SureSelect^{QXT} Target Enrichment for the Illumina Platform

Featuring Transposase-Based Library Prep Technology

Protocol

Version F2, July 2021

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

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CAUTION

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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{QXT} Target Enrichment system.

This protocol is specifically developed and optimized to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus prior to sample sequencing.

If you wish to prepare whole-genome libraries using the SureSelect^{QXT} system, instead see publication part number G9682-90000 at www.agilent.com.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps to prepare gDNA sequencing libraries for target enrichment.

3 Hybridization and Capture

This chapter describes the steps to hybridize and capture the prepared DNA library using a SureSelect or ClearSeq Probe.

4 Indexing and Sample Processing for Multiplexed Sequencing

This chapter describes the steps for post-capture amplification and guidelines for sequencing sample preparation.

5 Reference

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

What's New in Version F2

- Support for SureSelect XT HS Human All Exon V8 Probe and updated recommendation for use of SSel XT HS and XT Low Input Human All Exon V7 Probe (see Table 2 on page 14)
- Updates to instructions in the "Hybridization and Capture" chapter on page 35 to page 42 including addition of hybridization temperature considerations for probes designed for use with the SureSelect XT system (see footnote to Table 13 on page 38)
- Updates to downstream sequencing guidelines (see Table 22 on page 58)

What's New in Version F1

- Updates to downstream sequencing support information including sequencing kit selection and seeding concentration updates (see Table 22 on page 58) and support for the NovaSeq platform (see page 58 through page 65 and see page 71)
- Updates to instructions for adaptor trimming using SureCall (see page 63) or AGeNT (see page 65)

What's New in Version F0

- Support for revised SureSelect custom probe products, produced using an updated manufacturing process beginning August, 2020 (see Table 3 on page 15). Custom probes produced using the legacy manufacturing process are also fully supported by the protocols in this document. Probe information was reorganized (see Table 2 on page 14 and Table 3 on page 15), and probe nomenclature throughout document was updated.
- Updates to thermal cycler and plasticware recommendations (see *Caution* and Table 5 on page 16 and see step 4 on page 21 for example of updated usage instructions).

- Updates to ordering information for Dynabeads MyOne Streptavidin T1 beads, AMPure XP Kits, and 1X Low TE Buffer (see Table 1 on page 13) and for Eppendorf ThermoMixer C and Qubit Fluorometer (see Table 5 on page 16).
- Support for Agilent 4150 TapeStation system and Agilent 5200 Fragment Analyzer system (see Table 5 on page 16).
- Minor updates to Bioanalyzer and TapeStation assay use instructions and reference document links (see page 30, page 32, page 51, and page 53.
- Removal of reference information for expired SureSelect^{QXT} Reagent Kits p/n G9681A/G9681B, replaced by G9683A/G9683B in 2018 (see Table 1 on page 13, Table 34 on page 68, and Table 35 on page 68 for current Reagent Kit information).
- Updates to Technical Support contact information (see page 2)

Content

1 Before You Begin

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

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

Overview of the Workflow

The SureSelect^{QXT} target enrichment workflow is summarized in Figure 1.

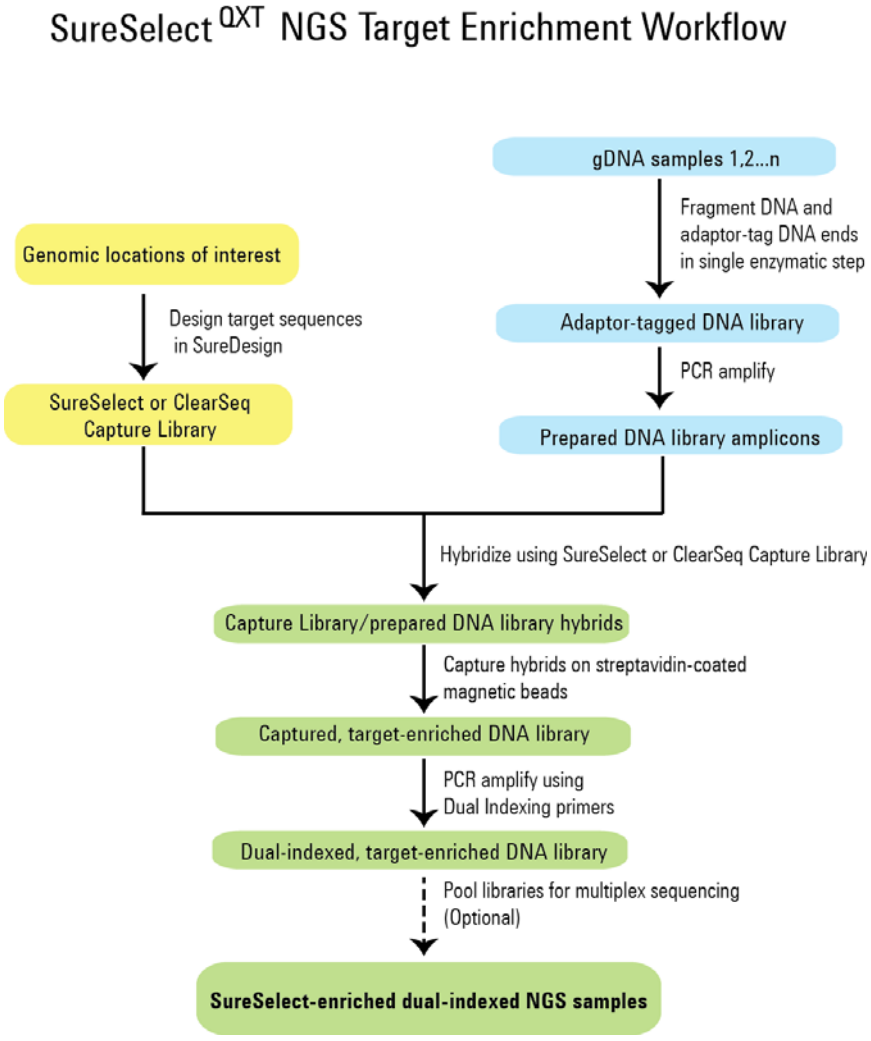

Figure 1 Overall target-enriched sequencing sample preparation workflow.

Procedural Notes

- The SureSelect^{QXT} system requires high-quality DNA samples for optimal performance. Use best practices for verifying DNA sample quality before initiating the workflow. For best practice, store diluted DNA solutions at 4°C to avoid repeated freeze-thaw cycles, which may compromise DNA quality.
- Performance of the SureSelect^{QXT} library preparation protocol is very sensitive to variations in amounts of DNA sample and other reaction components. It is important to quantify and dilute DNA samples as described on page 21. Carefully measure volumes for all reaction components, and combine components as described on page 22. Use best-practices for liquid handling, including regular pipette calibration, to ensure precise volume measurement.
- Use care in handling the SureSelect QXT Enzyme Mix. After removing the vial from storage at –20°C, keep on ice or in a cold block while in use. Return the vial to storage at –20°C promptly after use.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of domed 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.
- Use best-practices to prevent PCR product 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 pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  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.

- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.
- 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.
- In general, follow Biosafety Level 1 (BSL1) safety rules.

Safety Notes

CAUTION

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

Required Reagents

Table 1 Required Reagents

Description	Vendor and part number
SureSelect or ClearSeq Probe	Select the appropriate probe from Table 2 or Table 3
SureSelect^{QXT} Reagent Kit (for Illumina HiSeq, MiSeq, and NextSeq platforms)	Agilent
16 Samples	p/n G9683A
96 Samples	p/n G9683B
AMPure XP Kit	Beckman Coulter Genomics
5 ml	p/n A63880
60 ml	p/n A63881
450 ml	p/n A63882
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 ml	p/n 65601
10 ml	p/n 65602
50 ml	p/n 65604D
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Qubit dsDNA HS Assay Kit *or*	Thermo Fisher Scientific p/n Q32851
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

Table 2 Compatible Pre-Designed Probes

Probe	16 Reactions	96 Reactions
SureSelect XT HS Human All Exon V8	5191-6783	5191-6784
SSel XT HS and XT Low Input Human All Exon V7	5191-4028	5191-4029
SureSelect XT Human All Exon V6	5190-8863	5190-8864
SureSelect XT Human All Exon V6 + UTRs	5190-8881	5190-8882
SureSelect XT Human All Exon V6 + COSMIC	5190-9307	5190-9308
SureSelect XT Clinical Research Exome V2	5190-9491	5190-9492
SureSelect XT Focused Exome	5190-7787	5190-7788
SureSelect XT Mouse All Exon	5190-4641	5190-4642
ClearSeq Comprehensive Cancer XT	5190-8011	5190-8012
ClearSeq Inherited Disease XT	5190-7518	5190-7519
Pre-designed Probes customized with additional *Plus* custom content		
SSel XT HS and XT Low Input Human All Exon V7 Plus 1	Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.	
SSel XT HS and XT Low Input Human All Exon V7 Plus 2		
SureSelect XT Human All Exon V6 Plus 1		
SureSelect XT Human All Exon V6 Plus 2		
SureSelect XT Clinical Research Exome V2 Plus 1		
SureSelect XT Clinical Research Exome V2 Plus 2		
SureSelect XT Focused Exome Plus 1		
SureSelect XT Focused Exome Plus 2		
ClearSeq Comprehensive Cancer Plus XT		
ClearSeq Inherited Disease Plus XT		

Table 3 Compatible Custom Probes*

Probe	16 Reactions	96 Reactions
SureSelect Custom Tier1 1–499 kb	Please visit the SureDesign website to design Custom SureSelect probes and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance. Custom probes are also available in 480 Reaction size.	
SureSelect Custom Tier2 0.5 –2.9 Mb		
SureSelect Custom Tier3 3 –5.9 Mb		
SureSelect Custom Tier4 6 –11.9 Mb		
SureSelect Custom Tier5 12–24 Mb		

* Custom Probes designed August 2020 or later are produced using an updated manufacturing process; design size Tier is shown on labeling for these products. Custom Probes designed and ordered prior to August 2020 may be reordered, with these probes produced using the legacy manufacturing process; design-size Tier is not shown on labeling for the legacy-process products. Custom Probes of both categories use the same optimized target enrichment protocols detailed in this publication

Optional Reagents

Table 4 Optional Reagents

Description	Vendor and part number
Agilent QPCR NGS Library Quantification Kit (Illumina GA)	Agilent p/n G4880A

Required Equipment

CAUTION Sample volumes exceed 0.2 ml in certain steps of this protocol. Make sure that the plasticware used with the selected thermal cycler holds ≥0.25 ml per well.

Table 5 Required Equipment

Description	Vendor and part number
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, domed	Consult the thermal cycler manufacturer's recommendations
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-ml PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
96-well plate mixer	Eppendorf ThermoMixer C, p/n 5382000023 and Eppendorf SmartBlock 96 PCR, p/n 5306000006, or equivalent
Vortex mixer	general laboratory supplier
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
P10, P20, P200 and P1000 pipettes	Rainin Pipet-Lite Pipettes or equivalent

Table 5 Required Equipment

Description	Vendor and part number
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
DNA Analysis Platform and Consumables*	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200/4150 TapeStation	Agilent p/n G2991AA/G2992AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent†
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier

* DNA samples may also be analyzed using the Agilent 5200 Fragment Analyzer, p/n M5310AA, and associated NGS Fragment Kits (DNF-473-0500 and DNF-474-0500). Implement any sample dilution instructions provided in protocols in this document, and then follow the assay instructions provided for each NGS Fragment Kit.

† Select a magnetic separator configured to collect magnetic particles on one side of each well. Do not use a magnetic separator configured to collect the particles in a ring formation.

2 Sample Preparation

This section contains instructions for preparation of genomic DNA sequencing libraries prior to target enrichment, for subsequent sequencing on Illumina platforms.

Step 1. Fragment and adaptor-tag the genomic DNA samples

In this step, the gDNA is enzymatically fragmented and adaptors are added to ends of the fragments in a single reaction. This step uses the SureSelect^{QXT} Reagent Kit components listed in Table 6 in addition to some reagents obtained from other suppliers (see Table 1 on page 13).

Table 6 Reagents for DNA fragmentation and adaptor-tagging

Kit Component	Storage Location	Where Used
SureSelect QXT Stop Solution	SureSelect QXT Hyb Module Box 1, Room Temperature	page 20 (below)
SureSelect QXT Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 22
SureSelect QXT Enzyme Mix ILM	SureSelect QXT Library Prep Kit Box 2, –20°C	page 22

Before you begin, remove the SureSelect QXT Enzyme Mix ILM and the SureSelect QXT Buffer tubes from storage at –20°C and place on ice. Vortex each reagent vigorously to mix before use. Remove the AMPure XP beads from storage at 4°C and allow to warm up to room temperature.

NOTE While obtaining components for this step, also remove the DMSO vial from the SureSelect QXT Library Prep Kit Box 2 in –20°C storage. Leave the DMSO vial at room temperature in preparation for use on page 26.

For each DNA sample to be sequenced, prepare 1 library.

1 Verify that the SureSelect QXT Stop Solution contains 25% ethanol, by referring to the container label and the instructions below.

Before the first use of a fresh container, add 1.5 ml of ethanol to the provided bottle containing 4.5 ml of stop solution, for a final ethanol concentration of 25%. Seal the bottle then vortex well to mix. After adding the ethanol, be sure to mark the label for reference by later users.

Keep the prepared 1X SureSelect QXT Stop Solution at room temperature, tightly sealed, until it is used on page 23.

2 Prepare reagents for the purification protocols on page 24 and page 28.

   a Transfer the AMPure XP beads to room temperature. The beads should be held at room temperature for at least 30 minutes before use. *Do not freeze the beads at any time.*

   b Prepare 800 µl of fresh 70% ethanol per sample, plus excess, for use in the purification steps. The 70% ethanol may be used for multiple steps done on the same day, when stored in a sealed container.

3 Quantify and dilute gDNA samples using two serial fluorometric assays:

   a Use the Qubit dsDNA BR Assay or Qubit dsDNA HS Assay to determine the initial concentration of each gDNA sample. Follow the manufacturer's instructions for the specific assay kit and the Qubit instrument. This step is critical for successful preparation of input DNA at the required concentration to ensure optimal fragmentation.

   b Dilute each gDNA sample with nuclease-free water to a final concentration of 100 ng/µl in a LoBind tube.

   c Carefully measure the DNA concentration of each of the 100 ng/µl dilutions using a second Qubit dsDNA BR or HS Assay.

   d Adjust each gDNA sample with nuclease-free water to a final concentration of 25 ng/µl in a LoBind tube.

CAUTION The duration and temperature of incubation for DNA fragmentation must be precisely controlled for optimal results. Make sure to preprogram the thermal cycler as directed in step 4 before setting up the fragmentation reactions. **Do not exceed 10 minutes at 45°C**, as indicated in Table 7.

4 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 7. Immediately pause the program, and keep paused until samples are loaded in step 8.

Table 7 Thermal cycler program for DNA fragmentation

Step	Temperature	Time
Step 1	45°C	10 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

5 Before use, vortex the SureSelect QXT Buffer and SureSelect QXT Enzyme Mix ILM tubes vigorously at high speed. Note that the SSEL QXT Buffer is viscous and thorough and vigorous mixing is critical for optimal fragmentation.

These components are in liquid form when removed from –20°C storage and should be returned to –20°C storage promptly after use in step 6.

CAUTION Minor variations in volumes of the solutions combined in step 6 below may result in DNA fragment size variation.

The SureSelect QXT Buffer and Enzyme Mix solutions are highly viscous. Be sure to follow the dispensing and mixing instructions in the steps below. Thorough mixing of the reagents and reactions is critical for optimal performance.

6 Set up the fragmentation reactions on ice using a PCR plate or strip tube. Components must be added in the order listed below. Do not pre-mix the SureSelect QXT Buffer and Enzyme Mix.

- **a** To each sample well, add 17 µl of SureSelect QXT Buffer.
- **b** Add 2 µl of each DNA sample to its assigned sample well. While dispensing the DNA, be sure to place the pipette tip at the bottom of the well.
- **c** Add 2 µl of SureSelect QXT Enzyme Mix, ILM to each sample well. While dispensing the enzyme mixture, place the pipette tip at the bottom of the well. After dispensing of the 2 µl of enzyme mix, pipette up and down 8 to 10 times to ensure complete transfer of the viscous solution to the well.

7 Seal the wells, briefly spin, then **mix thoroughly by vortexing the plate or strip tube at high speed for 20 seconds**.

8 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 7.

9 During the 10-minute thermal cycler incubation, vigorously vortex the AMPure XP beads at high speed to ensure homogeneous distribution of beads throughout the solution so that the beads are ready for use on page 24.

10 When the thermal cycler has completed the 1-minute incubation at 4°C, immediately place the samples on ice and proceed to step 11.

11 Add 32 µl of 1X SureSelect QXT Stop Solution (containing 25% ethanol) to each fragmentation reaction. Seal the wells with fresh caps, then vortex at high speed for 5 seconds. Briefly spin the plate or strip tube to collect the liquid.

Incubate the samples at room temperature for 1 minute. Proceed directly to the purification protocol on page 24.

Step 2. Purify the adaptor-tagged library using AMPure XP beads

Before you begin, verify that the AMPure XP beads have been incubated at room temperature for at least 30 minutes and that fresh 70% ethanol has been prepared for use in step 6.

1. Verify that the AMPure XP bead suspension has been well mixed and appears homogeneous and consistent in color.
2. Add 52 µl of the homogeneous bead suspension to each well containing the DNA samples. Seal the wells with fresh caps, then vortex for 5 seconds. Briefly spin the samples to collect the liquid, without pelleting the beads.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
3. Incubate samples for 5 minutes at room temperature.
4. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
5. While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
6. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µl of fresh 70% ethanol in each sample well.
7. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
8. Repeat step 6 and step 7 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
9. Dry the samples on the thermal cycler (with lid open) at 37°C for 1 to 3 minutes. Do not overdry the samples.
10. Add 11 µl of nuclease-free water to each sample well.
11. Seal the sample wells with fresh caps, then mix well on a vortex mixer and briefly spin the plate or tubes to collect the liquid.
12. Incubate for 2 minutes at room temperature.
13. Put the plate or tubes in the magnetic stand and leave for 2 minutes or until the solution in each well is clear.

14 Remove each cleared supernatant (approximately 10 µl) to wells of a fresh plate or strip tube and keep on ice. You can discard the beads at this time.

Step 3. Amplify the adaptor-tagged DNA library

In this step, the adaptor-tagged gDNA library is repaired and PCR-amplified.

1 Thaw then vortex to mix the reagents listed in Table 8. Keep all reagents except DMSO on ice.

Table 8 Reagents for precapture amplification

Kit Component	Storage Location	Where Used
Herculase II Fusion DNA Polymerase	SureSelect QXT Library Prep Kit Box 2, –20°C	page 26 (below)
Herculase II 5× Reaction Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 26 (below)
100 mM dNTP Mix (25 mM each dNTP)	SureSelect QXT Library Prep Kit Box 2, –20°C	page 26 (below)
SureSelect QXT Primer Mix	SureSelect QXT Hyb Module Box 2, –20°C	page 26 (below)
DMSO	Transferred to Room Temperature storage on page 20	page 26 (below)

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 9, on ice. Mix well on a vortex mixer.

Table 9 Preparation of pre-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	25 µl	425 µl
Herculase II 5× Reaction Buffer	10 µl	170 µl
100 mM dNTP Mix (25 mM each dNTP)	0.5 µl	8.5 µl
DMSO	2.5 µl	42.5 µl
SureSelect QXT Primer Mix	1 µl	17 µl
Herculase II Fusion DNA Polymerase	1 µl	17 µl
Total	**40 µl**	**680 µl**

3 Add 40 µl of the pre-capture PCR reaction mix prepared in step 2 to each 10-µl purified DNA library sample.

Seal the wells with fresh caps and mix by vortexing gently for 5 seconds. Spin samples briefly to collect the liquid.

4 Incubate the plate in the thermal cycler (with the heated lid ON) and run the program in Table 10.

Table 10 Thermal cycler program for pre-capture PCR

Segment Number	Number of Cycles	Temperature	Time
1	1	68°C	2 minutes
2	1	98°C	2 minutes
3	8	98°C	30 seconds
		57°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	1	4°C	Hold

Step 4. Purify the amplified library with AMPure XP beads

Before you begin, verify that the AMPure XP beads have been kept at room temperature for at least 30 minutes and that fresh 70% ethanol has been prepared for use in step 6.

1. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
2. Transfer the samples to room temperature, then add 50 µl of the homogeneous bead suspension to each sample well containing the 50-µl amplified DNA samples. Seal the wells with fresh caps, then vortex for 5 seconds. Briefly spin the samples to collect the liquid without pelleting the beads.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
3. Incubate samples for 5 minutes at room temperature.
4. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
5. While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
6. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µl of fresh 70% ethanol in each sample well.
7. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
8. Repeat step 6 and step 7 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
9. Dry the samples on the thermal cycler (with lid open) at 37°C for 1 to 3 minutes. Do not overdry the samples.
10. Add 13 µl of nuclease-free water to each sample well.
11. Seal the sample wells with fresh caps, then mix well on a vortex mixer and briefly spin the plate or tubes to collect the liquid.
12. Incubate for 2 minutes at room temperature.
13. Put the plate or tubes in the magnetic stand and leave for 2 minutes or until the solution in each well is clear.

14 Remove each cleared supernatant (approximately 13 µl) to wells of a fresh plate or strip tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells and store the samples at 4°C short term or at –20°C for long term storage.

Step 5. Assess library DNA quantity and quality

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

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

NOTE

The presence of magnetic beads in the samples may adversely impact the Bioanalyzer results. If you suspect bead contamination in the samples, place the plate or strip tube on the magnetic rack before withdrawing samples for analysis.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Verify that the electropherogram shows the peak of DNA fragment size positioned between 245 to 325 bp. Sample electropherograms are shown in Figure 2. Variability of fragmentation profiles may be observed.

NOTE

A peak DNA fragment size significantly less than 245 bp may indicate too little gDNA in the fragmentation reaction and may be associated with increased duplicates in the sequencing data. In contrast, a peak DNA fragment size significantly greater than 325 bp may indicate too much gDNA in the fragmentation reaction and may be associated with decreased percent-on-target performance in sequencing results.

4. Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

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

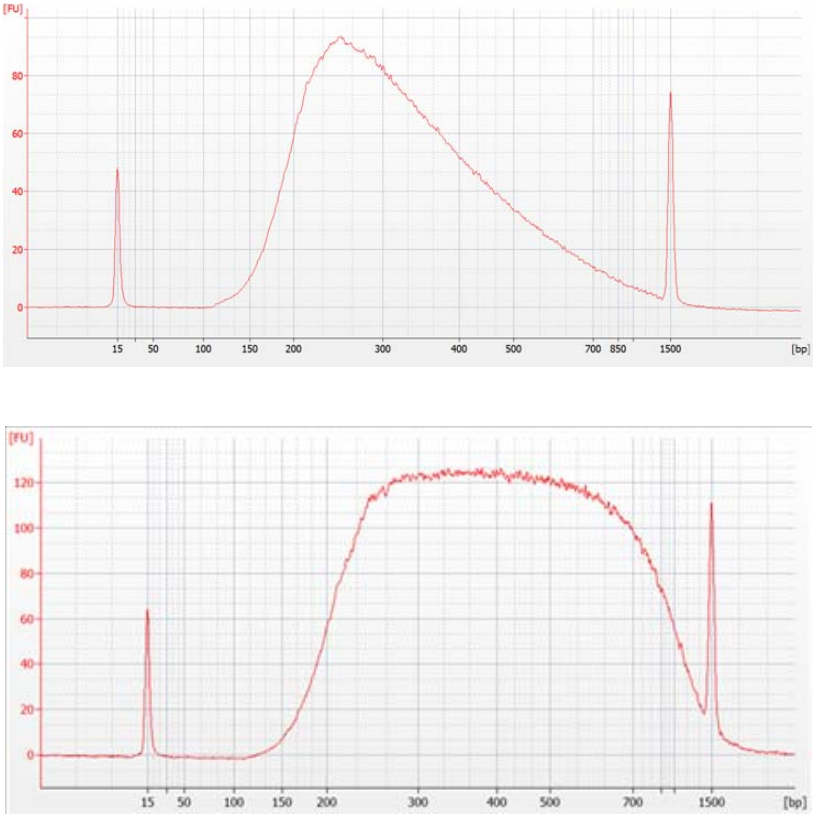

Figure 2 Representative sample electropherograms showing pre-capture analysis of amplified library DNA using the Agilent 2100 Bioanalyzer and a DNA 1000 Assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit. Perform the assay according to the Agilent D1000 Assay Quick Guide.

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

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

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

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 245 to 325 bp. Sample electropherograms are shown in Figure 3. Variability of fragmentation profiles may be observed.

NOTE A peak DNA fragment size significantly less than 245 bp may indicate too little gDNA in the fragmentation reaction and may be associated with increased duplicates in the sequencing data. In contrast, a peak DNA fragment size significantly greater than 325 bp may indicate too much gDNA in the fragmentation reaction and may be associated with decreased percent-on-target performance in sequencing results.

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

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

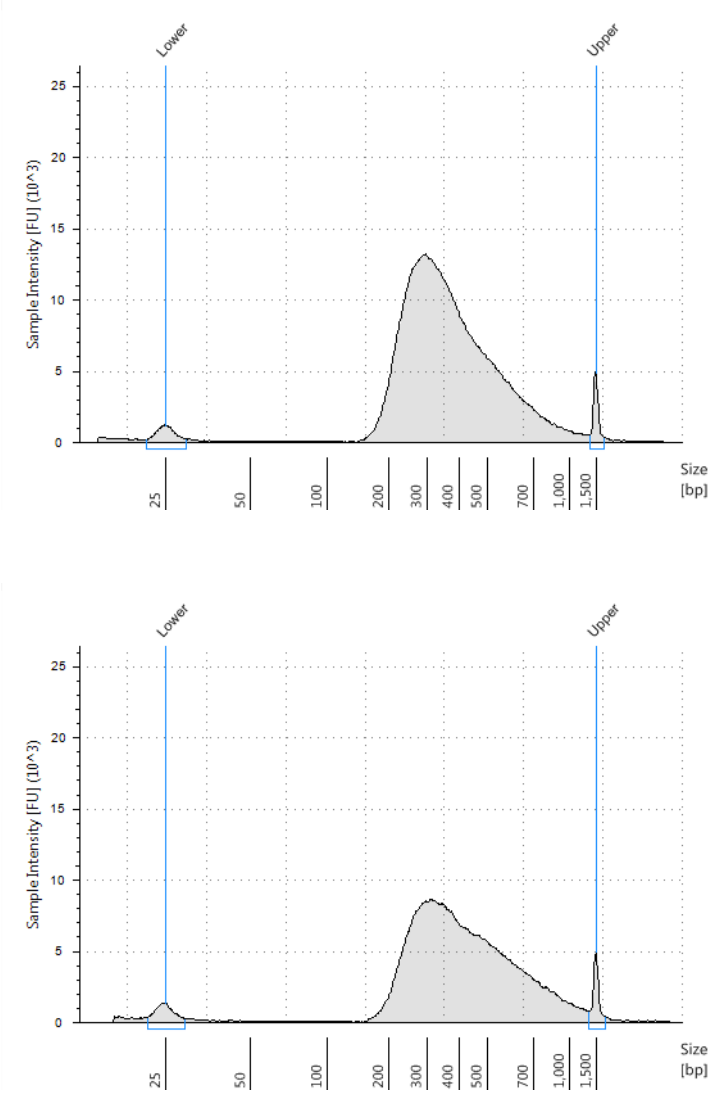

Figure 3 Representative sample electropherograms showing pre-capture analysis of amplified library DNA using a D1000 ScreenTape.

3 Hybridization and Capture

This chapter describes the steps to hybridize the prepared gDNA libraries with a target-specific probe. After hybridization, the targeted molecules are captured on streptavidin beads.

CAUTION The ratio of probe to gDNA library is critical for successful capture.

Step 1. Aliquot prepared DNA samples for hybridization

The amount of prepared gDNA library used in the hybridization reaction varies according to the design size of the probe used for hybridization as outlined in Table 11 below.

Table 11 Amount of adaptor-tagged DNA libraries used for hybridization

Probe size	Amount of prepared gDNA library used in hybridization	Volume of prepared gDNA library samples added to hybridization
Probes >3.0 Mb	750 to 1500 ng DNA	12 µl
Probes ≤3.0 Mb	500 to 750 ng DNA	12 µl

1 Place the prepared gDNA library samples into the hybridization plate or strip tube wells as described below. Use the maximum possible amount of each prepped DNA, within the range listed in Table 11.

   a For hybridization to probes >3 Mb, place up to 1500 ng of prepared DNA library into the designated well and then bring the final volume to 12 µl using nuclease-free water.

   b For hybridization to probes ≤3.0 Mb, place up to 750 ng of prepared DNA library into the designated well and then bring the final volume to 12 µl using nuclease-free water.

Step 2. Hybridize DNA samples to the probe

This step uses the SureSelect^{QXT} Reagent Kit components listed in Table 12. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin briefly to collect the liquid.

Table 12 Reagents for Hybridization and Capture

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect Fast Hybridization Buffer	SureSelect QXT Hyb Module Box 2, –20°C	Warm to Room Temperature (RT), then keep at RT	page 39
SureSelect QXT Fast Blocker Mix	SureSelect QXT Hyb Module Box 2, –20°C	Thaw on ice	page 37 (below)
SureSelect RNase Block	SureSelect QXT Hyb Module Box 2, –20°C	Thaw on ice	page 39
Probe	–80°C	Thaw on ice	page 39

CAUTION For each protocol step that requires removal of tube cap strips, make sure to reseal the tubes with a fresh strip of domed caps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during incubations.

1 To each adaptor-tagged DNA sample well, add 5 µl of SureSelect QXT Fast Blocker Mix. Pipette up and down 8 to 10 times to mix, then cap the wells. Vortex at high speed for 5 seconds, then spin the plate or strip tube briefly.

Sample wells now contain 17 µl of prepared DNA + Fast Blocker mixture.

2 Transfer the sealed prepared DNA + Blocker samples to the thermal cycler and start the program shown in Table 13, using a heated lid.

Important: The thermal cycler must be paused during Segment 3 to allow additional reagents to be added to the Hybridization wells in step 5 on page 40.

During Segments 1 and 2 of the thermal cycling program, begin preparing the additional reagents as described in step 3 on page 39 and step 4 on page 39. If needed, you can finish these preparation steps after pausing the thermal cycler in Segment 3.

Table 13 Thermal cycler program for Hybridization*

Segment #	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (**Pause cycler here** for reagent addition; see step 5 on page 40)
4	60	65°C†	1 minute
		37°C	3 seconds
5	1	65°C†	Hold briefly until ready to begin capture steps on page 42

* Use a reaction volume setting of 30 µl (final volume of hybridization reactions during cycling in Segment 4).

† Hybridization at 65°C is optimal for probes designed for the SureSelect XT HS2/XT HS/XT Low Input platforms. Reducing the hybridization temperature (Segments 4 and 5) may improve performance for probes designed for the SureSelect XT platform, including SureSelect XT Human All Exon V6 (62.5°C), SureSelect XT Clinical Research Exome V2 (62.5°C) and custom probes originally designed for use with SureSelect XT system (60°C–65°C).

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

3 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water), according to Table 14. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well. Keep the stock vial and diluted RNase Block on ice.

Table 14 Preparation of RNase Block solution

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)	Volume for 96 reactions (includes excess)
SureSelect RNase Block	0.5 µl	10 µl	50 µl
Nuclease-free water	1.5 µl	30 µl	150 µl
Total	**2 µl**	**40 µl**	**200 µl**

NOTE

Prepare the mixture described in step 4, below, just before pausing the thermal cycler in Segment 3 as described on page 38. Keep the mixture at room temperature briefly, until adding the mixture to sample wells in step 5 on page 40. Do not keep solutions containing the probe at room temperature for extended periods.

4 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 15 for probes ≥3 Mb or Table 16 for probes <3 Mb.

Combine the listed reagents at room temperature. Mix well by vortexing at high speed for 5 seconds then spin down briefly. Proceed immediately to step 5.

Table 15 Preparation of Probe Hybridization Mix for **probes ≥3 Mb**

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
25% RNase Block solution (from step 3)	2 µl	34 µl
Probe (with design ≥3 Mb)	5 µl	85 µl
SureSelect Fast Hybridization Buffer	6 µl	102 µl
Total	**13 µl**	**221 µl**

Table 16 Preparation of Probe Hybridization Mix for **probes <3 Mb**

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
25% RNase Block solution (from step 3)	2 µl	34 µl
Probe (with design <3 Mb)	2 µl	34 µl
SureSelect Fast Hybridization Buffer	6 µl	102 µl
Nuclease-free water	3 µl	51 µl
Total	**13 µl**	**221 µl**

5 Once the thermal cycler starts Segment 3 of the program in Table 13 (1 minute at 65°C), pause the program. With the cycler paused, and while keeping the DNA + Blocker samples in the cycler, transfer 13 µl of the room-temperature Probe Hybridization Mix from step 4 to each sample well.

Mix well by pipetting up and down 8 to 10 times.

6 Seal the wells with fresh domed strip caps. Make sure that all wells are completely sealed. Vortex at high speed for 5 seconds, and then spin the tubes or plate briefly and return the samples to the thermal cycler.

The hybridization reaction wells now contain approximately 30 µl.

7 Resume the thermal cycling program to allow hybridization of the prepared DNA samples to the probe.

CAUTION

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

Before you do the first experiment, make sure the plasticware and capping method are appropriate for the thermal cycler. Check that no more than 4 µl is lost to evaporation under the conditions used for hybridization.

Step 3. Prepare streptavidin-coated magnetic beads

The hybrid capture protocol uses the SureSelect^{QXT} Reagent Kit components in Table 17 in addition to the streptavidin-coated magnetic beads obtained from another supplier (see Table 1 on page 13).

Table 17 Reagents for hybrid capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect QXT Hyb Module Box 1, RT	page 41 (below)
SureSelect Wash Buffer 2	SureSelect QXT Hyb Module Box 1, RT	page 42
SureSelect Wash Buffer 1	SureSelect QXT Hyb Module Box 1, RT	page 43

NOTE

If you are equipped for higher-volume magnetic bead captures, the streptavidin beads may be batch-washed in an Eppendorf tube or conical vial. Start the batch-washing procedure using excess bead solution. After resuspending the washed beads in the appropriate volume of SureSelect Binding Buffer, aliquot 200 µl of the washed beads to plate or strip tube wells to be used for hybridization capture.

1. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.
2. For each hybridization sample, add 50 µl of the resuspended beads to wells of a fresh PCR plate or a strip tube.
3. Wash the beads:
   a. Add 200 µl of SureSelect Binding Buffer.
   b. Mix by pipetting up and down 10 times.
   c. Put the plate or strip tube into a magnetic separator device.
   d. Wait at least 5 minutes or until the solution is clear, then remove and discard the supernatant.
   e. Repeat step a through step d two more times for a total of 3 washes.
4. Resuspend the beads in 200 µl of SureSelect Binding Buffer.

Step 4. Capture the hybridized DNA using streptavidin-coated beads

1 After all streptavidin bead preparation steps are complete and with the hybridization thermal cycling program in the final 65°C hold segment (see Table 13 on page 38), transfer the samples to room temperature.

2 Immediately transfer the entire volume (approximately 30 µl) of each hybridization mixture to wells containing 200 µl of washed streptavidin beads. Seal the wells with fresh caps.

3 Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1800 rpm), for 30 minutes at room temperature.

Make sure the samples are properly mixing in the wells.

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

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

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

5 When the 30-minute incubation period initiated in step 3 is complete, collect the liquid at the bottom of wells using the method appropriate for your labware:

- For samples in strip tubes, spin the samples briefly.
- For samples in 96-well plates, collect the liquid in the wells manually, using a swift, sharp flicking motion.

CAUTION Do not spin the streptavidin bead-bound DNA samples held in 96-well plates using a plate spinner or centrifuge during the wash steps. Spinning the 96-well plates can over-pack the streptavidin beads, preventing complete resuspension during the washes and can adversely impact sample recovery.

6 Put the plate or strip tube in a magnetic separator to collect the beads from the suspension. Wait 1 minute for the solution to clear, then remove and discard the supernatant.

7 Resuspend the beads in 200 µl of SureSelect Wash Buffer 1 (held at room temperature) by pipetting up and down 8 to 10 times.

Make sure the beads are in suspension before proceeding.

8 Seal the wells with fresh caps, then mix by vortexing at high speed for 8 seconds. Collect the liquid at the bottom of wells using the method appropriate for your labware (spinning for strip tubes or manual collection for 96-well plates).

9 Put the plate or strip tube in a magnetic separator. Wait 1 minute for the solution to clear, then remove and discard the supernatant.

CAUTION Make sure that the Wash Buffer 2 is pre-warmed to 65°C before use in step 10 below.

10 **Remove the plate or strip tubes from the magnetic separator and transfer to a rack at room temperature.** Wash the beads with Wash Buffer 2, using the protocol steps below.

a Resuspend the beads in 200 µl of 65°C prewarmed Wash Buffer 2. Pipette up and down at least 10 times to resuspend the beads.

Make sure the beads are in suspension before proceeding.

b Seal the wells with fresh caps and then vortex at high speed for 5 seconds. Collect the liquid at the bottom of wells using the method appropriate for your labware (spinning for strip tubes or manual collection for 96-well plates).

c Incubate the samples for 10 minutes at 65°C in the thermal cycler with the heated lid ON.

d Put the plate or strip tube in the magnetic separator at room temperature.

e Wait 1 minute for the solution to clear, then remove and discard the supernatant.

f Repeat step a through step e two more times for a total of 3 washes.

11 After removing the supernatant from the final wash, spin the samples briefly, return the plate or tubes to the magnetic stand, and then remove any remaining wash buffer droplets.

12 Add 23 µl of nuclease-free water to each sample well. Place the capture plate or strip tube on ice until PCR reactions are set up on page 47. Proceed to "Indexing and Sample Processing for Multiplexed Sequencing" on page 45.

NOTE Captured DNA is retained on the streptavidin beads during the post-capture amplification step. Do not separate the supernatant from the beads at this step.

4 Indexing and Sample Processing for Multiplexed Sequencing

This chapter describes the steps to add index tags by amplification, and to purify and assess quality and quantity of the indexed libraries. Sample pooling instructions are provided to prepare the indexed samples for multiplexed sequencing, and guidelines are provided for downstream sequencing steps.

Step 1. Amplify the captured libraries to add index tags

In this step, the SureSelect-enriched DNA libraries are PCR amplified using the appropriate pair of dual indexing primers.

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

This step uses the components listed in Table 18. Thaw then vortex to mix the reagents listed below and keep on ice.

Table 18 Reagents for post-capture indexing by PCR amplification

Kit Component	Storage Location	Where Used
Herculase II Fusion DNA Polymerase	SureSelect QXT Library Prep Kit Box 2, –20°C	page 47
Herculase II 5× Reaction Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 47
100 mM dNTP Mix (25 mM each dNTP)	SureSelect QXT Library Prep Kit Box 2, –20°C	page 47
SureSelect QXT P7 and P5 dual indexing primers	SureSelect QXT Library Prep Kit Box 2, –20°C	page 47

Prepare one indexing amplification reaction for each DNA library.

1 Determine the appropriate index assignments for each sample. See the Reference section for sequences of the index portion of the P7 (page 70) and P5 (page 71) indexing primers used to amplify the DNA libraries in this step.

   Use a different indexing primer combination for each sample to be sequenced in the same lane.

NOTE For sample multiplexing, Agilent recommends maximizing index diversity on both P7 and P5 primers as required for color balance. For example, when 8-plexing, use eight different P7 index primers with two P5 index primers. See Table 41 on page 72 and Table 42 on page 73 for additional details.

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 19, on ice. Mix well on a vortex mixer.

Table 19 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	13.5 µl	229.5 µl
Herculase II 5× Reaction Buffer	10 µl	170 µl
100 mM dNTP Mix (25 mM each dNTP)	0.5 µl	8.5 µl
Herculase II Fusion DNA Polymerase	1 µl	17 µl
Total	**25 µl**	**425 µl**

3 Obtain the plate or strip tube containing the bead-bound target-enriched DNA samples from ice. Add 25 µl of the PCR reaction mix prepared in step 2 to the 23-µl of bead suspension in each sample well.

4 Add 1 µl of the appropriate P7 dual indexing primer (P7 i1 to P7 i12) to each PCR reaction mixture well. Add only one of the twelve possible P7 primers to each reaction well.

5 Add 1 µl of the appropriate P5 dual indexing primer (P5 i13 to P5 i20) to each PCR reaction mixture well. Add only one of the eight possible P5 primers to each reaction well.

6 Mix well by pipetting to ensure the beads are fully resuspended, then transfer the PCR plate or strip tube to a thermal cycler and run the PCR amplification program shown in Table 20.

Table 20 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	Probes >3 Mb: 10 Cycles	98°C	30 seconds
	Probes 1 to 3 Mb: 12 Cycles	58°C	30 seconds
	Probes <1 Mb: 14 Cycles	72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

7 When the PCR amplification program is complete, spin the plate or strip tube briefly. Remove the streptavidin-coated beads by placing the plate or strip tube on the magnetic stand at room temperature. Wait 2 minutes for the solution to clear, then remove each supernatant (approximately 50 µl) to wells of a fresh plate or strip tube.

The beads can be discarded at this time.

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

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µl of fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 60 µl of the homogeneous AMPure XP bead suspension to each 50-µl amplified DNA sample in the PCR plate or strip tube. Seal the wells with fresh caps, then vortex for 5 seconds. Briefly spin the samples to collect the liquid, without pelleting the beads.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
7. While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
8. Continue to keep the plate or tubes 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. Make sure to remove all of the ethanol at each wash step.
11. Dry the samples on the thermal cycler (with lid open) at 37°C for 1 to 3 minutes. Do not overdry the samples.
12. Add 25 µl of nuclease-free water to each sample well.
13. Seal the sample wells, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 minutes or until the solution is clear.

16 **Remove the cleared supernatant (approximately 25 µl) to a fresh LoBind tube.** You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the libraries at –20°C.

Step 3. Assess indexed library DNA quantity and quality

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

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

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis.
3. Load the prepared chip into the instrument and start the run within five minutes after preparation.
4. Verify that the electropherogram shows the peak of DNA fragment size positioned between 325 and 450 bp. A sample electropherogram is shown in Figure 4.
5. Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

If you wish to more-precisely quantify the target enriched samples prior to pooling, proceed to "Step 4. Quantify each index-tagged library by QPCR (optional)" on page 55.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 56.

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

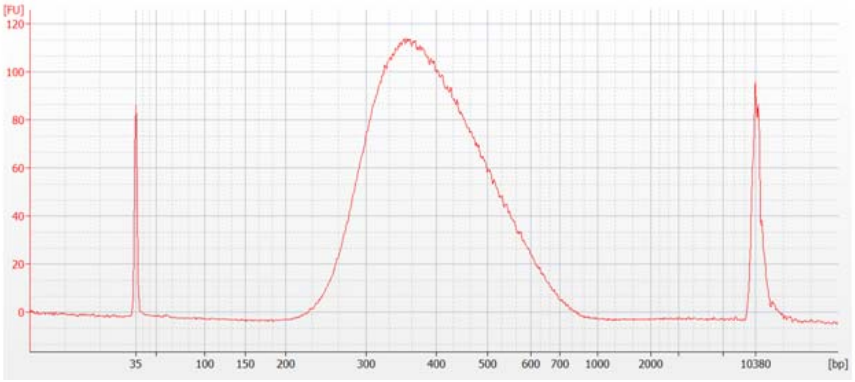

Figure 4 Post-capture analysis of amplified indexed library DNA using the 2100 Bioanalyzer and a High Sensitivity DNA Assay.

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

Use a High Sensitivity D1000 ScreenTape and associated reagent kit. Perform the assay according to the Agilent High Sensitivity D1000 Assay Quick Guide.

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

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

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

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 325 and 450 bp. A sample electropherogram is shown in Figure 5.

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

If you wish to more-precisely quantify the target enriched samples prior to pooling, proceed to "Step 4. Quantify each index-tagged library by QPCR (optional)" on page 55.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 56.

Stopping Point If you do not continue to the next step, store the libraries at 4°C overnight or at –20°C for up to one month.

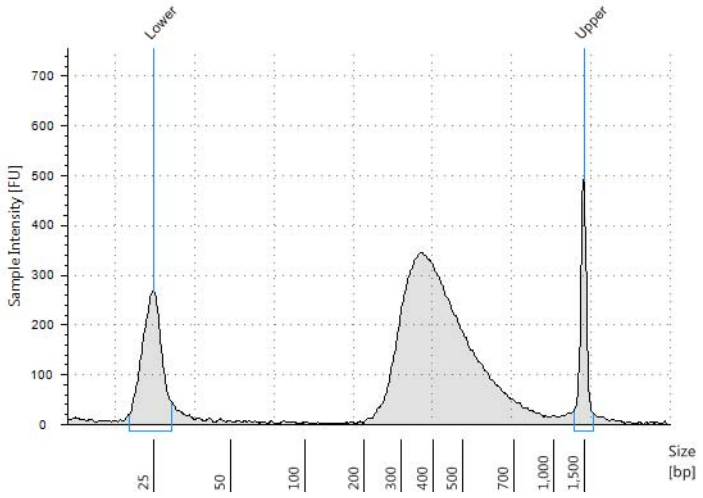

Figure 5 Post-capture analysis of amplified indexed library DNA using a High Sensitivity D1000 ScreenTape.

Step 4. Quantify each index-tagged library by QPCR (optional)

You can use the Agilent QPCR NGS Library Quantification Kit (for Illumina) to accurately determine the concentration of each index-tagged captured library. Refer to the protocol that is included with the Agilent QPCR NGS Library Quantification Kit (p/n G4880A) for more details to do this step.

1 Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.

2 Dilute each index-tagged captured library such that it falls within the range of the standard curve.

Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.

3 Prepare the QPCR master mix with Illumina adaptor-specific PCR primers according to instructions provided in the kit.

4 Add an aliquot of the master mix to PCR tubes and add template.

5 On a QPCR system, such as the Mx3005p, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.

6 Use the standard curve to determine the concentration of each unknown index-tagged library, in nM.

Step 5. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for 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.

Guidelines for optimal low-level multiplexing of samples indexed using the SureSelect^{QXT} dual indexes are provided on page 72.

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of indexed sample to use.

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

where *V(f)* is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool

is the number of indexes, and

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

Table 21 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM.

Table 21 Example of indexed sample volume calculation for total volume of 20 µl

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

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

Step 6. Prepare sequencing samples

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 22 for kit configurations compatible with the recommended read length plus reads for the SureSelect^{QXT} 8-bp dual indexes.

The optimal seeding concentration for SureSelect^{QXT} target-enriched libraries varies according to sequencing platform, run type and Illumina kit version. See Table 22 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality.

To do this step, refer to the manufacturer's instructions, using the modifications described on page 59 for use of the SureSelect^{QXT} Read Primers with the Illumina Paired-End Cluster Generation Kits. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 22 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length*	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	10–13 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	13–16 pM
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	10–13 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	14–19 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.3–1.8 pM
HiSeq 3000/4000	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1	300–400 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	200–400 pM

* If your application requires a different read length, verify that you have sufficient sequencing reagents to complete Reads 1 and 2 in addition to the dual 8-bp index reads.

Using the SureSelect^{QXT} Read Primers with Illumina's Paired-End Cluster Generation Kits

To sequence the SureSelect^{QXT} libraries on Illumina's sequencing platforms, you need to use the following custom sequencing primers, provided in SureSelect QXT Library Prep Kit Box 2:

- **SureSelect QXT Read Primer 1**
- **SureSelect QXT Read Primer 2**
- **SureSelect QXT Index 1 Read Primer**
- **SureSelect QXT Index 2 Read Primer** (this primer is used only for HiSeq 3000, HiSeq 4000, and NextSeq platforms and for NovaSeq platform runs using v1.5 chemistry)

These SureSelect^{QXT} custom sequencing primers are provided at 100 µM and must be diluted in the corresponding Illumina primer solution, using the platform-specific instructions below:

For the HiSeq 2500 platform, combine the primers as shown in Table 23 or Table 24 on page 60.

For the HiSeq 3000 or HiSeq 4000 platform, combine the primers as shown in Table 25 on page 60.

For the MiSeq platform, combine the primers as shown in Table 26 on page 61.

For the NextSeq platform, combine the primers as shown in Table 27 or Table 28 on page 61.

For the NovaSeq platform, combine the primers as shown in Table 29 through Table 32 on page 62.

NOTE

It is important to combine the primers precisely in the indicated ratios. Carefully follow the instructions indicated in Table 23 to Table 32. Where specified, add the custom primer volume directly to the solution already in cBot reagent plate wells. Otherwise, combine measured volumes of each solution; do not rely on volumes reported on vial labels or in Illumina literature. Vortex each mixture vigorously to ensure homogeneity for proper detection of the indexes using the custom read primers.

Table 23 HiSeq 2500 High Output custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	6 µl SureSelect QXT Read Primer 1 (brown cap)	1194 µl HP10	1.2 ml*
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	2985 µl HP12	3 ml
Read 2	15 µl SureSelect QXT Read Primer 2 (black cap)	2985 µl HP11	3 ml

* Aliquot the mixture as directed for HP6 or HP10 in Illumina's cluster generation protocol.

Table 24 HiSeq 2500 Rapid Mode custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	8.8 µl SureSelect QXT Read Primer 1 (brown cap)	1741.2 µl HP10	1.75 ml
Index	8.8 µl SureSelect QXT Index 1 Read Primer (clear cap)	1741.2 µl HP12	1.75 ml
Read 2	8.8 µl SureSelect QXT Read Primer 2 (black cap)	1741.2 µl HP11	1.75 ml

Table 25 HiSeq 3000 and HiSeq 4000 custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	TotalVolume	Reagent Rack Position
Read 1	1.5 µl SureSelect QXT Read Primer 1 (brown cap)	298.5 µl HP10*	0.3 ml per well	cBot Column 11
Read 2	15 µl SureSelect QXT Read Primer 2 (black cap)	2985 µl HP11	3 ml	16
Index 1+ Index 2	22.5 µl SureSelect QXT Index 1 Read Primer (clear cap) + 22.5 µl SureSelect QXT Index 2 Read Primer (purple cap)	4455 µl HP14	4.5 ml	17

* Use cBot recipe *HiSeq_3000_4000_HD_Exclusion_Amp_v1.0*. Add 1.5 µl SureSelect QXT Read Primer 1 to the 298.5 µl of HP10 in each well of column 11 in the cBot reagent plate.

Table 26 **MiSeq platform** custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume	Final Cartridge Position
Read 1	3 µl SureSelect QXT Read Primer 1 (brown cap)	597 µl HP10 (well 12)	0.6 ml	well 18
Index	3 µl SureSelect QXT Index 1 Read Primer (clear cap)	597 µl HP12 (well 13)	0.6 ml	well 19
Read 2	3 µl SureSelect QXT Read Primer 2 (black cap)	597 µl HP11 (well 14)	0.6 ml	well 20

Table 27 **NextSeq 500/550 High-Output v2 Kit** custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	3.9 µl SureSelect QXT Read Primer 1 (brown cap)	1296.1 µl BP10 (from well 20)	1.3 ml	well 7
Read 2	4.2 µl SureSelect QXT Read Primer 2 (black cap)	1395.8 µl BP11 (from well 21)	1.4 ml	well 8
Index 1+ Index 2	6 µl SureSelect QXT Index 1 Read Primer (clear cap) + 6 µl SureSelect QXT Index 2 Read Primer (purple cap)	1988 µl BP14 (from well 22)	2 ml	well 9

Table 28 **NextSeq 500/550 Mid-Output v2 Kit** custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	2.7 µl SureSelect QXT Read Primer 1 (brown cap)	897.3 µl BP10 (from well 20)	0.9 ml	well 7
Read 2	3.3 µl SureSelect QXT Read Primer 2 (black cap)	1096.7 µl BP11 (from well 21)	1.1 ml	well 8
Index 1+ Index 2	4.8 µl SureSelect QXT Index 1 Read Primer (clear cap) + 4.8 µl SureSelect QXT Index 2 Read Primer (purple cap)	1590.4 µl BP14 (from well 22)	1.6 ml	well 9

Table 29 NovaSeq 6000 using SP/S1/S2 flowcell with v1.0 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	12 µl SureSelect QXT Read Primer 1 (brown cap)	3988 µl BP10 (well 24)	4 ml	5
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	4985 µl BP14 (well 23)	5 ml	7
Read 2	6 µl SureSelect QXT Read Primer 2 (black cap)	1994 µl BP11 (well 13)	2 ml	6

Table 30 NovaSeq 6000 using S4 flowcell with v1.0 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	21.9 µl SureSelect QXT Read Primer 1 (brown cap)	7278.1 µl BP10 (well 24)	7.3 ml	5
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	4985 µl BP14 (well 23)	5 ml	7
Read 2	10.5 µl SureSelect QXT Read Primer 2 (black cap)	3489.5 µl BP11 (well 13)	3.5 ml	6

Table 31 NovaSeq 6000 using SP/S1/S2 flowcell with v1.5 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	12 µl SureSelect QXT Read Primer 1 (brown cap)	3988 µl BP10 (well 24)	4 ml	5
Index 1+ Index 2	15 µl SureSelect QXT Index 1 Read Primer (clear cap) + 15 µl SureSelect QXT Index 2 Read Primer (purple cap)	4970 µl VP14 (well 23)	5 ml	7
Read 2	6 µl SureSelect QXT Read Primer 2 (black cap)	1994 µl BP11 (well 13)	2 ml	6

Table 32 NovaSeq 6000 using S4 flowcell with v1.5 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	21.9 µl SureSelect QXT Read Primer 1 (brown cap)	7278.1 µl BP10 (well 24)	7.3 ml	5
Index 1+ Index 2	15 µl SureSelect QXT Index 1 Read Primer (clear cap) + 15 µl SureSelect QXT Index 2 Read Primer (purple cap)	4970 µl VP14 (well 23)	5 ml	7
Read 2	10.5 µl SureSelect QXT Read Primer 2 (black cap)	3489.5 µl BP11 (well 13)	3.5 ml	6

Step 7. Set up the sequencing run and trim adaptors from the reads

Refer to Illumina protocols to set up custom sequencing primer runs, using the additional guidelines outlined below.

For SureSelect^{QXT} dual index sequence information, see page 70.

Before aligning reads to the reference genome, SureSelect^{QXT} adaptor sequences must be trimmed from the reads. You can use SureCall, Agilent's NGS data analysis software, to perform adaptor trimming, alignment of reads and variant calling of FASTQ sequencing data files generated by Illumina sequencers. To download SureCall free-of-charge and for additional information, including tutorials on this software, visit the SureCall page at www.agilent.com.

To use SureCall to analyze SureSelect^{QXT} library data, you first need to define an analysis workflow. This analysis workflow identifies the libraries as SureSelect^{QXT} libraries and enables automated adaptor trimming. The trimmed FASTQ files can then be used for alignment to generate BAMs for downstream analysis.

To create the analysis workflow, refer to Figure 6 on page 64. Upon starting SureCall, click the **Analysis Workflow** tab. Choose the appropriate analysis type (single sample, paired, or trio analysis), and then click the **Import Unaligned Files** button. Using the menus near the top of the screen, choose the appropriate design description from the *Design* menu, select **Default SureSelect Method** from the *Analysis Method* menu, and select **QXT** from the *Library Prep* menu. Within the *Select Unaligned Sample Files* window, specify your read 1 and read 2 files using the **Add** buttons. Once done, refer to the SureCall guide for next steps on alignment and variant calling.

If using another pipeline for alignment and downstream analysis, refer to the platform-specific guidelines starting on page 64.

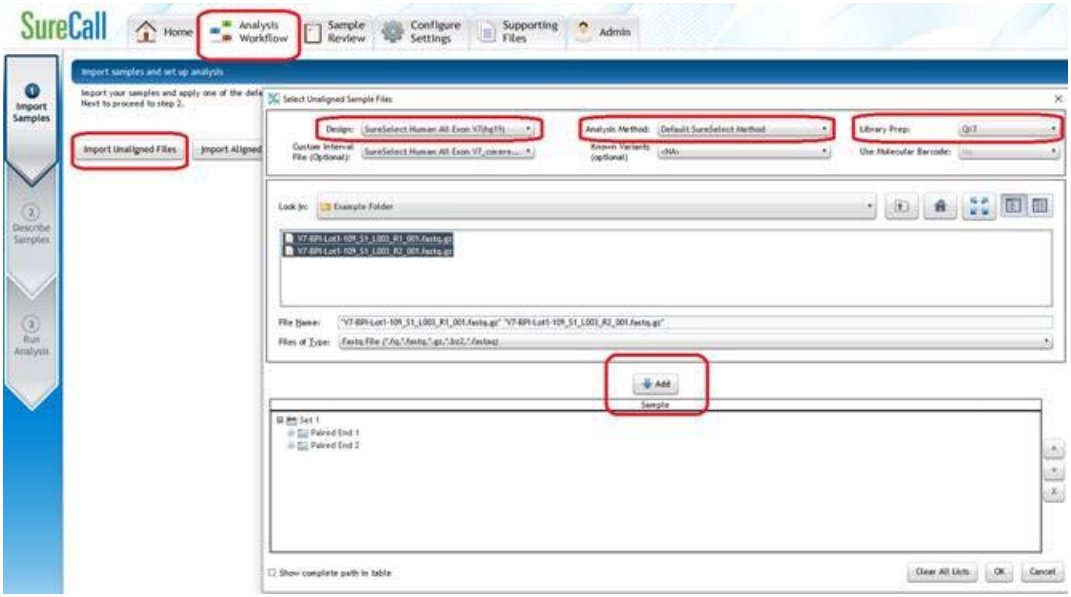

Figure 6 Analysis workflow setup in SureCall.

MiSeq platform sequencing run setup and adaptor trimming guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom primer Sample Sheet.

Set up the run to include adapter trimming using the IEM Sample Sheet Wizard. When prompted by the wizard, select the *Use Adapter Trimming* option, and specify **CTGTCTCTTGATCACA** as the adapter sequence. This enables the MiSeq Reporter software to identify the adaptor sequence and trim the adaptor from reads.

HiSeq/NextSeq/NovaSeq platform sequencing run setup and adaptor trimming guidelines

Set up sequencing runs using the settings shown in Table 33. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface. Since custom primers are spiked into the standard sequencing primer tubes, no additional specialized settings are required to accommodate the use of custom primers in the run.

For the NextSeq or NovaSeq platform, Cycle Number and custom sequencing primer settings can be specified on the *Run Configuration* screen of the instrument control software interface.

Table 33 Run Configuration screen Cycle Number settings

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

After the sequencing run is complete, generate demultiplexed FASTQ data following Illumina's instructions and then trim adaptor sequences from the reads using the Trimmer utility of the Agilent Genomics NextGen Toolkit (AGeNT). For additional information and to download this toolkit free-of-charge, visit the AGeNT page at www.agilent.com.

5 Reference

This chapter contains reference information, including component kit contents and reference information for use during the downstream sample sequencing steps.

Kit Contents

SureSelect^{QXT} Reagent Kits contain the following component kits:

Table 34 SureSelect^{QXT} Reagent Kit Contents

Component Kits	Storage Condition	G9683A 16 Samples	G9683B 96 Samples
SureSelect QXT Library Prep Kit, Box 2*	–20°C	5500-0126	5500-0127
SureSelect QXT Target Enrichment Kit, ILM Hyb Module, Box 1	Room Temperature	5190-7333	5190-7335
SureSelect QXT Target Enrichment Kit, ILM Hyb Module, Box 2	–20°C	5190-7332	5190-7334

* SureSelect QXT Library Prep Kit, Box 1 is not required for the workflow described in this manual.

The contents of each of the component kits listed in Table 34 are described in Table 35 to Table 37 below.

Table 35 SureSelect QXT Library Prep Kit Box 2 Content

Kit Component	16 Reactions	96 Reactions
SureSelect QXT Buffer	tube with white cap	bottle
SureSelect QXT Enzyme Mix ILM	tube with orange cap	tube with orange cap
Herculase II Fusion DNA Polymerase	tube with red cap	tube with red cap
Herculase II 5× Reaction Buffer	tube with clear cap	tube with clear cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap	tube with green cap
DMSO	tube with green cap	tube with green cap
SureSelect QXT Read Primer 1	tube with amber cap	tube with amber cap
SureSelect QXT Read Primer 2	tube with black cap	tube with black cap
SureSelect QXT Index 1 Read Primer	tube with clear cap	tube with clear cap
SureSelect QXT Index 2 Read Primer	tube with purple cap	tube with purple cap
SureSelect QXT P7 dual indexing primers	P7 i1 through P7 i8 provided in 8 tubes with yellow caps (one tube per primer)	P7 i1 through P7 i12 provided in 12 tubes with yellow caps (one tube per primer)
SureSelect QXT P5 dual indexing primers	P5 i13 and P5 i14 provided in 2 tubes with blue caps (one tube per primer)	P5 i13 through P5 i20 provided in 8 tubes with blue caps (one tube per primer)

Table 36 SureSelect QXT Hyb Module Box 1 Content

Kit Component	16 Reactions	96 Reactions
SureSelect QXT Stop Solution	bottle	bottle
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

Table 37 SureSelect QXT Hyb Module Box 2 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Fast Hybridization Buffer	bottle	bottle
SureSelect QXT Fast Blocker Mix	tube with blue cap	tube with blue cap
SureSelect QXT Primer Mix	tube with clear cap	tube with clear cap
SureSelect RNase Block	tube with purple cap	tube with purple cap

Nucleotide Sequences of SureSelect^{QXT} Dual Indexes

The nucleotide sequence of each SureSelect^{QXT} index is provided in the tables below.

Note that some index number assignments of the SureSelect^{QXT} P5 and P7 indexes differ from the index number assignments used by Illumina for indexes of similar or identical sequence.

Each index is 8 bases in length. Refer to Illumina's sequencing run setup instructions for sequencing libraries using 8-base indexes.

Table 38 SureSelect^{QXT} P7 Indexes 1 to 12

Index Number	Sequence
P7 Index 1 (P7 i1)	TAAGGCGA
P7 Index 2 (P7 i2)	CGTACTAG
P7 Index 3 (P7 i3)	AGGCAGAA
P7 Index 4 (P7 i4)	TCCTGAGC
P7 Index 5 (P7 i5)	GTAGAGGA
P7 Index 6 (P7 i6)	TAGGCATG
P7 Index 7 (P7 i7)	CTCTCTAC
P7 Index 8 (P7 i8)	CAGAGAGG
P7 Index 9 (P7 i9)	GCTACGCT
P7 Index 10 (P7 i10)	CGAGGCTG
P7 Index 11 (P7 i11)	AAGAGGCA
P7 Index 12 (P7 i12)	GGACTCCT

Table 39 SureSelect^{QXT} P5 Indexes 13 to 20 for HiSeq 2500, MiSeq, or NovaSeq (v1.0 chemistry) platform

Index Number	Sequence
P5 Index 13 (P5 i13)	TAGATCGC
P5 Index 14 (P5 i14)	CTCTCTAT
P5 Index 15 (P5 i15)	TATCCTCT
P5 Index 16 (P5 i16)	AGAGTAGA
P5 Index 17 (P5 i17)	GTAAGGAG
P5 Index 18 (P5 i18)	ACTGCATA
P5 Index 19 (P5 i19)	AAGGAGTA
P5 Index 20 (P5 i20)	CTAAGCCT

Table 40 SureSelect^{QXT} P5 Indexes 13 to 20 for HiSeq 3000/4000, NextSeq, or NovaSeq (v1.5 chemistry) platform*

Index Number	Sequence
P5 Index 13 (P5 i13)	GCGATCTA
P5 Index 14 (P5 i14)	ATAGAGAG
P5 Index 15 (P5 i15)	AGAGGATA
P5 Index 16 (P5 i16)	TCTACTCT
P5 Index 17 (P5 i17)	CTCCTTAC
P5 Index 18 (P5 i18)	TATGCAGT
P5 Index 19 (P5 i19)	TACTCCTT
P5 Index 20 (P5 i20)	AGGCTTAG

* When doing runs on these platforms through BaseSpace, use the reverse complement sequences provided in Table 39.

Guidelines for Multiplexing with Dual-Indexed Samples

Agilent recommends following the dual index sample pooling guidelines shown in Table 41 for 16 reaction kits and shown in Table 42 for 96 reaction kits. These are designed to maintain color balance at each cycle of the index reads on both ends. They also provide flexibility of demultiplexing as single or dual indexed samples in low-plexity experiments. One-base mismatches should be allowed during demultiplexing.

Table 41 Dual index sample pooling guidelines for 16 Reaction Kits

Plexity of Sample Pool	Recommended SureSelect^{QXT} P7 Indexes	Recommended SureSelect^{QXT} P5 Indexes
1-plex	Any P7 index (i1 to i8)	Either P5 index (i13 or i14)
2-plex	P7 i1 and P7 i2 OR P7 i2 and P7 i4	P5 i13 and P5 i14
3-plex	P7 i1, P7 i2 and P7 i4 OR P7 i3, P7 i4 and P7 i6 OR P7 i5, P7 i7 and P7 i8	P5 i13 and P5 i14 (as needed)
4- or 5-plex	P7 i1, P7 i2, P7 i4 and any additional P7 index(es) OR P7 i3, P7 i4, P7 i6 and any additional P7 index(es) OR P7 i5, P7 i7, P7 i8 and any additional P7 index(es)	P5 i13 and P5 i14 (as needed)
6- to 8-plex	Any combination of 6, 7, or 8 different P7 indexes	P5 i13 and P5 i14 (as needed)
9-to 16-plex	All eight P7 indexes (i1 to i8)	P5 i13 and P5 i14 (as needed)

Table 42 Dual index sample pooling guidelines for 96 Reaction Kits

Plexity of Sample Pool	Recommended SureSelect^{QXT} P7 Indexes	Recommended SureSelect^{QXT} P5 Indexes
1-plex	Any P7 index i1 to i12	Any P5 index (i13 to i20)
2-plex	P7 i1 and P7 i2 OR P7 i2 and P7 i4	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18
3-plex	P7 i1, P7 i2 and P7 i4 OR P7 i3, P7 i4 and P7 i6 OR P7 i5, P7 i7 and P7 i8	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
4-plex	P7 i1, P7 i2, P7 i3* and P7 i4 OR P7 i3, P7 i4, P7 i5* and P7 i6 OR P7 i5, P7 i6*, P7 i7 and P7 i8	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
5-plex	P7 i1, P7 i2, P7 i3*, P7 i4 and P7 i5* OR P7 i3, P7 i4, P7 i5*, P7 i6 and p7 i7* OR P7 i5, P7 i6*, P7 i7, P7 i8 and p7 i9*	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
6- to 12-plex	Any combination of P7 indexes i1 to i12 using each index only once	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
13-to 96-plex	All twelve P7 indexes (i1 to i12)	P5 i13 and P5 i14 and any other P5 index OR P5 i15 and P5 i16 and any other P5 index OR P5 i17 and P5 i18 and any other P5 index (as needed)

* The indicated indexes may be substituted with another index, as long as the substitute index differs from all others used in the sample pool.

Quick Reference Guide to SureSelect Protocol Differences

Key differences between the SureSelect^{QXT}, SureSelect^{XT}, and SureSelect^{XT2} protocols are summarized below.

Table 43 SureSelect protocol differences

Step/Feature	SureSelect^{QXT}	SureSelect^{XT}	SureSelect^{XT2}
		Library Prep	
DNA fragmentation	Transposase-mediated	Mechanical shearing (Covaris)	Mechanical shearing (Covaris)
Library Prep DNA input	50 ng	3 µg or 200 ng	1 µg or 100 ng
Library Prep steps	• Fragmentation and adaptor tagging in single step • PCR	• End repair • dA-tailing • Ligation • PCR	• End repair • Sequential dA-tailing and ligation • PCR
Number of AMPure XP purifications during Library Prep	2	5 (4 for low input protocol)	3
		Hybridization/Capture	
Hyb DNA input	750–1500 ng for >3 Mb 500–750 ng for ≤3 Mb	750 ng for all capture sizes	1500 ng total (pools of 8 or 16 indexed samples) for all capture sizes
Indexing	Dual index (post-capture)	Single index (post-capture)	Single index (pre-capture)
Library pooling	Post-capture	Post-capture	Pre-capture (pools of 16 for captures ≤24 Mb or of 8 for exome captures); post-capture pooling optional
Hybridization buffer	1 tube	4 tubes	1 tube
Blocker	1 tube	3 tubes	1 tube
Hybridization mix preparation temperature	Hyb buffer + probe mixed at RT	Hyb buffer + probe mixed at RT	Hyb buffer + probe mixed on ice
Hybridization protocol	Thermal cycling (90 minutes) using 60 cycles of 3 sec @37°C and 60 sec @65°C	Constant temperature (65°C) for 16 or 24 hours	Constant temperature (65°C) for 16 or 24 hours

Table 43 SureSelect protocol differences

Step/Feature	SureSelect^{QXT}	SureSelect^{XT}	SureSelect^{XT2}
Streptavidin bead addition conditions	Hybridization mixture transferred to RT and then added to beads	Hybridization mixture held at 65°C while added to beads	Hybridization mixture held at 65°C while added to beads
Number of capture washes	1 × Wash 1 3 × Wash 2	1 × Wash 1 3 × Wash 2	1 × Wash 1 6× Wash 2

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In This Book

This guide contains information to run the SureSelect^{QXT} Library Prep and Target Enrichment protocol.

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