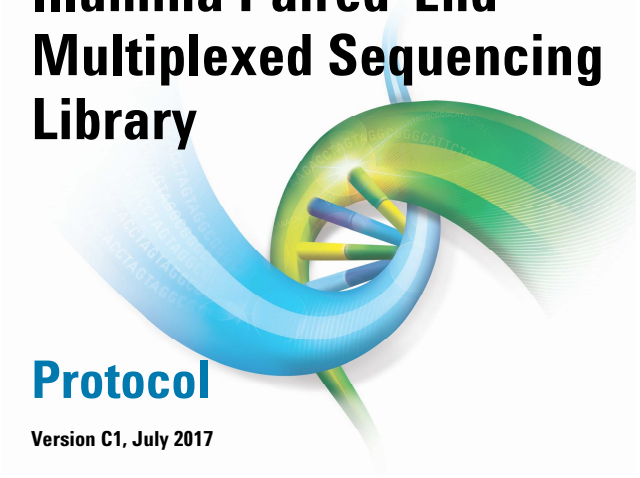
SureSelect^{XT} Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library



Protocol

Version C1, July 2017

SureSelect platform manufactured with Agilent SurePrint Technology

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

Before you begin, view hands-on videos of SureSelect procedures at
http://www.agilent.com/genomics/protocolvideos.

Agilent Technologies

Notices

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Acknowledgment

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In this Guide...

This guide provides an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{XT} Library Prep and Capture System.

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 (3 µg DNA Samples)

This chapter describes the steps to prepare libraries for target enrichment from 3-µg gDNA samples.

3 Sample Preparation (200 ng DNA Samples)

This chapter describes the steps to prepare libraries for target enrichment from 200-ng gDNA samples.

4 Hybridization and Capture

This chapter describes the steps to hybridize and capture the prepared library DNA.

5 Indexing and Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the sample libraries. Samples are pooled by mass prior to sequencing.

6 Appendix: Using FFPE-derived DNA Samples

This chapter contains recommended protocol modifications for FFPE-derived DNA samples.

7 Reference

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

What's New in Version C1

- Updates to sequencing kit selection and seeding concentration guidelines (see Table 40 on page 82)
- Updates to DNA input descriptions (see *Caution* on page 21, text on page 85, and Table 42 on page 86)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see page 19)
- Addition of Agilent 4200 Tapestation system-compatible plasticware ordering information (see page 19)
- Updates to product guarantee and support statement (see *Note* on page 9)

What's New in Version C0

- Support for Clinical Research Exome V2 Capture Libraries (see Table 3 on page 15).
- Removal of reference information for obsolete kits containing 6-bp indexing primers 1–16, provided in clear-capped tubes (typically received before February 2015). If you need assistance with kits containing these obsolete indexing primer components, please contact Technical Support.
- Updates to supplier name for materials purchased from Thermo Fisher Scientific (see Table 2 on page 14, Table 5 on page 17, and Table 6 on page 19)
- Kit use-by information removed. See product labels and the Certificate of Analysis for each component for expiration date information.

Content

Contents

1 Before You Begin

Make sure you have the most current protocol. Go to genomics.agilent.com and search for G7530-90000.

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 This protocol differs from the Illumina Multiplexed Paired-End sequencing manual and other SureSelect protocols at several steps. Pay close attention to the primers used for each amplification step and the blocking agents used during hybridization.

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^{XT} target enrichment workflow is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1.

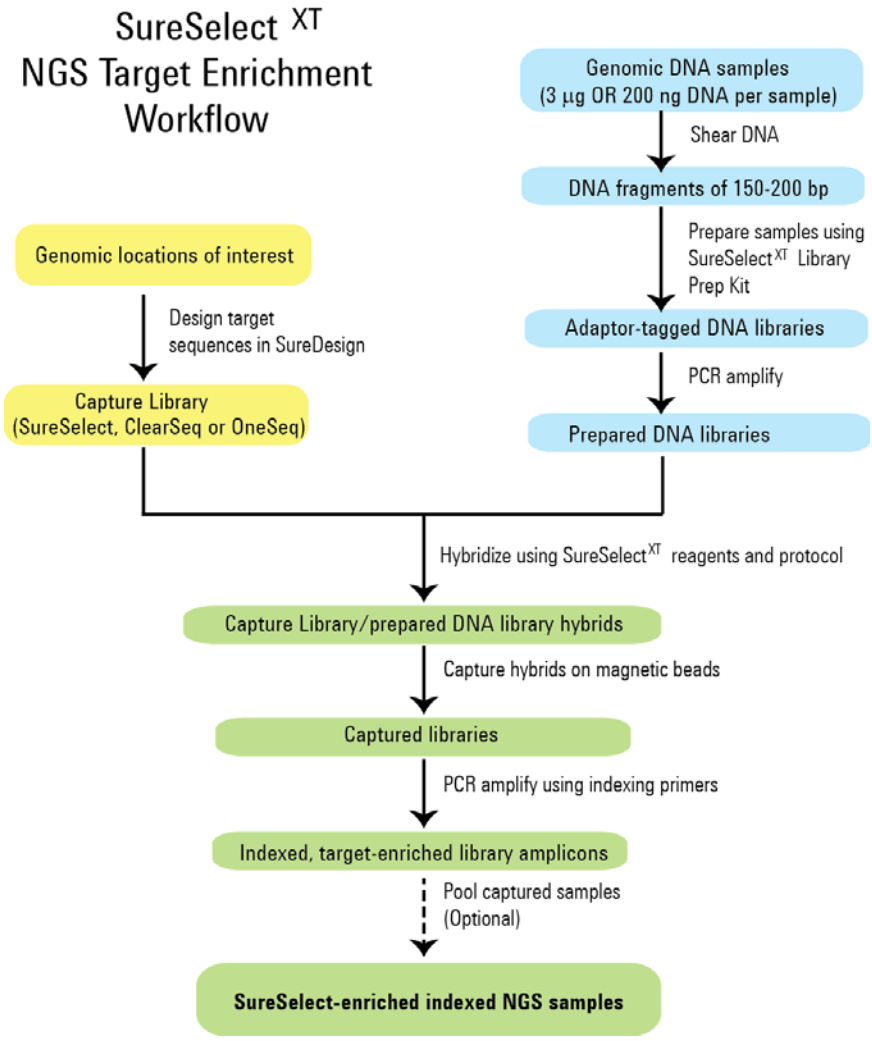

Figure 1 Overall target-enriched sequencing sample preparation workflow.

Table 1 Estimated time requirements (up to 16 sample run size)

Step	Time
Library Preparation	5 hours
Hybridization and Capture	16 or 24 hours
Post-capture amplification	1 hour
QC using Bioanalyzer or TapeStation and sample pooling	1.5 hours

Procedural Notes

- This protocol supports sample processing in the plates or strip tubes specified in Table 5 on page 17. Certain protocol steps include liquid volumes exceeding 0.2 ml which can be accommodated by the recommended plasticware. If using different plasticware, you must first verify that wells can accommodate at least 0.31 ml for processing of 3-µg DNA samples or can accommodate at least 0.28 ml for processing of 200-ng DNA samples. If needed, samples may be transferred to 1.5-ml tubes for the high-volume protocol steps, with possible impacts on sample throughput and yield.
- 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 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.
- Do not mix stock solutions of gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until 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.
- In general, follow Biosafety Level 1 (BL1) safety rules.
- 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.

Safety Notes

CAUTION

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

Required Reagents

Table 2 Required Reagents for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
SureSelect, ClearSeq or OneSeq Capture Library	Select the appropriate library from Table 3 or Table 4
SureSelect^{XT} Reagent Kit, Illumina (ILM) platforms*	Agilent
HiSeq platform, 16 reactions	p/n G9611A
HiSeq platform, 96 reactions	p/n G9611B
HiSeq platform, 480 reactions	p/n G9611C
MiSeq platform, 16 reaction	p/n G9612A
MiSeq platform, 96 reactions	p/n G9612B
MiSeq platform, 480 reactions	p/n G9612C
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
Herculase II Fusion DNA Polymerase (includes dNTPs and 5× Buffer)	Agilent
200 Reactions (processes 100 XT libraries)	p/n 600677
400 Reactions	p/n 600679
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 mL	p/n 65601
10 mL	p/n 65602
100 mL	p/n 65603
1X Low TE Buffer (10 mM Tris-HCl, pH 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 BR dsDNA Assay Kit	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

* HiSeq and MiSeq Reagent Kits are also compatible with the NextSeq 500 platform.

Table 3 SureSelect^{XT} Capture Libraries

Capture Library	16 Reactions	96 Reactions	480 Reactions
SureSelect^{XT} Clinical Research Exome V2	5190-9491	5190-9492	–
SureSelect^{XT} Clinical Research Exome V2 Plus 1	5190-9494	5190-9495	–
SureSelect^{XT} Clinical Research Exome V2 Plus 2	5190-9497	5190-9498	–
SureSelect^{XT} Clinical Research Exome	5190-7338	5190-7339	–
SureSelect^{XT} Focused Exome	5190-7787	5190-7788	–
SureSelect^{XT} Focused Exome Plus 1	5190-7790	5190-7791	–
SureSelect^{XT} Focused Exome Plus 2	5190-7793	5190-7795	–
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} Human All Exon v6 Plus 1	5190-8866	5190-8867	–
SureSelect^{XT} Human All Exon v6 Plus 2	5190-8869	5190-8870	–
SureSelect^{XT} Human All Exon v5	5190-6208	5190-6209	–
SureSelect^{XT} Human All Exon v5 + UTRs	5190-6213	5190-6214	–
SureSelect^{XT} Human All Exon v5 + lncRNA	5190-6446	5190-6447	–
SureSelect^{XT} Human All Exon v5 Plus	5190-6211	5190-6212	–
SureSelect^{XT} Human All Exon v4	5190-4631	5190-4632	5190-4634
SureSelect^{XT} Human All Exon v4 + UTRs	5190-4636	5190-4637	5190-4639
SureSelect^{XT} Mouse All Exon	5190-4641	5190-4642	5190-4644
SureSelect^{XT} Human X-Chromosome	5190-4651	5190-4652	5190-4653
SureSelect^{XT} Custom 1 kb up to 499 kb (reorder)	5190-4806 (5190-4811)	5190-4807 (5190-4812)	5190-4809 (5190-4814)
SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb (reorder)	5190-4816 (5190-4821)	5190-4817 (5190-4822)	5190-4819 (5190-4824)
SureSelect^{XT} Custom 3 Mb up to 5.9 Mb (reorder)	5190-4826 (5190-4831)	5190-4827 (5190-4832)	5190-4829 (5190-4834)
SureSelect^{XT} Custom 6 Mb up to 11.9 Mb (reorder)	5190-4836 (5190-4841)	5190-4837 (5190-4842)	5190-4839 (5190-4844)
SureSelect^{XT} Custom 12 Mb up to 24 Mb (reorder)	5190-4896 (5190-4901)	5190-4897 (5190-4902)	5190-4899 (5190-4904)

Table 4 Compatible ClearSeq and OneSeq Capture Libraries

Capture Library	16 Reactions	96 Reactions	480 Reactions
ClearSeq Comprehensive Cancer XT	5190-8011	5190-8012	–
ClearSeq Comprehensive Cancer Plus XT	5190-8014	5190-8015	–
ClearSeq Inherited Disease XT	5190-7518	5190-7519	–
ClearSeq Inherited Disease Plus XT	5190-7521	5190-7522	–
ClearSeq DNA Kinome XT	5190-4646	5190-4647	5190-4649
OneSeq 1Mb CNV Backbone + Custom 1–499 kb	5190-9462	5190-9463	–
OneSeq 1Mb CNV Backbone + Custom 0.5–2.9 Mb	5190-9465	5190-9466	–
OneSeq 1Mb CNV Backbone + Custom 3–5.9 Mb	5190-9468	5190-9469	–
OneSeq 1Mb CNV Backbone + Custom 6–11.9 Mb	5190-9471	5190-9472	–
OneSeq 1Mb CNV Backbone + Custom 12–24 Mb	5190-9474	5190-9475	–
OneSeq Constitutional Research Panel	5190-8702	5190-8703	–
OneSeq Hi Res CNV Backbone + Custom 1–499 kb	5190-8705	5190-8887	–
OneSeq Hi Res CNV Backbone + Custom 0.5 –2.9 Mb	5190-8889	5190-8890	–
OneSeq Hi Res CNV Backbone + Custom 3–5.9 Mb	5190-8892	5190-8893	–
OneSeq Hi Res CNV Backbone + Custom 6–11.9 Mb	5190-8895	5190-8896	–

Required Equipment

CAUTION Sample volumes exceed 0.2 ml in certain steps of this protocol. If you plan to use plates or strip tubes besides the SureCycler 8800-compatible plasticware listed in the table below, first make sure that the plasticware holds ≥0.31 ml per well when processing 3-µg DNA samples or ≥0.28 ml per well when processing 200-ng DNA samples. Samples may be transferred to 1.5-ml tubes for processing where required, with possible impacts on sample throughput and yield.

Table 5 Required Equipment for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
Qubit Fluorometer	Thermo Fisher Scientific p/n Q32857
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
Covaris Sample Preparation System, S-series or E-series model	Covaris
Covaris sample holders	
96 microTUBE plate (E-series only)	Covaris p/n 520078
microTUBE for individual sample processing	Covaris p/n 520045
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C 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

Table 5 Required Equipment for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
96-well plate mixer	Eppendorf ThermoMixer C, p/n 5382 000.015 and Eppendorf SmartBlock 96 PCR, p/n 5306 000.006, or equivalent
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 TapeStation*	Agilent p/n G2991AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
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†
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vortex mixer	
Vacuum concentrator	Savant SpeedVac, model DNA120, or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

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

† 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.

Optional Reagents and Equipment

Table 6 Optional materials for processing of all samples

Description	Vendor and part number
Ethylene Glycol	American Bioanalytical p/n AB00455
Tween 20	Sigma-Aldrich p/n P9416-50ML
Tube-strip capping tool	Agilent p/n 410099
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402A
Peelable Aluminum Seal for PlateLoc Sealer	Agilent p/n 24210-001
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific p/n L12-20
Agilent QPCR NGS Library Quantification Kit (Illumina GA)	Agilent p/n G4880A
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent

Table 7 Optional materials for processing of FFPE samples

Description	Vendor and part number
Agilent NGS FFPE QC Kit	Agilent
16 reactions	p/n G9700A
96 reactions	p/n G9700B
TapeStation Genomic DNA Analysis Consumables:	
Genomic DNA ScreenTape	Agilent p/n 5067-5365
Genomic DNA Reagents	Agilent p/n 5067-5366

2 Sample Preparation (3 µg DNA Samples)

CAUTION This section contains instructions for the preparation of gDNA libraries from 3 µg DNA samples. **For 200 ng DNA samples and FFPE-derived DNA samples, see the library preparation protocol on page 41.**

The sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual indexed library is prepared. For an overview of the SureSelect^{XT} target enrichment workflow, see Figure 1 on page 10.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads for all purification steps, and primers used for PCR. Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.

Step 1. Shear the DNA

NOTE Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Use the Qubit system to quantify genomic DNA before library preparation.

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

1 Set up the Covaris E-series or S-series instrument.

   a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

   b Check that the water covers the visible glass part of the tube.

   c On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use, or according to the manufacturer's recommendations.

   d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.

   e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide for more details.

2 Put a Covaris microTUBE into the loading and unloading station.

   Keep the cap on the tube.

NOTE You can use the 96 microTUBE plate (see Table 5 on page 17) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

3 Use the Qubit dsDNA Assay to determine the concentration of your gDNA sample.

   Follow the instructions for the instrument.

4 Dilute 3 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 130 µL.

5 Use a tapered pipette tip to slowly transfer the 130-µL DNA sample through the pre-split septa.

   Be careful not to introduce a bubble into the bottom of the tube.

6 Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 8.

The target DNA fragment size is 150 to 200 bp.

Table 8 Shear settings for Covaris instruments (SonoLab software v7 or later)

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

7 Put the Covaris microTUBE back into the loading and unloading station.

8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

9 Transfer each sheared DNA sample (approximately 130 µL) to a separate well of a 96-well plate or strip tube.

Step 2. Purify the sample using AMPure XP beads

NOTE

Instructions in this manual are for sample processing in 96-well PCR plates. When processing a small number of samples, you can instead use strip tubes or individual tubes that are compatible with the thermal cycler and magnetic separation device used in the protocol.

The total liquid volume is 0.31 ml in step 4 through step 7 of the protocol below. If you are using plates or strip tubes besides the recommended SureCycler 8800-compatible plasticware (see Table 5 on page 17), first make sure that wells hold this volume. Samples may be transferred to 1.5-ml tubes for this step, if needed.

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

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

NOTE

The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 2 mL of fresh 70% ethanol per sample.

3 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.

4 Add 180 µL of homogeneous AMPure XP beads to each sheared DNA sample (approximately 130 µL) in the PCR plate. Pipette up and down 10 times to mix.

5 Incubate samples for 5 minutes at room temperature.

6 Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).

7 Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

NOTE

If some magnetic beads remain suspended in solution after 5 minutes, carefully remove and discard 100 µl of cleared solution from near the bottom of the wells, and continue incubating the plate in the magnetic stand for an additional 3 minutes. After the remaining suspension has cleared, remove and discard the remaining cleared solution (approximately 210 µl) from the wells.

8 Continue to keep the plate in the magnetic stand while you dispense 200 µL of 70% ethanol in each sample well.

Use fresh 70% ethanol for optimal results.

9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

10 Repeat step 8 to step 9 once.

11 Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

12 Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.

NOTE Do not dry the bead pellet to the point that the pellet appears cracked during any of the bead drying steps in the protocol. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13 Add 50 µL nuclease-free water to each sample well.

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 48 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 3. Assess quality (optional)

Analysis of the purified sheared DNA samples prior to library preparation is optional. If you elect to include this step, follow the instructions below.

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

Use a DNA 1000 chip and reagent kit for analysis of the 3 µg sheared DNA samples using the 2100 Bioanalyzer. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Check that the electropherogram shows a DNA fragment size peak between 150–200 bp. A sample electropherogram is shown in Figure 2.

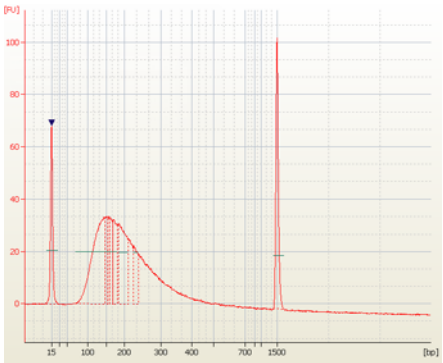

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using an Agilent 4200 TapeStation or 2200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and D1000 Reagents for analysis of the 3 µg sheared DNA samples. For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

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

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

3 Verify that the electropherogram shows a DNA fragment size peak between 150–200 bp. A sample electropherogram is shown in Figure 3.

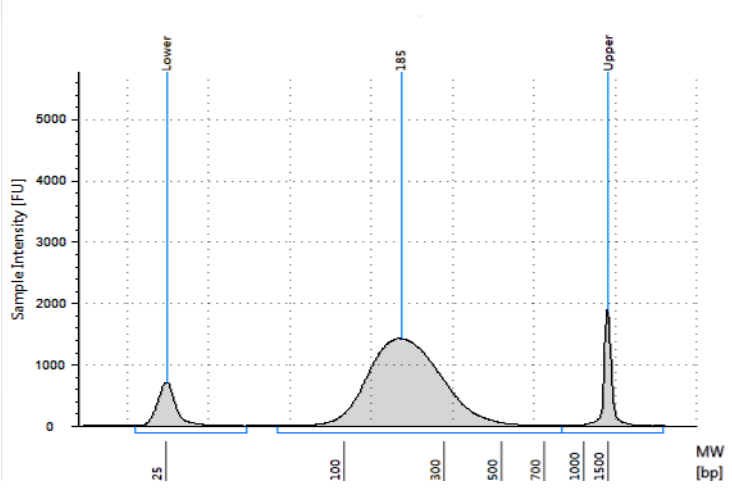

Figure 3 Analysis of sheared DNA using a D1000 ScreenTape.

Step 4. Repair the ends

Use the SureSelect XT Library Prep Kit ILM for this step.

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 16 samples (including excess) are shown in each table as an example.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of End Repair master mix, as described in Table 9, on ice. Mix well on a vortex mixer.

Table 9 Preparation of End Repair master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	35.2 µL	580.8 µL
10× End Repair Buffer (clear cap)	10 µL	165 µL
dNTP Mix (green cap)	1.6 µL	26.4 µL
T4 DNA Polymerase (purple cap)	1 µL	16.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	33 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	36.3 µL
Total	**52 µL**	**858 µL**

2 Add 52 µL of the master mix to each PCR plate sample well containing purified, sheared DNA. Mix by pipetting up and down.

3 Incubate the plate in the thermal cycler and run the program in Table 10. Do not use a heated lid.

Table 10 End-Repair Thermal Cycler Program

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

Step 5. Purify the sample 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. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 180 µL of homogeneous AMPure XP beads to each 100-µL end-repaired DNA sample in the PCR plate. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.
12. Add 32 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, 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 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 6. Adenylate the 3' end of the DNA fragments

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Adenylation master mix, as described in Table 11, on ice. Mix well on a vortex mixer.

Table 11 Preparation of Adenylation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	11 µL	181.5 µL
10× Klenow Polymerase Buffer (blue cap)	5 µL	82.5 µL
dATP (green cap)	1 µL	16.5 µL
Exo(–) Klenow (red cap)	3 µL	49.5 µL
Total	**20 µL**	**330 µL**

2 Add 20 µL of the Adenylation master mix to each end-repaired, purified DNA sample (approximately 30 µL).

3 Mix well by pipetting up and down.

4 Incubate the plate in the thermal cycler and run the program in Table 12. Do not use a heated lid.

Table 12 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 7. Purify the sample 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. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL dA-tailed DNA sample in the PCR plate. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 15 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, 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 to 3 minutes, until the solution is clear.
16. Remove 13 µL of the cleared supernatant to a fresh PCR plate well. You can discard the beads at this time.
17. Proceed immediately to the next step, Step 8. Ligate the paired-end adaptor.

Step 8. Ligate the paired-end adaptor

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Ligation master mix, as described in Table 13, on ice. Mix well on a vortex mixer.

Table 13 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	15.5 µL	255.75 µL
5× T4 DNA Ligase Buffer (green cap)	10 µL	165 µL
SureSelect Adaptor Oligo Mix (brown cap)	10 µL	165 µL
T4 DNA Ligase (red cap)	1.5 µL	24.75 µL
Total	**37 µL**	**610.5 µL**

2 Add 37 µL of the Ligation master mix to each dA-tailed, purified DNA sample (13 µL) in the PCR plate wells.

3 Mix well by pipetting up and down.

4 Incubate the plate in the thermal cycler and run the program in Table 14. Do not use a heated lid.

Table 14 Ligation Thermal Cycler Program

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

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

Step 9. Purify the sample 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. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each adaptor-ligated DNA sample in the PCR plate (50 µL). Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 32 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, 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 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 32 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 10. Amplify the adaptor-ligated library

This step uses the components listed in Table 15. Thaw the reagents listed below and keep on ice.

Table 15 Reagents for pre-capture PCR amplification

Component	Storage Location
SureSelect Primer	SureSelect XT Library Prep Kit ILM, –20°C
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
Herculase II Fusion DNA Polymerase	Herculase II Fusion DNA Polymerase kit, –20°C
5× Herculase II Reaction Buffer	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix	Herculase II Fusion DNA Polymerase kit*, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

This protocol uses half of the adaptor-ligated library for amplification. The remainder can be saved at –20°C for future use, if needed.

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 16, on ice. Mix well on a vortex mixer.

Table 16 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	21 µL	346.5 µL
SureSelect Primer (brown cap)	1.25 µL	20.6 µL
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (clear cap)	1.25 µL	20.6 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	165 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.25 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	16.5 µL
Total	**35 µL**	**577.5 µL**

2 Combine 35 µL of the PCR reaction mixture prepared in Table 16 and 15 µL of each purified DNA library sample from step 16 on page 33. Add a single DNA library sample to each well of the plate or strip tube. Mix by pipetting.

3 Run the program in Table 17 in a thermal cycler.

Table 17 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	4–6	98°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

NOTE Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, five cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low, or too high (where non-specific high molecular weight products are observed), adjust the number of cycles accordingly to amplify the remaining library template.

Step 11. Purify the amplified library with AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL amplified DNA sample in the PCR plate. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 30 µL nuclease-free water to each sample well.
13. Seal the wells, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 12. Assess quality and quantity

Quality assessment can be done with either the 2100 Bioanalyzer instrument or an Agilent TapeStation instrument.

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

See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 4.

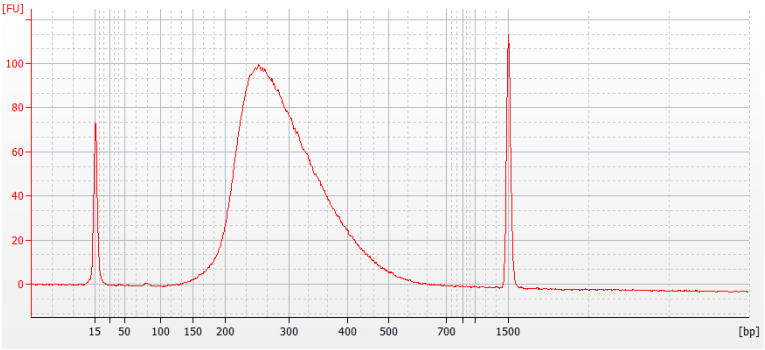

Figure 4 Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using an Agilent 4200 TapeStation or 2200 TapeStation and D1000 ScreenTape

For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

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

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

3 Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 5.

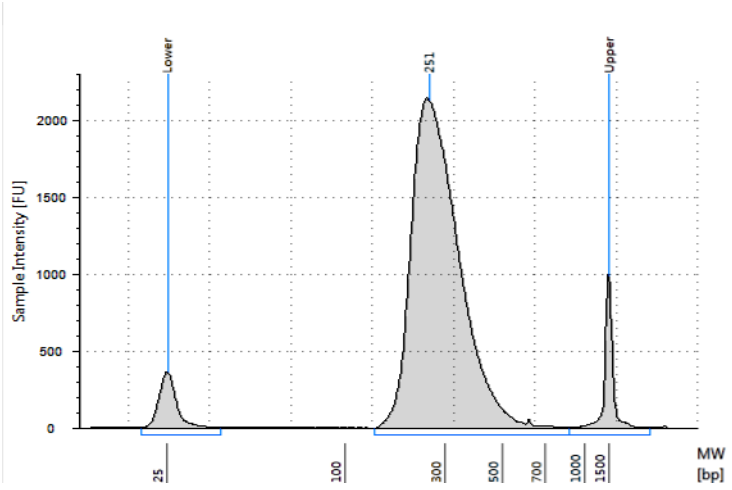

Figure 5 Analysis of amplified library DNA using a D1000 ScreenTape.

3 Sample Preparation (200 ng DNA Samples)

CAUTION This section contains instructions for the preparation of gDNA libraries from 200 ng DNA samples. **For higher input (3 µg) DNA samples, see the library preparation protocol on page 21.**

The sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual indexed library is prepared. For an overview of the SureSelect^{XT} target enrichment workflow, see Figure 1 on page 10.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads for all purification steps, and primers used for PCR. Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.

Step 1. Shear the DNA

NOTE

Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Use the Qubit system to quantify genomic DNA before library preparation.

For FFPE-derived DNA samples, review the protocol modifications detailed in the Appendix on page 85 before starting the library preparation protocol.

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

1 Set up the Covaris E-series or S-series instrument.

   a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

   b Check that the water covers the visible glass part of the tube.

   c On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use, or according to the manufacturer's recommendations.

   d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.

   e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide for more details.

2 Put a Covaris microTUBE into the loading and unloading station.

   Keep the cap on the tube.

NOTE

You can use the 96 microTUBE plate (see Table 5 on page 17) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

3 Use the Qubit dsDNA Assay to determine the concentration of your gDNA sample.

   Follow the instructions for the instrument.

NOTE

For FFPE-derived DNA samples with significantly degraded DNA, use the concentration of amplifiable DNA as determined by qPCR and use the maximum amount of DNA available in the range of 100–200 ng. See Chapter 6 for more information on when protocol modifications are appropriate for FFPE samples.

4 Dilute 200 ng of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 50 µL.

5 Use a tapered pipette tip to slowly transfer the 50-µL DNA sample through the pre-split septa.

Be careful not to introduce a bubble into the bottom of the tube.

6 Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 18.

The target DNA fragment size is 150 to 200 bp.

NOTE For FFPE-derived DNA samples, reduce the duration of shearing from 6 minutes to 4 minutes. See Chapter 6 for a complete list of modifications recommended for FFPE samples.

Table 18 Shear settings for Covaris instruments (SonoLab software v7 or later)

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds*
Bath Temperature	4° to 8° C

* For more complete shearing, the 360-second treatment time may be completed in two rounds of 180 seconds each. After completing the first round of shearing for 180 seconds, spin the microTUBE briefly to collect the liquid, then shear the DNA for an additional 180 seconds.

7 Put the Covaris microTUBE back into the loading and unloading station.

8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

9 Transfer each sheared DNA sample (approximately 50 µL) to a separate well of a 96-well plate or strip tube.

NOTE Instructions in this manual are for sample processing in 96-well PCR plates. When processing a small number of samples, you can instead use strip tubes or individual tubes that are compatible with the thermal cycler and magnetic separation device used in the protocol.

Step 2. Assess quality (optional)

This step is optional.

Quality assessment can be done with the 2100 Bioanalyzer instrument.

For analysis of **200 ng sheared DNA samples, Use a High Sensitivity DNA chip and reagent kit.** See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the High Sensitivity DNA assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Check that the electropherogram shows a DNA fragment size peak at approximately 150 bp. A sample electropherogram is shown in Figure 6.

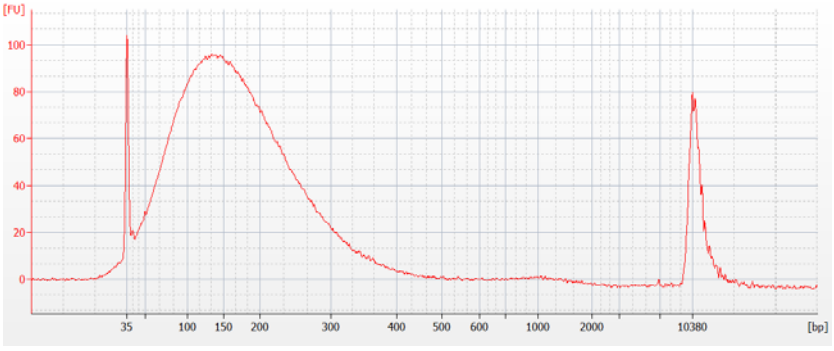

Figure 6 Analysis of sheared DNA using a High Sensitivity DNA Bioanalyzer assay.

Step 3. Repair the ends

Use the SureSelect XT Library Prep Kit ILM for this step.

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 16 samples (including excess) are shown in each table as an example.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of End Repair master mix, as described in Table 19, on ice. Mix well on a vortex mixer.

Table 19 Preparation of End Repair master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	35.2 µL	580.8 µL
10× End Repair Buffer (clear cap)	10 µL	165 µL
dNTP Mix (green cap)	1.6 µL	26.4 µL
T4 DNA Polymerase (purple cap)	1 µL	16.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	33 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	36.3 µL
Total	**52 µL**	**858 µL**

2 Add 52 µL of the master mix to each PCR plate well containing the sheared DNA samples (approximately 48–50 µL). Mix by pipetting up and down.

3 Incubate the plate in the thermal cycler and run the program in Table 20. Do not use a heated lid.

Table 20 End-Repair Thermal Cycler Program

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

Step 4. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 1.6 mL of fresh 70% ethanol per sample.

3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 180 µL of homogeneous AMPure XP beads to each end-repaired DNA sample (approximately 100 µL) in the PCR plate. Pipette up and down 10 times to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
7. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

NOTE If some magnetic beads remain suspended in solution after 5 minutes, carefully remove and discard 100 µl of cleared solution from near the bottom of the wells, and continue incubating the plate in the magnetic stand for an additional 3 minutes. After the remaining suspension has cleared, remove and discard the remaining cleared solution (approximately 180 µl) from the wells.

8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of 70% ethanol in each sample well.

   Use fresh 70% ethanol for optimal results.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 to step 9 once.

11 Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

12 Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.

NOTE Do not dry the bead pellet to the point that the pellet appears cracked during any of the bead drying steps in the protocol. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13 Add 32 µL nuclease-free water to each sample well.

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 5. Adenylate the 3' end of the DNA fragments

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Adenylation master mix, as described in Table 21, on ice. Mix well on a vortex mixer.

Table 21 Preparation of Adenylation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	11 µL	181.5 µL
10× Klenow Polymerase Buffer (blue cap)	5 µL	82.5 µL
dATP (green cap)	1 µL	16.5 µL
Exo(–) Klenow (red cap)	3 µL	49.5 µL
Total	**20 µL**	**330 µL**

2 Add 20 µL of the Adenylation master mix to each end-repaired, purified DNA sample (approximately 30 µL).

3 Mix well by pipetting up and down.

4 Incubate the plate in the thermal cycler and run the program in Table 22. Do not use a heated lid.

Table 22 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 6. Purify the sample 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. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL dA-tailed DNA sample in the PCR plate. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 15 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, 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 to 3 minutes, until the solution is clear.
16. Remove 13 µL of the cleared supernatant to a fresh PCR plate well. You can discard the beads at this time.
17. Proceed immediately to the next step, Step 7. Ligate the paired-end adaptor.

Step 7. Ligate the paired-end adaptor

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Dilute the SureSelect Adaptor Oligo Mix (brown cap) 1:10 in nuclease-free water immediately before use. Use the diluted oligo mix when preparing the Ligation master mix in the next step.

NOTE

For FFPE-derived DNA samples, skip this dilution step and use the undiluted SureSelect Adaptor Oligo Mix in step 2 below. See Chapter 6 for a complete list of modifications recommended for FFPE samples.

2 Prepare the appropriate volume of Ligation master mix, as described in Table 23, on ice. Mix well on a vortex mixer.

Table 23 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	15.5 µL	255.75 µL
5× T4 DNA Ligase Buffer (green cap)	10 µL	165 µL
Diluted SureSelect Adaptor Oligo Mix from step 1	10 µL	165 µL
T4 DNA Ligase (red cap)	1.5 µL	24.75 µL
Total	**37 µL**	**610.5 µL**

3 Add 37 µL of the Ligation master mix to each dA-tailed, purified DNA sample (13 µL) in the PCR plate wells.

4 Mix well by pipetting up and down.

5 Incubate the plate in the thermal cycler and run the program in Table 24. Do not use a heated lid.

Table 24 Ligation Thermal Cycler Program

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

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

Step 8. Purify the sample 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. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each adaptor-ligated DNA sample in the PCR plate (50 µL). Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 32 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, 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 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 9. Amplify the adaptor-ligated library

This step uses the components listed in Table 25. Thaw the reagents listed below and keep on ice.

Table 25 Reagents for pre-capture PCR amplification

Component	Storage Location
SureSelect Primer (brown cap)	SureSelect XT Library Prep Kit ILM, –20°C
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (clear cap)	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
Herculase II Fusion DNA Polymerase (red cap)	Herculase II Fusion DNA Polymerase kit, –20°C
5× Herculase II Reaction Buffer (clear cap)	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix (green cap)	Herculase II Fusion DNA Polymerase kit*, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 26, on ice. Mix well on a vortex mixer.

Table 26 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	6 µL	99.0 µL
SureSelect Primer (brown cap)	1.25 µL	20.6 µL
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (clear cap)	1.25 µL	20.6 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	165 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.3 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	16.5 µL
Total	**20 µL**	**330 µL**

2 Add 20 µL of the PCR reaction mixture prepared in Table 26 to each purified DNA library sample (30 µL) in the PCR plate wells.

Mix by pipetting.

3 Run the program in Table 27 in a thermal cycler.

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Table 27 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10	98°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

NOTE FFPE-derived DNA samples may require a different cycle number for amplification, depending on DNA integrity. See page 87 or page 89 for DNA integrity score-based cycle number recommendations.

Step 10. Purify the amplified library with AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL amplified DNA sample in the PCR plate. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 30 µL nuclease-free water to each sample well.
13. Seal the wells, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

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

Step 11. Assess quality and quantity

Sample analysis can be done with either the 2100 Bioanalyzer instrument or an Agilent TapeStation instrument.

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

See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 7.

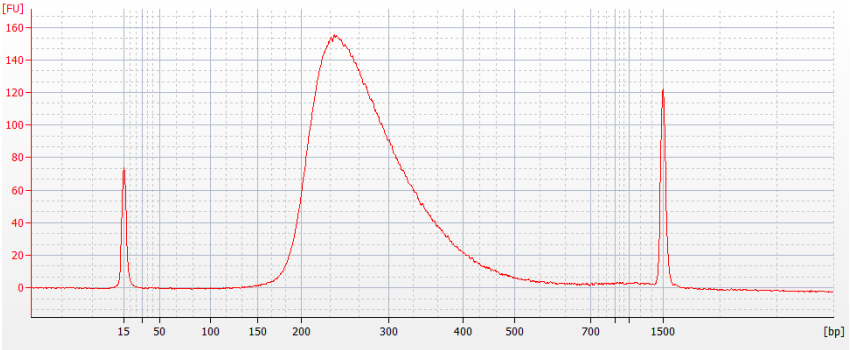

Figure 7 Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using an Agilent 4200 TapeStation or 2200 TapeStation and D1000 ScreenTape

For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

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

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

3 Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 8.

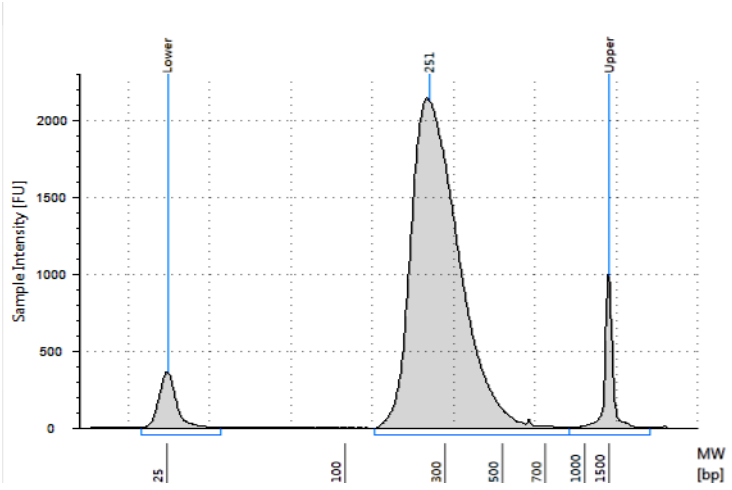

Figure 8 Analysis of amplified library DNA using a D1000 ScreenTape.

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

4 Hybridization and Capture

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

Each DNA library sample must be hybridized and captured individually prior to addition of the indexing tag by PCR.

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

Step 1. Hybridize DNA samples to the Capture Library

In this step, the prepared gDNA libraries are hybridized to a target-specific Capture Library.

This step uses the SureSelect^{XT} Reagent Kit components listed in Table 28. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 28 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect Hyb 1	SureSelect Target Enrichment-Box 1, RT	—	page 62
SureSelect Hyb 2	SureSelect Target Enrichment-Box 1, RT	—	page 62
SureSelect Hyb 3	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Warm to Room Temperature (RT)	page 62
SureSelect Hyb 4	SureSelect Target Enrichment-Box 1, RT	—	page 62
SureSelect Indexing Block 1	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 62
SureSelect Block 2	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 62
SureSelect ILM Indexing Block 3	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 62
SureSelect RNase Block	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 63
Capture Library	–80°C	Thaw on ice	page 64

For each sample library prepared, do one hybridization and capture. Do not pool samples at this stage.

The hybridization reaction requires 750 ng of prepared DNA in a volume of 3.4 µL (initial concentration of 221 ng/µL).

NOTE

For FFPE-derived DNA samples, add the maximum amount of DNA available in range of 500–750 ng DNA. See Chapter 6 for a complete list of modifications recommended for FFPE samples.

1. For prepped libraries with DNA concentrations above 221 ng/µL, prepare 3.4 µL of a 221 ng/µL dilution of each library.
2. For prepped libraries with DNA concentrations below 221 ng/µL, use a vacuum concentrator to concentrate the samples at ≤ 45°C.
   a. Add the entire 30-µL volume of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

      You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.
   b. Dehydrate using a vacuum concentrator on low heat (less than 45°C).
   c. Reconstitute with nuclease-free water to a final concentration of 221 ng/µL. Pipette up and down along the sides of the tube for optimal recovery.
   d. Mix well on a vortex mixer and spin in a centrifuge for 1 minute.
3. Transfer each 3.4-µL gDNA library sample (750 ng) to a separate well of a 96-well plate or strip tube. Seal the wells and keep on ice.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 16 or 24 hour incubation.

If you want to use a different combination of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape), first test the conditions. Incubate 27 µL of water at 65°C for 24 hours as a test. Include water in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 4 µL.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 97.

4 Prepare the Hybridization Buffer by mixing the components in Table 29 at room temperature.

If a precipitate forms, warm the Hybridization Buffer at 65°C for 5 minutes.

Keep the prepared Hybridization Buffer at room temperature until it is used in step 9.

Table 29 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction*	Volume for 16 reactions (includes excess)
SureSelect Hyb 1 (orange cap or bottle)	6.63 µL	116 µL
SureSelect Hyb 2 (red cap)	0.27 µL	4.7 µL
SureSelect Hyb 3 (yellow cap or bottle)	2.65 µL	46.4 µL
SureSelect Hyb 4 (black cap or bottle)	3.45 µL	60.4 µL
Total	**13 µL**	**227.5**

* Prepare Hybridization Buffer for at least 5 reaction equivalents per run to allow accurate pipetting volumes.

5 Prepare the SureSelect Block Mix by mixing the components in Table 30. Keep the mixture on ice until it is used in step 6.

Table 30 Preparation of SureSelect Block Mix

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

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

6 To each gDNA library sample well prepared in step 3 on page 61, add 5.6 µL of the SureSelect Block Mix prepared in Table 30. Pipette up and down to mix.

7 Cap the wells, then transfer the sealed plate or strip tube to the thermal cycler and run the following program shown in Table 31.

Use a heated lid, set at 105°C, to hold the temperature at 65°C.

Make sure that the DNA + Block Mix samples are held at 65°C for at least 5 minutes before adding the remaining hybridization reaction components in step 10 below.

Table 31 Thermal cycler program for DNA + Block Mix prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold (at least 5 minutes)

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

8 Prepare the appropriate dilution of SureSelect RNase Block, based on the size of your Capture Library, according to Table 32. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Keep the mixture on ice until it is used in step 9.

Table 32 Preparation of RNase Block dilution

Capture Library Size	RNase Block dilution (parts RNase Block:parts water)	Volume of dilute RNase Block Required per hybridization reaction
≥3.0 Mb	25% (1:3)	2 µL
<3.0 Mb	10% (1:9)	5 µL

NOTE

Prepare the Capture Library mixture described in step 9, below, near the end of the 65°C hold step of >5 minute duration) described in Table 31. Keep the mixture at room temperature briefly, until adding the mixture to sample wells in step 10. Do not keep solutions containing the Capture Library at room temperature for extended periods.

9 Prepare the Capture Library Hybridization Mix appropriate for your Capture Library size according to Table 33 (Capture Libraries≥3 Mb), or Table 34 (Capture Libraries<3 Mb).

Mix well by vortexing at high speed for 5 seconds then spin down briefly. Keep the mixture at room temperature briefly, until use in step 10.

Table 33 Preparation of Capture Library Hybridization Mix for ≥**3 Mb Capture Libraries**

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 4	13 µL	221 µL
25% RNase Block solution from step 8	2 µL	34 µL
Capture Library ≥3 Mb	5 µL	85 µL
Total	**20 µL**	**340 µL**

Table 34 Preparation of Capture Library Hybridization Mix for **<3 Mb Capture Libraries**

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 4	13 µL	221 µL
10% RNase Block solution from step 8	5 µL	85 µL
Capture Library <3 Mb	2 µL	34 µL
Total	**20 µL**	**340 µL**

10 Maintain the gDNA library + Block Mix plate or strip tube at 65°C while you add 20 µL of the Capture Library Hybridization Mix from step 9 to each sample well. Mix well by pipetting up and down 8 to 10 times.

The hybridization reaction wells now contain approximately 27 to 29 µL, depending on the degree of evaporation during the thermal cycler incubation.

11 Seal the wells with strip caps or using the PlateLoc Thermal Microplate Sealer. Make sure that all wells are completely sealed.

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

When using the SureCycler 8800 thermal cycler and sealing with strip caps, make sure to use domed strip caps and to place a compression mat over the PCR plate or strip tubes in the thermal cycler.

12 Incubate the hybridization mixture for 16 or 24 hours at 65°C with a heated lid at 105°C.

Step 2. Prepare streptavidin-coated magnetic beads

The hybrid capture protocol uses reagents provided in SureSelect Target Enrichment Box 1 (stored at room temperature) in addition to the streptavidin-coated magnetic beads obtained from another supplier (see Table 2 on page 14).

1. Prewarm SureSelect Wash Buffer 2 at 65°C in a circulating water bath or heat block for use in "Step 3. Capture the hybridized DNA using streptavidin-coated beads" on page 67.
2. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.
3. For each hybridization sample, add 50 µL of the resuspended beads to wells of a fresh PCR plate or strip tube.
4. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix by pipetting up and down until beads are fully resuspended.
   c. Put the plate or strip tube into a magnetic separator device.
   d. Wait 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.
5. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

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.

Step 3. Capture the hybridized DNA using streptavidin-coated beads

1 Estimate and record the volume of hybridization solution that remains after the 24 hour incubation.

2 Maintain the hybridization plate or strip tube at 65°C while you use a multichannel pipette to transfer the entire volume (approximately 25 to 29 µL) of each hybridization mixture to the plate or strip tube wells containing 200 µL of washed streptavidin beads.

   Mix well by slowly pipetting up and down until beads are fully resuspended.

NOTE Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 52 on page 97 for tips to minimize evaporation.

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

   Make sure the samples are properly mixing in the wells.

4 Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner.

5 Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear, then remove and discard the supernatant.

6 Resuspend the beads in 200 µL of SureSelect Wash Buffer 1. Mix by pipetting up and down until beads are fully resuspended.

7 Incubate the samples for 15 minutes at room temperature.

8 Briefly spin in a centrifuge or mini-plate spinner.

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

CAUTION It is important to maintain bead suspensions at 65°C during the washing procedure below to ensure specificity of capture.

Make sure that the SureSelect Wash Buffer 2 is pre-warmed to 65°C before use.

Do not use a tissue incubator, or other devices with significant temperature fluctuations, for the incubation steps.

10 Wash the beads with SureSelect Wash Buffer 2:

- **a** Resuspend the beads in 200 µL of 65°C prewarmed Wash Buffer 2. Pipette up and down until beads are fully resuspended.
- **b** Cap the wells, then incubate the sample plate or strip tube for 10 minutes at 65°C on the thermal cycler.
- **c** Put the plate or strip tube in the magnetic separator. Wait for the solution to clear, then remove and discard the supernatant.
- **d** Repeat step a through step c for a total of 3 washes.

  Make sure all of the wash buffer has been removed during the final wash.

11 Add 30 µL of nuclease-free water to each sample well. Pipette up and down to resuspend the beads.

Keep the samples on ice until they are used on page 71.

NOTE Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

5 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 captured libraries. Sample pooling instructions are provided to prepare the indexed samples for multiplexed sequencing.

Step 1. Amplify the captured libraries with indexing primers

In this step, the SureSelect-enriched DNA libraries are PCR amplified in PCR reactions that include the appropriate indexing primer for each sample.

This step uses the components listed in Table 35. Thaw then vortex to mix the reagents listed below and keep on ice.

Table 35 Reagents for post-capture indexing by PCR amplification

Kit Component	Storage Location
5× Herculase II Reaction Buffer	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix (25 mM each dNTP)	Herculase II Fusion DNA Polymerase kit*, –20°C
Herculase II Fusion DNA Polymerase	Herculase II Fusion DNA Polymerase kit, –20°C
SureSelect ILM Indexing Post-Capture Forward PCR Primer	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
SureSelect 8 bp Indexes (reverse primers)	SureSelect XT Library Prep Kit ILM, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

Prepare one indexing amplification reaction for each DNA library.

NOTE

When processing FFPE-derived DNA samples, some details of this step should be modified. See Table 42 on page 86 for more information.

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.

1 Determine the appropriate index assignments for each sample. See Table 51 in the "Reference" chapter for sequences of the index portion of the SureSelect 8 bp Indexes A01 through H12 indexing primers used to amplify the DNA libraries in this step.

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

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 36, on ice. Mix well on a vortex mixer.

Table 36 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	18.5 µL	314.5 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	170 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.5 µL
SureSelect ILM Indexing Post-Capture Forward PCR Primer (orange cap)	1 µL	17 µL
Total	**31 µL**	**527 µL**

3 Add 31 µL of the PCR reaction mix prepared in Table 36 to each sample well of a fresh PCR plate or strip tube.

4 Add 5 µL of the appropriate indexing primer (SureSelect 8 bp Indexes A01 through H02, provided in white-capped tubes *or* A01 through H12, provided in blue plate) to each well. Add only one of the 16 or 96 possible indexing primers to each reaction well.

5 Add the DNA library samples to the PCR reactions:

a Obtain the PCR plate or strip tube containing 30 µL of bead-bound target-enriched DNA samples from ice (prepared on page 68).

b Pipette each DNA sample up and down until the bead suspension is homogeneous, then transfer 14 µL of the sample to the appropriate well of the PCR plate or strip tube containing PCR reaction mix and indexing primer.

c Mix the PCR reactions well by pipetting.

d Store the remaining library-bound beads at –20°C for future use, if needed.

6 Transfer the PCR plate or strip tube to a thermal cycler and run the PCR amplification program shown in Table 37.

Table 37 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 Cycles	98°C	30 seconds
	See Table 38 for recommendations based on Capture Library size	57°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 38 Post-capture PCR cycle number recommendations

Capture Library	Cycles
1 kb up to 0.5 Mb	16 cycles
0.5 Mb up to 1.49 Mb	14 cycles
>1.5 Mb	12 cycles
All Exon and Exome libraries	10 to 12 cycles
OneSeq Constitutional Research Panel	10 cycles
OneSeq Hi Res CNV Backbone-based custom designs	10 cycles
OneSeq 1Mb CNV Backbone-based custom designs	10 to 12 cycles

7 When the PCR amplification program is complete, spin the plate or strip tube briefly.

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

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 9.
3. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 90 µL of the homogeneous AMPure XP bead suspension to each 50-µL amplified DNA sample bead suspension in the PCR plate or strip tube.
5. Mix thoroughly by pipetting up and down.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
6. Incubate samples for 5 minutes at room temperature.
7. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
8. 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.
9. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µL of freshly prepared 70% ethanol in each sample well.
10. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
11. Repeat step 9 and step 10 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
12. Seal the wells with strip caps, then briefly spin to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
13. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
14. Add 30 µL of nuclease-free water to each sample well.

15 Seal the sample wells, then mix well on a vortex mixer and briefly spin to collect the liquid.

16 Incubate for 2 minutes at room temperature.

17 Put the plate or strip tube in the magnetic stand and leave for 2 minutes or until the solution is clear.

18 Remove the cleared supernatant (approximately 30 µL) to a fresh well. You can discard the beads at this time.

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

Step 3. Assess indexed library DNA quantity and quality

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

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified indexed DNA. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
3. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
4. Verify that the electropherogram shows the peak of DNA fragment size positioned between 250 and 350 bp. A sample electropherogram is shown in Figure 9.
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 79.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 80.

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

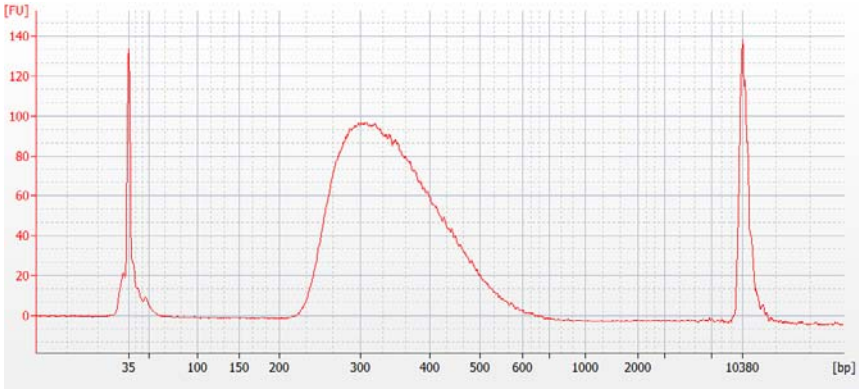

Figure 9 Post-capture analysis of amplified indexed library DNA using the 2100 Bioanalyzer and a High Sensitivity DNA Assay.

Option 2: Analysis using an Agilent 4200 TapeStation or 2200 TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the amplified indexed DNA. For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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

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

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

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 250 and 350 bp. A sample electropherogram is shown in Figure 10.

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 79.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 80.

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.

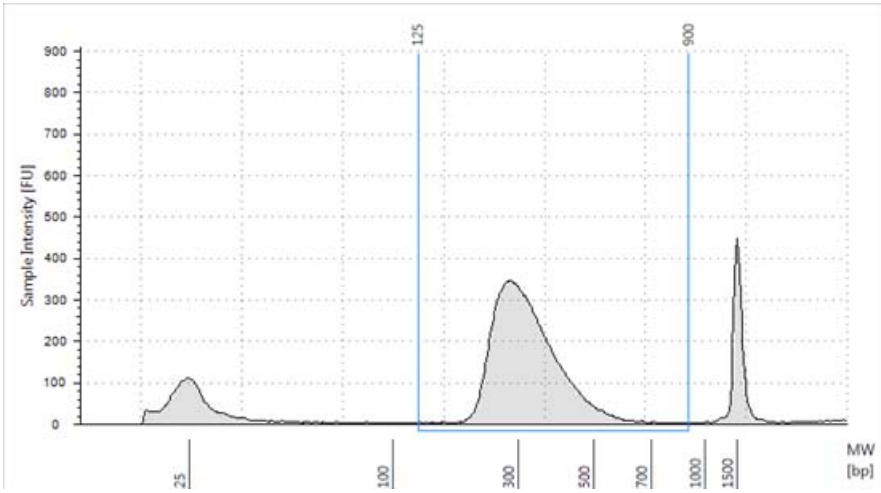

Figure 10 Post-capture analysis of amplified indexed library DNA using the 2200 TapeStation with a High Sensitivity D1000 ScreenTape.

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.

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 39 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 39 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

NOTE The sequencing workflow for FFPE-derived DNA libraries should employ the modifications discussed in the Appendix, starting on page 85. Modifications include the requirement for adapter trimming and increased sequencing depth for lower-integrity DNA samples.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 40 for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect^{XT} target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 40 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.

Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 40 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	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	200 Cycle Kit	v3	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	12–14 pM
HiSeq 2000	All Runs	2 × 100 bp	200 Cycle Kit	v3	6–9 pM
HiSeq 2000	All Runs	2 × 100 bp	250 Cycle Kit	v4	8–12 pM
MiSeq	All Runs	2 × 100 bp	300 Cycle Kit	v2	9–10 pM
MiSeq	All Runs	2 × 76 bp	150 Cycle Kit	v3	12–16 pM
NextSeq 500/550	All Runs	2 × 100 bp	300 Cycle Kit	v2	1.5–1.8 pM
HiSeq 3000/4000	All Runs	2 × 100 bp	300 Cycle Kit	v1	180–190 pM

Sequencing run setup guidelines for 8-bp indexes

Sequencing runs must be set up to perform an 8-bp index read. See the Reference chapter for complete index sequence information.

For the HiSeq 2500 and NextSeq 500 (v1) platforms, use the *Cycles* settings shown in Table 41. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

Table 41 Cycle Number settings for HiSeq and NextSeq platforms

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	9
Index 2 (i5)	0
Read 2	100

For the MiSeq platform, use the Illumina Experiment Manager (IEM) software to generate a Sample Sheet that includes the run parameters specified in Table 42.

Table 42 Run parameters for MiSeq platform Sample Sheet

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

6 Appendix: Using FFPE-derived DNA Samples

FFPE-derived DNA samples may be used in the Library Preparation protocol for 200 ng samples and subsequent Target Enrichment protocol after making the minor protocol modifications detailed in this chapter.

Protocol modifications that should be applied to all FFPE samples are detailed on page 86.

Additional protocol modifications may be appropriate, depending on the integrity of the FFPE sample DNA. DNA integrity may be assessed using the Agilent NGS FFPE QC Kit or using the Agilent 4200 TapeStation or 2200 TapeStation system and Genomic DNA ScreenTape.

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample and a ΔΔCq DNA integrity score. Protocol modifications based on ΔΔCq scores for individual samples are detailed on page 87.

The Agilent 4200 TapeStation or 2200 TapeStation system, combined with the Genomic DNA ScreenTape assay, provides a microfluidics-based method for determination of a DNA Integrity Number (DIN) score. Protocol modifications based on DIN scores for individual samples are detailed on page 89.

Modifications for all FFPE DNA samples

SureSelect^{XT} Protocol Modifications

Protocol modifications that should be applied to all FFPE samples are detailed in Table 42.

Table 42 SureSelect^{XT} protocol modifications for all FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
Library Preparation using 200 ng DNA, page 43	Duration of DNA Shearing	6 minutes	4 minutes
Library Preparation using 200 ng DNA, page 50	Dilution of SureSelect Adaptor Oligo Mix for Ligation reaction	Use 1:10 dilution of SureSelect Adaptor Oligo Mix	Use undiluted SureSelect Adaptor Oligo Mix
Hybridization, page 61	Amount of prepared library added to Hybridization	750 ng	500–750 ng (use maximum available in range)
Post-capture PCR, page 71	Amount of captured DNA bead suspension added to PCR	14 µl	30 µl (decrease the amount of water added to post-capture PCR by 16 µl to compensate for greater volume of captured DNA)

Downstream Sequencing Modifications

For all FFPE sample-derived libraries, set up the sequencing run to include adapter trimming.

To do this step, use the IEM Sample Sheet Wizard. When prompted by the wizard, select the **Use Adapter Trimming** and **Use Adapter Trimming Read 2** options. This enables the MiSeq Reporter software to identify the adaptor sequence and trim the adaptor from reads.

Modifications for samples assessed using the Agilent NGS FFPE QC Kit

Before applying protocol modifications in this section, use the Agilent NGS FFPE QC Kit to determine the ΔΔCq DNA integrity score and the quantity of amplifiable DNA for each FFPE DNA sample. For the complete Agilent NGS FFPE QC Kit protocol, go to genomics.agilent.com and search for document part number G9700-90000.

SureSelect^{XT} Protocol Modifications

Protocol modifications that should be applied to FFPE samples based on the ΔΔCq score determined for each sample are detailed in Table 43.

Table 43 SureSelect^{XT} protocol modifications based on ΔΔCq DNA integrity score

Protocol Step and Parameter	non-FFPE Samples	FFPE Samples		
		ΔΔCq≤1*	ΔΔCq between 1 and 4	ΔΔCq>4
DNA input for Library Preparation, page 42	200 ng, based on Qubit Assay	200 ng, based on Qubit Assay	100 to 200 ng of amplifiable DNA, based on qPCR quantification	100 to 200 ng of amplifiable DNA, based on qPCR quantification
Pre-capture PCR cycle number, page 55	10 cycles	10 cycles	10 cycles	13 cycles

* FFPE samples with ΔΔCq scores ≤1 should be treated like non-FFPE samples at these steps. For samples of this type, make sure to use the DNA concentration determined by the Qubit Assay, instead of the concentration determined by qPCR, to calculate the volume required for 200 ng DNA.

Downstream Sequencing Modifications

After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines in Table 44 to determine the amount of extra sequencing output required for FFPE DNA samples, based on the ΔΔCq DNA integrity score.

For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with ΔΔCq score of 1 requires 150–200 Mb of sequencing output to achieve the same coverage.

Table 44 Recommended sequencing augmentation for FFPE-derived DNA samples

ΔΔCq value	Recommended fold increase for FFPE-derived sample
<0.5	No extra sequencing output
between 0.5 and 2	Increase sequencing allocation by 1.5× to 2×
between 2 and 3.5	Increase sequencing allocation by 3×
between 3.5 and 5	Increase sequencing allocation by 4× to 5×
>5	Increase sequencing allocation by 6× to 10×

Modifications for samples assessed using Agilent's Genomic DNA ScreenTape

Before applying protocol modifications in this section, use the Agilent 4200 TapeStation or 2200 TapeStation system and Genomic DNA ScreenTape to determine the DNA Integrity Number (DIN) score for each sample. For more information on how to obtain DIN numbers using the TapeStation system, go to genomics.agilent.com and search for document part number G5991-5442.

Use the DIN score to determine whether additional SureSelect^{XT} protocol or downstream sequencing modifications are appropriate for each sample.

SureSelect^{XT} Protocol Modifications

Protocol modifications that should be applied to FFPE samples based on DIN score are detailed in Table 45.

Table 45 SureSelect^{XT} protocol modifications based on DIN score

Protocol Step and Parameter	non-FFPE Samples	FFPE Samples		
		DIN ≥5	DIN between 2 and 5	DIN <2
DNA input for Library Preparation, page 42	200 ng, based on Qubit Assay	200 ng, based on Qubit Assay	100 to 200 ng of amplifiable DNA, based on qPCR quantification*	100 to 200 ng of amplifiable DNA, based on qPCR quantification
Pre-capture PCR cycle number, page 55	10 cycles	10 cycles	10 cycles	13 cycles

* Use the Agilent NGS FFPE QC Kit for qPCR-based sample quantification. See Table 7 on page 19 for ordering information.

Downstream Sequencing Modifications

After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines in Table 46 below to determine the amount of extra sequencing output required for FFPE DNA samples, based on the ΔΔCq DNA integrity score.

For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with DIN score of 4 requires approximately 200 Mb of sequencing output to achieve the same coverage.

Table 46 Recommended sequencing augmentation for FFPE-derived DNA samples

DIN value	Recommended fold increase for FFPE-derived sample
≥8	No extra sequencing output
between 5 and 8	Increase sequencing allocation by 1.5×
between 3 and 5	Increase sequencing allocation by 2×
between 1.5 and 3	Increase sequencing allocation by 3× to 5×
<1.5	Increase sequencing allocation by 6× to 10×

7 Reference

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

Kit Contents

Each SureSelect^{XT} Reagent Kit contains the following component kits:

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect XT Library Prep Kit ILM	–20°C	5500-0132	5500-0133	5 x 5500-0133
SureSelect Target Enrichment-Box 1	Room Temperature	5190-8645	5190-8646	5 x 5190-8646
SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2	–20°C	5190-4455	5190-4456	5190-4457

The contents of each of the component kits listed in Table 47 are described in the tables below.

Table 47 SureSelect XT Library Prep Kit ILM Content

Kit Component	16 Reactions	96 or 480 Reactions
10X End Repair Buffer	tube with clear cap	tube with clear cap
10X Klenow Polymerase Buffer	tube with blue cap	tube with blue cap
5X T4 DNA Ligase Buffer	tube with green cap	tube with green cap
T4 DNA Ligase	tube with red cap	tube with red cap
Exo(-) Klenow	tube with red cap	tube with red cap
T4 DNA Polymerase	tube with purple cap	tube with purple cap
Klenow DNA Polymerase	tube with yellow cap	tube with yellow cap
T4 Polynucleotide Kinase	tube with orange cap	tube with orange cap
dATP	tube with green cap	tube with green cap
dNTP Mix	tube with green cap	tube with green cap
SureSelect Adaptor Oligo Mix	tube with brown cap	tube with brown cap
SureSelect Primer (forward primer)	tube with brown cap	tube with brown cap
SureSelect^{XT} 8 bp Index reverse primers*	SureSelect 8bp Indexes A01 through H02, provided in 16 white-capped tubes	SureSelect 8bp Indexes A01 through H12, provided in blue 96-well plate†

* See Table 52 on page 96 for index sequences.

† See Table 50 on page 95 for a plate map.

Table 48 SureSelect Target Enrichment-Box 1 Content

Kit Component	16 Reactions	96 or 480 Reactions
SureSelect Hyb 1	tube with orange cap	tube with orange cap
SureSelect Hyb 2	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

Table 49 SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2 Content

Kit Component	16 Reactions	96 Reactions	480 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap	bottle
SureSelect Indexing Block 1	tube with green cap	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap	tube with blue cap
SureSelect ILM Indexing Block 3	tube with brown cap	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap	tube with purple cap
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	tube with clear cap	tube with clear cap	tube with clear cap
SureSelect ILM Indexing Post-Capture Forward PCR Primer	tube with orange cap	tube with orange cap	tube with orange cap

Table 50 Plate map for SureSelect 8bp Indexes A01 through H12, provided in blue plate in Library Prep kit p/n 5500-0133

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

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

Each index is 8 nt in length. See page 83 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 51 SureSelect^{XT} Indexes, for indexing primers provided in blue 96-well plate or white capped tubes

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

Alternative Capture Equipment Combinations

Table 52 below lists combinations of thermal cyclers, lid temperatures, plates or strip tubes and sealing methods that have shown minimal evaporation when used for the Hybridization protocol on page 60.

Refer to this list for additional equipment and plasticware combination options for hybridization, beyond the combinations used for protocol optimization and supported by Agilent. Note that minimal evaporation is required to ensure optimal capture results.

Table 52 Tested options that show minimal evaporation

PCR Machine	Plate/Strips	Cover	Comments
Agilent Mx3005P Real-Time PCR System	Mx3005P Strip Tubes (Agilent p/n 401428)	Mx3005P Optical Strip Caps (Agilent p/n 401425)	Heated lid
Agilent Mx3005P Real-Time PCR System	Thermo Fisher Scientific ABI MicroAmp Optical 96-well plates (p/n N8010560)	MicroAmp clear adhesive film (p/n 4306311)	Heated lid; use ABI compression pad (4312639); use two layers of film
ABI GeneAmp 9700	Thermo Fisher Scientific ABI MicroAmp Optical 96-well plates (p/n N8010560)	MicroAmp caps (p/n N8010535)	Heated lid
ABI Veriti (p/n 4375786)	Thermo Fisher Scientific ABI MicroAmp Optical 96-well plates (p/n N8010560)	MicroAmp clear adhesive film (p/n 4306311)	Heated lid; use ABI compression pad (4312639); use two layers of film
Eppendorf Mastercycler	Eppendorf 8-Tube PCR Tubes	Attached caps	Lid heating set to 75°C
BioRad (MJ Research) PTC-200	Mx4000 Strip Tubes (Agilent p/n 410022)	Mx4000 Optical Caps (Agilent p/n 401024)	Heated lid
BioRad (MJ Research) PTC-200	Mx4000 Strip Tubes (Agilent p/n 410022)	Mx3005P Optical Strip Caps (Agilent p/n 401425)	Heated lid
BioRad (MJ Research) PTC-200	Mx3005P 96-well plate (Agilent p/n 410088)	Mx3005P Optical Strip Caps (Agilent p/n 401425)	Heated lid

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In This Book

This guide contains information to run the SureSelect^{XT} target enrichment protocol.

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