



SureSelect^{XT} RNA Direct

For Preparation of Strand-Specific Sequencing Libraries from High-Quality or FFPE-Derived RNA Samples for the Illumina Platform



Protocol

Version A1, March 2019

SureSelect platform manufactured with Agilent SurePrint Technology

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



Agilent Technologies

Notices

© Agilent Technologies, Inc. 2017, 2019

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

G9691-90050

Edition

Version A1, March 2019

Printed in USA

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

Acknowledgment

Oligonucleotide sequences © 2006, 2008, and 2011 Illumina, Inc. All rights reserved. Only for use with the Illumina sequencer systems and associated assays.

Technical Support

For US and Canada

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

Or send an e-mail to:

ngs.support@agilent.com

For Europe, Middle East, Africa, and India

Call 00800 345 600 (toll free) or
+49 69 8679 7730

Or send an e-mail to:

genomics_tech_europe@agilent.com

For all other regions

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

Or send an e-mail to:

ngs.support@agilent.com

Warranty

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

Technology Licenses

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

Restricted Rights Legend

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

Notice to Purchaser: Limited License

Limited Use Label License: This product and its use are the subject of one or more issued and/or pending U.S. and foreign patent applications owned by Max Planck Gesellschaft exclusively licensed to New England Biolabs, Inc. and sublicensed to Agilent Technologies. The purchase of this product from Agilent Technologies, Inc., its affiliates, or its authorized resellers and distributors conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any claims in the foregoing patents or patent applications directed to producing the product. The buyer cannot sell or otherwise transfer this product or its components to a third party or otherwise use this product for the following COMMERCIAL PURPOSES: (1) use of the product or its components in manufacturing; or (2) use of the product or its components for therapeutic or prophylactic purposes in humans or animals.

Safety Notices

CAUTION

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

WARNING

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

In this Guide...

This guide describes an optimized protocol for 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 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 12.
- 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	9
	Procedural Notes	10
	Safety Notes	10
	Required Reagents	11
	Required Equipment	13
2	Sample Preparation	15
	Step 1. Prepare and assess quality of total RNA	18
	Step 2. Fragment total RNA and anneal primers	20
	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 and quantity	36
3	Hybridization	41
	Step 1. Hybridize the library	42
	Step 2. Prepare streptavidin beads	47
	Step 3. Capture hybrids using streptavidin beads	48

Contents

4	Indexing and Sample Processing for Multiplexed Sequencing	51
	Step 1. Amplify the captured libraries to add index tags	52
	Step 2. Purify the amplified captured libraries using AMPure XP beads	55
	Step 3. Assess indexed library DNA quantity and quality	57
	Step 4. Pool samples for multiplexed sequencing	61
	Step 5. Prepare and analyze sequencing samples	63
5	Reference	67
	Kit Contents	68
	Nucleotide Sequences of SureSelect ^{XT} Indexes A01 to H12	72



1 Before You Begin

Procedural Notes	10
Safety Notes	10
Required Reagents	11
Required Equipment	13

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 Target Enrichment

Description	Vendor and part number
SureSelect or ClearSeq RNA Target Enrichment Probe Library	Select one library 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
Agencourt 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. The aliquots may be stored for up to one year before use. See [page 17](#) for additional information.

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.

1 Before You Begin

Required Reagents

Table 2 Compatible SureSelect and ClearSeq Target Enrichment Probe Libraries

Target Enrichment Probe Library	16 Samples	96 Samples
Custom RNA Target Enrichment Probes 1–499 kb (reorder)	5190-4934 (5190-4939)	5190-4935 (5190-4940)
Custom RNA Target Enrichment Probes 0.5–2.9 Mb (reorder)	5190-4944 (5190-4949)	5190-4945 (5190-4950)
Custom RNA Target Enrichment Probes 3– 5.9 Mb (reorder)	5190-4954 (5190-4959)	5190-4955 (5190-4960)
SureSelect^{XT} 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 Target Enrichment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
Compression mats	Agilent p/n 410187
Low-Adhesion Tubes (RNase, DNase, and DNA-free)	USA Scientific
1.5 mL	p/n 1415-2600
0.5 mL	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
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

1 Before You Begin

Required Equipment

Table 3 Equipment for RNA Direct Target Enrichment

Description	Vendor and part number
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent*
Labnet MPS1000 Mini Plate Spinner (Optional)	Labnet International p/n C1000
Nucleic Acid Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
RNA 6000 Pico Kit	Agilent p/n 5067-1513
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200 TapeStation [†]	Agilent p/n G2991AA
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
High Sensitivity RNA ScreenTape	Agilent p/n 5067-5579
High Sensitivity RNA ScreenTape Sample Buffer	Agilent p/n 5067-5580
High Sensitivity RNA ScreenTape Ladder	Agilent p/n 5067-5581
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585

* 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.

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



2 Sample Preparation

- Step 1. Prepare and assess quality of total RNA 18
- Step 2. Fragment total RNA and anneal primers 20
- 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 and quantity 36

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.



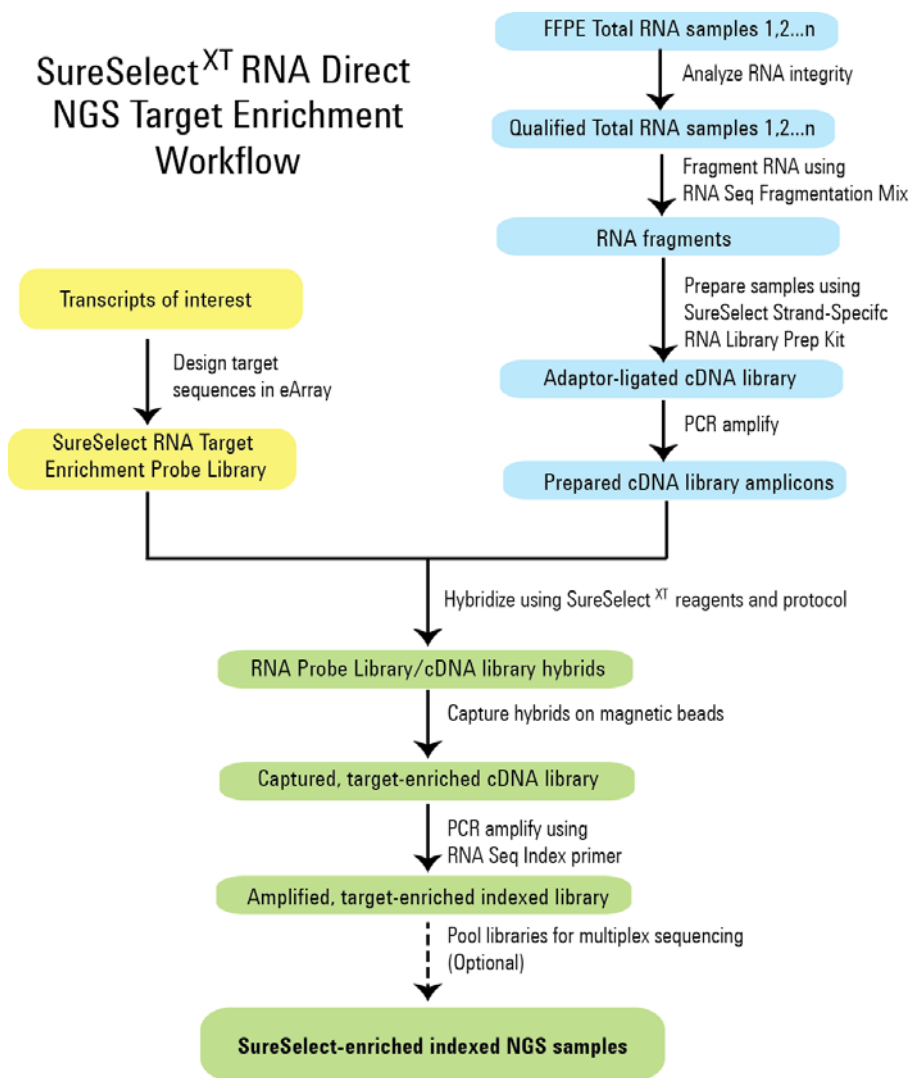


Figure 1 Overall RNA sequencing library preparation and target enrichment workflow.

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 4](#) on ice.

Table 4 Reagents thawed and held on ice before use in protocol

Kit Component	Storage Location	Where Used in Protocol
RNA Seq Fragmentation Mix (red cap or bottle)	RNA Library Prep Kit Box 1, –20°C	page 21
RNA Seq First Strand Master Mix (orange cap)	RNA Library Prep Kit Box 1, –20°C	page 24
RNA Seq Second-Strand + End-Repair Enzyme Mix (blue cap or bottle)	RNA Library Prep Kit Box 1, –20°C	page 27
RNA Seq Second-Strand + End-Repair Oligo Mix (yellow cap)	RNA Library Prep Kit Box 1, –20°C	page 27

2 Sample Preparation

Step 1. Prepare and assess quality of total RNA

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 the 4200 TapeStation instrument and the appropriate assay system described in [Table 5](#).

Using either platform, determine the percentage of RNA in the sample that is >200 nt using the analysis mode described in [Table 5](#). 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 5 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 2200 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.

Step 1. Prepare and assess quality of total RNA

- 3 Grade each RNA sample based on the percentage of RNA in the sample >200 nucleotides, according to [Table 6](#).

Table 6 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

2 Sample Preparation

Step 2. Fragment total RNA and anneal primers

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 RNA Seq 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 RNA Seq Fragmentation Mix, this step is required, even for highly degraded FFPE samples.**

Step 2. Fragment total RNA and anneal primers

- 1 Preprogram a SureCycler 8800 thermal cycler (with the heated lid ON) with the appropriate program in Table 7, based on the RNA integrity grade for the samples. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you prepare the RNA samples.

Table 7 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.

2 Sample Preparation

Step 2. Fragment total RNA and anneal primers

- 3 Completely lyophilize the total RNA samples using a plate or strip tube-compatible vacuum concentrator at $\leq 45^{\circ}\text{C}$. To preserve sample integrity, use the minimum amount of time required to dry each sample.
- 4 Add 19 μL of RNA Seq 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 RNA Seq 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 7](#) 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 Place the samples in the SureCycler 8800 thermal cycler, then press the *Play* button to resume the thermal cycling program in [Table 7](#).

During the thermal cycler incubation steps in [Table 7](#), 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 8](#).

Table 8 Preparation of 120 ng/μl Actinomycin D

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

CAUTION

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

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

2 Sample Preparation

Step 3. Synthesize first-strand cDNA

- 3 Prepare the appropriate amount of RNA Seq First Strand Master Mix + Actinomycin D mixture, according to [Table 9](#). Mix by vortexing at high speed for 5 seconds, then spin briefly and keep on ice.

Table 9 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
RNA Seq First Strand Master Mix (orange cap)	8.0 μ L	136 μ L
Total	8.5 μL	144.5 μL

- 4 Once the thermal cycler program in [Table 7](#) 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 SureCycler 8800 thermal cycler (with the heated lid ON) with the program in [Table 10](#). Start the program, then immediately press the *Pause* button.
- 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 Place the samples in the thermal cycler, then press the *Play* button to resume the thermal cycling program in [Table 10](#) (with heated lid ON).

Table 10 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.

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

CAUTION

The RNA Seq Second Strand + End Repair 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 RNA Seq Second Strand + End Repair Enzyme Mix (blue capped tube or bottle) and RNA Seq Second Strand + End Repair Oligo Mix (yellow capped tube) at high speed for 5 seconds to ensure homogeneity.
- 2 Add 25 μL of RNA Seq Second Strand + End Repair Enzyme Mix to each 20- μL purified first-strand cDNA sample. Keep on ice.
- 3 Add 5 μL of RNA Seq Second Strand + End Repair Oligo Mix to each sample well, for a total reaction volume of 50 μL . Keep on ice.
- 4 Seal the wells. Mix thoroughly by vortexing the plate or strip tube at high speed for 5 seconds or by agitating the plate or strip tube on a plate shaker at approximately 1600 rpm for 5 seconds.
- 5 Spin the plate or strip tube briefly to collect the liquid.
- 6 Incubate the plate or strip tube in the thermal cycler and run the program in [Table 11](#). **Do not use a heated lid.**

Table 11 Thermal cycler program for second-strand synthesis and end repair

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

Table 12 Reagents thawed and held on ice before use in protocol

Kit Component	Storage Location	Where Used in Protocol
RNA Seq dA Tailing Master Mix (green cap or bottle)	RNA Library Prep Kit Box 1, -20°C	page 29
SureSelect Ligation Master Mix (purple cap)	RNA Library Prep Kit Box 1, -20°C	page 31
SureSelect Oligo Adaptor Mix (blue cap)	RNA Library Prep Kit Box 1, -20°C	page 31
RNA Seq ILM Reverse PCR Primer (black cap)	RNA Library Prep Kit Box 1, -20°C	page 34
RNA Seq PCR Master Mix (red cap or bottle)	RNA Library Prep Kit Box 1, -20°C	page 34
Uracil DNA Glycosylase (UDG) (yellow cap)	RNA Library Prep Kit Box 1, -20°C	page 34
SureSelect Primer (brown cap)	RNA Library Prep Kit Box 1, -20°C	page 34

CAUTION

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

- 1 Vortex the thawed vial of RNA Seq dA Tailing Master Mix (green-capped tube or bottle) 5 seconds at high speed to ensure homogeneity.
- 2 Add 20 μ L of RNA Seq dA Tailing Master Mix to each 20- μ L 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.

2 Sample Preparation

Step 7. dA-Tail the cDNA 3' ends

- 5 Incubate the plate or strip tube in the thermal cycler and run the program in [Table 13](#). **Do not use a heated lid.**

Table 13 Thermal cycler program for 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 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 the thermal cycler and run the program in [Table 14](#). **Do not use a heated lid.**

Table 14 Thermal cycler program for adaptor ligation

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

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 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 RNA Seq PCR Master Mix used at this step is highly viscous and thorough mixing is critical for optimal kit performance. Mix by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions in [Table 16](#). Pipetting up and down is not sufficient to mix this reagent.

- 1 Preprogram a SureCycler 8800 thermal cycler (with the heated lid ON) with the program in [Table 15](#). Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you prepare the PCR reactions.

Table 15 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 Sample Preparation

Step 10. Amplify the adaptor-ligated cDNA library

- 2 Vortex the thawed vial of RNA Seq PCR Master Mix 5 seconds at high speed to ensure homogeneity.
- 3 Prepare the appropriate volume of PCR reaction mix, as described in [Table 16](#), on ice. Mix well by vortexing at high speed then spin briefly to collect the liquid.

Table 16 Preparation of pre-capture PCR Reaction Mix

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

- 4 Add 28 μ L of the pre-capture PCR reaction mix prepared in [step 3](#) to each 22- μ L 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 Place the samples in the thermal cycler, then press the *Play* button to resume the thermal cycling program in [Table 15](#).

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 either the 2100 Bioanalyzer instrument or an Agilent 4200 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).

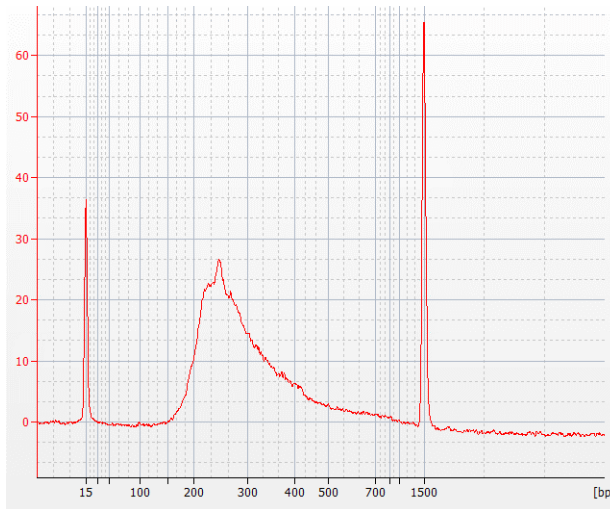


Figure 2 Amplified library DNA prepared from a high-quality RNA sample analyzed using a D1000 Assay.

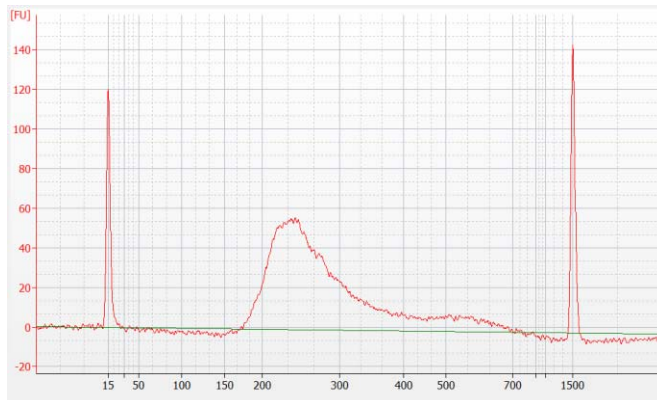


Figure 3 Amplified library DNA prepared from an FFPE RNA sample analyzed using a D1000 Assay.

2 Sample Preparation

Step 12. Assess quality and quantity

Option 2: Analysis using an Agilent 4200 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).

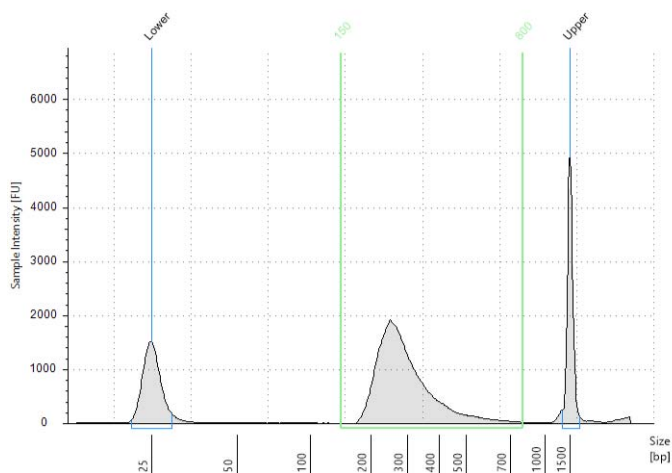


Figure 4 Amplified library DNA prepared from a high-quality RNA sample analyzed using a D1000 ScreenTape.

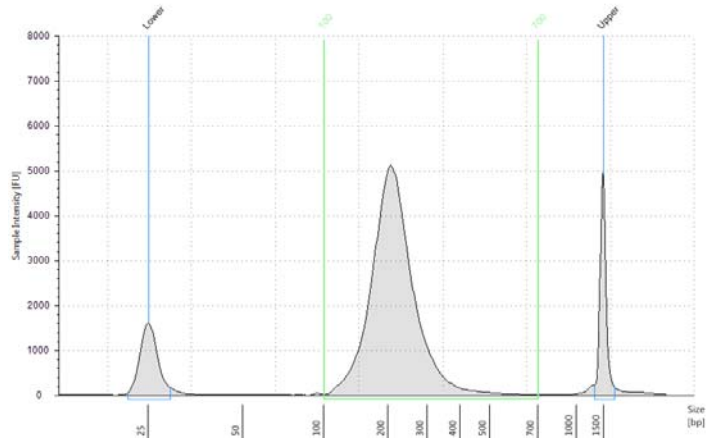


Figure 5 Amplified library DNA prepared from an FFPE RNA sample analyzed using a D1000 ScreenTape.

2 Sample Preparation

Step 12. Assess quality and quantity



3 Hybridization

- Step 1. Hybridize the library 42
- Step 2. Prepare streptavidin beads 47
- Step 3. Capture hybrids using streptavidin beads 48

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

CAUTION

The ratio of Probe Library to prepped 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 Probe Library.

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

CAUTION

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

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 $\leq 45^\circ\text{C}$.
 - 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 SureCycler 8800 thermal cycler (with the heated lid ON) with the program in [Table 17](#). Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the reactions.

Table 17 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 18](#) 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 18 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.

3 Hybridization

Step 1. Hybridize the library

- 6 Prepare the SureSelect Block Mix by mixing the components in [Table 19](#). Keep the mixture on ice until it is used in [step 7](#).

Table 19 Preparation of SureSelect Block Mix

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

- 7 To each cDNA library sample well prepared in [step 3](#) on [page 42](#), add 5.6 μ L of the SureSelect Block Mix prepared in [Table 19](#). Pipette up and down to mix.
- 8 Cap the wells, then transfer the sealed sample plates or strips to the thermal cycler and press the *Play* button to resume the thermal cycling program in [Table 17](#). 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 17](#). 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 20](#). Keep on ice.

Table 20 Preparation of 25% RNase Block solution

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

NOTE

Prepare the Probe Library mixture described in [step 10](#), below, near the end of the Segment 2 (5 minutes at 65°C) in [Table 17](#). 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 Library at room temperature for extended periods.

10 Prepare the Probe Library Hybridization Mix by combining the solutions listed in [Table 21](#), 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 21 Preparation of Probe Library 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
Probe Library*	5 µL	85 µL
Total	20 µL	340 µL

* These conditions are optimal for all Probe Libraries listed in [Table 2](#) on page 12, 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 Library volume. For probe libraries ≥3 Mb, begin optimization using 5 µl Probe Library in the hybridization as shown in [Table 21](#). For probe libraries <3 Mb, begin optimization by adding 2 µl Probe Library to the hybridization, and compensate for the volume difference by adding dilute RNase Block as 5 µl of a 10% solution.

3 Hybridization

Step 1. Hybridize the library

11 Maintain the DNA library + Block Mix plate or strip tube at 65°C while you add 20 µL of the Probe Library 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 SureCycler thermal cycler, with compression mat, for 24 hours at 65°C with a heated lid at 105°C.

Step 2. Prepare streptavidin beads

In this step, Dynabeads M-270 Streptavidin magnetic beads (see [Table 1](#) on page 11 for ordering information) are prepared for use in capturing the cDNA library/Probe Library hybrids.

The reagents listed in [Table 22](#) are used to prepare the Dynabeads M-270 Streptavidin magnetic beads and for subsequent steps in the capture protocol.

Table 22 Reagents for post-hybridization capture using streptavidin beads

Kit Component	Storage Location	Where Used in Protocol
SureSelect Binding Buffer (bottle)	SureSelect Target Enrichment Box 1, Room Temperature	page 47
SureSelect Wash Buffer 1 (bottle)	SureSelect Target Enrichment Box 1, Room Temperature	page 48
SureSelect Wash Buffer 2 (bottle)	SureSelect Target Enrichment Box 1, Room Temperature	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/Probe Library hybrids are captured on the prepared streptavidin magnetic beads.

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

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

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

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

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

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

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

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

- b** Cap the wells then incubate in the thermal cycler, with heated lid ON, held at 65°C until used in [step 10](#).

- 5** At the end of the 30-minute incubation for capture, briefly spin the sample plate or strip tube in a centrifuge or mini-plate spinner.
- 6** Put the plate or strip tube on the magnetic stand at room temperature for approximately five minutes. Remove and discard the supernatant.
- 7** Resuspend the beads in 200 μL of SureSelect Wash Buffer 1. Pipette up and down until beads are resuspended. Cap the wells then mix on a vortex mixer for 5 seconds.
- 8** Incubate the samples for 15 minutes at room temperature.

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 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.

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 52
- Step 2. Purify the amplified captured libraries using AMPure XP beads 55
- Step 3. Assess indexed library DNA quantity and quality 57
- Step 4. Pool samples for multiplexed sequencing 61
- Step 5. Prepare and analyze sequencing samples 63

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.

CAUTION

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

Use reagents from the SureSelect Strand Specific RNA Library Prep Kit, Box 1 for this step. Prepare 1 indexing amplification reaction for each cDNA library.

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

See [Table 34](#) 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 RNA Seq PCR Master Mix used at this step is highly viscous and thorough mixing is critical for optimal kit performance. Mix by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions as directed in [Table 23](#). Pipetting up and down is not sufficient to mix this reagent.

- 2 Thaw the reagents listed in [Table 23](#) 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 23](#). Mix well using a vortex mixer and keep on ice.

Table 23 Preparation of Post-capture PCR Reaction Mix

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

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 24](#). Use a heated lid on the thermal cycler at 105°C.

Table 24 Post-Capture PCR indexing thermal cycler program

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	12 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.

4 Indexing and Sample Processing for Multiplexed Sequencing

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

13 Add 30 μ L 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 μ L 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 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 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 μ L 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.

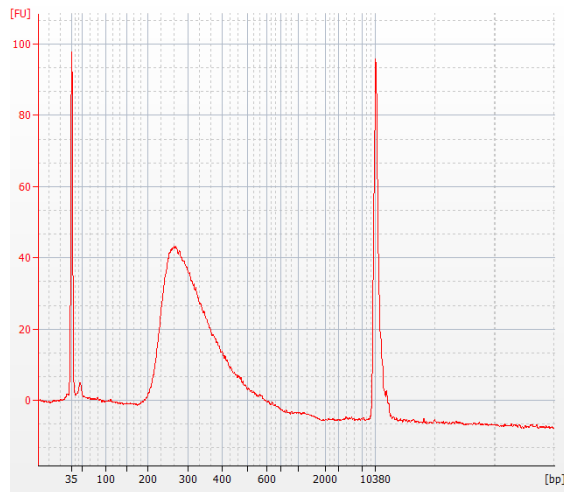


Figure 6 Post-capture amplified library DNA prepared from a high-quality RNA sample analyzed using a High Sensitivity DNA Assay.

4 Indexing and Sample Processing for Multiplexed Sequencing
Step 3. Assess indexed library DNA quantity and quality

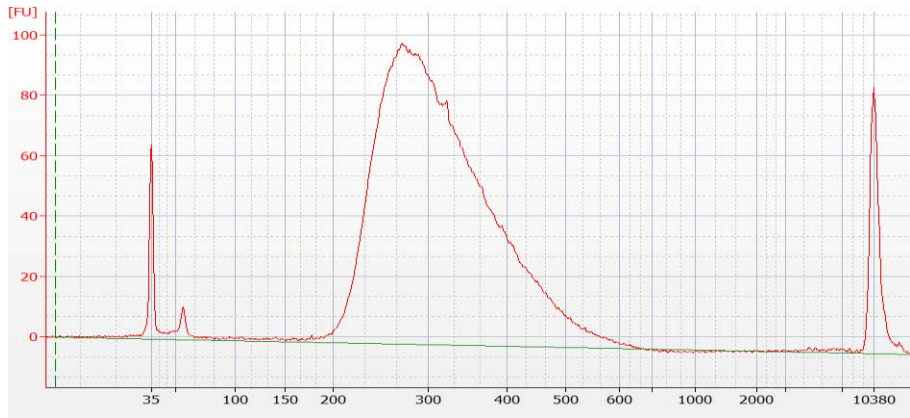


Figure 7 Post-capture amplified library DNA prepared from an FFPE RNA sample analyzed using a High Sensitivity DNA Assay.

Option 2: Analysis using an Agilent 4200 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 μ L of each indexed DNA sample diluted with 2 μ L 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.

4 Indexing and Sample Processing for Multiplexed Sequencing

Step 3. Assess indexed library DNA quantity and quality

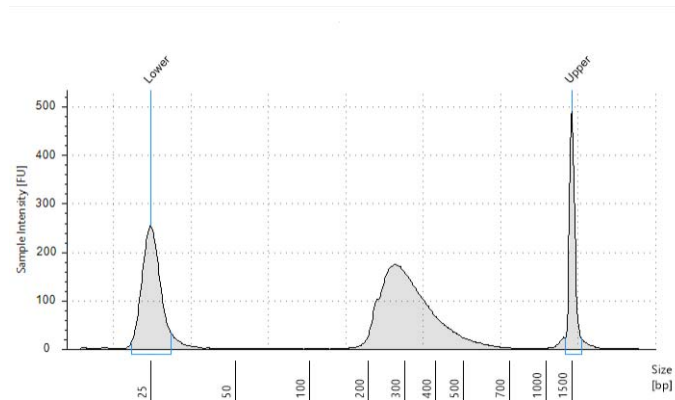


Figure 8 Post-capture amplified library DNA prepared from a high-quality RNA sample analyzed using a High Sensitivity D1000 ScreenTape.

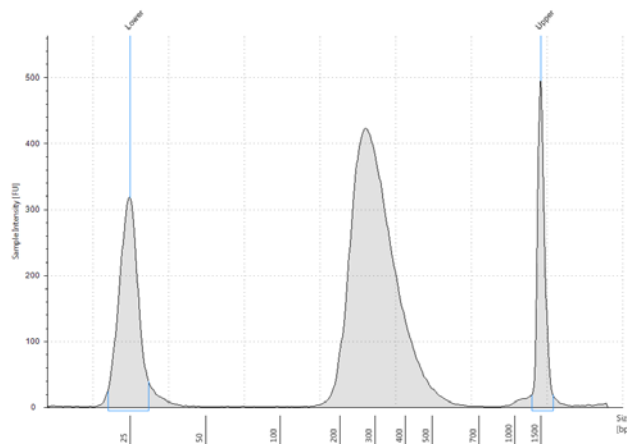


Figure 9 Post-capture amplified library DNA prepared from an FFPE RNA sample analyzed using a High Sensitivity D1000 ScreenTape.

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 25 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 25 Example of indexed sample volume calculation for total volume of 20 μL

Component	V(f)	C(i)	C(f)	#	Volume to use (μL)
Sample 1	20 μL	20 nM	10 nM	4	2.5
Sample 2	20 μL	10 nM	10 nM	4	5
Sample 3	20 μL	17 nM	10 nM	4	2.9
Sample 4	20 μL	25 nM	10 nM	4	2
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.

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.

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 26](#) 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 26](#) 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 26 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 27](#). 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 27 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 28](#).

Table 28 Run parameters for MiSeq platform Sample Sheet

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

Sequence analysis guidelines

The SureSelect^{XT} RNA sequencing library preparation method preserves RNA strandedness as described here. The first strand of cDNA is the reverse complement of the poly(A) RNA transcript strand. Since the second strand of cDNA is eliminated before PCR, the sequence of read 1, which starts at the P5 end, matches only the first strand of cDNA. Read 2, which starts at the P7 end, matches the second strand of cDNA (the poly(A) RNA transcript strand). When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (<http://picard.sourceforge.net/>) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY= SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

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



5 Reference

Kit Contents 68

Nucleotide Sequences of SureSelect^{XT} Indexes A01 to H12 72

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



Kit Contents

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

Table 29 SureSelect^{XT} RNA Direct Reagent Kit Content

Component Kits	Storage Condition	16 Samples	96 Samples
SureSelect Strand Specific RNA Library Prep, ILM, Box 1	-20°C	5500-0134	5500-0135
SureSelect Target Enrichment Box 1	Room Temperature	5190-8645	5190-8646
SureSelect Target Enrichment Box 2	-20°C	5190-6261	5190-6262

The contents of each of the component kits listed in [Table 29](#) are described in the tables below.

Table 30 SureSelect Strand Specific RNA Library Prep, ILM, Box 1 Content

Kit Component	16 Reactions (p/n 5500-0134)	96 Reactions (p/n 5500-0135)
RNA Seq Fragmentation Mix	tube with red cap	bottle
RNA Seq First Strand Master Mix	tube with orange cap	tube with orange cap
RNA Seq Second-Strand + End-Repair Enzyme Mix	tube with blue cap	bottle
RNA Seq Second-Strand + End-Repair Oligo Mix	tube with yellow cap	tube with yellow cap
RNA Seq dA Tailing Master Mix	tube with green cap	bottle
SureSelect Ligation Master Mix	tube with purple cap	tube with purple cap
SureSelect Oligo Adaptor Mix	tube with blue cap	tube with blue cap
RNA Seq PCR Master Mix	tube with red cap	bottle
Uracil DNA Glycosylase (UDG)	tube with yellow cap	tube with yellow cap
SureSelect Primer	tube with brown cap	tube with brown cap
RNA Seq ILM Reverse PCR Primer	tube with black cap	tube with black cap
RNA Seq ILM Post-capture PCR Primer	tube with green cap	tube with green cap
SureSelect ^{XT} Indexes, 8 bp [*]	SureSelect 8 bp Indexes A01 through H02, provided in 16 tubes with white caps	SureSelect 8 bp Indexes A01 through H12, provided in blue 96-well plate [†]

* See [Table 34](#) on page 72 for index sequences.

† See [Table 33](#) on page 71 for a plate map.

5 Reference

Kit Contents

Table 31 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 32 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 33 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 34 SureSelect RNA Seq Indexes, for indexing primers in white-capped tubes or blue 96-well plates

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

www.agilent.com

In This Book

This guide contains information to run the SureSelect^{XT} RNA Direct protocol.

© Agilent Technologies, Inc. 2017, 2019

Version A1, March 2019



p/n G9691-90050



Agilent Technologies