



SureSelect Target Enrichment System for Sequencing on Ion Proton

Protocol

Version C0, December 2016

SureSelect platform manufactured with Agilent
SurePrint Technology

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

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



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In this Guide...

This guide describes the recommended operational procedures to capture the genomic regions of interest using the Agilent SureSelect Target Enrichment System for sequencing on the Ion Proton platform.

1 Before You Begin

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

2 Sample Preparation

This chapter describes the steps to prepare the DNA samples for target enrichment.

3 Hybridization and Capture

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

4 Post-Hybridization Amplification 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.

5 Reference

This chapter contains reference information.

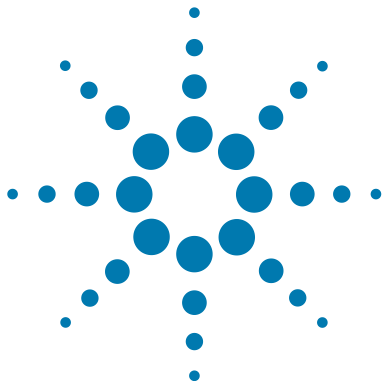
What's New in Version C0

- Support for ClearSeq Capture Libraries, including ClearSeq Comprehensive Cancer XT Libraries (see [Table 4](#) on page 13).
- Support for Clinical Research Exome V2 and All Exon v6 Capture Libraries (see [Table 3](#) on page 12).
- Updates to format and content of reagent and equipment specification tables (see [Table 2](#) on page 11 through [Table 6](#) on page 15).
- Relocation of the overall workflow figure and time requirements table (see [page 8](#) to [page 9](#)).
- Updates to “[Procedural Notes](#)” on page 10.
- Updates to library preparation setup instructions, including updates to reaction mixture tables for 16 reaction run sizes (for example, see [Table 9](#) on page 23). Revisions do not affect the final composition of the reaction mixtures.
- Updates to hybridization instructions (see [page 33](#) to [page 38](#)). Revisions do not affect the final composition of the hybridization reaction mixture, but format and order of certain preparation steps have been modified.
- Updates to post-capture amplification setup instructions (see [page 44](#) to [page 46](#)). Revisions do not affect the final composition of the amplification reaction mixture or cycling conditions.
- Updates to sample purification instructions (see [page 20](#), [page 24](#), [page 28](#), [page 47](#), and [page 53](#)). Revisions do not affect the amounts of reagents used or the final composition of the purified samples.
- Updates to workflow guidelines for order of library pooling and post-capture size selection (see [page 48](#)).
- Updates to “[Step 5. Size-select the DNA fragments](#)” on page 51.
- Update to amplified library storage instructions (exclusion of Tween 20; see [step](#) on [page 50](#)).
- Kit use-by information removed. See product labels and the Certificate of Analysis for each component for expiration date information.

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Make sure you have the most current protocol. Go to genomics.agilent.com and search for G7530-90005.

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 Ion Xpress Plus Fragment Library Kit 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 cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols or instruments to process samples for enrichment.



Overview of the Workflow

The SureSelect Target Enrichment for Ion Proton sequencing workflow is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1.

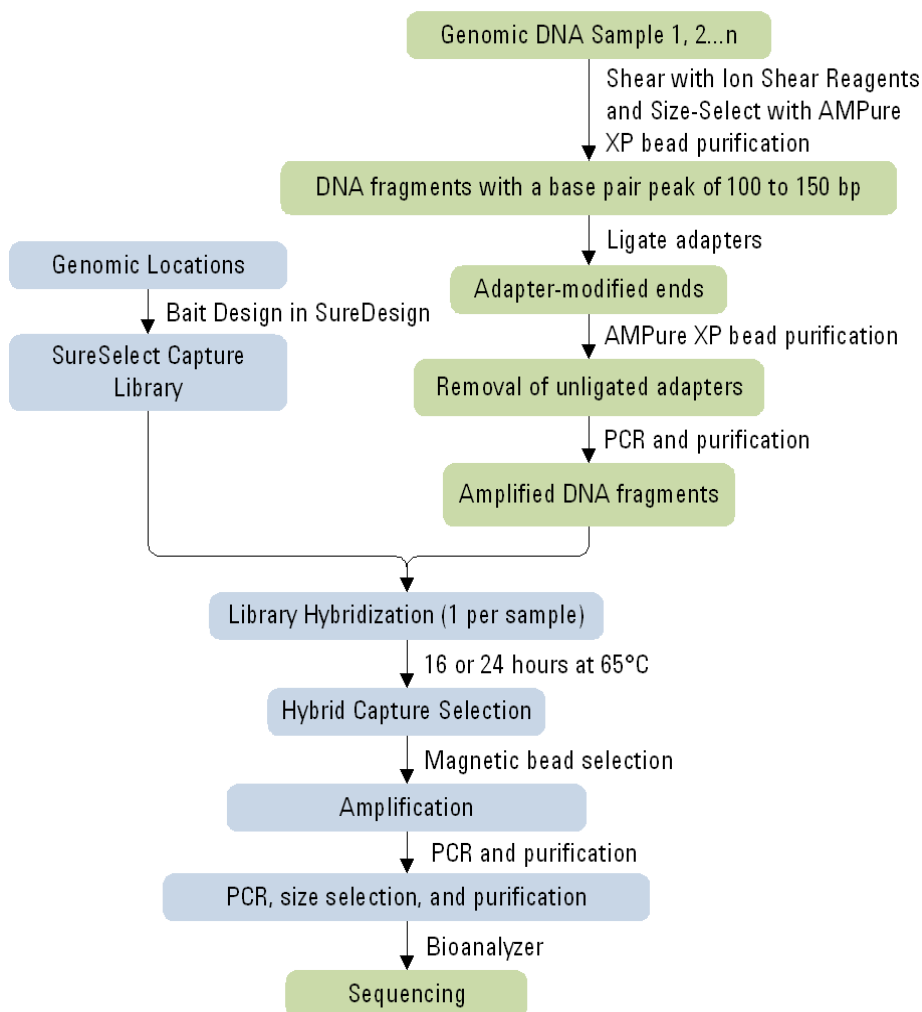


Figure 1 Overall target-enriched sequencing sample preparation workflow.

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

Step	Time
Ion Xpress Plus Library Preparation	1 day
Library Hybridization	16 or 24 hours
Bead preparation	30 minutes
Capture and washing	2 hours
Post-hybridization amplification	1 hour
PCR purification	30 minutes
E-gel size selection	30 minutes
QC using Bioanalyzer	1 hour

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- 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.
- Avoid repeated freeze-thaw cycles for stock and diluted gDNA solutions.
- 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.
- Some thermal cycler incubation steps must be run without a heated lid. If the heated lid of your cycler cannot be turned off, do these incubation steps with the lid open.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

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

Required Reagents

Table 2 Required Reagents for SureSelect Target Enrichment for Ion Proton Sequencing

Description	Vendor and part number
SureSelect or ClearSeq Capture Library	Select the appropriate library from Table 3 or Table 4
SureSelect Target Enrichment Reagent Kit, PTN*	Agilent
16 reactions	p/n G9605A
96 reactions	p/n G9605B
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
Ion Xpress Plus Fragment Library Kit	Thermo Fisher Scientific p/n 4471269
Ion Xpress Barcode Adapters 1-16 Kit	Thermo Fisher Scientific p/n 4471250
50-bp DNA Ladder	Thermo Fisher Scientific p/n 10416-014
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 mL	p/n 65601
10 mL	p/n 65602
100 mL	p/n 65603
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Qubit BR dsDNA Assay Kit	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

1 Before You Begin

Required Reagents

Table 3 SureSelect^{XT} Capture Libraries

Capture Library	16 Reactions	96 Reactions	480 Reactions
SureSelect ^{XT} Clinical Research Exome V2	5190-9491	5190-9492	–
SureSelect ^{XT} Clinical Research Exome V2 Plus 1	5190-9494	5190-9495	–
SureSelect ^{XT} Clinical Research Exome V2 Plus 2	5190-9497	5190-9498	–
SureSelect ^{XT} Clinical Research Exome	5190-7338	5190-7339	–
SureSelect ^{XT} Focused Exome	5190-7787	5190-7788	–
SureSelect ^{XT} Focused Exome Plus 1	5190-7790	5190-7791	–
SureSelect ^{XT} Focused Exome Plus 2	5190-7793	5190-7795	–
SureSelect ^{XT} Human All Exon v6	5190-8863	5190-8864	–
SureSelect ^{XT} Human All Exon v6 + UTRs	5190-8881	5190-8882	–
SureSelect ^{XT} Human All Exon v6 + COSMIC	5190-9307	5190-9308	–
SureSelect ^{XT} Human All Exon v6 Plus 1	5190-8866	5190-8867	–
SureSelect ^{XT} Human All Exon v6 Plus 2	5190-8869	5190-8870	–
SureSelect ^{XT} Human All Exon v5	5190-6208	5190-6209	–
SureSelect ^{XT} Human All Exon v5 + UTRs	5190-6213	5190-6214	–
SureSelect ^{XT} Human All Exon v5 + lncRNA	5190-6446	5190-6447	–
SureSelect ^{XT} Human All Exon v5 Plus	5190-6211	5190-6212	–
SureSelect ^{XT} Human All Exon v4	5190-4631	5190-4632	5190-4634
SureSelect ^{XT} Human All Exon v4 + UTRs	5190-4636	5190-4637	5190-4639
SureSelect ^{XT} Mouse All Exon	5190-4641	5190-4642	5190-4644
SureSelect ^{XT} Human X-Chromosome	5190-4651	5190-4652	5190-4653
SureSelect ^{XT} Custom 1 kb up to 499 kb (reorder)	5190-4806 (5190-4811)	5190-4807 (5190-4812)	5190-4809 (5190-4814)
SureSelect ^{XT} Custom 0.5 Mb up to 2.9 Mb (reorder)	5190-4816 (5190-4821)	5190-4817 (5190-4822)	5190-4819 (5190-4824)
SureSelect ^{XT} Custom 3 Mb up to 5.9 Mb (reorder)	5190-4826 (5190-4831)	5190-4827 (5190-4832)	5190-4829 (5190-4834)
SureSelect ^{XT} Custom 6 Mb up to 11.9 Mb (reorder)	5190-4836 (5190-4841)	5190-4837 (5190-4842)	5190-4839 (5190-4844)
SureSelect ^{XT} Custom 12 Mb up to 24 Mb (reorder)	5190-4896 (5190-4901)	5190-4897 (5190-4902)	5190-4899 (5190-4904)

Table 4 Compatible ClearSeq Capture Libraries

Capture Library	16 Reactions	96 Reactions	480 Reactions
ClearSeq Comprehensive Cancer XT	5190-8011	5190-8012	–
ClearSeq Comprehensive Cancer Plus XT	5190-8014	5190-8015	–
ClearSeq Inherited Disease XT	5190-7518	5190-7519	–
ClearSeq Inherited Disease Plus XT	5190-7521	5190-7522	–
ClearSeq DNA Kinome XT	5190-4646	5190-4647	5190-4649

Required Equipment

Table 5 Required Equipment for Library Prep and Target Enrichment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
Agilent 2100 Bioanalyzer and Consumables	
Agilent 2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
Agilent 2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
Qubit Fluorometer	Thermo Fisher Scientific p/n Q32857
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent

1 Before You Begin

Required Equipment

Table 5 Required Equipment for Library Prep and Target Enrichment

Description	Vendor and part number
E-Gel iBase and E-Gel Safe Imager Combo Kit or Safe Imager Real-Time Transilluminator <i>and</i> E-Gel iBase Power System	Thermo Fisher Scientific p/n G6465 Thermo Fisher Scientific p/n G6500 Thermo Fisher Scientific p/n G6400
E-Gel SizeSelect 2% Agarose Gel	Thermo Fisher Scientific p/n G6610-02
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Nutator plate mixer	BD Diagnostics p/n 421105, or equivalent
Magnetic separator	Thermo Fisher Scientific p/n 12321D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Multichannel Pipette	Pipetman or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, or equivalent
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Heat blocks or water baths held at 37°C and 65°C	

Optional Reagents and Equipment

Table 6 Optional materials for processing

Description	Vendor and part number
Tube-strip capping tool	Agilent p/n 410099
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402A
Peelable Aluminum Seal for PlateLoc Sealer	Agilent p/n 24210-001
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific p/n 4306311

Sequencing Reagents and Equipment

Table 7 Required materials for template preparation and sequencing

Description	Vendor and part number
Ion OneTouch 2 System	Thermo Fisher Scientific p/n 4474779
Ion PI Template OT2 200 Kit v2	Thermo Fisher Scientific p/n 4485146
Ion PI Sequencing 200 Kit v2	Thermo Fisher Scientific p/n 4485149

1 Before You Begin
Sequencing Reagents and Equipment



2 Sample Preparation

- Step 1. Fragment the DNA with Ion Shear Plus reagents 18
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- Step 6. Amplify the adapter-ligated library 25
- Step 7. Purify the amplified library with AMPure XP beads 28
- Step 8. Assess quality and quantity 29

This section contains instructions for prepped library production specific to the Ion Proton sequencing platform. For each sample to be sequenced, an individual barcoded library is prepared. For an overview of the SureSelect target enrichment for Ion Proton workflow, see [Figure 1](#) on page 8.

The steps in this section differ from the Ion Xpress protocol in the use of the Herculase II enzyme for amplification.

Refer to the *Ion Xpress Plus Fragment Library Kit* protocol (Thermo Fisher Scientific p/n 4471269) for more information.



2 Sample Preparation

Step 1. Fragment the DNA with Ion Shear Plus reagents

Step 1. Fragment the DNA with Ion Shear Plus reagents

NOTE

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

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

Use reagents from the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific p/n 4471269).

- 1 Use the Qubit dsDNA BR Assay to determine the concentration of your gDNA sample.

Follow the instructions for the instrument.

- 2 Dilute 1 μg of high-quality gDNA with nuclease-free water in a 1.5-mL LoBind tube to a total volume of 10 μL .

CAUTION

The final EDTA concentration must be < 0.1 mM in the gDNA dilution.

- 3 For each sample, combine the reagents in [Table 8](#).

Table 8 Ion Shear Reaction

Reagent	Volume for 1 reaction
Nuclease-free water	25 μL
Diluted gDNA	10 μL
Ion Shear Plus 10 \times Reaction Buffer	5 μL
Total	40 μL

- 4 Add 10 μL of Ion Shear Plus Enzyme Mix and mix well by pipetting.

CAUTION

Do not create bubbles.

Step 1. Fragment the DNA with Ion Shear Plus reagents

- 5** Incubate the reactions in a heat block or water bath at 37°C for 50 minutes. Adjust incubation time between 50 to 60 minutes to optimize for your laboratory conditions to obtain approximately 130-bp peaks.
- 6** Add 5 µL of Ion Shear Plus Stop Buffer immediately after the incubation time, and mix thoroughly on a vortex mixer for 5 seconds.
- 7** Store the reaction tubes on ice.

2 Sample Preparation

Step 2. Purify and size-select the sample using AMPure XP beads

Step 2. Purify and size-select 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 12](#).

NOTE

The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 2.4 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 60.5 μL of homogeneous AMPure XP beads to each sheared DNA sample (approximately 55 μL). Mix well on a vortex mixer.
- 5 Incubate samples for 5 minutes at room temperature.
- 6 Put the tube into a magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
- 7 Keep the tube in the magnetic stand. Carefully remove 110 μL of the cleared solution to a fresh LoBind tube. You can discard the beads at this time.
- 8 Add 38.5 μL of homogeneous AMPure XP beads to the collected solution. Mix well on a vortex mixer.
- 9 Incubate samples for 5 minutes at room temperature.
- 10 Put the tube into a magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
- 11 Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution from the tube. Do not touch the beads while removing the solution.
- 12 Continue to keep the tube in the magnetic stand while you dispense 200 μL of 70% ethanol in each tube.
Use fresh 70% ethanol for optimal results.
- 13 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 14 Repeat [step 12](#) to [step 13](#) once.

Step 2. Purify and size-select the sample using AMPure XP beads

15 Briefly spin the tube to collect the residual ethanol. Return the tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

16 Dry the sample by placing the unsealed tube in the 37°C heat block for 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.

17 Add 27 µL nuclease-free water. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.

18 Incubate for 2 minutes at room temperature.

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

20 Remove the cleared supernatant (approximately 27 µL) to a fresh 1.5-ml LoBind tube. You can discard the beads at this time.

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

2 Sample Preparation

Step 3. Assess quality with the 2100 Bioanalyzer

Step 3. Assess quality with the 2100 Bioanalyzer

Use a DNA 1000 chip and reagent kit for this step. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μL of each sample for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the DNA 1000 assay from the drop down list. Start the run. Enter sample names and comments in the Data and Assay context.
- 6 Check that the electropherogram shows a distribution between 50–250 bp. A sample electropherogram is shown in [Figure 2](#).
- 7 Determine the DNA concentration by integrating under the peak. If the concentration is $>15 \text{ ng}/\mu\text{L}$, adjust the concentration to $15 \text{ ng}/\mu\text{L}$ with nuclease-free water before use in the next step.

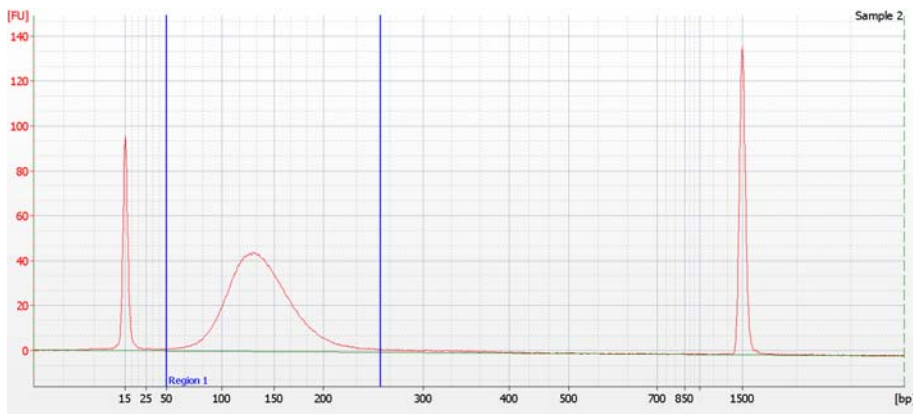


Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Stopping Point

If you do not continue to the next step, store the samples at 4°C overnight or at -20°C for prolonged storage.

Step 4. Ligate the adapter and barcode the sample

Use reagents from the Ion Xpress Plus Fragment Library Kit and the Ion Xpress Barcode Adapters Kit for this step.

- 1 Prepare the appropriate volume of Ligation master mix, as described in [Table 9](#), on ice. Mix well by pipetting.

Table 9 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	31 μL	511.5 μL
10 \times Ligase Buffer	10 μL	165 μL
Ion Xpress P1 Adapter	10 μL	165 μL
Nick Repair Polymerase	8 μL	132 μL
DNA Ligase	4 μL	66 μL
dNTP Mix	2 μL	33 μL
Total	65 μL	1072.5 μL

- 2 For each sample, place 65 μL of the Ligation master mix in a PCR tube or plate well.
- 3 Add 10 μL of one of Ion Xpress Barcode 1 through 16 (from the Ion Xpress Barcode Adapters Kit) to each aliquot of Ligation master mix.
- 4 Add 25 μL of purified sheared DNA sample to each mixture. Mix well by pipetting up and down. Change pipette tips between samples.
- 5 Incubate the reactions in the thermal cycler and run the program in [Table 10](#). Do not use a heated lid.

Table 10 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	25°C	15 minutes
Step 2	72°C	5 minutes
Step 3	4°C	Hold

- 6 Continue immediately to the next step.

2 Sample Preparation

Step 5. Purify the sample using AMPure XP beads

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 a 1.5-ml LoBind tube, and add the adapter-ligated library (approximately 100 μL). Mix well on a vortex mixer.
- 4 Incubate samples for 5 minutes at room temperature.
- 5 Put the tube into a magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
- 6 Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution. Do not touch the beads while removing the solution.
- 7 Continue to keep the tube in the magnetic stand while you dispense 500 μL of freshly-prepared 70% ethanol in each tube.
- 8 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 9 Repeat [step 7](#) and [step 8](#) once.
- 10 Briefly spin the tube to collect the residual ethanol. Return the tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 11 Dry the sample by placing the unsealed tube in the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
- 12 Add 50 μL nuclease-free water. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.
- 13 Incubate for 2 minutes at room temperature.
- 14 Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 15 Remove the cleared supernatant (approximately 50 μL) to a fresh 1.5-ml LoBind tube. You can discard the beads at this time.

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

Step 6. Amplify the adapter-ligated library

This step uses the components listed in [Table 11](#). Thaw the reagents listed below and keep on ice.

Table 11 Reagents for pre-capture PCR amplification

Component	Storage Location
SureSelect PTN PCR Primer Mix	SureSelect Target Enrichment Kit PTN 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.

2 Sample Preparation

Step 6. Amplify the adapter-ligated library

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 12](#), on ice. Mix well on a vortex mixer.

Table 12 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	11 μ L	181.5 μ L
5 \times Herculase II Reaction Buffer (clear cap)	10 μ L	165 μ L
SureSelect PTN PCR Primer Mix (clear cap)	2.5 μ L	41.25 μ L
Herculase II Fusion DNA Polymerase (red cap)	1 μ L	16.5 μ L
100 mM dNTP Mix (green cap)	0.5 μ L	8.25 μ L
Total	25 μL	412.5 μL

- 2 Dispense 25 μ L of the PCR reaction mixture into each sample well of a PCR plate or strip tube.
- 3 Add 25 μ L of each DNA sample from [step 15](#) on [page 24](#). Add a single DNA library sample to each well of the plate or strip tube.
Mix by pipetting. Change pipette tips between samples.

NOTE

The optimal amount of adapter-ligated library in the PCR is <200 ng. Follow the sample dilution guidelines provided in [step 7](#) on [page 22](#) to ensure that the appropriate amount of DNA is added to the amplification reaction.

4 Place the samples in a thermal cycler and run the program in [Table 13](#).

Table 13 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	7–9	98°C	30 seconds
		60°C	10 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, 8 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. The small amount of high molecular weight PCR products around 400 bp does not have a significant impact on the capture performance.

As an alternative, you can prepare one PCR master mix as outlined in [Table 12](#). Use this master mix to prepare three small-scale 10 µL PCR reactions and run for 7, 8, or 9 cycles. Clean these PCR reactions using the AMPure XP protocol outlined in “[Step 5. Purify the sample using AMPure XP beads](#)” with these modifications: Use 30 µL of AMPure XP beads and elute with 20 µL of nuclease-free water. Run these cleaned samples on the Bioanalyzer, as described in “[Step 8. Assess quality and quantity](#)”.

Use the optimal cycle number to repeat PCR at the 50 µL reaction scale.

2 Sample Preparation

Step 7. Purify the amplified library with AMPure XP beads

Step 7. 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 a 1.5-ml LoBind tube, and add the amplified library (50 μL). Mix well on a vortex mixer.
- 4 Incubate samples for 5 minutes at room temperature.
- 5 Put the tube into a magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
- 6 Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution from the tube. Do not touch the beads while removing the solution.
- 7 Continue to keep the tube in the magnetic stand while you dispense 500 μL of freshly-prepared 70% ethanol into the tube.
- 8 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 9 Repeat [step 7](#) and [step 8](#) once.
- 10 Briefly spin the tube to collect the residual ethanol. Return the tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 11 Dry the sample by placing the unsealed tube in the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
- 12 Add 25 μL nuclease-free water. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.
- 13 Incubate for 2 minutes at room temperature.
- 14 Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 15 Remove the cleared supernatant (approximately 25 μL) to a fresh 1.5-ml LoBind tube. You can discard the beads at this time.

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

Step 8. Assess quality and quantity

Use a DNA 1000 chip and reagent kit for this step. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μL of each sample for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the DNA 1000 assay from the drop down list. Start the run. Enter sample names and comments in the Data and Assay context.
- 6 Check that the electropherogram shows a distribution with a peak sized at approximately 220 bp. Note that high molecular weight PCR products sized around 400 bp do not affect capture performance. A sample electropherogram is shown in [Figure 3](#).
- 7 Determine the DNA concentration by integrating under the peak.

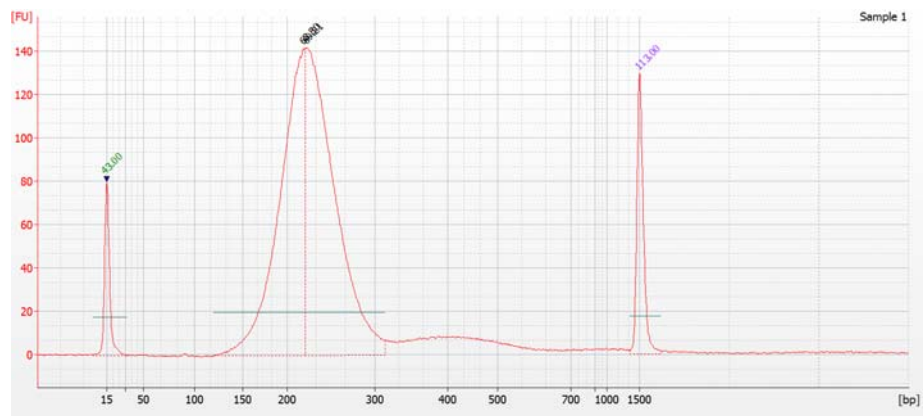
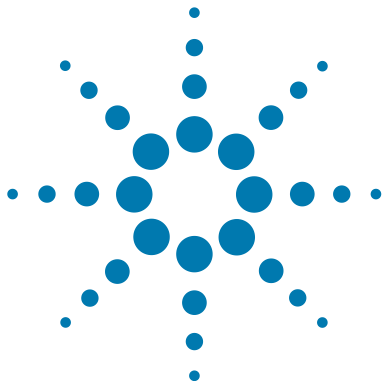


Figure 3 Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

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

2 Sample Preparation

Step 8. Assess quality and quantity



3 Hybridization and Capture

- Step 1. Hybridize DNA samples to the Capture Library 33
- Step 2. Prepare streptavidin-coated magnetic beads 39
- Step 3. Capture the hybridized DNA using streptavidin-coated beads 40

This chapter describes the steps to hybridize the prepared gDNA libraries with a target-specific Capture Library. After hybridization, the targeted molecules are captured on streptavidin beads. Each DNA library sample must be hybridized and captured individually. The hybridization and capture process is summarized in [Figure 4](#) on page 32.

CAUTION

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



3 Hybridization and Capture

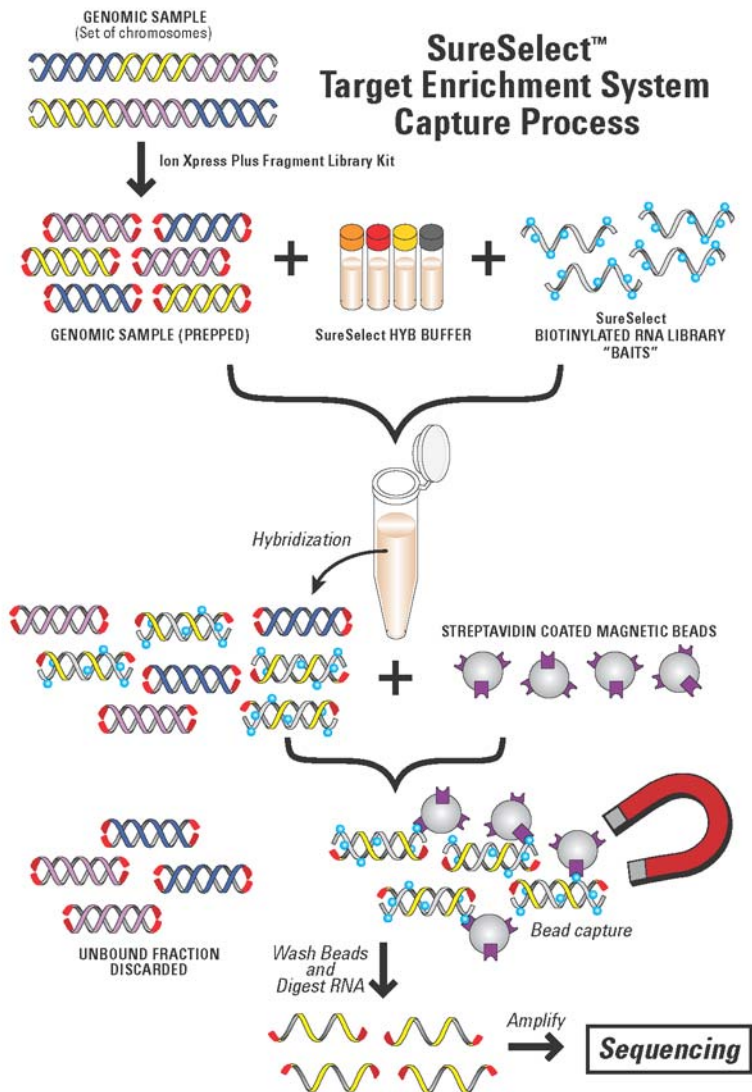


Figure 4 SureSelect Target Enrichment System Capture Process

Step 1. Hybridize DNA samples to the Capture Library

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

This step uses the SureSelect Reagent Kit components listed in [Table 14](#). Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 14 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect Hyb 1	SureSelect Target Enrichment Kit PTN Hyb Module Box 1, RT	—	page 35
SureSelect Hyb 2	SureSelect Target Enrichment Kit PTN Hyb Module Box 1, RT	—	page 35
SureSelect Hyb 3	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, -20°C	Warm to Room Temperature (RT)	page 35
SureSelect Hyb 4	SureSelect Target Enrichment Kit PTN Hyb Module Box 1, RT	—	page 35
SureSelect PTN Block 1	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, -20°C	Thaw on ice	page 35
SureSelect Block 2	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, -20°C	Thaw on ice	page 35
SureSelect PTN Block 3	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, -20°C	Thaw on ice	page 35
SureSelect RNase Block	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, -20°C	Thaw on ice	page 36
Capture Library	-80°C	Thaw on ice	page 37

3 Hybridization and Capture

Step 1. Hybridize DNA samples to the Capture Library

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

CAUTION

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

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

For a partial list of tested options showing minimal evaporation, refer to “[Alternative Capture Equipment Combinations](#)” on page 61.

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

- 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 $\leq 45^\circ\text{C}$.
 - a Add the entire volume of prepped library (approximately 24 μL) 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 until samples are used in [step 6](#) on [page 36](#).

Step 1. Hybridize DNA samples to the Capture Library

- 4 Prepare the Hybridization Buffer by mixing the components in [Table 15](#) 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 15 Preparation of Hybridization Buffer

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

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

- 5 Prepare the SureSelect Block Mix by mixing the components in [Table 16](#). Keep the mixture on ice until it is used in [step 6](#).

Table 16 Preparation of SureSelect Block Mix

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

3 Hybridization and Capture

Step 1. Hybridize DNA samples to the Capture Library

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.

- To each gDNA library sample well prepared in [step 3](#) on [page 34](#), add 5.6 μL of the SureSelect Block Mix prepared in [Table 16](#). Pipette up and down to mix.
- Cap the wells, then transfer the sealed plate or strip tube to the thermal cycler and run the following program shown in [Table 17](#).
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 17 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.

- Prepare the appropriate dilution of SureSelect RNase Block, based on the size of your Capture Library, according to [Table 18](#). 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 18 Preparation of RNase Block dilution

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

NOTE

Prepare the Capture Library mixture described in [step 9](#), below, near the end of the 65°C hold step of >5 minute duration) described in [Table 17](#). Keep the mixture at room temperature briefly, until adding the mixture to sample wells in [step 10](#). Do not keep solutions containing the Capture Library at room temperature for extended periods.

- 9** Prepare the Capture Library Hybridization Mix appropriate for your Capture Library size according to [Table 19](#) (Capture Libraries ≥ 3 Mb), or [Table 20](#) (Capture Libraries < 3 Mb).

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

Table 19 Preparation of Capture Library Hybridization Mix for ≥ 3 Mb Capture Libraries

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

Table 20 Preparation of Capture Library Hybridization Mix for < 3 Mb Capture Libraries

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

3 Hybridization and Capture

Step 1. Hybridize DNA samples to the Capture Library

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

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

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

CAUTION

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

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

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

Step 2. Prepare streptavidin-coated magnetic beads

The hybrid capture protocol uses reagents provided in SureSelect Target Enrichment Kit PTN Hyb Module Box 1 (stored at room temperature) in addition to the streptavidin-coated magnetic beads obtained from another supplier (see [Table 2](#) on page 11).

- 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 40.
- 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 a 1.5-mL LoBind tube.
- 4** Wash the beads:
 - a** Add 200 µL of SureSelect Binding Buffer.
 - b** Mix on a vortex mixer for 5 seconds.
 - c** Put the tubes 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 1.5-mL LoBind tubes to be used for hybridization capture.

3 Hybridization and Capture

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

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 at 65°C while you transfer the entire volume (approximately 25 to 29 µL) of each hybridization mixture to a tube containing 200 µL of washed streptavidin beads from [step 5](#) on [page 39](#).
Mix well by inverting the tubes 3 to 5 times or until beads are fully resuspended.

NOTE

Excessive evaporation (exceeding 4 µL of the hybridization mixture), can indicate suboptimal capture performance. See the *Caution* notice on [page 34](#) for tips to test the hybridization conditions and minimize evaporation.

- 3 Incubate the capture reactions on a Nutator mixer, or equivalent, for 30 minutes at room temperature.
Make sure the samples are properly mixing in the tubes.
- 4 Briefly spin the tubes in a centrifuge, then put the tubes in a magnetic separator to collect the beads.
- 5 Wait until the solution is clear, then remove and discard the supernatant.
- 6 Resuspend the beads in 500 µL of SureSelect Wash Buffer 1. Mix on a vortex mixer for 5 seconds.
- 7 Incubate the samples for 15 minutes at room temperature, mixing occasionally by vortexing.
- 8 Briefly spin the tubes in a centrifuge, then put the tubes in a magnetic separator to collect the beads.
- 9 Wait until the solution is clear, then remove and discard the supernatant.

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

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 500 μ L of 65°C prewarmed SureSelect Wash Buffer 2. Mix on a vortex mixer for 5 seconds.
- b** Incubate the samples for 10 minutes at 65°C in a recirculating water bath, heat block or equivalent, mixing occasionally by vortexing.
- c** Briefly spin the tubes in a centrifuge, then put the tubes in a magnetic separator to collect the beads. 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 μ L of nuclease-free water to each tube. Mix on a vortex mixer for 5 seconds.

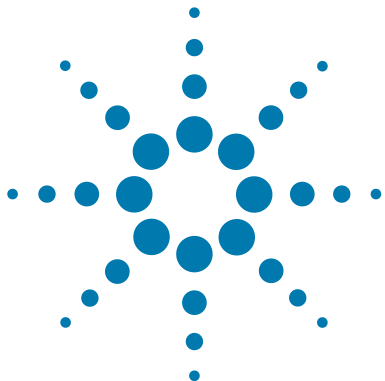
Keep the samples on ice until they are used on [page 45](#).

NOTE

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

3 Hybridization and Capture

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



4 Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing

- Step 1. Amplify the captured library 44
- Step 2. Purify the sample using AMPure XP beads 47
- Step 3. Assess DNA quantity and quality 48
- Step 4. Pool samples for multiplexed sequencing 50
- Step 5. Size-select the DNA fragments 51
- Step 6. Purify the sample using AMPure XP beads 53
- Step 7. Assess DNA quantity and quality 54
- Step 8. Prepare templates for sequencing 55

This chapter describes the steps to amplify, purify and assess quality and quantity of the captured libraries. Instructions are also provided for sample pooling, size-selection of the pooled samples, and diluting samples for cluster amplification.



4 Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured library

Step 1. Amplify the captured library

In this step, the SureSelect-enriched DNA libraries are PCR amplified. This step uses the components listed in [Table 21](#). Thaw then vortex to mix the reagents listed below and keep on ice.

Table 21 Reagents for post-capture 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 PTN PCR Primer Mix	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, –20°C

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

CAUTION

Do not use amplification enzymes other than Herculase II Fusion DNA Polymerase. Other enzymes have not been validated.

Prepare one amplification reaction for each hybrid capture DNA library. Include a negative no-template control.

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 Prepare the appropriate volume of PCR reaction mix, as described in [Table 22](#), on ice. Mix well on a vortex mixer.

Table 22 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	22.5 μ L	382.5 μ L
5 \times Herculase II Reaction Buffer (clear cap)	10 μ L	170 μ L
SureSelect PTN PCR Primer Mix (clear cap)	2 μ L	34 μ L
Herculase II Fusion DNA Polymerase (red cap)	1 μ L	17 μ L
100 mM dNTP Mix (green cap)	0.5 μ L	8.5 μ L
Total	36 μL	612 μL

- 2 Add 36 μ L of the PCR reaction mix prepared in [Table 22](#) to each sample well of a fresh PCR plate or strip tube.
- 3 Add the DNA library samples to the PCR reactions:
 - a Obtain the hybrid capture DNA samples from ice (prepared on [page 41](#)).
 - b Pipette each DNA sample up and down until the bead suspension is homogeneous, then transfer 14 μ L of the sample to the appropriate well of the PCR plate or strip tube containing PCR reaction mix.
 - c Mix the PCR reactions well by pipetting.
 - d Store the remaining library-bound beads at -20°C for future use, if needed.

4 Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured library

- 4 Transfer the PCR plate or strip tube to a thermal cycler and run the PCR amplification program shown in [Table 23](#).

Table 23 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 11 Cycles See Table 24 for recommendations based on Capture Library size	98°C	30 seconds
		60°C	10 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 24 Post-capture PCR cycle number recommendations

Capture Size	Cycles
1 kb up to 3.2 Mb	11 cycles
>3.2 Mb	10 cycles
All Exon and Exome libraries	9 cycles

- 5 When the PCR amplification program is complete, spin the plate or strip tube briefly.
Proceed to “[Step 2. Purify the sample using AMPure XP beads](#)” on page 47.

Step 2. 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 1 mL of fresh 70% ethanol per sample, plus excess, for use in [step 8](#).
- 3 Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
- 4 Add 90 μL of homogeneous AMPure XP beads to a 1.5-ml LoBind tube, and add the amplified library (50 μL). Mix well on a vortex mixer.
- 5 Incubate sample for 5 minutes at room temperature.
- 6 Put the tube into a magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
- 7 Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution from the tube. Do not touch the beads while removing the solution.
- 8 Continue to keep the tube in the magnetic stand while you dispense 500 μL of freshly-prepared 70% ethanol into the tube.
- 9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10 Repeat [step 8](#) and [step 9](#) step once.
- 11 Briefly spin the tube to collect the residual ethanol. Return the tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 12 Dry the sample by placing the unsealed tube in the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
- 13 Add 20 μL nuclease-free water or Low TE Buffer. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.
- 14 Incubate for 2 minutes at room temperature.
- 15 Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 16 Remove the cleared supernatant (approximately 20 μL) to a fresh 1.5-ml LoBind tube. You can discard the beads at this time.

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

Step 3. Assess DNA quantity and quality

Use the Bioanalyzer High Sensitivity DNA Assay to quantify and assess the quality and size range of the amplified DNA. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

- 1 Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
- 2 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μ L of each sample for the analysis.
- 3 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 4 Determine the DNA concentration by integrating under the peak.
- 5 Verify that the electropherogram shows a peak of DNA fragment size at approximately 220 bp. A sample electropherogram is shown in [Figure 5](#). Primer-dimer peaks can be expected around 40 bp and are removed during the size-selection step ([page 51](#)).

If all samples to be multiplexed show the same DNA fragment size on the electropherograms, proceed to “[Step 4. Pool samples for multiplexed sequencing](#)” on page 50. After pooling, do the size-selection of the pooled DNA in a single lane and subsequent purification and qualification steps using the pooled libraries.

If samples to be multiplexed show differently-sized DNA peaks, then the individual libraries must be size-selected prior to pooling. In this case, proceed to “[Step 5. Size-select the DNA fragments](#)” on page 51. Load each DNA library in a separate lane for size selection, then purify and qualify each library separately in “[Step 6. Purify the sample using AMPure XP beads](#)” and “[Step 7. Assess DNA quantity and quality](#)”, before doing “[Step 4. Pool samples for multiplexed sequencing](#)” on page 50.

Stopping Point

If you do not continue to the next step, store the libraries at 4°C overnight or at -20°C for longer periods.

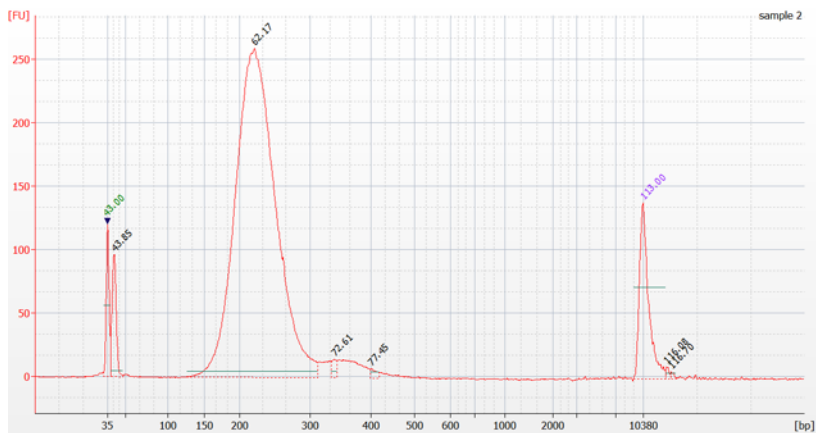


Figure 5 Post-capture analysis using the Bioanalyzer High Sensitivity DNA Assay.

Step 4. Pool samples for multiplexed sequencing

Prepare a 20 µL-volume pool of the DNA samples to be multiplexed, such that each barcode-tagged sample is present in equimolar amounts in the pool.

- 1 Use the formula below to determine the amount of each sample to include in the pool.

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

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 samples to be combined, and

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

Table 25 shows an example of the amount of 2 barcoded samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

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

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	10 nM	10 nM	2	10
Sample 2	20 µL	12.5 nM	10 nM	2	8
Low TE					2

- 2 Adjust the final volume of the pooled library to the desired final concentration.
 - If the final volume of the combined barcode-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 barcode-tagged samples is greater than the final desired volume, $V(f)$, lyophilize and reconstitute to the desired volume.

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

Step 5. Size-select the DNA fragments

This section describes the basic protocol to size-select the DNA fragments by agarose gel electrophoresis using the integrated E-Gel system (see supplier information on [page 14](#)).

- 1 Set up the integrated E-Gel system according to the manufacturer's instructions and select the **SizeSelect 2%** program using the arrow buttons on the E-Gel iBase Power System.
- 2 Remove an E-Gel SizeSelect 2% Agarose Gel from its package and gently remove both of the combs from the cassette.
- 3 Seat the gel cassette in the E-Gel iBase Power System according to the manufacturer's instructions. Do not pre-run the gel.
- 4 Dilute the 1 µg/µL 50-bp ladder to 25 ng/µL (1:40) in 1X Low TE Buffer. Load 10 µL (250 ng) of the diluted DNA ladder into the smaller middle lane of the gel (Lane M).
- 5 Load 20 µL of the pooled DNA sample into one well.

When doing size-selection before sample pooling, load each 20-µL sample into a separate well.

NOTE

Do not use the wells on the gel edges as they tend to run slower and will affect size selection. Do not use the wells next to the ladder.

- 6 Add 25 µL of nuclease-free water to all unused wells in the top row.
- 7 Add 25 µL of nuclease-free water to all of the large wells in the bottom row, and add 10 µL to the smaller center well (Lane M).
- 8 Place the amber filter unit on top of the E-Gel iBase Power System.
- 9 Press **Mode** to set the run time to 11 to 13 minutes. If you are inexperienced with this system, select a shorter time.
- 10 Press **Go** to start the electrophoresis.
- 11 Run the electrophoresis until the PCR product band reaches the reference line on the gel. Press **Go** to stop the electrophoresis.

If time runs out before the PCR product band reaches the reference line, run the gel for a few additional minutes by repeating [step 9](#) and [step 10](#) using a 2 to 3 minute run time.

4 Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing

Step 5. Size-select the DNA fragments

- 12** Migrate the PCR product band into the collection well using the following steps:
 - a** Change the run time to 1 to 2 minutes using **Mode**.
 - b** Press **Go** to resume electrophoresis. Carefully monitor the migration of the PCR product and 200 bp band of the ladder. The target selection size is approximately 220 bp, which is similar to the expected PCR product size.
 - c** When the PCR product band migrates into the collection well, press **Go** to stop the electrophoresis.
- 13** Take care not to pierce the bottoms of the wells as you use a pipette to collect approximately 10 μ L of the solution from the collection well.
- 14** Adjust the total volume to 20 μ L using nuclease-free water.

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 Prepare 400 μL of 70% ethanol per sample, plus excess, for use in [step 8](#).
- 3 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 4 Add 36 μL of homogeneous AMPure XP beads to a 1.5 ml LoBind tube. Add the size-selected DNA sample (20 μL). Mix well on a vortex mixer.
- 5 Incubate samples for 5 minutes at room temperature.
- 6 Put the tube into a magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
- 7 Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution from the tube. Do not touch the beads while removing the solution.
- 8 Continue to keep the tube in the magnetic stand while you dispense 200 μL of 70% ethanol in each tube.
- 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 Briefly spin the tube to collect the residual ethanol. Return the tube to the magnetic stand for 30 seconds. Remove the residual ethanol.
- 12 Dry the sample by placing the unsealed tube in the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

NOTE

Do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 13 Add 25 μL nuclease-free water. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.
- 14 Incubate for 2 minutes at room temperature.
- 15 Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 16 Remove the cleared supernatant (approximately 25 μL) to a fresh 1.5-ml LoBind tube. You can discard the beads at this time.

Step 7. Assess DNA quantity and quality

Use the Bioanalyzer High Sensitivity DNA Assay for this step. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information. For accurate quantification, make sure that the concentration falls within the linear range of the assay (see [Specifications](#)). If needed, reanalyze the sample using a 1:10 dilution.

- 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 μ L of each sample for the analysis.
- 3 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 4 Determine the DNA concentration by integrating under the peak.
- 5 Verify that the electropherogram shows a peak of DNA fragment size at approximately 220 bp. A sample electropherogram is shown in [Figure 6](#).

For samples that were size-selected and purified as individual libraries, do “[Step 4. Pool samples for multiplexed sequencing](#)” on page 50 before you do “[Step 8. Prepare templates for sequencing](#)” on page 55.

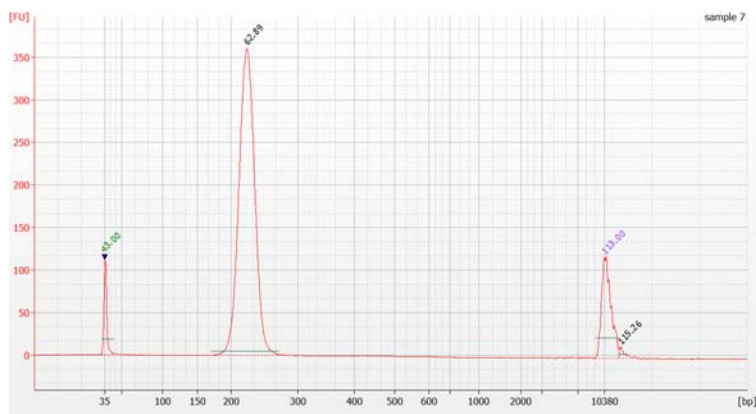


Figure 6 Analysis of size-selected, target enriched DNA using the Bioanalyzer High Sensitivity DNA Assay.

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

Step 8. Prepare templates for sequencing

Use the Ion OneTouch 2 System with the Ion PI Template OT2 200 Kit v2 and Ion PI Sequencing 200 Kit v2 to prepare the templates for the sequencer.

4 Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing
Step 8. Prepare templates for sequencing



5 Reference

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This chapter contains reference information.



SureSelect Reagent Kit Content

Each SureSelect Target Enrichment Reagent Kit for Ion Proton contains the following component kits:

Table 26 SureSelect Reagent Kit Contents

Product	Storage Condition	16 Reactions	96 Reactions
SureSelect Target Enrichment Kit PTN Hyb Module Box 1	Room Temperature	5190-6542	5190-6543
SureSelect Target Enrichment Kit PTN Hyb Module Box 2	-20°C	5190-6514	5190-6515

The contents of each of the component kits listed in [Table 26](#) are described in the tables below.

Table 27 SureSelect Target Enrichment Kit PTN Hyb Module Box 1 Content

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

Table 28 SureSelect Target Enrichment Kit PTN Hyb Module Box 2 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap
SureSelect PTN Block 1	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap
SureSelect PTN Block 3	tube with brown cap	tube with brown cap
SureSelect PTN PCR Primer Mix	tube with clear cap	tube with clear cap
SureSelect RNase Block	tube with purple cap	tube with purple cap

Other Reagent Kit Content

These reagents are from kits other than the SureSelect Reagent kit. Make sure you use only the reagents listed here.

Table 29 Herculase II Fusion DNA Polymerase (Agilent)

Kit Component	Format
DMSO	tube with green cap
100 mM dNTP Mix	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Buffer	tube with clear cap

Table 30 Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific p/n 4471269)

Kit Component
Ion Shear Plus Enzyme Mix
Ion Shear Plus 10× Reaction Buffer
Ion Shear Plus Stop Buffer
10× Ligase Buffer
DNA Ligase
Adapters
Nick Repair Polymerase
dNTP Mix

Table 31 Ion Xpress Barcode Adapters Kit

Kit Component
Ion Xpress P1 Adapter
Ion Xpress Barcode 1 through 16

Alternative Capture Equipment Combinations

Table 32 below lists combinations of thermal cyclers, lid temperatures, plates or strip tubes and sealing methods that have shown minimal evaporation when used for the Hybridization protocol on page 60.

Refer to this list for additional equipment and plasticware combination options for hybridization. Note that minimal evaporation is required to ensure optimal capture results.

Table 32 Tested options that show minimal evaporation

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

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In This Book

This guide contains information to run the SureSelect Target Enrichment for Sequencing on Ion Proton protocol.

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