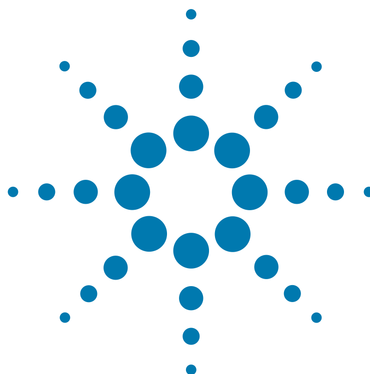

SureSelect Target Enrichment System for Sequencing on Ion Proton

Version C1, December 2018

SureSelect platform manufactured with Agilent SurePrint Technology

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Agilent Technologies

Notices

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

Technical Support

For US and Canada

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

Or send an e-mail to:
ngs.support@agilent.com

For Europe, Middle East, Africa, and India
Call 00800 345 600 (toll free) or
+49 69 8679 7730

Or send an e-mail to:
genomics_tech_europe@agilent.com

For all other regions
Agilent's world-wide sales and support center telephone numbers can be obtained at www.agilent.com/genomics under Contact Us.

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CAUTION

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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 C1

- Support for All Exon v7 Capture Libraries (see Table 3 on page 12) and reformatting of Capture Library selection tables (see Table 3 on page 12 through Table 5 on page 13)
- Updates to ordering information for certain non-Agilent reagent and equipment items required for the protocol (see Table 2 on page 11, Table 6 on page 14, and Table 8 on page 15)
- Updates to guidelines for DNA size selection step (see page 49)
- Updates to sequencing setup guidelines (see page 52)
- Removal of figure depicting the hybridization process (see page 31 to page 32). Revisions do not affect the instructions for performing hybridization steps.
- Updates to Technical Support contact information (see page 2)

Content

1 **Before You Begin** 7

Overview of the Workflow 8
Procedural Notes 10
Safety Notes 10
Required Reagents 11
Required Equipment 14
Optional Reagents and Equipment 15
Sequencing Reagents and Equipment 15

2 **Sample Preparation** 17

Step 1. Fragment the DNA with Ion Shear Plus reagents 18
Step 2. Purify and size-select the sample using AMPure XP beads 20
Step 3. Assess quality with the 2100 Bioanalyzer 22
Step 4. Ligate the adapter and barcode the sample 23
Step 5. Purify the sample using AMPure XP beads 24
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

3 **Hybridization and Capture** 31

Step 1. Hybridize DNA samples to the Capture Library 32
Step 2. Prepare streptavidin-coated magnetic beads 38
Step 3. Capture the hybridized DNA using streptavidin-coated beads 39

4 Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing 41

Step 1. Amplify the captured library 42

Step 2. Purify the sample using AMPure XP beads 45

Step 3. Assess DNA quantity and quality 46

Step 4. Pool samples for multiplexed sequencing 48

Step 5. Size-select the DNA fragments 49

Step 6. Purify the sample using AMPure XP beads 50

Step 7. Assess DNA quantity and quality 51

Step 8. Prepare templates for sequencing 52

5 Reference 53

SureSelect Reagent Kit Content 54

Other Reagent Kit Content 55

Alternative Capture Equipment Combinations 56

1
Before You Begin

Overview of the Workflow 8
Procedural Notes 10
Safety Notes 10
Required Reagents 11
Required Equipment 14
Optional Reagents and Equipment 15
Sequencing Reagents and Equipment 15

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.

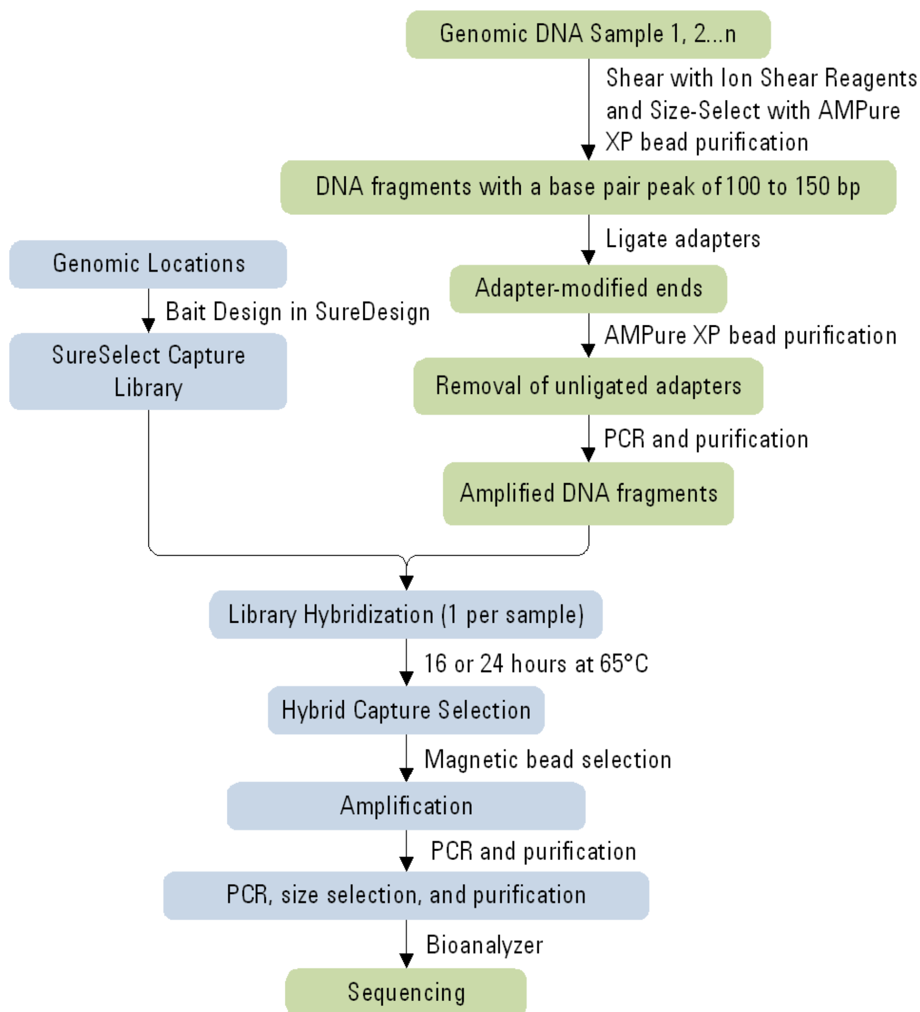


Figure 1 Overall target-enriched sequencing sample preparation workflow.

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, Table 4, or Table 5
SureSelect Target Enrichment Reagent Kit, PTN* 16 reactions 96 reactions	Agilent p/n G9605A p/n G9605B
Herculase II Fusion DNA Polymerase (includes dNTPs and 5× Buffer) 200 Reactions (processes 100 XT libraries) 400 Reactions	Agilent p/n 600677 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 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1 2 mL 10 mL 100 mL	Thermo Fisher Scientific p/n 65601 p/n 65602 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 100 assays 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

Table 3 SureSelect^{XT} Pre-Designed 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 V7	5191-4004	5191-4005	–
SureSelect^{XT} Human All Exon V7 Plus 1	5191-4010	5191-4011	–
SureSelect^{XT} Human All Exon V7 Plus 2	5191-4016	5191-4017	–
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

Table 4 Compatible ClearSeq Capture Libraries

Capture Library	16 Reactions	96 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

Table 5 Custom Design SureSelect^{XT} Capture Libraries

Capture Library	16 Reactions	96 Reactions	480 Reactions
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)

Required Equipment

Table 6 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 Q33226
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
E-Gel Power Snap Electrophoresis Device	Thermo Fisher Scientific p/n G8100
E-Gel SizeSelect II 2% Agarose Gel	Thermo Fisher Scientific p/n G661012
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

Table 6 Required Equipment for Library Prep and Target Enrichment

Description	Vendor and part number
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 7 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 8 Required materials for template preparation and sequencing

Description	Vendor and part number
Ion Proton or Ion PGM sequencing reagent kit	Consult Thermo Fisher Scientific for ordering information

2
Sample Preparation

Step 1. Fragment the DNA with Ion Shear Plus reagents 18
Step 2. Purify and size-select the sample using AMPure XP beads 20
Step 3. Assess quality with the 2100 Bioanalyzer 22
Step 4. Ligate the adapter and barcode the sample 23
Step 5. Purify the sample using AMPure XP beads 24
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.

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 9.

Table 9 Ion Shear Reaction

Reagent	Volume for 1 reaction
Nuclease-free water	25 µL
Diluted gDNA	10 µL
Ion Shear Plus 10× 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.

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 on 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.

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.

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.

Step 3. Assess quality with the 2100 Bioanalyzer

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

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 ng/µL, adjust the concentration to 15 ng/µL with nuclease-free water before use in the next step.

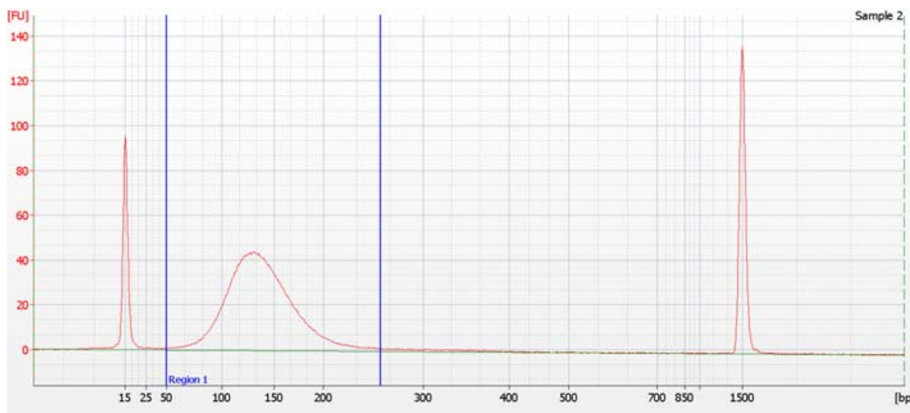


Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

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 10, on ice. Mix well by pipetting.

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

Table 11 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.

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 12. Thaw the reagents listed below and keep on ice.

Table 12 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.

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

Table 13 Preparation of SureSelect Pre-Capture PCR Reaction Mix

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

2 Dispense 25 µL of the PCR reaction mixture into each sample well of a PCR plate or strip tube.

3 Add 25 µL 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 14.

.

Table 14 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 13. 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.

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 Bioanalyzer DNA 1000 chip and reagent kit for this step. Perform the assay according to the Agilent DNA 1000 Kit Guide.

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.

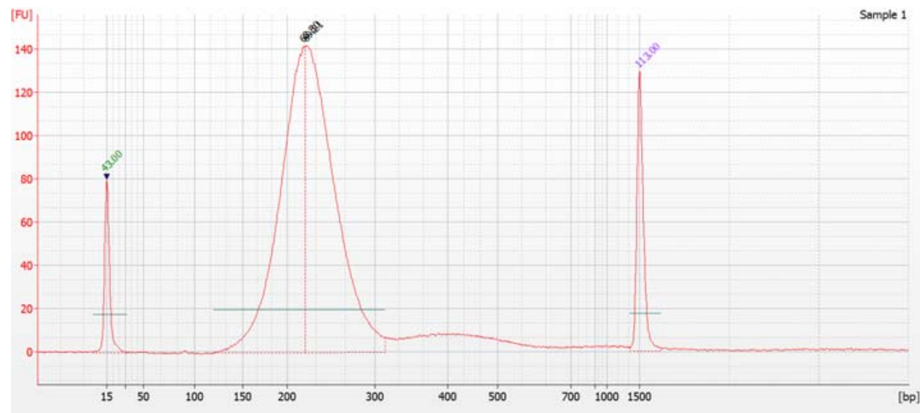


Figure 3 Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

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.

3
Hybridization and Capture

Step 1. Hybridize DNA samples to the Capture Library 32
Step 2. Prepare streptavidin-coated magnetic beads 38
Step 3. Capture the hybridized DNA using streptavidin-coated beads 39

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.

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

Step 1. Hybridize DNA samples to the Capture Library

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

This step uses the SureSelect Reagent Kit components listed in Table 15. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 15 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 34
SureSelect Hyb 2	SureSelect Target Enrichment Kit PTN Hyb Module Box 1, RT	—	page 34
SureSelect Hyb 3	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, –20°C	Warm to Room Temperature (RT)	page 34
SureSelect Hyb 4	SureSelect Target Enrichment Kit PTN Hyb Module Box 1, RT	—	page 34
SureSelect PTN Block 1	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, –20°C	Thaw on ice	page 34
SureSelect Block 2	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, –20°C	Thaw on ice	page 34
SureSelect PTN Block 3	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, –20°C	Thaw on ice	page 34
SureSelect RNase Block	SureSelect Target Enrichment Kit PTN Hyb Module Box 2, –20°C	Thaw on ice	page 35
Capture Library	–80°C	Thaw on ice	page 36

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 56.

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 ≤ 45°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 35.

4 Prepare the Hybridization Buffer by mixing the components in Table 16 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 16 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 17. Keep the mixture on ice until it is used in step 6.

Table 17 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**

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 33, add 5.6 µL of the SureSelect Block Mix prepared in Table 17. 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 18.

   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 18 Thermal cycler program for DNA + Block Mix prior to hybridization

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

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

8. Prepare the appropriate dilution of SureSelect RNase Block, based on the size of your Capture Library, according to Table 19. 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 19 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 18. 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 20 (Capture Libraries≥3 Mb), or Table 21 (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 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 µL	221 µL
25% RNase Block solution from step 8	2 µL	34 µL
Capture Library ≥3 Mb	5 µL	85 µL
Total	**20 µL**	**340 µL**

Table 21 Preparation of Capture Library Hybridization Mix for **<3 Mb Capture Libraries**

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

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

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

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

CAUTION

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

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

---

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

Step 2. Prepare streptavidin-coated magnetic beads

The hybrid capture protocol uses reagents provided in SureSelect Target Enrichment 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 39.
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.

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 38.

   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 33 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.

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 µL 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.

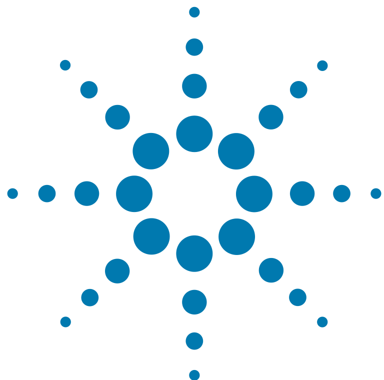
  Make sure all of the wash buffer has been removed during the final wash.

11 Add 30 µL 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 43.

NOTE

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



4
Post-Hybridization Amplification and Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured library 42
Step 2. Purify the sample using AMPure XP beads 45
Step 3. Assess DNA quantity and quality 46
Step 4. Pool samples for multiplexed sequencing 48
Step 5. Size-select the DNA fragments 49
Step 6. Purify the sample using AMPure XP beads 50
Step 7. Assess DNA quantity and quality 51
Step 8. Prepare templates for sequencing 52

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.

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

Table 22 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 23, on ice. Mix well on a vortex mixer.

Table 23 Preparation of post-capture PCR Reaction mix

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

2 Add 36 µL of the PCR reaction mix prepared in Table 23 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 40).

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.

c Mix the PCR reactions well by pipetting.

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

4 Transfer the PCR plate or strip tube to a thermal cycler and run the PCR amplification program shown in Table 24.

Table 24 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 11 Cycles See Table 25 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 25 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 45.

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 for more information on doing this step.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
3. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
4. 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 4. Primer-dimer peaks can be expected around 40 bp and are removed during the size-selection step (page 49).

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 48. 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 49. 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 48.

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.

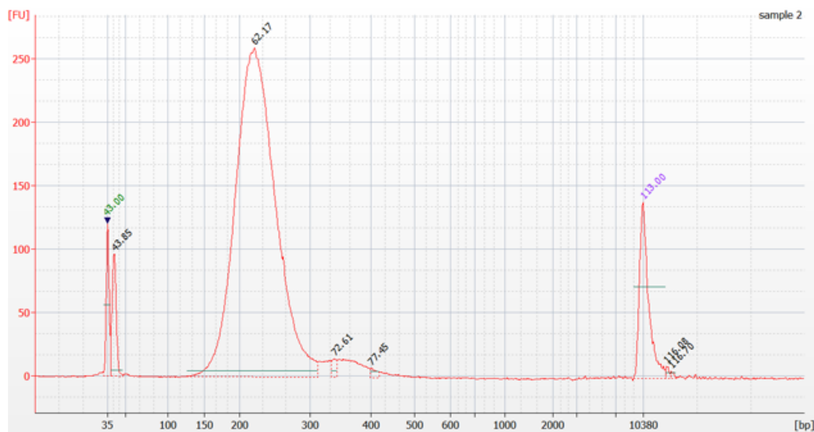


Figure 4 Post-capture analysis using the Bioanalyzer High Sensitivity DNA Assay.

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 26 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 26 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

Size-select the DNA library fragments by electrophoresis using the E-Gel system with a 2% Agarose Gel (see supplier information on page 14). Migrate the library PCR product band, containing fragments sized approximately 220 bp, into the recovery well using the 200-bp band of the 50-bp ladder for reference. Collect the size-selected DNA library in 20 µL of 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 for more information on doing this step. 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 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 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.

For samples that were size-selected and purified as individual libraries, do “Step 4. Pool samples for multiplexed sequencing” on page 48 before you do “Step 8. Prepare templates for sequencing” on page 52.

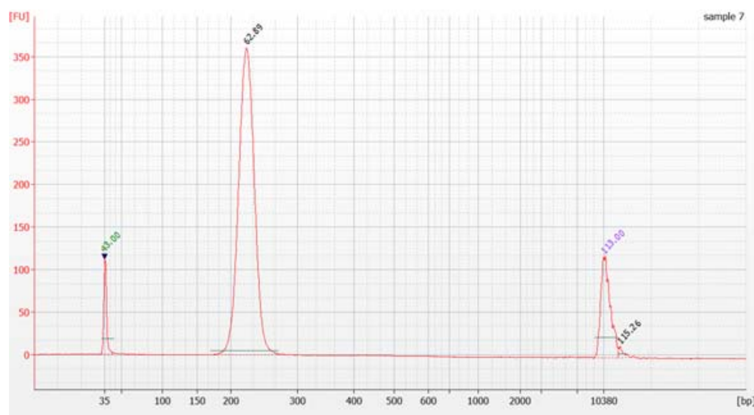
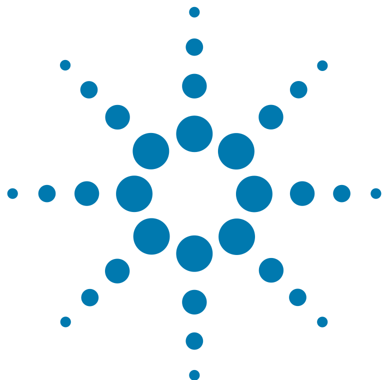


Figure 5 Analysis of size-selected, target enriched DNA using the Bioanalyzer High Sensitivity DNA Assay.

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

Follow the recommendations for diluting your library for template preparation in the appropriate Ion PGM or Ion Proton user guide.



5
Reference

SureSelect Reagent Kit Content 54
Other Reagent Kit Content 55
Alternative Capture Equipment Combinations 56

This chapter contains reference information.

SureSelect Reagent Kit Content

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

Table 27 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 27 are described in the tables below.

Table 28 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 29 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 30 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 31 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 32 Ion Xpress Barcode Adapters Kit

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

Alternative Capture Equipment Combinations

Table 33 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 33 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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