# SureSelect$^{XT}$ Target Enrichment System for the Illumina Platform

## Protocol

**Version D1, July 2021**

**SureSelect platform manufactured with Agilent SurePrint Technology**

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

Agilent Technologies

# Notices



## Manual Part Number

G7530-90000

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

## Acknowledgment



## Technical Support

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

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**CAUTION**

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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 D1

- Support for SureSelect XT Human All Exon V8 Probe (see Table 3 on page 17)
- Minor updates to instructions in the"Hybridization and Capture" chapter on page 63 to page 71
- Updates to downstream sequencing platform and kit support information (see Table 41 on page 86 and footnote to Table 2 on page 16)

## What's New in Version D0

- Support for revised SureSelect custom probe products, produced using an updated manufacturing process beginning August, 2020 (see Table 4 on page 18). Custom probes produced using the legacy manufacturing process are also fully supported by the protocols in this document. Probe information was reorganized (see Table 3 on page 17 through Table 5 on page 18), and probe nomenclature throughout document was updated.
- Updates to thermal cycler and plasticware recommendations (see Table 6 on page 19, *Caution* on page 19 and on page 65, *Procedural Notes* on page 14, and Table 55 on page 101).
- Updates to ordering information for Dynabeads MyOne Streptavidin T1 beads, 1X Low TE Buffer, and AMPure XP Kits (Table 2 on page 16) and for Eppendorf ThermoMixer C and Qubit Fluorometer (Table 6 on page 19).
- Updates to optional materials in Table 7 on page 21, including removal of ethylene glycol supplier information (see page 24 and page 44 for related updates to DNA shearing set up instructions).
- Updates to Agilent TapeStation 4200/4150 ordering information (Table 6 on page 20) and sample mixing information (for example, see *Caution* on page 61).
- Support for 5200 Fragment Analyzer (see footnote to Table 6 on page 20).

- Correction to “Downstream Sequencing Modifications" on page 93 to indicate that FFPE sample adjustments based on DIN score.
- Updates to Technical Support contact information (see page 2)

# Content

# 1 Before You Begin



Make sure you have the most current protocol. Go to 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.

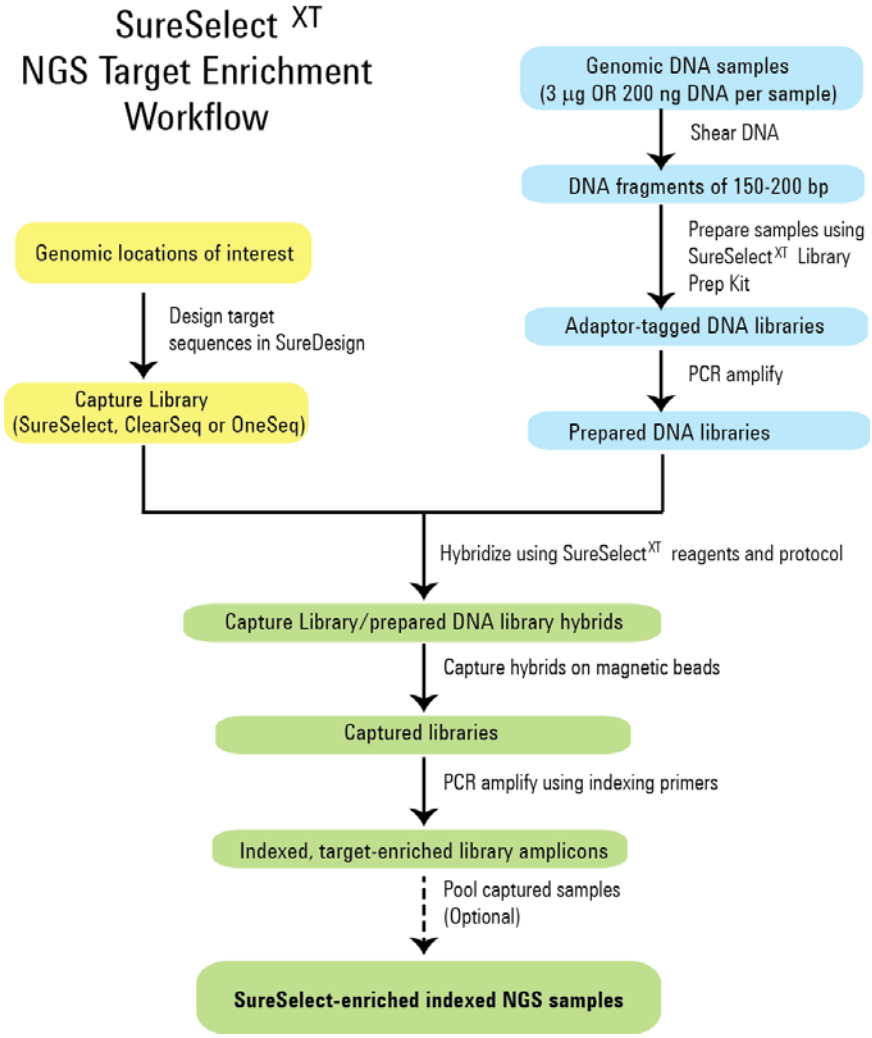


**Figure 1** Overall target-enriched sequencing sample preparation workflow.

**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 includes sample processing steps in PCR plates or strip tubes, with certain protocol steps using liquid volumes exceeding 0.2 ml. Before you begin, you must verify that the wells of the plasticware selected for use 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 (BSL1) 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

| Description | Vendor and part number |
|---|---|
| SureSelect, ClearSeq or OneSeq Probe | Select the appropriate probe from Table 3, Table 4, or Table 5 |
| 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 |
| 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 |
| 50 mL | p/n 65604D |
| 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA) | Thermo Fisher Scientific p/n 12090-015, or equivalent |
| 100% Ethanol, molecular biology grade | Sigma-Aldrich p/n E7023 |
| Qubit dsDNA HS Assay Kit *or* | Thermo Fisher Scientific p/n Q32851 |
| Qubit BR dsDNA Assay Kit | Thermo Fisher Scientific |
| 100 assays | p/n Q32850 |
| 500 assays | p/n Q32853 |
| Nuclease-free Water (not DEPC-treated) | Thermo Fisher Scientific p/n AM9930 |

* HiSeq and MiSeq Reagent Kits are also compatible with the NextSeq 500 and NovaSeq 6000 platforms.

**Table 3** Compatible Pre-Designed Probes

| Probe | 16 Reactions | 96 Reactions |
|---|---|---|
| **SureSelect XT Human All Exon V8** | 5191-6879 | 5191-6891 |
| **SureSelect XT Human All Exon V7** | 5191-4004 | 5191-4005 |
| **SureSelect XT Human All Exon V6** | 5190-8863 | 5190-8864 |
| **SureSelect XT Human All Exon V6 + UTRs** | 5190-8881 | 5190-8882 |
| **SureSelect XT Human All Exon V6 + COSMIC** | 5190-9307 | 5190-9308 |
| **SureSelect XT Clinical Research Exome V2** | 5190-9491 | 5190-9492 |
| **SureSelect XT Focused Exome** | 5190-7787 | 5190-7788 |
| **SureSelect XT Mouse All Exon** | 5190-4641 | 5190-4642 |
| **SureSelect XT Human X-Chromosome** | 5190-4651 | 5190-4652 |
| **ClearSeq Comprehensive Cancer XT** | 5190-8011 | 5190-8012 |
| **ClearSeq Inherited Disease XT** | 5190-7518 | 5190-7519 |
| **Pre-designed probes customized with additional *Plus* custom content** | | |
| **SureSelect XT Human All Exon V7 Plus 1** | Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance. | |
| **SureSelect XT Human All Exon V7 Plus 2** | | |
| **SureSelect XT Human All Exon V6 Plus 1** | | |
| **SureSelect XT Human All Exon V6 Plus 2** | | |
| **SureSelect XT Clinical Research Exome V2 Plus 1** | | |
| **SureSelect XT Clinical Research Exome V2 Plus 2** | | |
| **SureSelect XT Focused Exome Plus 1** | | |
| **SureSelect XT Focused Exome Plus 2** | | |
| **ClearSeq Comprehensive Cancer Plus XT** | | |
| **ClearSeq Inherited Disease Plus XT** | | |

**Table 4** Compatible Custom Probes*

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

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

**Table 5** Compatible OneSeq CNV Probes

| Probe | 16 Reactions | 96 Reactions |
|---|---|---|
| **OneSeq 300kb CNV Backbone + Human All Exon V7** | 5191-4022 | 5191-4023 |
| **OneSeq 1Mb CNV Backbone + Human All Exon V7** | 5191-4025 | 5191-4026 |
| **OneSeq Constitutional Research Panel** | 5190-8702 | 5190-8703 |
| **Pre-designed probes customized with additional *Plus* custom content** | | |
| **OneSeq 1Mb CNV Backbone + Custom 1–499 kb** | Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance. | |
| **OneSeq 1Mb CNV Backbone + Custom 0.5–2.9 Mb** | | |
| **OneSeq 1Mb CNV Backbone + Custom 3–5.9 Mb** | | |
| **OneSeq 1Mb CNV Backbone + Custom 6–11.9 Mb** | | |
| **OneSeq 1Mb CNV Backbone + Custom 12–24 Mb** | | |
| **OneSeq Hi Res CNV Backbone + Custom 1–499 kb** | | |
| **OneSeq Hi Res CNV Backbone + Custom 0.5 –2.9 Mb** | | |
| **OneSeq Hi Res CNV Backbone + Custom 3–5.9 Mb** | | |
| **OneSeq Hi Res CNV Backbone + Custom 6–11.9 Mb** | | |

# Required Equipment

**CAUTION** Sample volumes exceed 0.2 ml in certain steps of this protocol. Make sure that the plasticware used with the selected thermal cycler holds ≥0.31 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 6** Required Equipment

| Description | Vendor and part number |
|---|---|
| Thermal Cycler with 96-well, 0.2 ml block | Various suppliers |
| Plasticware compatible with the selected thermal cycler:<br>96-well plates or 8-well strip tubes<br>Tube cap strips, domed | Consult the thermal cycler manufacturer's recommendations |
| Qubit Fluorometer | Thermo Fisher Scientific p/n Q33238 |
| Qubit Assay Tubes | Thermo Fisher Scientific p/n Q32856 |
| Covaris Sample Preparation System, S-series or E-series model | Covaris |
| Covaris sample holders<br>96 microTUBE plate (E-series only)<br>microTUBE for individual sample processing | <br>Covaris p/n 520078<br>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 |
| 96-well plate mixer | Eppendorf ThermoMixer C, p/n 5382000023 and Eppendorf SmartBlock 96 PCR, p/n 5306000006, or equivalent |

**Table 6** Required Equipment

| Description | Vendor and part number |
|---|---|
| DNA Analysis Platform and Consumables* | |
| Agilent 2100 Bioanalyzer Instrument | Agilent p/n G2939BA |
| Agilent 2100 Expert SW Laptop Bundle (optional) | Agilent p/n G2953CA |
| DNA 1000 Kit | Agilent p/n 5067-1504 |
| High Sensitivity DNA Kit | Agilent p/n 5067-4626 |
| OR | |
| Agilent 4200/4150 TapeStation | Agilent p/n G2991AA/G2992AA |
| 96-well sample plates | Agilent p/n 5042-8502 |
| 96-well plate foil seals | Agilent p/n 5067-5154 |
| 8-well tube strips | Agilent p/n 401428 |
| 8-well tube strip caps | Agilent p/n 401425 |
| D1000 ScreenTape | Agilent p/n 5067-5582 |
| D1000 Reagents | Agilent p/n 5067-5583 |
| High Sensitivity D1000 ScreenTape | Agilent p/n 5067-5584 |
| High Sensitivity D1000 Reagents | Agilent p/n 5067-5585 |
| Magnetic separator | Thermo Fisher Scientific p/n 12331D or equivalent† |
| Vacuum concentrator | Savant SpeedVac, model DNA120, or equivalent |
| Multichannel pipette | Rainin Pipet-Lite Multi Pipette or equivalent |
| P10, P20, P200 and P1000 pipettes | Rainin Pipet-Lite Pipettes or equivalent |
| Sterile, nuclease-free aerosol barrier pipette tips | general laboratory supplier |
| Vortex mixer | general laboratory supplier |
| Ice bucket | general laboratory supplier |
| Powder-free gloves | general laboratory supplier |

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

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

# Optional Reagents and Equipment

**Table 7** Optional materials for processing of all samples

| Description | Vendor and part number |
|---|---|
| Tween 20 | Sigma-Aldrich p/n P9416-50ML |
| PlateLoc Thermal Microplate Sealer with Small Hotplate and Peelable Aluminum Seal for PlateLoc Sealer | Please contact the SureSelect support team (see page 2) or your local representative for ordering information |
| MicroAmp Clear Adhesive Film | Thermo Fisher Scientific p/n 4311971 |
| 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 8** 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 43.**

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 12.

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.

This protocol has been optimized using a Covaris model E220 instrument and 130-µl Covaris microTUBE for shearing 130-µl DNA samples to a target DNA fragment size of 150 to 200 bp. To shear using a different Covaris instrument model/sample holder, or if your NGS workflow requires a different DNA fragment size, consult the manufacturer's literature for recommended shearing conditions.

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. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.

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 6 on page 19) 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 (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) 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 septum.

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 9.

The target DNA fragment size is 150 to 200 bp.

**Table 9** 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 septum, 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. Before you begin, 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 Bioanalyzer DNA 1000 chip and reagent kit and perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Check that the electropherogram shows a DNA fragment size peak between 150–200 bp. A sample electropherogram is shown in Figure 2.

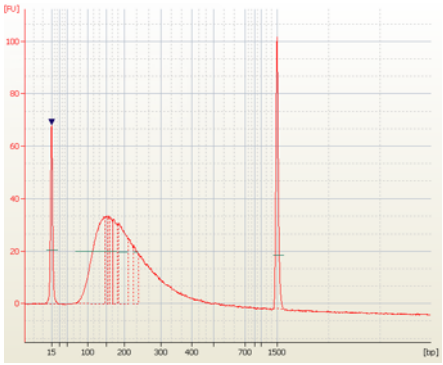


**Figure 2** Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

### Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit for analysis of the 3 µg sheared DNA samples. Perform the assay according to the Agilent D1000 Assay Quick Guide.

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

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

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

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

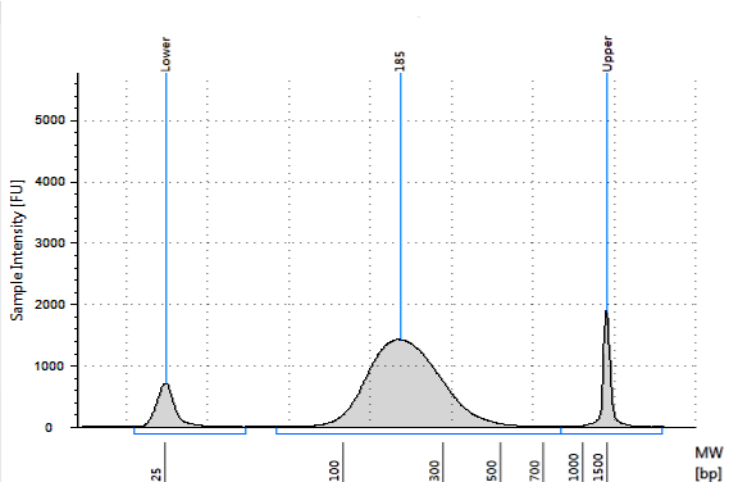


**Figure 3** Analysis of sheared DNA using a D1000 ScreenTape.

## 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 10, on ice. Mix well on a vortex mixer.

**Table 10** 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 11. Do not use a heated lid.

**Table 11** 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. dA-tail 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 dA-Tailing master mix, as described in Table 12, on ice. Mix well on a vortex mixer.

**Table 12** Preparation of dA-Tailing 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 dA-Tailing 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 13. Do not use a heated lid.

**Table 13** 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 14, on ice. Mix well on a vortex mixer.

**Table 14** 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 15. Do not use a heated lid.

**Table 15** 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 16. Thaw the reagents listed below and keep on ice.

**Table 16** Reagents for pre-capture PCR amplification

| Component | Storage Location |
|---|---|
| SureSelect Primer | SureSelect XT Library Prep Kit ILM, –20°C |
| SureSelect ILM Index 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 17, on ice. Mix well on a vortex mixer.

**Table 17** 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 Index 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 17 and 15 µL of each purified DNA library sample from step 16 on page 35. Add a single DNA library sample to each well of the plate or strip tube. Mix by pipetting.

**3** Run the program in Table 18 in a thermal cycler.

.

**Table 18** 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 Agilent TapeStation instrument.

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

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

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Verify that the electropherogram shows 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.

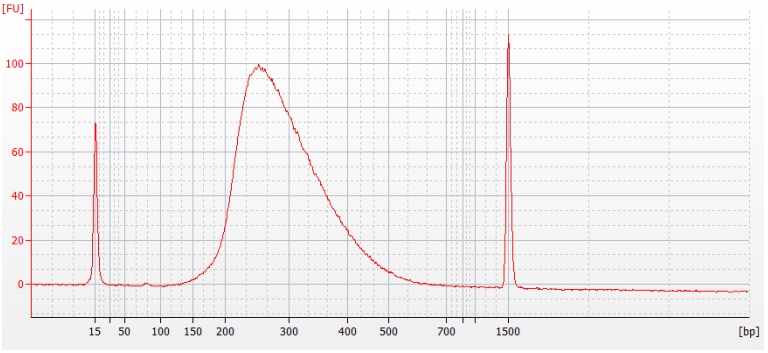


**Figure 4** Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

### Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

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

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

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

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

3 Verify that the electropherogram shows 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.

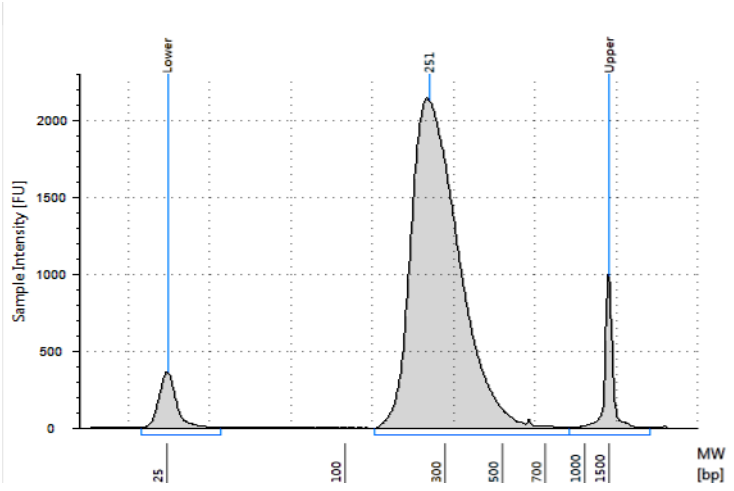


**Figure 5** Analysis of amplified library DNA using a D1000 ScreenTape.

# 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 23.**

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 12.

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 89 before starting the library preparation protocol.

This protocol has been optimized using a Covaris model E220 instrument and 130-µl Covaris microTUBE for shearing 50-µl DNA samples to a target DNA fragment size of 150 to 200 bp. To shear using a different Covaris instrument model/sample holder (e.g. 50-µl microTUBE), or if your NGS workflow requires a different DNA fragment size, consult the manufacturer's literature for recommended shearing conditions.

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

1 Set up the Covaris E-series or S-series instrument. Refer to the Covaris instrument user guide for details.

   **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. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.

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 6 on page 19) 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 (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) 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 septum.

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 19.

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 19** 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 septum, 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.** Perform the assay according to the High Sensitivity DNA Kit Guide.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Check that the electropherogram shows a DNA fragment size peak at approximately 150 bp. A sample electropherogram is shown in Figure 6.

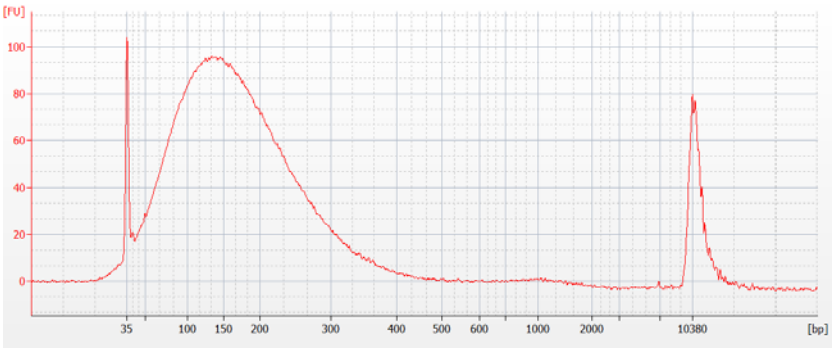


**Figure 6** Analysis of sheared DNA using a High Sensitivity DNA Bioanalyzer assay.

## 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 20, on ice. Mix well on a vortex mixer.

**Table 20** 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 21. Do not use a heated lid.

**Table 21** 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. dA-tail 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 dA-Tailing master mix, as described in Table 22, on ice. Mix well on a vortex mixer.

**Table 22** Preparation of dA-Tailing 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 dA-Tailing 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 23. Do not use a heated lid.

**Table 23** 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 ten-fold in nuclease-free water immediately before use. For example, for a 16-reaction run, combine 17 µL SureSelect Adaptor Oligo Mix (brown cap) with 153 µL of nuclease-free water.

   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 24, on ice. Mix well on a vortex mixer.

**Table 24** 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 25. Do not use a heated lid.

**Table 25** 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 26. Thaw the reagents listed below and keep on ice.

**Table 26** Reagents for pre-capture PCR amplification

| Component | Storage Location |
|---|---|
| SureSelect Primer (brown cap) | SureSelect XT Library Prep Kit ILM, –20°C |
| SureSelect ILM Index 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 27, on ice. Mix well on a vortex mixer.

**Table 27** 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 Index 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 27 to each purified DNA library sample (30 µL) in the PCR plate wells.

Mix by pipetting.

**3** Run the program in Table 28 in a thermal cycler.

.

**Table 28** 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 91 or page 93 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

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

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
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 7.

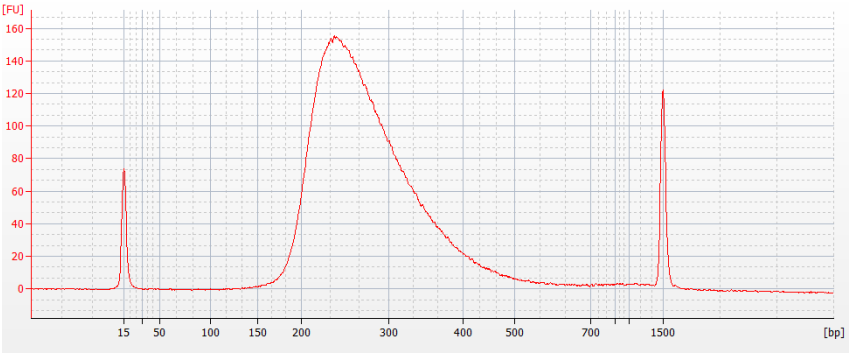


**Figure 7** Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

### Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

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

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

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

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

3 Verify that the electropherogram shows 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.

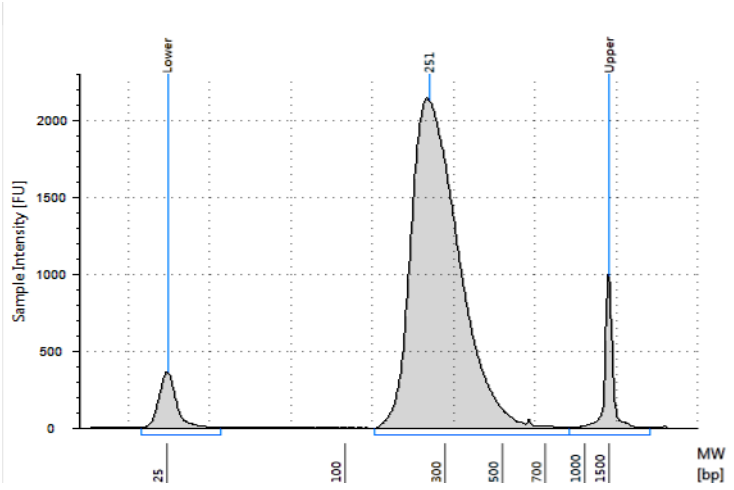


**Figure 8** Analysis of amplified library DNA using a D1000 ScreenTape.

**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 probe. After hybridization, the targeted molecules are captured on streptavidin beads.

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

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

# Step 1. Hybridize DNA samples to the probe

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

This step uses the components listed in Table 29. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

**Table 29** Reagents for Hybridization

| Kit Component | Storage Location | Thawing Conditions | Where Used |
|---|---|---|---|
| SureSelect Hyb 1 | SureSelect Target Enrichment-Box 1, RT | — | page 66 |
| SureSelect Hyb 2 | SureSelect Target Enrichment-Box 1, RT | — | page 66 |
| SureSelect Hyb 3 | SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C | Warm to Room Temperature (RT) | page 66 |
| SureSelect Hyb 4 | SureSelect Target Enrichment-Box 1, RT | — | page 66 |
| SureSelect Indexing Block 1 | SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C | Thaw on ice | page 66 |
| SureSelect Block 2 | SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C | Thaw on ice | page 66 |
| SureSelect Indexing Block 3 | SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C | Thaw on ice | page 66 |
| SureSelect RNase Block | SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C | Thaw on ice | page 67 |
| Probe | –80°C | Thaw on ice | page 68 |

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.

Before you do the first experiment, make sure the plasticware and sealing method (strip caps or sealing tape), are appropriate for the thermal cycler. To test evaporation under the conditions used for hybridization, incubate 27 µL of water at 65°C for 24 hours. Include water in each well that you might use, including center and edge wells. Check that no more than 4 µL is lost to evaporation.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 101.

4 Prepare the Hybridization Buffer by mixing the components in Table 30 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 30** Preparation of Hybridization Buffer

| Reagent | Volume for 1 reaction* | Volume for 16 reactions (includes excess) |
|---|---|---|
| SureSelect Hyb 1 (orange cap) | 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) | 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 31. Keep the mixture on ice until it is used in step 6.

**Table 31** 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 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 65, add 5.6 µL of the SureSelect Block Mix prepared in Table 31. 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 32.

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 32** 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 design size of your probe, according to Table 33. 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 33** Preparation of RNase Block dilution

| Probe Size | RNase Block dilution (parts RNase Block: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 mixture described in step 9, below, near the end of the 65°C hold step of >5 minute duration described in Table 32. Keep the mixture at room temperature briefly, until adding the mixture to sample wells in step 10. Do not keep solutions containing the probe at room temperature for extended periods.

**9** Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 34 for probes≥3 Mb or Table 35 for probes<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 34** Preparation of Probe Hybridization Mix for **probes ≥3 Mb**

| 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 |
| Probe (with design ≥3 Mb) | 5 µL | 85 µL |
| **Total** | **20 µL** | **340 µL** |

**Table 35** Preparation of Probe Hybridization Mix for **probes<3 Mb**

| 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 |
| Probe (with design <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 Probe 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 or 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 16).

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 71.
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 16 or 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 55 on page 101 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 all of 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 all of 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 75.

**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 36. Thaw then vortex to mix the reagents listed below and keep on ice.

**Table 36** 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 44 on page 90 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 54 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 37, on ice. Mix well on a vortex mixer.

**Table 37** 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 37 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 72).

**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 38.

**Table 38** 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 39 for recommendations for specific Probes | 57°C | 30 seconds |
| | | 72°C | 1 minute |
| 3 | 1 | 72°C | 10 minutes |
| 4 | 1 | 4°C | Hold |

**Table 39** Post-capture PCR cycle number recommendations

| Probe Design Size/Description | 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. Perform the assay according to the High Sensitivity DNA Kit Guide.

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 83.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 84.

**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.

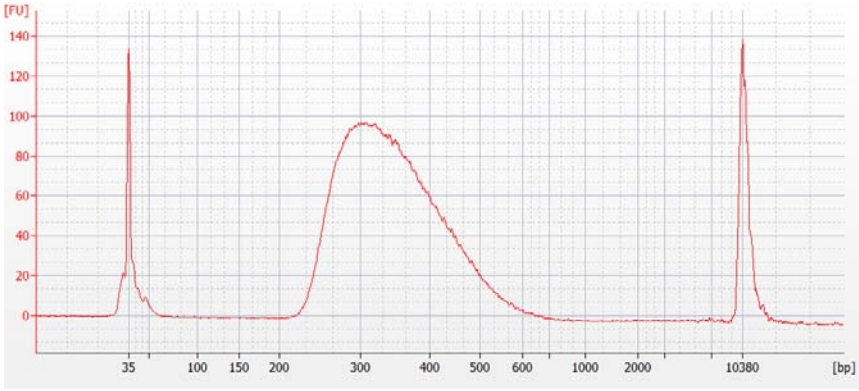


**Figure 9** Post-capture analysis of amplified indexed library DNA using the 2100 Bioanalyzer and a High Sensitivity DNA Assay.

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

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

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

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

2 Load the sample plate or tube strips from step 1, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation instrument as instructed in the reagent kit guide. 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 83.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 84.

**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.

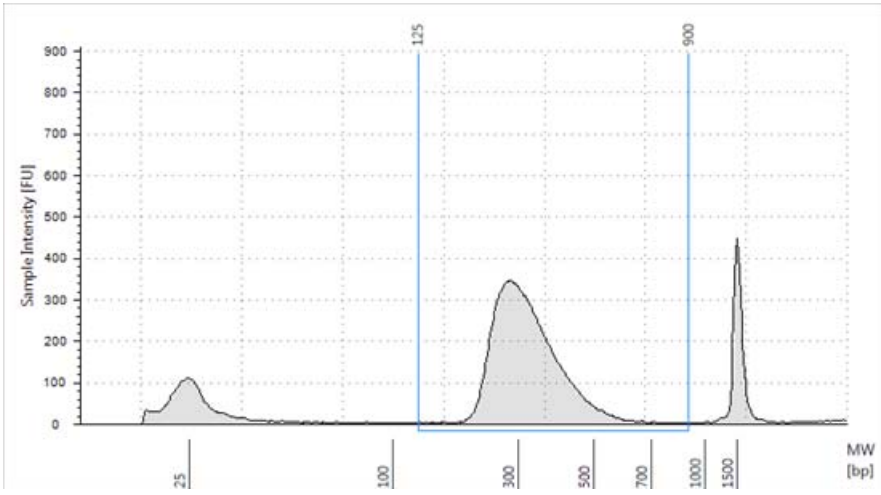


**Figure 10** Post-capture analysis of amplified indexed library DNA using a High Sensitivity D1000 ScreenTape.

## 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 40 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 40** 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 89. 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 41 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 41 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. Begin optimization using a seeding concentration in the middle of the range listed in Table 41.

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

**Table 41** Illumina Kit Configuration and Seeding Concentration 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 | 250 Cycle Kit | v4 | 12–14 pM |
| MiSeq | All Runs | 2 × 100 bp | 300 Cycle Kit | v2 | 9–10 pM |
| MiSeq | All Runs | 2 × 75 bp | 150 Cycle Kit | v3 | 12–16 pM |
| NextSeq 500/550 | All Runs | 2 × 100 bp | 300 Cycle Kit | v2.5 | 1.2–1.5 pM |
| HiSeq 3000/4000 | All Runs | 2 × 100 bp | 300 Cycle Kit | v1 | 300–400 pM |
| NovaSeq 6000 | Standard Workflow Runs | 2 × 100 bp | 300 Cycle Kit | v1.0 or v1.5 | 300–600 pM |
| NovaSeq 6000 | Xp Workflow Runs | 2 × 100 bp | 300 Cycle Kit | v1.0 or v1.5 | 200–400 pM |

### Sequencing run setup guidelines for 8-bp indexes

Sequencing runs must be set up to perform an 8-bp index read. For complete index sequence information, see the Table 54 on page 100.

For the HiSeq platform, **Cycles** settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the **Index Type** selection buttons. Use the **Cycles** settings shown in Table 42.

For the NextSeq and NovaSeq platforms, the **Read Length** settings shown in Table 42 can be specified on the *Run Setup* screen of the instrument control software interface. In the **Custom Primers** section of the NextSeq or NovaSeq platform *Run Setup* screen, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*)

**Table 42** Cycle Number settings for HiSeq/NextSeq/NovaSeq platforms

| Run Segment | Cycles/Read Length |
|---|---|
| Read 1 | 100 |
| Index 1 (i7) | 8 |
| Index 2 (i5) | 0 |
| Read 2 | 100 |

For the MiSeq platform, use the Illumina Experiment Manager (IEM) software to generate a Sample Sheet that includes the run parameters specified in Table 43.

**Table 43** Run parameters for MiSeq platform Sample Sheet

| Parameter | Entry |
|---|---|
| Workflow | GenerateFASTQ |
| Cycles for Read 1 | 100 for v2 chemistry<br>75 for v3 chemistry |
| Cycles for Read 2 | 100 for v2 chemistry<br>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 54 on page 100). |

# 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 90.

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 TapeStation instrument 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 91.

TThe Agilent TapeStation instrument, combined with the Genomic DNA ScreenTape assay, provides an automated electrophoresis method for determination of a DNA Integrity Number (DIN) score. Protocol modifications based on DIN scores for individual samples are detailed on page 93.

# Modifications for all FFPE DNA samples

## SureSelect$^{XT}$ Protocol Modifications

Protocol modifications that should be applied to all FFPE samples are detailed in Table 44.

**Table 44** 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 45 | Duration of DNA Shearing | 6 minutes | 4 minutes |
| Library Preparation using 200 ng DNA, page 53 | Dilution of SureSelect Adaptor Oligo Mix for Ligation reaction | Use ten-fold dilution of SureSelect Adaptor Oligo Mix | Use undiluted SureSelect Adaptor Oligo Mix |
| Hybridization, page 65 | Amount of prepared library added to Hybridization | 750 ng | 500–750 ng (use maximum available in range) |
| Post-capture PCR, page 75 | 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 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 45.

**Table 45** 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 44 | 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 58 | 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 46 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 46** 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 TapeStation instrument 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 agilent.com and search for document part number 5991-5442.

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 47.

**Table 47** 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 44 | 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 58 | 10 cycles | 10 cycles | 10 cycles | 13 cycles |

* Use the Agilent NGS FFPE QC Kit for qPCR-based sample quantification. See Table 8 on page 21 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 48 below to determine the amount of extra sequencing output required for FFPE DNA samples, based on the DNA integrity (DIN) 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 48** 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:

**Table 49** 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 49 are described in the tables below.

**Table 50** 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 54 on page 100 for index sequences.

† See Table 53 on page 99 for a plate map.

**Table 51** 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 52** 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 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 Index 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 53** 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 87 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

**Table 54 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 55 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 64. Note that minimal evaporation is required to ensure optimal capture results.

**Table 55** Hybridization equipment options that show minimal evaporation

| PCR Machine | Plate/Strips | Cover | Comments |
|---|---|---|---|
| Agilent SureCycler 8800 Thermal Cycler, p/n G8800A | 96-well plates, p/n 410088<br>8-well strip tubes, p/n 410092 | Domed cap strips, p/n 410096 | Heated lid |
| 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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