



SureSelect Max mRNA Library Preparation

For Illumina Platform NGS

Protocol

Version B0 January 2026

SureSelect platform manufactured with Agilent SurePrint technology.

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Acknowledgment

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In this Guide...

This guide provides an optimized protocol for preparation of Illumina paired-end multiplexed mRNA sequencing libraries using the SureSelect Max mRNA Library Preparation Module. The workflow supported by this guide includes input RNA preparation, including poly-A purification, through library preparation using adaptors with optional duplex molecular barcodes or MBCs. Libraries are PCR-indexed using SureSelect Max UDI primers. The prepared libraries are ready for next-generation sequencing (NGS) using an Illumina platform.

1 Before You Begin

This section contains information that you should read and understand before you start an experiment.

2 mRNA Enrichment, Fragmentation, and Conversion to cDNA

This section describes the steps to prepare total RNA samples, enrich for poly- A mRNA, fragment the mRNA, and convert the RNA fragments to cDNA.

3 cDNA Library Preparation

This section describes the steps to prepare dual-indexed sequencing libraries from the cDNA fragments. Libraries can be prepared with either MBC-tagged or MBC-free adaptors. Libraries prepared using this protocol are ready for NGS using an Illumina sequencing platform.

4 Appendix: NGS and Analysis Guidelines

This appendix provides guidelines for completing NGS using the Illumina platform and for analysis of the SureSelect Max mRNA libraries.

5 Reference

This section contains reference information, including component kit contents and troubleshooting information.

What's New in Version B0

- Added details to footnotes for [Table 1](#) on page 9 and [Table 13](#) on page 23 regarding the kit size and run configuration details.
- Revised preparative steps for the supplied Amplification Master Mix ([Table 12](#) on page 22) and the user-prepared ligation master mix ([page 23](#)).
- Revised *Note* on [page 25](#) regarding addition of Adaptor Oligo Mix and Ligation Master Mix.
- Minor updates to instructions for obtaining NGS support for SureSelect Max for ILM library sequencing guidance ([page 36](#)).
- Fully updated "[Analysis Pipeline Guidelines](#)" on page 37.
- New *Note* below [Table 1](#) on page 9 explaining use of AMPure XP Beads as a replacement for SureSelect Max Purification Beads.

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Before You Begin

Overview of the Workflow [8](#)
SureSelect Max Modules Used in the Workflow [9](#)
Additional Materials Used in the Workflow [10](#)
Procedural and Safety Notes [12](#)

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents before you start an experiment.

NOTE

Agilent guarantees performance and provides technical support for the SureSelect Max reagents required for this workflow only when used as directed in this Protocol.

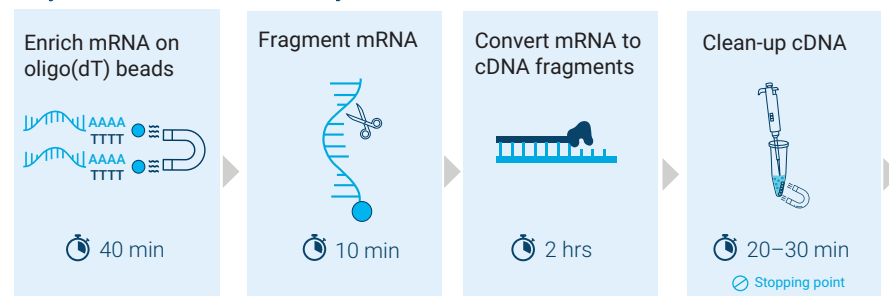
Overview of the Workflow

The SureSelect Max system offers flexible workflow options for preparation and target enrichment of DNA or RNA libraries for NGS in a modular format. This publication provides optimized protocols for the mRNA library preparation workflow, summarized in [Figure 1](#). The mRNA libraries can be constructed to include or exclude duplex molecular barcodes (MBCs) by using different library adaptors and can be indexed using 384 unique dual indexing (UDI) primers.

Detailed mRNA library preparation protocols begin on [page 13](#). Guidelines for downstream NGS are also provided in this guide.

SureSelect Max mRNA Library Preparation Workflow 5.5 Hours

Poly-A Selection and cDNA Synthesis Modules



SureSelect Max Library Preparation Module



Figure 1 Summary of the SureSelect Max mRNA library preparation workflow, including optional stopping points and estimated time requirements. Estimates are guidelines for 16 reaction runs using 1000 ng input RNA. Timing for runs using different protocol parameters may vary.

SureSelect Max reagents are not interchangeable with SureSelect XT HS2 or other SureSelect system reagents.

SureSelect Max Modules Used in the Workflow

This publication provides optimized protocols for the mRNA library preparation workflow. Agilent's SureSelect Max reagents required to complete the protocols are summarized in [Table 1](#).

Table 1 SureSelect Max Kits Used in the mRNA Library Prep Workflow

Description	16 Reaction Kits*	96 Reaction Kits†
SureSelect Max mRNA Library Prep Kit, including the following modules: SureSelect Poly-A Selection Module SureSelect cDNA Module SureSelect Max Library Preparation Module	G9665A	G9665B
SureSelect Max Adaptors and UDI Primers Kit for ILM (Select One): MBC Adaptors and UDI Primers 1-16 MBC Adaptors and UDI Primers 17-32 MBC Adaptors and UDI Primers 1-96 MBC Adaptors and UDI Primers 97-192 MBC Adaptors and UDI Primers 193-288 MBC Adaptors and UDI Primers 289-384	G9667A G9667B	G9668A G9668B G9668C G9668D
MBC-Free Adaptors and UDI Primers 1-16 MBC-Free Adaptors and UDI Primers 17-32 MBC-Free Adaptors and UDI Primers 1-96 MBC-Free Adaptors and UDI Primers 97-192 MBC-Free Adaptors and UDI Primers 193-288 MBC-Free Adaptors and UDI Primers 289-384	G9669A G9669B	G9673A G9673B G9673C G9673D
SureSelect Max Purification Beads	G9962A (5 mL)	G9962B (30 mL)

* 16-reaction kits contain enough reagents for 2 runs containing 8 samples per run.

† 96-reaction kits contain enough reagents for 4 runs containing 24 samples per run.

NOTE

AMPure XP Beads can replace SureSelect Max Purification Beads in the protocols provided in this user guide (see [Table 2](#) on page 10 for ordering information).

Both types of magnetic purification beads have similar overall performance. The bead types are not, however, identical in composition and the average fragment length and yield results may differ slightly. Use a single bead source in validated protocols.

Additional Materials Used in the Workflow

See [Table 2](#) and [Table 3](#) for additional reagents and equipment used in the workflow.

Table 2 Ordering Information for Additional Reagents and Equipment

Description	Vendor and Part Number	Usage Notes
Nucleic acid analysis system	Select from Table 3 on page 11	Required for QC of input RNA and prepared cDNA libraries
Small-volume spectrophotometer	NanoDrop 2000, Thermo Fisher Scientific p/n ND-2000 or equivalent	—
Thermal Cycler with 96-well, 0.2 mL block	Various suppliers	—
Plasticware compatible with the selected thermal cycler: 96-well plates or 8-well strip tubes Tube cap strips	Consult the thermal cycler manufacturer's recommendations	—
Low-adhesion tubes (RNase/DNase/DNA-free) 1.5-mL 0.5-mL	USA Scientific (or equivalent) p/n 1415-2600 p/n 1405-2600	—
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent	—
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 or equivalent	Requires adapter, p/n C1000-ADAPT, for use with strip tubes
Multichannel and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent	—
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, ice bucket, and powder-free gloves	General laboratory supplier	—
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent	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 ring formation.
QPCR Human Reference Total RNA	Agilent p/n 750500	Control input RNA (optional)
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015 or equivalent	Solvent for adaptor dilution
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated.
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276	—
Optional: AMPure XP Kit (5 mL)	Beckman Coulter Genomics p/n A63880	Optional alternative to SureSelect Max Purification Beads (See Table 1)

Table 3 Recommended Analysis Systems

Analysis System	Vendor and Part Number Information	Usage Notes
Agilent 4200/4150 TapeStation Instrument Consumables:	Agilent p/n G2991AA/G2992AA	Recommended systems for qualification of input RNA samples and QC of prepared cDNA libraries prior to NGS. Library QC may also be performed using the Agilent 2100 Bioanalyzer instrument and DNA 1000 Kit, p/n 5067-1504.
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	
RNA ScreenTape	p/n 5067-5576	
RNA ScreenTape Sample Buffer	p/n 5067-5577	
RNA ScreenTape Ladder	p/n 5067-5578	
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	
Agilent 5200/5300/5400 Fragment Analyzer Instrument	Agilent p/n M5310AA/M5311AA/M5312AA	
Consumables:		
RNA Kit (15NT)	p/n DNF-471-0500	
HS RNA Kit (15NT)	p/n DNF-472-0500	
NGS Fragment Kit (1-6000 bp)	p/n DNF-473-0500	

Procedural and Safety 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.
- Use of the following best-practices is recommended to prevent PCR product and nuclease contamination of samples throughout the workflow:
 - 1 Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
 - 2 Maintain clean work areas. Clean the surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
 - 3 Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
 - 4 Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- Several reagent solutions used in the SureSelect Max protocols are highly viscous. Follow the mixing instructions provided in the protocols.
- Avoid introducing bubbles into reaction mixtures during mixing steps. Before adding sample vials to the thermal cycler for incubation or PCR steps, verify the absence of bubbles at the bottom of the sample wells. If present, spin samples briefly to release the bubbles.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

CAUTION

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

2 mRNA Enrichment, Fragmentation, and Conversion to cDNA

- Step 1. Prepare and qualify RNA samples 15
- Step 2. Select poly-A mRNA from total RNA 15
- Step 3. Fragment the mRNA 17
- Step 4. Synthesize first-strand cDNA 18
- Step 5. Synthesize second-strand cDNA 18
- Step 6. Purify cDNA using magnetic purification beads 19

This section describes the steps to prepare mRNA-enriched cDNA for the RNA sequencing library preparation protocol in the following section.

The protocol is compatible with intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE- derived RNA samples.

The protocol requires 10 ng to 1000 ng of input total RNA, with higher RNA input requirements for lower-quality samples. For optimal sequencing results, use the maximum amount of input RNA available in the 10–1000 ng range.

The protocol produces cDNA fragments suitable for 2 x 100 or 2 x 150 read length NGS.

The mRNA enrichment workflow segment uses the components in [Table 4](#) and the RNA fragmentation and cDNA synthesis workflow segment uses the components in [Table 5](#). Remove the listed reagents from cold storage and prepare as directed before use (refer to the *Where Used* column).

Table 4 Reagents brought to room temperature before use in mRNA enrichment steps

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Poly-A Selection Module (Pre PCR), 4°C	Oligo(dT) Microparticles (tube with brown cap or bottle)	Equilibrate to room temperature. Vortex well immediately before use.	page 15
	Bead Washing Buffer (bottle)	Equilibrate to room temperature, then ready-to-use.	page 16
	Bead Elution Buffer (tube with green cap or bottle)	Equilibrate to room temperature, then ready-to-use.	page 16
	Bead Binding Buffer (tube with purple cap or bottle)	Equilibrate to room temperature, then ready-to-use.	page 17

Table 5 Reagents thawed before use in fragmentation and cDNA synthesis steps

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect cDNA Module (Pre PCR), -20°C	2X Priming Buffer (tube with purple cap)	Thaw on ice then keep on ice, vortex to mix.	page 17
	First Strand Master Mix (amber tube with amber cap)*	Thaw on ice for 30 minutes then keep on ice, vortex to mix.	page 18
	Second Strand Enzyme Mix (tube with blue cap or bottle)	Thaw on ice then keep on ice, vortex to mix.	page 19
	Second Strand Oligo Mix (tube with yellow cap)	Thaw on ice then keep on ice, vortex to mix.	page 19
+4°C	SureSelect Max Purification Beads OR AMPure XP Beads	Equilibrate at room temperature for at least 30 minutes before use, vortex to mix.	page 19

* The First Strand Master Mix contains actinomycin D and is provided ready-to-use. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Step 1. Prepare and qualify RNA samples

The library preparation protocol requires 10 ng to 1 µg high-quality total RNA. A minimum RNA input of 50 ng is required for samples with reduced RNA integrity, as detailed below.

NOTE

The protocol in this publication is suitable for intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE-derived RNA samples.

Consider preparing an additional sequencing library in parallel, using a high-quality control RNA sample, such as Agilent's QPCR Human Reference Total RNA (p/n 750500, supplied at 1 µg/µL). Use of this control is especially recommended during the first run of the protocol, to verify that all protocol steps are being successfully performed. Routine use of this control is helpful for any required troubleshooting, in order to differentiate any performance issues related to RNA input from other factors.

- 1 Prepare total RNA for each sample in the run in nuclease-free water.
- 2 Determine the RNA concentration using a small volume spectrophotometer. Verify that the 260/280 and 260/230 absorbance ratio values for the sample are both approximately 1.8 to 2.0. A significant deviation from ratios of 2.0 indicates that the sample may require further purification before use in NGS library preparation.
- 3 Analyze RNA integrity by determining the RNA Integrity Number (RIN), or equivalent, using one of the RNA qualification platforms listed in [Table 3](#) on page 11. The RIN/RQN quality scores reported by these Agilent platforms are equivalent measures of RNA quality. Select the specific RNA assay for your platform based on the concentration determined in [step 2](#).

For optimal performance, total RNA samples should have RIN>8. For samples with RIN>8, the amount of total RNA needed for the library preparation protocol is 10 ng to 1 µg.

Samples with RIN of 6 to 8 may be used in the protocol, using a minimum RNA input of 50 ng.

NOTE

Libraries prepared using lower-quality RNA samples with RIN of 6 to 8 also require an additional purification step at a later stage of the protocol (see [page 25](#)).

Samples with RIN<6 are not suitable for use in this protocol; instead consider Agilent's SureSelect Max RNA system (see publication [G9664-90000](#) at [www.agilent.com](#)).

- 4 Place each total RNA sample in a separate well of a 96-well plate or strip tube in 25 µL of nuclease-free water and hold on ice.

Step 2. Select poly-A mRNA from total RNA

In this step, the samples are selectively enriched for poly-A tailed mRNA using two serial rounds of binding to oligo(dT) magnetic particles.

- 1 Vortex the Oligo(dT) Microparticles until the suspension appears homogeneous and consistent in color. If bead aggregates are still present after vortexing, mix thoroughly by pipetting up and down until the suspension appears homogeneous.
- 2 Add 25 µL of the homogeneous Oligo(dT) bead suspension to each total RNA sample well.

- 3 Seal the wells, then gently vortex for 5 seconds and briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.
- 4 To denature the RNA, transfer the sample plate or strip tube to a thermal cycler and run the program in [Table 6](#). Set the heated lid to 105°C.

Table 6 Thermal cycler program for RNA denaturation (50 µL vol)

Step	Temperature	Time
Step 1	65°C	5 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

- 5 After the thermal cycler reaches the 4°C Hold step, remove the plate or strip tube and incubate at room temperature for 5 minutes, to allow poly-A mRNA binding to the oligo(dT) beads.
- 6 Move the plate or strip tube to a magnetic separation device at room temperature. Wait for the solution to clear (approximately 2 to 5 minutes).
- 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 or disturb the beads while removing the solution.
- 8 Remove the plate or strip tube from the magnetic stand. Gently add 200 µL of Bead Washing Buffer to each well.

Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µL, without introducing bubbles.

CAUTION

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

- 9 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).
- 10 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.
- 11 Remove the plate or strip tube from the magnetic stand. Add 25 µL of Bead Elution Buffer to each sample well.
- 12 Seal the wells, then gently vortex for 5 seconds. Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid.
- 13 To elute the RNA, transfer the sample plate or strip tube to a thermal cycler and run the program in [Table 7](#). Set the heated lid to 105°C.

Table 7 Thermal cycler program for RNA elution (25 µL vol)

Step	Temperature	Time
Step 1	80°C	2 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

- 14 After the thermal cycler reaches the 4°C Hold step, remove the plate or strip tube and add 25 µL of Bead Binding Buffer to each sample well.
- 15 Seal the wells, then gently vortex for 5 seconds. Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid.
- 16 Incubate the samples at room temperature for 5 minutes, to allow poly-A mRNA to re-bind the beads.
- 17 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).
- 18 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.
- 19 Remove the plate or strip tube from the magnetic stand. Gently add 200 µL of Bead Washing Buffer to each well.

Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µL, without introducing bubbles. If bubbles or foam are present, spin the plate or strip tube briefly before continuing.
- 20 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).
- 21 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.
- 22 Remove the bead-bound RNA sample plate or strip tube from the magnetic stand. Add 10 µL of nuclease-free water to each sample well and keep the samples on ice.
- 23 Proceed immediately to **“Step 3. Fragment the mRNA”** below.

Step 3. Fragment the mRNA

In this step, the poly-A mRNA-enriched samples are chemically-fragmented by treatment with metal ions at elevated temperature to a size appropriate for RNA sequencing library preparation. The 2X Priming Buffer used in this step includes both fragmentation agents and primers for cDNA synthesis used in the next step.

- 1 Preprogram a thermal cycler as shown in [Table 8](#). Set the heated lid to 105°C.

Table 8 Thermal cycler program for fragmentation of mRNA samples (20 µL vol)

Step	Temperature	Time
Step 1	94°C	4 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

- 2 Add 10 µL of 2X Priming Buffer to each sample well containing 10 µL of bead-bound RNA.
- 3 Cap the wells and vortex at high speed to resuspend the beads. Spin briefly to collect the liquid and remove bubbles.
- 4 Place the plate or strip tube in the thermal cycler and run the program in [Table 8](#). During this step the RNA is simultaneously fragmented and eluted from the oligo(dT) beads.

- 5 Once the thermal cycler program in [Table 8](#) reaches the 4°C Hold step, transfer the fragmented RNA sample plate or strip tube to the magnetic stand at room temperature. Watch for the bead suspension to clear, then transfer each supernatant (approximately 20 µL) to wells of a fresh plate or strip tube. Keep the eluted RNA samples on ice. You can discard the oligo(dT) beads at this time.

Minimize the processing time at this step to avoid rebinding of RNA to the beads. Proceed immediately to “[Step 4. Synthesize first-strand cDNA](#)”.

Step 4. Synthesize first-strand cDNA

CAUTION

The First Strand Master Mix used in this step is viscous. Mix thoroughly by vortexing where directed in the protocol. Pipetting up and down is not sufficient to mix this reagent.

The First Strand Master Mix is provided with actinomycin D already supplied in the mixture. Do not supplement with additional actinomycin D.

- 1 Preprogram a thermal cycler as shown in [Table 9](#). Set the heated lid to 105°C.

Table 9 Thermal cycler program for first-strand cDNA synthesis (24 µL vol)

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

- 2 Vortex the thawed vial of First Strand Master Mix for 5 seconds at high speed to ensure homogeneity.
- 3 Add 4 µL of First Strand Master Mix to each RNA sample well.
- 4 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.
- 5 Place the sample plate or strip tube in the thermal cycler and run the program in [Table 9](#).

Step 5. Synthesize second-strand cDNA

CAUTION

The Second Strand Enzyme Mix used in this step is viscous. Mix thoroughly by vortexing where directed in the protocol. Pipetting up and down is not sufficient to mix this reagent.

- 1 Once the thermal cycler program in [Table 9](#) begins the 4°C hold step, transfer the samples to ice.

2 Preprogram the thermal cycler as shown in [Table 10](#). Set the heated lid to 105°C.

Table 10 Thermal cycler program for second-strand synthesis (54 µL vol)

Step	Temperature	Time
Step 1	16°C	60 minutes
Step 2	4°C	Hold

- 3 Vortex the thawed vials of Second Strand Enzyme Mix and of Second Strand Oligo Mix at high speed for 5 seconds to ensure homogeneity.
- 4 Add 25 µL of Second Strand Enzyme Mix to each sample well. Keep on ice.
- 5 Add 5 µL of Second Strand Oligo Mix to each sample well, for a total reaction volume of 54 µL. Keep on ice.
- 6 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.
- 7 Place the sample plate or strip tube in the thermal cycler and run the program in [Table 10](#).

NOTE

The magnetic purification beads used in the next step must be equilibrated to room temperature for at least 30 minutes before use.

Step 6. Purify cDNA using magnetic purification beads

Once the thermal cycler program in [Table 10](#) reaches the 4°C hold step, purify the cDNA using room-temperature (RT) SureSelect Max Purification Beads or AMPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in [Table 11](#).

Table 11 Magnetic purification bead cDNA cleanup parameters

Parameter	Value
Volume of RT purification bead suspension added to each sample well	105 µL
Final elution solvent and volume	52 µL nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 50 µL

- 1 Prepare 400 µL of 70% ethanol per sample, plus excess, for use in [step 8](#).

NOTE

The freshly-prepared 70% ethanol may be used for all purification steps run on the same day.

- 2 Mix the room-temperature purification beads well until homogeneous and consistent in color.
- 3 Transfer the cDNA samples from the thermal cycler to room temperature, then add 105 µL of the bead suspension to each sample well.
- 4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
- 5 Incubate the bead suspensions for 5 minutes at room temperature.

- 6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 to 10 minutes).
- 7 Keep 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 Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

NOTE

In the drying step below, do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 12 Dry the samples by keeping the unsealed plate or strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.
- 13 Elute the cDNA by adding 52 μL of nuclease-free water to each sample well.
- 14 Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed 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. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
- 15 Incubate for 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer cDNA fragments.
- 16 Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- 17 Transfer the cleared supernatant (approximately 50 μL) to a fresh well. Keep on ice.

You can discard the beads at this time.

The purified cDNA is ready for NGS library preparation; proceed to [“cDNA Library Preparation”](#) on page 21.

Stopping Point

If you do not continue to the next step, seal the wells and store at 4°C overnight or at –20°C for prolonged storage.

3 cDNA Library Preparation

- Step 1. Prepare the ligation master mix [23](#)
- Step 2. Repair and dA-tail the cDNA 3' ends [23](#)
- Step 3. Ligate the adaptor [24](#)
- Step 4. Purify libraries using magnetic purification beads [25](#)
- Step 5. Amplify and index the libraries [28](#)
- Step 6. Purify amplified libraries using magnetic purification beads [29](#)
- Step 7. QC and quantify the libraries [30](#)
- Step 8. Pool libraries for multiplex NGS [32](#)

Use the instructions in this section to prepare mRNA-enriched cDNA libraries for NGS.

For each sample to be sequenced, an individual dual-indexed library is prepared. To process multiple samples together, the protocol includes steps for preparation of reagent mixtures for 8 or 24 samples with overage, followed by distribution to individual cDNA samples.

This workflow segment uses the components listed in [Table 12](#). Remove the listed reagents from cold storage and prepare as directed just before use (see the *Where Used* column).

Table 12 Reagents thawed before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Library Preparation Module, stored at -20°C	Ligation Buffer (purple cap or bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix.	page 23
	T4 DNA Ligase (blue cap)	Place on ice just before use, invert to mix.	page 23
	End Repair-A Tailing Buffer (yellow cap or bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix.	page 24
	End Repair-A Tailing Enzyme Mix (orange cap)	Place on ice just before use, invert to mix.	page 24
	Amplification Master Mix (red cap or bottle)	Thaw on ice then keep on ice. Mix thoroughly by inversion 10X. Do not vortex.	page 28
SureSelect Max Adaptors and UDI Primers Kit for ILM, stored at -20°C	For MBC-tagged libraries: SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap) OR For MBC-free libraries: SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)	Thaw on ice then keep on ice, vortex to mix. Dilute 5-fold with 1X Low TE Buffer before use. Prepare a fresh dilution for each run.	page 24
	SureSelect Max UDI Primers for ILM (select the specific set of indexes to be used in the run): Index Pairs 1-8 (blue strip) Index Pairs 9-16 (white strip) Index Pairs 17-24 (black strip) Index Pairs 25-32 (red strip) Index Pairs 1-96 (orange plate) Index Pairs 97-192 (blue plate) Index Pairs 193-288 (green plate) Index Pairs 289-384 (red plate)	Thaw on ice then keep on ice, vortex to mix.	page 28
+4°C	SureSelect Max Purification Beads OR AMPure XP Beads	Equilibrate at room temperature (RT) for at least 30 minutes before use, vortex to mix. Beads may be retained at RT between purification steps.	page 25 and page 29

Step 1. Prepare the ligation master mix

Prepare the ligation master mix to allow equilibration to room temperature while you are completing the end repair/dA-tailing step. Leave cDNA samples on ice while completing this step.

CAUTION

The Ligation Buffer used in this step is viscous. Make sure to follow the mixing instructions in [step 1](#) and [step 2](#) below.

- 1 Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed just before use.
- 2 Prepare the appropriate volume of ligation master mix by combining the reagents in [Table 13](#).

Slowly pipette the Ligation Buffer into a 1.5-mL tube, ensuring that the full volume is dispensed. Slowly add the T4 DNA Ligase, rinsing the enzyme tip with buffer solution after addition. Mix well by slowly pipetting up and down 15–20 times with a pipette set to at least 80% of the mixture volume, or seal the tube and vortex at high speed for 10–20 seconds. Spin briefly.

Keep at room temperature for 30–45 minutes before use on [page 25](#).

Table 13 Preparation of ligation master mix

Reagent	Volume for 1 reaction	Volume for 8 reactions* (includes excess)	Volume for 24 reactions† (includes excess)
Ligation Buffer (purple cap or bottle)	23 µL	207 µL	598 µL
T4 DNA Ligase (blue cap)	2 µL	18 µL	52 µL
Total	25 µL	225 µL	650 µL

* 16 reaction Library Preparation Kits contain enough reagents for 2 runs of 8 samples each using the indicated excess volume. Reagents may be depleted after fewer than 16 reactions when using smaller run sizes.

† 96 reaction Library Preparation Kits contain enough reagents for 4 runs of 24 samples each using the indicated excess volume. Reagents may be depleted after fewer than 96 reactions when using smaller run sizes.

Step 2. Repair and dA-tail the cDNA 3' ends

CAUTION

The End Repair-A Tailing Buffer used in this step is viscous. Make sure to follow the mixing instructions in [step 2](#) and [step 3](#) below.

- 1 Preprogram a thermal cycler as shown in [Table 14](#). Set the heated lid to 105°C.

Table 14 Thermal cycler program for end repair/dA-tailing (70 µL vol)

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	65°C	15 minutes
Step 3	4°C	Hold

- Vortex the thawed vial of End Repair-A Tailing Buffer for 15 seconds at high speed to ensure homogeneity. Visually inspect the solution; if any solids are observed, continue vortexing until all solids are dissolved.
- Prepare the appropriate volume of end repair/dA-tailing master mix by combining the reagents in [Table 15](#).

Slowly pipette the End Repair-A Tailing Buffer into a 1.5-mL tube, ensuring that the full volume is dispensed. Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition. Mix well by pipetting up and down 15–20 times with a pipette set to at least 80% of the mixture volume, or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly and keep on ice.

Table 15 Preparation of end repair/dA-tailing master mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µL	144 µL	416 µL
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	36 µL	104 µL
Total	20 µL	180 µL	520 µL

- Add 20 µL of the end repair/dA-tailing master mix to each sample well containing 50 µL of purified cDNA. Mix by pipetting up and down 15–20 times using a pipette set to 50 µL or cap the wells and vortex at high speed for 5–10 seconds.
- Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and run the program in [Table 14](#).

Step 3. Ligate the adaptor

- Prepare a 5-fold dilution of the appropriate SureSelect Max Adaptor Oligo Mix as shown in [Table 16](#).

Table 16 SureSelect Max Adaptor Oligo Mix dilution for cDNA libraries

Reagent	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap) OR SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)	10 µL	30 µL
1X Low TE Buffer	40 µL	120 µL
Total	50 µL	150 µL

- Once the end repair/dA-tailing thermal cycler program ([Table 14](#)) reaches the 4°C Hold step, transfer the samples to ice. Preprogram the cycler as show in [Table 17](#) with the heated lid off.

Table 17 Thermal cycler program for ligation (100 µL vol)

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

- To each end-repaired/dA-tailed cDNA sample (approximately 70 µL), add 25 µL of the ligation master mix that was prepared on [page 23](#) and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70 µL or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.
- Add 5 µL of the diluted SureSelect Max Adaptor Oligo Mix (prepared in [step 1](#) on [page 24](#)) to each sample.

Mix by pipetting up and down 15–20 times using a pipette set to 70 µL or cap the wells and vortex at high speed for 5–10 seconds.

NOTE

Make sure to add the ligation master mix and the Adaptor Oligo Mix dilution to the samples in separate addition steps, mixing after each addition, as directed above. Adding these components together may increase adaptor-dimer formation and decrease kit performance.

- Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and run the program in [Table 17](#).

NOTE

The magnetic purification beads used in the next step must be equilibrated to room temperature for at least 30 minutes before use. Beads can be kept at room temperature through the final purification step on [page 29](#).

Step 4. Purify libraries using magnetic purification beads

CAUTION

The bead volume used at this step differs in various SureSelect system protocols. Adhere to the instructions provided below; do not use protocols provided for other SureSelect kits.

In this step, the adaptor-ligated cDNA libraries are purified using room-temperature (RT) SureSelect Max Purification Beads or AMPure XP Beads. Libraries prepared from <100 ng total RNA input or prepared from lower quality RNA samples (RIN 6–8) are subjected to an additional round of purification.

Critical purification protocol parameters are summarized for experienced users in [Table 18](#) for single-round purification and in [Table 19](#) for two-round purification.

Start the purification procedure once the thermal cycler program in [Table 17](#) reaches the 4°C hold step.

Table 18 Parameters for 1× magnetic bead purification of libraries from input RNA \geq 100 ng **AND** RIN \geq 8

Parameter	Value
Rounds of purification required	1×
Volume of 70% ethanol to be prepared	400 μ L per sample
Volume of RT purification bead suspension added to each sample well	80 μ L
Final elution solvent and volume	21 μ L nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 20 μ L

Table 19 Parameters for 2× magnetic bead purification of libraries from input RNA $<$ 100 ng **OR** RIN 6–8

Parameter	Value
Rounds of purification required	2×
Volume of 70% ethanol to be prepared	800 μ L per sample
Volume of RT purification bead suspension added to each sample well	Round 1: 80 μ L Round 2: 60 μ L
Final elution solvent and volume	Round 1: 50 μ L nuclease-free water Round 2: 21 μ L nuclease-free water
Amount of eluted sample transferred to fresh well	Round 1: 50 μ L Round 2: Approximately 20 μ L

- 1 Prepare fresh 70% ethanol for wash steps. See [Table 18](#) or [Table 19](#) for volumes needed.
- 2 Verify that the purification beads were held at RT for at least 30 minutes before use. Mix the beads well, until homogeneous and consistent in color.
- 3 Transfer the cDNA library samples from the thermal cycler to room temperature, then add 80 μ L of the bead suspension to each sample well.
- 4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
- 5 Incubate the bead suspensions for 5 minutes at room temperature.
- 6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 to 10 minutes).
- 7 Keep 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 μ L 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 Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

NOTE

In the drying step below, do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 12 Dry the samples by keeping the unsealed plate or strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.
- 13 Elute the library by adding the appropriate volume of nuclease-free water to each sample well, according to [Table 20](#).

Table 20 Elution volume based on RNA input characteristics

Total RNA Input Quantity/Quality	Elution Volume	Rounds of Purification Required
≥100 ng AND RIN≥8	21 µL	1×
<100 ng OR RIN 6–8	50 µL	2×

- 14 Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed 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. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
- 15 Incubate for 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer cDNA fragments.
- 16 Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- 17 Transfer the cleared supernatant (20 µL or 50 µL, see [Table 21](#)) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

Table 21 Supernatant transfer volume based on RNA input characteristics

Total RNA Input Quantity/Quality	Supernatant Volume Collected*	Rounds of Purification Required
≥100 ng AND RIN≥8	20 µL	1×
<100 ng OR RIN 6–8	50 µL	2×

* For samples with single round of purification, avoid bead carryover by removing 20 µL of the 21 µL elution volume. Small amounts of bead carryover are acceptable for samples subjected to a second round of purification; collect the entire 50 µL of supernatant for these samples.

- 18 For samples prepared from ≥100 ng total RNA input with RIN≥8, proceed directly to **“Step 5. Amplify and index the libraries”** below.

For lower-quantity or lower-quality input samples, complete [step 19](#) and [step 20](#) below.

For low input (<100 ng) or lower-quality (RIN 6–8) libraries only:

- 19 Add 60 µL of homogeneous purification beads to each 50 µL sample in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
- 20 Repeat the remaining purification steps, beginning with [step 4](#) on [page 26](#). Elute the re-purified cDNA library in 21 µL of nuclease-free water, and transfer 20 µL of the re-purified supernatant to a fresh well.

Proceed directly to **“Step 5. Amplify and index the libraries”**.

Step 5. Amplify and index the libraries

- 1 Determine the appropriate SureSelect Max UDI Primer assignment for each sample. See [page 41](#) for information on the UDI primers used to amplify the cDNA libraries in this step.

Use a different UDI number for each sample to be sequenced in the same lane.

NOTE

Agilent's SureSelect 8-bp dual indexes use a uniform numbering system across kit formats and between SureSelect Max for ILM and SureSelect XT HS2 platforms. Do not combine samples indexed with the same-numbered dual index pair from different kit formats for multiplex sequencing.

CAUTION

The UDI primers are provided in single-use aliquots. To avoid cross-contamination of libraries, do not retain and re-use any residual volume for subsequent experiments.

- 2 Preprogram a thermal cycler as shown in [Table 22](#). Set the heated lid to 105°C. Prewarm the instrument before samples are loaded in [step 6](#) on [page 29](#).

Table 22 Library indexing/amplification thermal cycler program (50 µL vol)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	8 to 14 based on input RNA quantity (see Table 23)	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

Table 23 Amplification program cycle number recommendations

Quantity of Input RNA	Cycles
1000 ng	8 cycles
250 ng	9 cycles
100 ng	10 cycles
50 ng	12 cycles
10 ng	14 cycles

NOTE

To avoid cross-contamination, it is recommended to set up PCR reactions (all components except the library) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

- 3 Mix the thawed Amplification Master Mix (red cap or bottle) thoroughly by inversion 10X then spin briefly.

- 4 Add 25 μL of the Amplification Master Mix to each sample well containing purified cDNA library fragments (20 μL).
- 5 Add 5 μL of the appropriate SureSelect Max UDI primer pair to each reaction.
Cap the wells then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid and release any bubbles.
- 6 Place the sample plate or strip tube in the thermal cycler and run the program in [Table 22](#).

CAUTION

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

Stopping Point If you do not continue to the next step, seal the sample wells and store at 4°C overnight or at -20°C for prolonged storage.

Step 6. Purify amplified libraries using magnetic purification beads

Once the thermal cycler program in [Table 22](#) reaches the 4°C hold step, purify the libraries using room-temperature SureSelect Max Purification Beads or AMPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in [Table 24](#).

Table 24 Magnetic purification bead cleanup parameters after amplification

Parameter	Value
Volume of RT purification bead suspension added to each sample well	50 μL
Final elution solvent and volume	15 μL 1X Low TE Buffer
Amount of eluted sample transferred to fresh well	Approximately 14 μL

- 1 Prepare 400 μL of 70% ethanol per sample, plus excess, for use in [step 8](#).
- 2 Mix the room-temperature purification beads well until homogeneous and consistent in color.
- 3 Transfer the amplified library samples from the thermal cycler to room temperature, then add 50 μL of the bead suspension to each sample well.
- 4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
- 5 Incubate the bead suspensions for 5 minutes at room temperature.
- 6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 minutes).
- 7 Keep 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** Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

NOTE

In the drying step below, do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 12** Dry the samples by keeping the unsealed plate or strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.
- 13** Elute the library by adding 15 µL of 1X Low TE Buffer to each sample well.
- 14** Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed 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. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
- 15** Incubate for 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer cDNA fragments.
- 16** Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- 17** Transfer the cleared supernatant (approximately 14 µL) to a fresh well. Keep on ice.
You can discard the beads at this time.

Stopping Point If you do not plan to continue to the library pooling step on same day, seal the wells and store at 4°C overnight or at –20°C for prolonged storage. Remove an aliquot for QC analysis before storage, if appropriate.

Step 7. QC and quantify the libraries

Analyze a sample of each library using one of the platforms listed in [Table 25](#). Follow the instructions in the linked user guide provided for each assay.

Table 25 Library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	D1000 ScreenTape	Agilent D1000 Assay Quick Guide	1 µL of five-fold dilution
Agilent 5200/5300/5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	Agilent NGS Fragment Kit (1-6000 bp) Kit Guide	2 µL of five-fold dilution

Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See [Table 26](#) for fragment size distribution guidelines. A representative electropherogram generated using the TapeStation system is provided in [Figure 2](#) to illustrate typical results.

Using the 150 bp to 1000 bp region of the electropherogram, determine the average fragment size and the library DNA concentration.

Table 26 Expected library fragment size guidelines

Input type	Expected average fragment size (150–1000 bp region)
Intact RNA	200 to 700 bp

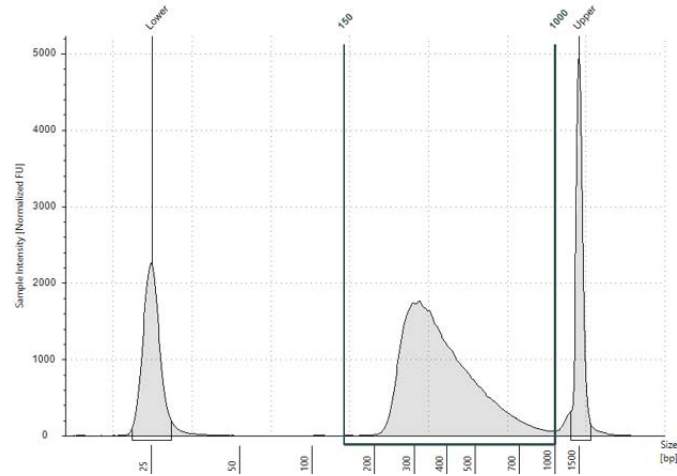


Figure 2 Library prepared from a high-quality RNA sample analyzed using a D1000 ScreenTape assay.

Stopping Point If you do not continue to the next step, seal the sample wells and store at 4°C overnight or at –20°C for prolonged storage.

Step 8. Pool libraries for multiplex NGS

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the sequencer used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

NOTE

SureSelect Max UDI strip and plate layouts are designed to provide the proper color balance for Illumina's two-channel and four-channel systems. A minimum plexity of four is recommended to ensure that library pools are color balanced. Pools containing any four consecutive SureSelect Max UDIs meet Illumina's guidance for optimal color balance and sequencing performance. Consult Illumina's guidelines for additional color balance and pooling strategy information including two-plex or three-plex pooling considerations.

Combine the library samples such that each indexed library is present in equimolar amounts in the NGS pool using one of the following methods. Use the diluent specified by your sequencing provider, such as Low TE, for the dilution steps.

Method 1: Dilute each sample to be pooled to the same final concentration (typically 4–15 nM, or the concentration of the most dilute sample) then combine equal volumes of all samples to create the final NGS pool.

Method 2: Starting with samples at different concentrations, add the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using Low TE. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

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 (typically 4 nM–15 nM or the concentration of the most dilute sample)

$\#$ 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 Low TE needed for a final volume of 20 μL at 10 nM DNA.

Table 27 Example of volume calculation for total volume of 20 μL at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use (μL)
Sample 1	20 μL	20 nM	10 nM	4	2.5
Sample 2	20 μL	10 nM	10 nM	4	5
Sample 3	20 μL	17 nM	10 nM	4	2.9
Sample 4	20 μL	25 nM	10 nM	4	2
Low TE					7.6

Store the library pool under the conditions specified by your sequencing provider. Typically, libraries should be stored at -20°C for short-term storage.

4 Appendix: NGS and Analysis Guidelines

SureSelect Max library composition	34
Sequencing setup and run guidelines	35
Analysis Pipeline Guidelines	37

This appendix provides guidelines for completing NGS using the Illumina platform and for analysis of the SureSelect Max mRNA libraries.

SureSelect Max library composition

The final SureSelect Max library fragments prepared using Agilent’s Illumina-compatible kit modules are shown in [Figure 3](#). Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using Illumina sequencers. The libraries are ready for sequencing using standard Illumina paired-end primers and chemistry.

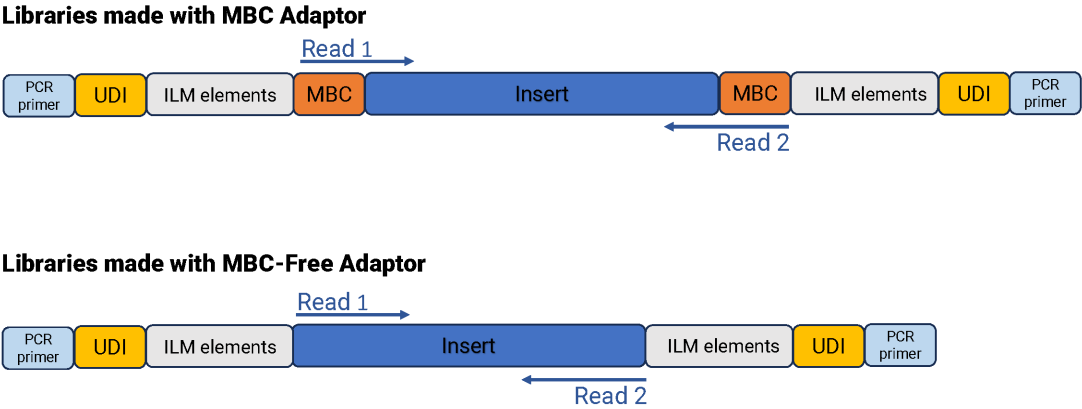


Figure 3 Content of SureSelect Max for ILM sequencing libraries. Each fragment contains one target insert (dark blue) surrounded by the following elements: Illumina paired-end sequencing elements (gray), unique dual indexes (gold), library PCR primers (light blue) and optional molecular barcode (MBC) sequences. MBCs include a 3-bp degenerate barcode plus dark bases.

Sequencing setup and run guidelines

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. [Table 28](#) provides guidelines for use of several Illumina instrument and chemistry combinations suitable for processing the SureSelect Max mRNA NGS libraries. For other Illumina NGS platforms, consult Illumina's documentation for kit configuration and seeding concentration guidelines.

Table 28 Illumina Kit Configuration Selection Guidelines

Instrument	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	12–16 pM
iSeq 100	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	50–150 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NextSeq 1000/2000	All Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	Standard SBS or XLEAP-SBS	650–1000 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.5	200–400 pM
NovaSeq X	All runs	2 × 100 bp or 2 × 150 bp	100, 200 or 300 Cycle Kit	v1	90–180 pM

Seeding concentration and cluster density may also need to be optimized based on the cDNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in [Table 28](#) or provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument's software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate **Cycles** or **Read Length** value for your library read length and using 8-bp dual index reads. See [Table 29](#) showing example settings for 2x150 bp sequencing.

Table 29 Run settings for 2x150 bp sequencing

Run Segment	Cycles/Read Length
Read 1	151*
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	151*

* Follow Illumina's recommendation to add one (1) extra cycle to the desired read length.

Follow Illumina's instructions for each platform and setup software option, incorporating the additional setup guidelines below:

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see [page 45](#).
- No custom primers are used for SureSelect Max library sequencing. Leave all *Custom Primers* options for *Read 1*, *Read 2*, *Index 1* and *Index 2* primers cleared/deselected during run setup.
- For MBC-tagged libraries, turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps to ensure proper processing of the degenerate MBC sequences in the adaptors.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the SureSelect Max index sequences can be obtained by downloading the [SureSelect Max Index Sequence Resource](#) Excel spreadsheet from Agilent.com. The provided sequences should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need additional SureSelect Max for ILM library sequencing guidance, contact the SureSelect support team (see [page 2](#)) or your local representative.

Analysis Pipeline Guidelines

Guidelines are provided below for typical NGS read processing and analysis pipeline steps appropriate for SureSelect Max mRNA libraries. Your NGS pipeline may vary.

MBC-free libraries

- 1 Demultiplexing:** Generate demultiplexed FASTQ files for each sample using Illumina's bcl2fastq, BCL Convert, or a similar software application. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes.
- 2 Adaptor trimming:** Turn on the adaptor trimming tools in the selected Illumina demultiplexing software to complete adaptor trimming at this step.
- 3 Alignment:** The trimmed reads should be aligned using a suitable RNA library data alignment tool such as STAR.

The resulting BAM files are ready for downstream analysis including variant and gene expression discovery.

MBC-tagged libraries

- 1 Demultiplexing:** Generate demultiplexed FASTQ files for each sample using Illumina's bcl2fastq, BCL Convert, or a similar software application. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes.

Turn off the MBC/UMI trimming options in Illumina's demultiplexing software to allow proper adaptor processing and use of the MBCs by downstream tools.

NOTE

If your libraries are MBC-tagged but your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis.

If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in [Table 29](#) on page 35). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in [Table 29](#) on page 35). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

Alternatively, the first 5 bases may be trimmed from the demultiplexed FASTQ files using a suitable processing tool (e.g., fgbio).

- 2 MBC-adaptor processing:** Use a suitable processing tool of your choice to trim and collect inline MBCs from each sequencing read. For example, MBC processing could be conducted with the fgbio best practice consensus pipeline, as described in: <https://github.com/fulcrumgenomics/fgbio/blob/main/docs/best-practice-consensus-pipeline.md>. The output includes trimmed, deduplicated reads in unaligned BAM format with single-stranded MBC consensus reads. For generation of double-stranded MBC consensus reads, refer to <https://fulcrumgenomics.github.io/fgbio/tools/latest/CallDuplexConsensusReads.html>.
 - Inline MBCs are added to both ends of the DNA inserts in the assay. To collect the MBCs and process them for analysis, one approach is to trim off 5 bases at the beginning of each read, take the first 3 of the 5 bases as MBC, and discard the remaining 2.
- 3 Alignment:**
 - RNA library alignment should be performed after read trimming and deduplication using a suitable tool such as STAR.

The resulting aligned BAM files are ready for downstream analysis including variant and gene expression discovery.

Strandedness guidelines

The SureSelect Max mRNA 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

Reagent Kit Contents	40
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This section contains reference information, including Reagent Kit contents, index sequences, troubleshooting information and a quick-reference protocol for experienced users.

Reagent Kit Contents

The SureSelect Max mRNA Library Preparation workflow uses the kits listed in [Table 30](#). Detailed contents of each of the multi- part component modules are listed in [Table 31](#) through [Table 35](#).

Table 30 Kits for SureSelect Max mRNA Library Preparation

Purchased Kit	Included Component Kits	Component Kit Part Number		Storage Condition
		16 Reactions	96 Reactions	
SureSelect Max mRNA Library Preparation Kit	SureSelect Poly-A Selection Module	5190-6410	5190-6411	+4°C
	SureSelect cDNA Module	5500-0148	5500-0149	-20°C
	SureSelect Max Library Preparation Module	5280-0065	5280-0066	-20°C
SureSelect Max Adaptors and UDI Primers Kit for ILM	SureSelect Max MBC Adaptor Oligo Mix for ILM	5282-0124	5282-0125	-20°C
	OR SureSelect Max MBC-Free Adaptor Oligo Mix for ILM	OR 5282-0126	OR 5282-0127	
	SureSelect Max UDI Primers for ILM	5282-0138 (Index 1-16) 5282-0119 (Index 17-32)	5282-0120 (Index 1-96) 5282-0121 (Index 97-192) 5282-0122 (Index 193-288) 5282-0123 (Index 289-384)	-20°C
SureSelect Max Purification Beads		5282-0225	5282-0226	+4°C

Table 31 SureSelect Poly-A Selection Module content

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

Table 32 SureSelect cDNA Module content

Kit Component	16 Reaction Kit (p/n 5500-0148)	96 Reaction Kit (p/n 5500-0149)
2X Priming Buffer	tube with purple cap	tube with purple cap
First Strand Master Mix*	amber tube with amber cap	amber tube with amber cap
Second Strand Enzyme Mix	tube with blue cap	bottle
Second Strand Oligo Mix	tube with yellow cap	tube with yellow cap

* The First Strand Master Mix contains actinomycin D. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Table 33 SureSelect Max Library Preparation Module content

Kit Component	16 Reaction Kit (p/n 5280-0065)	96 Reaction Kit (p/n 5280-0066)
End Repair-A Tailing Enzyme Mix	tube with orange cap	tube with orange cap
End Repair-A Tailing Buffer	tube with yellow cap	bottle
T4 DNA Ligase	tube with blue cap	tube with blue cap
Ligation Buffer	tube with purple cap	bottle
Amplification Master Mix	tube with red cap	bottle

Table 34 SureSelect Max Adaptor Oligo Mix for ILM options

Kit Component	16 Reaction Kits	96 Reaction Kits
SureSelect MBC Adaptor Oligo Mix for ILM	tube with white cap	tube with white cap
SureSelect MBC-Free Adaptor Oligo Mix for ILM	tube with black cap	tube with black cap

Table 35 SureSelect Max UDI Primers for ILM options

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect Max UDI Primers for ILM*	Blue 8-well strip tube (index pairs 1-8), AND White 8-well strip tube (index pairs 9-16) OR Black 8-well strip tube (index pairs 17-24) AND Red 8-well strip tube (index pairs 25-32)	Orange 96-well plate (index pairs 1–96), OR Blue 96-well plate (index pairs 97–192), OR Green 96-well plate (index pairs 193–288), OR Red 96-well plate (index pairs 289–384)

* See [page 42](#) through [page 44](#) for index strip and plate position maps; see [page 45](#) through [page 52](#) for index pair sequence information.

SureSelect Max UDI Primers Information

The SureSelect Max unique dual indexing (UDI) Primers are provided in pre-combined pairs of indexes. Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. One primer pair is provided in each well of 8-well strip tubes (16 reaction kits; see [Figure 4](#)) or of 96-well plates (96 reaction kits; see [page 43](#) to [page 44](#) for plate maps). Each well contains a single-use aliquot of a specific pair of forward plus reverse primers.

The nucleotide sequence of the index portion of each primer is provided in [Table 40](#) on page 45 through [Table 47](#) on page 52. Index sequences can also be obtained by downloading the [SureSelect Max Index Sequence Resource](#) Excel spreadsheet from Agilent.com.

NOTE

Clicking the Excel spreadsheet link from Agilent.com automatically downloads the index spreadsheet to the default folder for downloaded files saved by your web browser. Locate the file in the folder and open it in Microsoft Excel or another compatible spreadsheet program. The first tab of the spreadsheet provides instructions for use of the spreadsheet contents.

In [Table 40](#) through [Table 47](#) and in the downloadable Excel spreadsheet, P7 indexes are shown in forward orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in both forward and reverse complement orientations. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

Index Primer Pair Strip Tube and Plate Maps

SureSelect Max UDI Primers 1-16 and 17-32 (provided with 16 reaction kits) are supplied in sets of two 8-well strip tubes as detailed below.

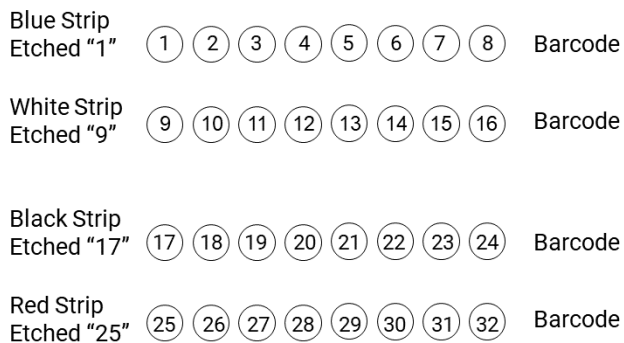


Figure 4 Map of the SureSelect Max UDI Primers for ILM strip tubes in 16 reaction kits

The blue strip contains Index Primer Pairs 1-8, with pair #1 supplied in the well proximal to the numeral **1** etched on the strip's plastic end tab.

The white strip contains Index Primer Pairs 9-16, with pair #9 supplied in the well proximal to the numeral **9** etched on the strip's plastic end tab.

The black strip contains Index Primer Pairs 17-24, with pair #17 supplied in the well proximal to the numeral **17** etched on the strip's plastic end tab.

The red strip contains Index Primer Pairs 25-32, with pair #25 supplied in the well proximal to the numeral **25** etched on the strip's plastic end tab.

When using the strip tube-supplied index primer pairs in the library preparation protocol, pierce the foil seal of the appropriate well with a pipette tip just before pipetting the solution. If the foil seal for any unused wells is disrupted during use, the unused wells may be re-sealed using the provided fresh foil seal strips. The provided foil strips may also be used to re-seal used wells to prevent index primer cross-contamination during subsequent use.

[Table 36](#) through [Table 39](#) show the plate positions of the SureSelect Max UDI Primers for ILM provided with 96 reaction kits.

CAUTION

The SureSelect Max UDI Primers are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well in only one library preparation reaction. Do not retain and re-use any residual volume for subsequent experiments.

Table 36 Plate map for SureSelect Max UDI Primers 1-96, provided in orange plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Table 37 Plate map for SureSelect Max UDI Primers 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Table 38 Plate map for SureSelect Max UDI Primers 193-288, provided in green plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Table 39 Plate map for SureSelect Max UDI Primers 289-384, provided in red plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

SureSelect Max Index Sequences

Table 40 SureSelect Max Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
1	A01	CAAGGTGA	ATGGTTAG	CTAACCAT	25	A04	AGATGGAT	TGGCACCA	TGGTGCCA
2	B01	TAGACCAA	CAAGGTGA	TCACCTTG	26	B04	GAATTGTG	AGATGGAT	ATCCATCT
3	C01	AGTCGCGA	TAGACCAA	TTGGTCTA	27	C04	GAGCACTG	GAATTGTG	CACAATTC
4	D01	CGGTAGAG	AGTCGCGA	TCGCGACT	28	D04	GTTGCGGA	GAGCACTG	CAGTGCTC
5	E01	TCAGCATC	AAGGAGCG	CGCTCCTT	29	E04	AATGGAAC	GTTGCGGA	TCCGCAAC
6	F01	AGAAGCAA	TCAGCATC	GATGCTGA	30	F04	TCAGAGGT	AATGGAAC	GTTCCATT
7	G01	GCAGGTTC	AGAAGCAA	TTGCTTCT	31	G04	GCAACAAT	TCAGAGGT	ACCTCTGA
8	H01	AAGTGTCT	GCAGGTTC	GAACCTGC	32	H04	GTGATCG	GCAACAAT	ATTGTTGC
9	A02	CTACCGAA	AAGTGTCT	AGACACTT	33	A05	ATGGTAGC	GTGATCG	CGATCGAC
10	B02	TAGAGCTC	CTACCGAA	TTCGGTAG	34	B05	CGCCAATT	ATGGTAGC	GCTACCAT
11	C02	ATGTCAAG	TAGAGCTC	GAGCTCTA	35	C05	GACAATTG	CGCCAATT	AATTGGCG
12	D02	GCATCATA	ATGTCAAG	CTTGACAT	36	D05	ATATTCCG	GACAATTG	CAATTGTC
13	E02	GACTTGAC	GCATCATA	TATGATGC	37	E05	TCTACCTC	ATATTCCG	CGGAATAT
14	F02	CTACAATG	GACTTGAC	GTCAAGTC	38	F05	TCGTCGTG	TCTACCTC	GAGGTAGA
15	G02	TCTCAGCA	CTACAATG	CATTGTAG	39	G05	ATGAGAAC	TCGTCGTG	CACGACGA
16	H02	AGACACAC	TCTCAGCA	TGCTGAGA	40	H05	GTCCTATA	ATGAGAAC	GTTTCAT
17	A03	CAGGTCTG	AGACACAC	GTGTGTCT	41	A06	AATGACCA	GTCCTATA	TATAGGAC
18	B03	AATACGCG	CAGGTCTG	CAGACCTG	42	B06	CAGACGCT	AATGACCA	TGGTCATT
19	C03	GCACACAT	AATACGCG	CGCGTATT	43	C06	TCGAACTG	CAGACGCT	AGCGTCTG
20	D03	CTTGCATA	GCACACAT	ATGTGTGC	44	D06	CGCTTCCA	TCGAACTG	CAGTTCGA
21	E03	ATCCTCTT	CTTGCATA	TATGCAAG	45	E06	TATTCCTG	CGCTTCCA	TGGAAGCG
22	F03	GCACCTAA	ATCCTCTT	AAGAGGAT	46	F06	CAAGTTAC	TATTCCTG	CAGGAATA
23	G03	TGCTGCTC	GCACCTAA	TTAGGTGC	47	G06	CAGAGCAG	CAAGTTAC	GTAACCTG
24	H03	TGGCACCA	TGCTGCTC	GAGCAGCA	48	H06	CGCGCAAT	CAGAGCAG	CTGCTCTG

Table 41 SureSelect Max Index Primer Pairs 49–96, provided in orange 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
49	A07	TGAGGAGT	CGCGCAAT	ATTGCGCG	73	A10	AACGCATT	ATAGTGAC	GTCACTAT
50	B07	ATGACGAA	TGAGGAGT	ACTCCTCA	74	B10	CAGTTGCG	AACGCATT	AATGCGTT
51	C07	TACGGCGA	ATGACGAA	TTCGTCAT	75	C10	TGCCTCGA	CAGTTGCG	CGCAACTG
52	D07	AGCGAGTT	TACGGCGA	TCGCCGTA	76	D10	AAGGCTTA	TGCCTCGA	TCGAGGCA
53	E07	TGTATCAC	AGCGAGTT	AACTCGCT	77	E10	GCAATGAA	AAGGCTTA	TAAGCCTT
54	F07	GATCGCCT	TGTATCAC	GTGATACA	78	F10	AAGAACCT	GCAATGAA	TTCATTGC
55	G07	GACTCAAT	GATCGCCT	AGGCGATC	79	G10	CTGTGCCT	AAGAACCT	AGGTTCTT
56	H07	CAGCTTGC	GACTCAAT	ATTGAGTC	80	H10	TACGTAGC	CTGTGCCT	AGGCACAG
57	A08	AGCTGAAG	CAGCTTGC	GCAAGCTG	81	A11	AAGTGGAC	TACGTAGC	GCTACGTA
58	B08	ATTCCGTG	AGCTGAAG	CTTCAGCT	82	B11	CAACCGTG	AAGTGGAC	GTCCACTT
59	C08	TATGCCGC	ATTCCGTG	CACGGAAT	83	C11	CTGTTGTT	CAACCGTG	CACGGTTG
60	D08	TCAGCTCA	TATGCCGC	GCGGCATA	84	D11	GCACGATG	CTGTTGTT	AACAACAG
61	E08	AACTGCAA	TCAGCTCA	TGAGCTGA	85	E11	GTACGGAC	GCACGATG	CATCGTGC
62	F08	ATTAGGAG	AACTGCAA	TTGCAGTT	86	F11	CTCCAAGC	GTACGGAC	GTCCGTAC
63	G08	CAGCAATA	ATTAGGAG	CTCCTAAT	87	G11	TAGTCTGA	CTCCAAGC	GCTTGGAG
64	H08	GCCAAGCT	CAGCAATA	TATTGCTG	88	H11	TTCGCCGT	TAGTCTGA	TCAGACTA
65	A09	TCCGTTAA	GCCAAGCT	AGCTTGGC	89	A12	GAACTAAG	ATACGAAG	CTTCGTAT
66	B09	GTGCAACG	TCCGTTAA	TTAACGGA	90	B12	AAGCCATC	GAGATTCA	TGAATCTC
67	C09	AGTAACGC	GTGCAACG	CGTTGCAC	91	C12	AACTCTTG	AAGCCATC	GATGGCTT
68	D09	CATAGCCA	AGTAACGC	GCGTACT	92	D12	GTAGTCAT	AACTCTTG	CAAGAGTT
69	E09	CACTAGTA	CATAGCCA	TGGCTATG	93	E12	CTCGCTAG	GTAGTCAT	ATGACTAC
70	F09	TTAGTGCG	CACTAGTA	TACTAGTG	94	F12	AGTCTTCA	CAGTATCA	TGATACTG
71	G09	TCGATACA	TTAGTGCG	CGCACTAA	95	G12	TCAAGCTA	CTTCGTAC	GTACGAAG
72	H09	ATAGTGAC	TCGATACA	TGTATCGA	96	H12	CTTATCCT	TCAAGCTA	TAGCTTGA

Table 42 SureSelect Max Index Primer Pairs 97–144, provided in blue 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
97	A01	TCATCCTT	CTTATCCT	AGGATAAG	121	A04	CAGGCAGA	AGACGCCT	AGGCGTCT
98	B01	AACACTCT	TCATCCTT	AAGGATGA	122	B04	TCCGCGAT	CAGGCAGA	TCTGCCTG
99	C01	CACCTAGA	AACACTCT	AGAGTGTT	123	C04	CTCGTACG	TCCGCGAT	ATCGCGGA
100	D01	AGTTCATG	CACCTAGA	TCTAGGTG	124	D04	CACACATA	CTCGTACG	CGTACGAG
101	E01	GTTGGTGT	AGTTCATG	CATGAACT	125	E04	CGTCAAGA	CACACATA	TATGTGTG
102	F01	GCTACGCA	GTTGGTGT	ACACCAAC	126	F04	TTCGCGCA	CGTCAAGA	TCTTGACG
103	G01	TCAACTGC	GCTACGCA	TGCGTAGC	127	G04	CGACTACG	TTCGCGCA	TGCGCGAA
104	H01	AAGCGAAT	TCAACTGC	GCAGTTGA	128	H04	GAAGGTAT	CGACTACG	CGTAGTCG
105	A02	GTGTTACA	AAGCGAAT	ATTCGCTT	129	A05	TTGGCATG	GAAGGTAT	ATACCTTC
106	B02	CAAGCCAT	GTGTTACA	TGTAACAC	130	B05	CGAATTCA	TTGGCATG	CATGCCAA
107	C02	CTCTCGTG	CAAGCCAT	ATGGCTTG	131	C05	TTAGTTGC	CGAATTCA	TGAATTCG
108	D02	TCGACAAC	CTCTCGTG	CACGAGAG	132	D05	GATGCCAA	TTAGTTGC	GCAACTAA
109	E02	TCGATGTT	TCGACAAC	GTTGTCGA	133	E05	AGTTGCCG	GATGCCAA	TTGGCATC
110	F02	CAAGGAAG	TCGATGTT	AACATCGA	134	F05	GTCCACCT	AGTTGCCG	CGGCAACT
111	G02	ATTGATGC	AGAGAATC	GATTCTCT	135	G05	ATCAAGGT	GTCCACCT	AGGTGGAC
112	H02	TCGCAGAT	TTGATGGC	GCCATCAA	136	H05	GAACCAGA	ATCAAGGT	ACCTTGAT
113	A03	GCAGAGAC	TCGCAGAT	ATCTGCGA	137	A06	CATGTTCT	GAACCAGA	TCTGGTTC
114	B03	CTGCGAGA	GCAGAGAC	GTCTCTGC	138	B06	TCACTGTG	CATGTTCT	AGAACATG
115	C03	CAACCAAC	CTGCGAGA	TCTCGCAG	139	C06	ATTGAGCT	TCACTGTG	CACAGTGA
116	D03	ATCATGCG	CAACCAAC	GTTGGTTG	140	D06	GATAGAGA	ATTGAGCT	AGCTCAAT
117	E03	TCTGAGTC	ATCATGCG	CGCATGAT	141	E06	TCTAGAGC	GATAGAGA	TCTCTATC
118	F03	TCGCCTGT	TCTGAGTC	GAATCAGA	142	F06	GAATCGCA	TCTAGAGC	GCTCTAGA
119	G03	GCGCAATT	TCGCCTGT	ACAGGCGA	143	G06	CTTCACGT	GAATCGCA	TGCGATTG
120	H03	AGACGCCT	GCGCAATT	AATTGCGC	144	H06	CTCCGTT	CTTCACGT	ACGTGAAG

Table 43 SureSelect Max Index Primer Pairs 145–192, provided in blue 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
145	A07	TGTGACTA	CTCCGGTT	AACCGGAG	169	A10	CGCTCAGA	CTAACAAG	CTTGTTAG
146	B07	GCTTCCAG	TGTGACTA	TAGTCACA	170	B10	TAACGACA	CGCTCAGA	TCTGAGCG
147	C07	CATCCTGT	GCTTCCAG	CTGGAAGC	171	C10	CATACTTG	TAACGACA	TGTCGTTA
148	D07	GTAATACG	CATCCTGT	ACAGGATG	172	D10	AGATACGA	CATACTTG	CAAGTATG
149	E07	GCCAACAA	GTAATACG	CGTATTAC	173	E10	AATCCGAC	AGATACGA	TCGTATCT
150	F07	CATGACAC	GCCAACAA	TTGTTGGC	174	F10	TGAAGTAC	AATCCGAC	GTCGGATT
151	G07	TGCAATGC	CATGACAC	GTGTCATG	175	G10	CGAATCAT	TGAAGTAC	GTACTIONA
152	H07	CACATTCG	TGCAATGC	GCATTGCA	176	H10	TGATTGGC	CGAATCAT	ATGATTTCG
153	A08	CAATCCGA	CACATTCG	CGAATGTG	177	A11	TCGAAGGA	TGATTGGC	GCCAATCA
154	B08	CATCGACG	CAATCCGA	TCGGATTG	178	B11	CAGTCATT	TCGAAGGA	TCCTTCGA
155	C08	GTGCGCTT	CATCGACG	CGTCGATG	179	C11	CGCGAACA	CAGTCATT	AATGACTG
156	D08	ATAGCGTT	GTGCGCTT	AAGCGCAC	180	D11	TACGGTTG	CGCGAACA	TGTTTCGCG
157	E08	GAGTAAGA	ATAGCGTT	AACGCTAT	181	E11	AGAACCGT	TACGGTTG	CAACCGTA
158	F08	CTGACACA	GAGTAAGA	TCTTACTC	182	F11	AGGTGCTT	AGAACCGT	ACGGTTCT
159	G08	ATACGTGT	CTGACACA	TGTGTCAG	183	G11	ATCGCAAC	AGGTGCTT	AAGCACCT
160	H08	GACCGAGT	ATACGTGT	ACACGTAT	184	H11	GCCTCTCA	ATCGCAAC	GTTGCGAT
161	A09	GCAGTTAG	GACCGAGT	ACTCGGTC	185	A12	TCGCGTCA	GCCTCTCA	TGAGAGGC
162	B09	CGTTCGTC	GCAGTTAG	CTAACTGC	186	B12	GAGTGCCT	TCGCGTCA	TGACGCGA
163	C09	CGTTAACG	CGTTCGTC	GACGAACG	187	C12	CGAACACT	GCATAAGT	ACTTATGC
164	D09	TCGAGCAT	CGTTAACG	CGTTAACG	188	D12	TAAGAGTG	AGAAGACG	CGTCTTCT
165	E09	GCCGTAAC	TCGAGCAT	ATGCTCGA	189	E12	TGGATTGA	TAAGAGTG	CACTCTTA
166	F09	GAGCTGTA	GCCGTAAC	GTTACGGC	190	F12	AGGACATA	TGGATTGA	TCAATCCA
167	G09	AGGAAGAT	GAGCTGTA	TACAGCTC	191	G12	GACATCCT	AGGACATA	TATGTCCT
168	H09	CTAACAAG	AGGAAGAT	ATCTTCCT	192	H12	GAAGCCTC	GACATCCT	AGGATGTC

Table 44 SureSelect Max Index Primer Pairs 193–240, provided in green 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
193	A01	GTCTCTTC	GAAGCCTC	GAGGCTTC	217	A04	GCGGTATG	CACGAGCT	AGCTCGTG
194	B01	AGTCACTT	GTCTCTTC	GAAGAGAC	218	B04	TCTATGCG	GCGGTATG	CATACCGC
195	C01	AGCATACA	AGTCACTT	AAGTGACT	219	C04	AGGTGAGA	TCTATGCG	CGCATAGA
196	D01	TCAGACAA	AGCATACA	TGTATGCT	220	D04	CACAACTT	AGGTGAGA	TCTCACCT
197	E01	TTGGAGAA	TCAGACAA	TTGTCTGA	221	E04	TTGTGTAC	CACAACTT	AAGTTGTG
198	F01	TTAACGTG	TTGGAGAA	TTCTCAA	222	F04	TCACAAGA	TTGTGTAC	GTACACAA
199	G01	CGTCTGTG	TTAACGTG	CACGTAA	223	G04	GAAGACCT	TCACAAGA	TCTTGTGA
200	H01	AACCTAAC	CGTCTGTG	CACAGACG	224	H04	AGTTCTGT	GAAGACCT	AGGTCTTC
201	A02	AGAGTGCT	AACCTAAC	GTTAGGTT	225	A05	GCAGTGTT	AGTTCTGT	ACAGAACT
202	B02	TTATCTCG	AGAGTGCT	AGCACTCT	226	B05	AGGCATGC	GCAGTGTT	AACACTGC
203	C02	CATCAGTC	TTATCTCG	CGAGATAA	227	C05	AAGGTACT	AGGCATGC	GCATGCCT
204	D02	AAGCACAA	CATCAGTC	GACTGATG	228	D05	CACTAAGT	AAGGTACT	AGTACCTT
205	E02	CAGTGAGC	AAGCACAA	TTGTGCTT	229	E05	GAGTCCTA	CACTAAGT	ACTTAGTG
206	F02	GTCGAAGT	CAGTGAGC	GCTCACTG	230	F05	AGTCCTTC	GAGTCCTA	TAGGACTC
207	G02	TCTCATGC	GTCGAAGT	ACTTCGAC	231	G05	TTAGGAAC	AGTCCTTC	GAAGGACT
208	H02	CAGAAGAA	TCTCATGC	GCATGAGA	232	H05	AAGTCCAT	TTAGGAAC	GTTCCCTAA
209	A03	CGGATAGT	CAGAAGAA	TTCTTCTG	233	A06	GAATACGC	AAGTCCAT	ATGGACTT
210	B03	CACGTGAG	CGGATAGT	ACTATCCG	234	B06	TCCAATCA	GAATACGC	GCGTATTC
211	C03	TACGATAC	CACGTGAG	CTCACGTG	235	C06	CGACGGTA	TCCAATCA	TGATTGGA
212	D03	CGCATGCT	TACGATAC	GTATCGTA	236	D06	CATTGCAT	CGACGGTA	TACCGTCG
213	E03	GCTTGCTA	CGCATGCT	AGCATGCG	237	E06	ATCTGCGT	CATTGCAT	ATGCAATG
214	F03	GAACGCAA	GCTTGCTA	TAGCAAGC	238	F06	GTACCTTG	ATCTGCGT	ACGCAGAT
215	G03	ATCTACCA	GAACGCAA	TTGCGTTC	239	G06	GAGCATAC	GTACCTTG	CAAGGTAC
216	H03	CACGAGCT	ATCTACCA	TGGTAGAT	240	H06	TGCTTACG	GAGCATAC	GTATGCTC

Table 45 SureSelect Max Index Primer Pairs 241–288, provided in green 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
241	A07	AAGAGACA	TGCTTACG	CGTAAGCA	265	A10	CAATGCTG	CATGAATG	CATTCATG
242	B07	TAGCTATG	AAGAGACA	TGTCTCTT	266	B10	CTTGATCA	CAATGCTG	CAGCATTG
243	C07	TCTGCTAC	TAGCTATG	CATAGCTA	267	C10	GCGAATTA	CTTGATCA	TGATCAAG
244	D07	GTCACAGA	TCTGCTAC	GTAGCAGA	268	D10	GTTGAGC	GCGAATTA	TAATTCGC
245	E07	CGATTGAA	GTCACAGA	TCTGTGAC	269	E10	GCCAGTAG	GTTGAGC	GCTCGAAC
246	F07	GAGAGATT	CGATTGAA	TTCAATCG	270	F10	AAGGTCGA	GCCAGTAG	CTACTGGC
247	G07	TCATACCG	GAGAGATT	AATCTCTC	271	G10	AGTGAAGT	CACTTATG	CATAAGTG
248	H07	TCCGAACT	TCATACCG	CGGTATGA	272	H10	GTTGCAAG	ATAACGGC	GCCGTTAT
249	A08	AGAGAGAA	TCCGAACT	AGTTCGGA	273	A11	AGCCGGAA	GTTGCAAG	CTTGCAAC
250	B08	GATCGTTA	AGAGAGAA	TTCTCTCT	274	B11	AACAGCCG	AGCCGGAA	TTCCGGCT
251	C08	GCGCTAGA	GATCGTTA	TAACGATC	275	C11	CTAGTGTA	AACAGCCG	CGGCTGTT
252	D08	ATGACTCG	GCGCTAGA	TCTAGCGC	276	D11	GAGGCTCT	CTAGTGTA	TACTACTAG
253	E08	CAATAGAC	ATGACTCG	CGAGTCAT	277	E11	CTCCGCAA	GAGGCTCT	AGAGCCTC
254	F08	CGATATGC	CAATAGAC	GTCTATTG	278	F11	CGCTATTG	CTCCGCAA	TTGCGGAG
255	G08	GTCAGAAT	CGATATGC	GCATATCG	279	G11	GTGTTGAG	CGCTATTG	CAATAGCG
256	H08	CATAAGGT	GCACTACT	AGTAGTGC	280	H11	TCACCGAC	GTGTTGAG	CTCAACAC
257	A09	TGTTGGTT	GATTCGGC	GCCGAATC	281	A12	CGGTAATC	TCACCGAC	GTCGGTGA
258	B09	ATACTCGC	TGTTGGTT	AACCAACA	282	B12	GTGACTGC	CGGTAATC	GATTACCG
259	C09	AATGCTAG	ATACTCGC	GCGAGTAT	283	C12	CGACTTGT	GTGACTGC	GCAGTCAC
260	D09	GCCTAGGA	AATGCTAG	CTAGCATT	284	D12	GATAGGAC	CGACTTGT	ACAAGTCG
261	E09	GCAACCGA	GCCTAGGA	TCCTAGGC	285	E12	AAGTACTC	GATAGGAC	GTCCTATC
262	F09	ATACTGCA	GCAACCGA	TCGTTGTC	286	F12	GCTCTCTC	AAGTACTC	GAGTACTT
263	G09	TCTCCTTG	ATACTGCA	TGCAGTAT	287	G12	CTACCAGT	GCTCTCTC	GAGAGAGC
264	H09	CATGAATG	TCTCCTTG	CAAGGAGA	288	H12	GATGAGAT	CTACCAGT	ACTGGTAG

Table 46 SureSelect Max Index Primer Pairs 289–336, provided in red 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
289	A01	AGATAGTG	GATGAGAT	ATCTCATC	313	A04	AGCTACAT	GATCCATG	CATGGATC
290	B01	AGAGGTTA	AGATAGTG	CACTATCT	314	B04	CGCTGTAA	AGCTACAT	ATGTAGCT
291	C01	CTGACCGT	AGAGGTTA	TAACCTCT	315	C04	CACTACCG	CGCTGTAA	TTACAGCG
292	D01	GCATGGAG	CTGACCGT	ACGGTCAG	316	D04	GCTCACGA	CACTACCG	CGGTAGTG
293	E01	CTGCCTTA	GCATGGAG	CTCCATGC	317	E04	TGGCTTAG	GCTCACGA	TCGTGAGC
294	F01	GCGTCACT	CTGCCTTA	TAAGGCAG	318	F04	TCCAGACG	TGGCTTAG	CTAAGCCA
295	G01	GCGATTAC	GCGTCACT	AGTGACGC	319	G04	AGTGGCAT	TCCAGACG	CGTCTGGA
296	H01	TCACCACG	GCGATTAC	GTAATCGC	320	H04	TGTACCGA	AGTGGCAT	ATGCCACT
297	A02	AGACCTGA	TCACCACG	CGTGGTGA	321	A05	AAGACTAC	TGTACCGA	TCGGTACA
298	B02	GCCGATAT	AGACCTGA	TCAGGTCT	322	B05	TGCCGTTA	AAGACTAC	GTAGTCTT
299	C02	CTTATTGC	GCCGATAT	ATATCGGC	323	C05	TTGGATCT	TGCCGTTA	TAACGGCA
300	D02	CGATACCT	CTTATTGC	GCAATAAG	324	D05	TCCTCAA	TTGGATCT	AGATCCAA
301	E02	CTCGACAT	CGATACCT	AGGTATCG	325	E05	CGAGTCGA	TCCTCAA	TTGGAGGA
302	F02	GAGATCGC	CTCGACAT	ATGTGCGAG	326	F05	AGGCTCAT	CGAGTCGA	TCGACTCG
303	G02	CGGTCTCT	GAGATCGC	GCGATCTC	327	G05	GACGTGCA	AGGCTCAT	ATGAGCCT
304	H02	TAACACAC	CGGTCTCT	AGAGACCG	328	H05	GAACATGT	GACGTGCA	TGCACGTC
305	A03	CACAATGA	TAACACAC	GTGAGTTA	329	A06	AATTGGCA	GAACATGT	ACATGTTC
306	B03	GACTGACG	CACAATGA	TCATTGTG	330	B06	TGGAGACT	AATTGGCA	TGCCAATT
307	C03	CTTAAGAC	GACTGACG	CGTCAGTC	331	C06	AACTCACA	TGGAGACT	AGTCTCCA
308	D03	GAGTGTAG	CTTAAGAC	GTCTTAAG	332	D06	GTAGACTG	AACTCACA	TGTGAGTT
309	E03	TGCACATC	GAGTGTAG	CTACACTC	333	E06	CGTAGTTA	GTAGACTG	CAGTCTAC
310	F03	CGATGTCG	TGCACATC	GATGTGCA	334	F06	CGTCAGAT	CGTAGTTA	TAACACG
311	G03	AACACCGA	CGATGTCG	CGACATCG	335	G06	AACGGTCA	CGTCAGAT	ATCTGACG
312	H03	GATCCATG	AACACCGA	TCGGTGTT	336	H06	GCCTTCAT	AACGGTCA	TGACCGTT

Table 47 SureSelect Max Index Primer Pairs 337–384, provided in red 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
337	A07	TGAGACGC	GCCTTCAT	ATGAAGGC	361	A10	CTGAGCTA	GCACAGTA	TACTGTGC
338	B07	CATCGGAA	TGAGACGC	GCGTCTCA	362	B10	CTTGCGAT	CTGAGCTA	TAGCTCAG
339	C07	TAGGACAT	CATCGGAA	TTCCGATG	363	C10	GAAGTAGT	CTTGCGAT	ATCGCAAG
340	D07	AACACAAG	TAGGACAT	ATGTCCTA	364	D10	GTTATCGA	GAAGTAGT	ACTACTTC
341	E07	TTCGACTC	AACACAAG	CTTGTGTT	365	E10	TGTCGTCG	GTTATCGA	TCGATAAC
342	F07	GTCGGTAA	TTCGACTC	GAGTCGAA	366	F10	CGTAACTG	TGTCGTCG	CGACGACA
343	G07	GTTTCATTC	GTCGGTAA	TTACCGAC	367	G10	GCATGCCT	CGTAACTG	CAGTTACG
344	H07	AAGCAGTT	GTTTCATTC	GAATGAAC	368	H10	TCGTACAC	GCATGCCT	AGGCATGC
345	A08	ATAAGCTG	AAGCAGTT	AACTGCTT	369	A11	CACAGGTG	TCGTACAC	GTGTACGA
346	B08	GCTTAGCG	ATAAGCTG	CAGCTTAT	370	B11	AGCAGTGA	CACAGGTG	CACCTGTG
347	C08	TTCCAACA	GCTTAGCG	CGCTAAGC	371	C11	ATTCCAGA	AGCAGTGA	TCACTGCT
348	D08	TACCGCAT	TTCCAACA	TGTTGGAA	372	D11	TCCTTGAG	ATTCCAGA	TCTGGAAT
349	E08	AGGCAATG	TACCGCAT	ATGCGGTA	373	E11	ATACCTAC	TCCTTGAG	CTCAAGGA
350	F08	GCCTCGTT	AGGCAATG	CATTGCCT	374	F11	AGACCATT	ATACCTAC	GTAGGTAT
351	G08	CACGGATC	GCCTCGTT	AACGAGGC	375	G11	CGTAAGCA	AGACCATT	AATGGTCT
352	H08	GAGACACG	CACGGATC	GATCCGTG	376	H11	TCTGTCAG	CGTAAGCA	TGCTTACG
353	A09	AGAGTAAG	GAGACACG	CGTGTCTC	377	A12	CACAGACT	TCTGTCAG	CTGACAGA
354	B09	AGTACGTT	AGAGTAAG	CTTACTCT	378	B12	GTCGCCTA	CACAGACT	AGTCTGTG
355	C09	AACGCTGC	AGTACGTT	AACGTAAT	379	C12	TGCGCTCT	GTCGCCTA	TAGGCGAC
356	D09	GTAGAGCA	AACGCTGC	GCAGCGTT	380	D12	GCTATAAG	TGCGCTCT	AGAGCGCA
357	E09	TCCTGAGA	GTAGAGCA	TGCTCTAC	381	E12	CAACAATC	GCTATAAG	CTTATAGC
358	F09	CTGAATAG	TCCTGAGA	TCTCAGGA	382	F12	AGAGAATC	CTCTCACT	AGTGAGAG
359	G09	CAAGACTA	CTGAATAG	CTATTCAG	383	G12	TAATGGTC	AGACGAGC	GCTCGTCT
360	H09	GCACAGTA	CAAGACTA	TAGTCTTG	384	H12	GTTGTATC	TAATGGTC	GACCATTA

Troubleshooting Guide

If yield of libraries is low

- ✓ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✓ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the PCR cycle number by 1 to 2 cycles.
- ✓ Yield from the magnetic bead purification step may be suboptimal. Consider the factors below:
 - Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.
 - Ensure that the magnetic beads are not over-dried just prior to sample elution (see [step 12 on page 27](#) and [page 30](#)). Monitor the bead pellets frequently while drying and conclude the drying step immediately after the residual ethanol has evaporated.
 - Increasing the elution incubation time (up to 10 minutes) may improve recovery, especially for longer cDNA fragments.

If solids observed in the End Repair-A Tailing Buffer

- ✓ Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

If library fragment size is different than expected in electropherograms

- ✓ cDNA fragment size selection during magnetic bead purification steps depends upon using the correct ratio of sample to beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color. Verify that you are using the recommended bead volume at each stage of the protocol.

If low molecular weight adaptor-dimer peak is present in library electropherograms

- ✓ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed with library samples for which adaptor-dimers are observed in the electropherogram at low abundance. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries. If excessive adaptor-dimers are observed, verify that the adaptor ligation protocol is being performed as directed on [page 25](#). In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the 5-fold diluted SureSelect Max Adaptor Oligo Mix to the mixture. Do not add the Ligation master mix and the Adaptor Oligo Mix to the sample in a single step.
- ✓ Perform an additional round of purification using AMPure XP beads after the ligation step, as described on [page 25](#). Two serial purifications are used in the standard protocol for low-input or lower-quality libraries, but two serial purification steps may be implemented in the library preparation protocol for RNA input of any quantity and quality.

If your libraries are MBC-tagged but your sequence analysis pipeline excludes MBCs

- ✓ You can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis using one of the options below:
 - If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y***. If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y***. For both methods, * is replaced with value equal to the remaining read length after masking or trimming. For example, use **N5Y146,I8,I8,N5Y146** for 2x150 NGS with 151 cycles (as shown in [Table 29](#) on page 35). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

- The first 5 bases may be trimmed from the demultiplexed FASTQ files using the AGeNT Trimmer module while trimming adaptor sequences or using a suitable processing tool of your choice, such as seqtk. Non-Agilent adaptor trimmers may fail to remove the MBC sequences from the opposite adaptor (refer to [Figure 3](#)), which may affect alignment quality.

Quick Reference Protocol: mRNA Library Prep

An abbreviated protocol summary is provided below for experienced users. Use the complete protocol on [page 14](#) to [page 31](#) until you are familiar with all of the protocol details.

Step	Summary of Conditions
PolyA-RNA Selection	
Prepare and qualify RNA samples	Prepare total RNA samples in nuclease-free H ₂ O. Qualify integrity (RIN). For RIN>8 use 10–1000 ng RNA input. For RIN 6 to 8, use 50–1000 ng RNA input. Samples with RIN<6 are not recommended for use in this protocol. Place the 10–1000 ng RNA samples in 25 µL nuclease-free H ₂ O in strip tube or plate wells.
Denature and bind poly-A mRNA to oligo(dT) beads	25 µL total RNA sample + 25 µL Oligo(dT) Microparticles suspension Incubate in thermal cycler for RNA denaturation: 5 min @ 65°C, 1 min @ 4°C, Hold @ 4°C. Incubate 5 min at room temperature (RT) for bead binding.
Wash and elute bead-bound mRNA	Collect Oligo(dT) beads with magnetic stand> discard supernatant. Wash beads with 200 µL Bead Washing Buffer> collect beads with magnetic stand> discard supernatant. Resuspend beads with 25 µL Bead Elution Buffer. Incubate in thermal cycler for RNA elution: 2 min @ 80°C, 1 min @ 4°C, Hold @ 4°C.
Re-bind poly-A mRNA to oligo(dT) beads	25 µL eluted RNA in Oligo(dT) bead suspension + 25 µL Bead Binding Buffer Incubate 5 min at RT for bead re-binding.
Wash and elute enriched mRNA	Collect Oligo(dT) beads with magnetic stand> discard supernatant. Wash beads with 200 µL Bead Washing Buffer> collect beads with magnetic stand> discard supernatant. Add 10 µL nuclease-free H ₂ O, retaining beads and liquid in sample well> keep on ice.
RNA Fragmentation and cDNA Synthesis	
Fragment mRNA and prime cDNA synthesis	10 µL bead-bound enriched poly-A mRNA + 10 µL 2× Priming Buffer Fragment using thermal cycler program 4 min @ 94°C, 1min @ 4°C, Hold @ 4°C. Collect Oligo(dT) beads with magnetic stand> transfer 20 µL supernatant to fresh well.
Synthesize first-strand cDNA	20 µL primed RNA fragments + 4 µL First Strand Master Mix Mix> spin> incubate in thermal cycler: 10 min @ 25°C, 40 min @ 37°C, Hold @ 4°C.
Synthesize second-strand cDNA	24 µL first-strand cDNA + 25 µL Second Strand Enzyme Mix + 5 µL Second Strand Oligo Mix Prepare on ice> mix> spin> incubate in thermal cycler: 60 min @ 16°C, Hold @ 4°C.
Purify cDNA	54 µL cDNA sample + 105 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 52 µL nuclease-free H ₂ O> mix> incubate 2-5 minutes> collect beads> transfer 50 µL supernatant to fresh well> keep on ice.
cDNA Library Preparation	
Prepare Ligation master mix	Per 8 reactions: 207 µL Ligation Buffer + 18 µL T4 DNA Ligase Per 24 reactions: 598 µL Ligation Buffer + 52 µL T4 DNA Ligase Prepare at RT> mix> spin> keep at RT 30–45 min before use.
Prepare End-Repair/dA-Tailing master mix	Per 8 reactions: 144 µL End Repair-A Tailing Buffer + 36 µL End Repair-A Tailing Enzyme Mix Per 24 reactions: 416 µL End Repair-A Tailing Buffer + 104 µL End Repair-A Tailing Enzyme Mix Prepare on ice> mix> spin> keep on ice.

Step	Summary of Conditions
End-Repair and dA-Tail the cDNA fragments	50 µL cDNA fragments + 20 µL End Repair/dA-Tailing master mix Mix> spin> incubate in thermal cycler: 15 min @ 20°C, 15 min @ 65°C, Hold @ 4°C.
Dilute then ligate adaptor	Dilute the SureSelect Max Adaptor Oligo Mix (MBC or MBC-Free) 5-fold with 1X Low TE Buffer. 70 µL cDNA sample + 25 µL Ligation master mix> mix> spin. Add 5 µL diluted SureSelect Max Adaptor Oligo Mix> mix> spin. Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C.
Purify cDNA library	For high quality RNA samples (1x purification): 100 µL cDNA library + 80 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 21 µL nuclease-free H ₂ O> mix> incubate 2-5 minutes> collect beads> transfer 20 µL supernatant to fresh well> keep on ice. For low quality RNA samples (2x purification): 100 µL cDNA library + 80 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 50 µL nuclease-free H ₂ O> mix> incubate 2-5 minutes> collect beads> transfer 50 µL supernatant to fresh well> keep on ice. 50 µL cDNA library supernatant + 60 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 21 µL nuclease-free H ₂ O> mix> incubate 2-5 minutes> collect beads> transfer 20 µL supernatant to fresh well> keep on ice.
Index and amplify library	20 µL cDNA library + 25 µL Amplification Master Mix + 5 µL SureSelect Max UDI Primers for ILM Vortex> spin> amplify in thermal cycler using program in Table 48 .
Purify amplified library	50 µL amplified cDNA + 50 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 15 µL 1X Low TE Buffer> mix> incubate 2-5 minutes> collect beads> transfer 14 µL supernatant to fresh well.
Quantify and qualify final cDNA library	Analyze quantity and quality using TapeStation or Fragment Analyzer System.

Table 48 Library indexing/amplification thermal cycler program (50 µL vol; heated lid at 105°C)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	1000 ng RNA input-8 cycles	98°C	15 seconds
	250 ng RNA input-9 cycles	60°C	30 seconds
	100 ng RNA input-10 cycles	72°C	30 seconds
	50 ng RNA input-12 cycles		
	10 ng RNA input-14 cycles		
3	1	72°C	1 minute
4	1	4°C	Hold

In This Book

This guide provides instructions for SureSelect Max mRNA Library Preparation for Illumina sequencing.

