocol
SureSelect Target Enrichment Box 1, Room Temperature	SureSelect Binding Buffer (bottle)	page 47
	SureSelect Wash Buffer 1 (bottle)	page 48
	SureSelect Wash Buffer 2 (bottle)	page 48

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.

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 53.

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.

Before you begin, thaw the reagents listed in Table 24 and keep on ice.

Table 24 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 52
	PCR Master Mix† (red cap or bottle)	page 53
	ILM Post-Capture PCR Primer‡ (green cap)	page 53

* 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 35 on page 72 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 25. Pipetting up and down is not sufficient to mix this reagent.

2 Thaw the reagents listed in Table 25 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 25. Mix well using a vortex mixer and keep on ice.

Table 25 Preparation of Post-capture PCR Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
PCR Master Mix (red cap or bottle)	25 µL	425 µL
ILM Post-Capture PCR Primer (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 mixture from step 3 in a PCR plate well.

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 49).
- **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 26. Use a heated lid on the thermal cycler at 105°C.

Table 26 Post-Capture PCR indexing thermal cycler program

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

* Cycle number may require optimization. If PCR yield is too low, repeat the PCR using the remaining volume of bead-bound cDNA library with up to 15 cycles. If sequencing results indicate excessive duplications, repeat the PCR using the remaining volume of bead-bound cDNA library with 10 or 11 cycles.

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 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 on the magnetic stand at room temperature. Wait for the solution to clear (approximately 5 minutes).
8. While keeping the plate in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
9. Continue to keep the plate 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.

NOTE

If libraries will be stored for an extended period prior to sequencing, instead elute the libraries using 30 µL of Buffer EB (not supplied; see Table 1 on page 11 for ordering information).

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 plate and store at –20°C.

Step 3. Assess indexed library DNA quantity and quality

Option 1: Analysis using the Agilent 2100 Bioanalyzer and High Sensitivity DNA Assay

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified indexed DNA. Perform the assay according to the High Sensitivity DNA Kit Guide.

1. OSet up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
3. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
4. Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 to 350 bp. Sample electropherograms are shown in Figure 6 (library prepared from high-quality RNA) and Figure 7 (library prepared from FFPE RNA).
5. Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

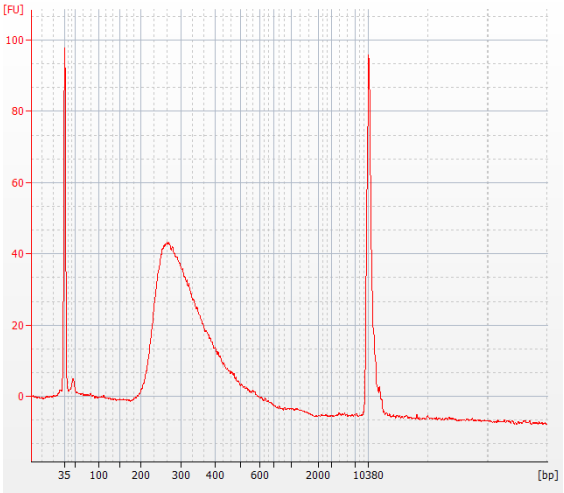

Figure 6 Post-capture amplified library DNA prepared from a high-quality RNA sample analyzed using a High Sensitivity DNA Assay.

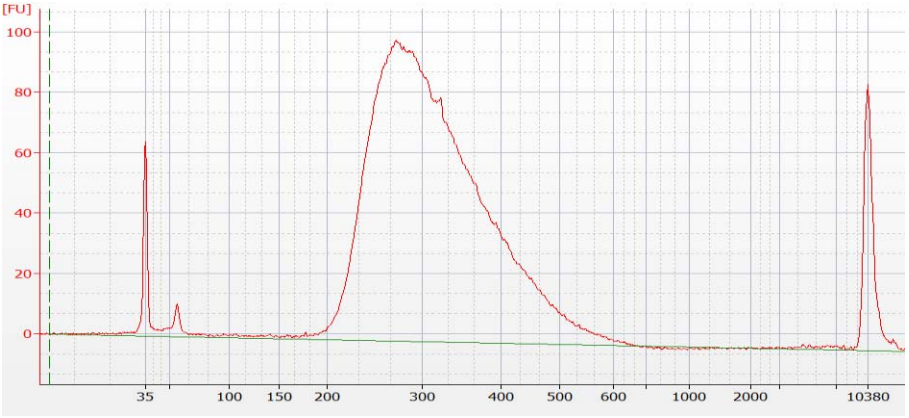

Figure 7 Post-capture amplified library DNA prepared from an FFPE RNA sample analyzed using a High Sensitivity DNA Assay.

Option 2: Analysis using an Agilent 4200/4150 TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape and associated reagent kit. For more information to do this step, see the Agilent High Sensitivity D1000 Assay Quick Guide.

1 Prepare the TapeStation samples as instructed in reagent kit guide. Use 2 µL of each indexed DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 to 350 bp. Sample electropherograms are shown in Figure 8 (library prepared from high-quality RNA) and Figure 9 (library prepared from FFPE RNA).

4 Measure the concentration of each library by integrating under the entire peak.

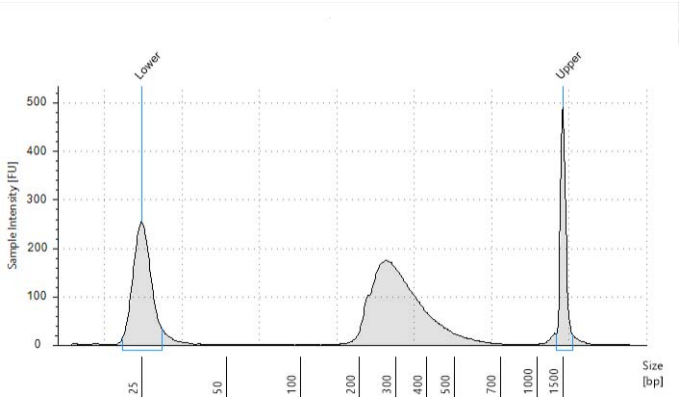

Figure 8 Post-capture amplified library DNA prepared from a high-quality RNA sample analyzed using a High Sensitivity D1000 ScreenTape.

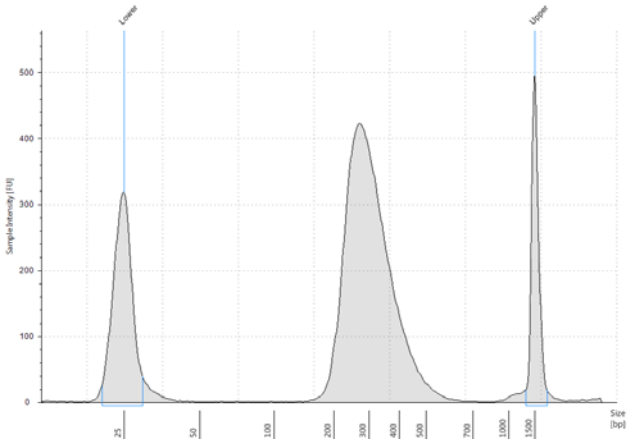

Figure 9 Post-capture amplified library DNA prepared from an FFPE RNA sample analyzed using a High Sensitivity D1000 ScreenTape.

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 (10 nM for the standard Illumina sequencing protocol)

is the number of indexes, and

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

Table 27 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Buffer EB needed for a final volume of 20 µL at 10 nM.

Table 27 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
Buffer EB					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 Buffer EB 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.

The pooled libraries can be stored in a DNA LoBind tube at –20°C for up to six months. Do not subject the libraries to multiple freeze-thaw cycles prior to sequencing.

Step 5. Prepare and analyze sequencing samples

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 28 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 28 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 28 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 29. 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 29 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 30.

Table 30 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.

5 Reference

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

Kit Contents

The SureSelect^{XT} RNA Direct Reagent Kits include the component kits listed in Table 30. The contents of each component kit are detailed in Table 31 through Table 33.

Table 30 SureSelect^{XT} RNA Direct Reagent Kit Content

Component Kits	Storage Condition	16 Samples	96 Samples
SureSelect RNA Library Prep, ILM (Pre PCR)*	–20°C	5500-0134	5500-0135
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*.

Table 31 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 35 on page 72 for index sequences.

** See Table 34 on page 71 for a plate map.

Table 32 SureSelect Target Enrichment Box 1 Content

Kit Component	16 Reactions	96 Reactions
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 33 SureSelect Target Enrichment Box 2 Content

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

Table 34 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 64 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 35 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 Direct protocol.

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