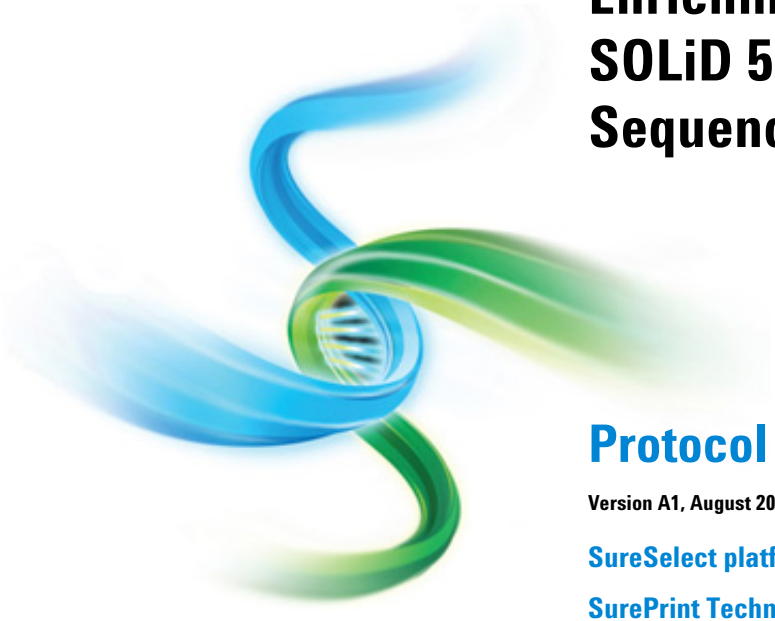

SureSelect^{XT} Target Enrichment System for SOLiD 5500 Multiplexed Sequencing

Protocol

Version A1, August 2015

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

Manual Part Number

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

Acknowledgement

Technical Support

For technical product support, contact your local Agilent Support Services representative.

For US and Canada, call (800) 227-9770 (option 3,4,4). For other countries, find your support center telephone numbers at www.agilent.com/chem/contactus.

Or send an e-mail to:
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SureSelect capture libraries and reagents must be used within one year of receipt.

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CAUTION

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In this Guide...

This guide describes the recommended operational procedures to capture genomic regions of interest using the Agilent SureSelect^{XT} Target Enrichment System Kit for SOLiD 5500 Multiplex Sequencing. This protocol is specifically developed and optimized to use Biotinylated RNA oligomer libraries, or Bait, to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus.

The SureSelect^{XT} Target Enrichment System Kit for SOLiD 5500 Multiplex Sequencing is designed to work on the SOLiD 5500 system. For SOLiD 4 systems, refer to the Target Enrichment kits and protocols for SOLiD Multiplexed Sequencing.

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 contains instructions for prepped library production specific to the Life Technologies SOLiD 5500 System.

3 Hybridization

This chapter describes the steps to combine the prepped library with the blocking agents and the SureSelect capture library.

4 Addition of Barcode Tags by Post-Hybridization Amplification

This chapter describes the steps to amplify, purify, quantify, and pool the barcoded sample libraries after target enrichment hybridization.

5 Reference

This chapter contains reference information.

Content

1 Before You Begin

Make sure you have the most current protocol. Go to the Next-Gen Sequencing User Manuals page on genomics.agilent.com and search for manual number G7530-90004.

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

NOTE Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols to process samples for enrichment.

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.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- Avoid repeated freeze-thaw cycles of 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.
- 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 1 Required Reagents for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
For use with 2100 Bioanalyzer:	
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
For use with 2200 TapeStation System:	
D1K ScreenTape	Agilent p/n 5067-5361
D1K Reagents	Agilent p/n 5067-5362
High Sensitivity D1K ScreenTape	Agilent p/n 5067-5363
High Sensitivity D1K Reagents	Agilent p/n 5067-5364
QPCR NGS Library Quantification Kit (SOLiD)	Agilent p/n G4881A
Herculase II Fusion DNA Polymerase (includes dNTP mix and 5x Buffer) 200 reactions 400 reactions (Each library requires 4 reactions for pre-capture amplification and 2 reactions for post-capture amplification.)	Agilent p/n 600677 p/n 600679
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Agencourt AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 4389764
Qubit dsDNA HS Assay Kit *or*	Life Technologies p/n Q32851
Qubit dsDNA BR Assay Kit 100 assays, 2-1000 ng 500 assays, 2-1000 ng 1000 assays, 2-1000 ng	 Life Technologies p/n Q32850 Life Technologies p/n Q32853 Life Technologies p/n Q33130
Qubit assay tubes	Life Technologies p/n Q32856
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

Table 2 SureSelect Reagent Kit*

Reagent Kits	16 Reactions	96 Reactions	480 Reactions
SureSelect^{XT} Reagent Kit, 5500	G9615A	G9615B	N/A

* SureSelect reagents must be used within one year of receipt.

Table 3 SureSelect Capture Library (select one)*

Capture Libraries	16 Reactions	96 Reactions	480 Reactions
SureSelect^{XT} Human All Exon 50Mb	5190-4626	5190-4627	5190-4629
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} Custom 1 kb up to 499 Kb	5190-4806	5190-4807	5190-4809
(reorder)	5190-4811	5190-4812	5190-4814
SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb	5190-4816	5190-4817	5190-4819
(reorder)	5190-4821	5190-4822	5190-4824
SureSelect^{XT} Custom 3 Mb up to 5.9 Mb	5190-4826	5190-4827	5190-4829
(reorder)	5190-4831	5190-4832	5190-4834
SureSelect^{XT} Custom 6 Mb up to 11.9 Mb	5190-4836	5190-4837	5190-4839
(reorder)	5190-4841	5190-4842	5190-4844
SureSelect^{XT} Custom 12 Mb up to 24 Mb	5190-4896	5190-4897	5190-4899
(reorder)	5190-4901	5190-4902	5190-4904

* SureSelect capture libraries must be used within one year of receipt.

Table 4 Required Reagents for Hybridization

Description	Vendor and part number
Dynabeads MyOne Streptavidin T1	Life Technologies
2 mL	Cat #65601
10 mL	Cat #65602
100 mL	Cat #65603
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

Optional Reagents

Table 5 Optional Reagents

Description	Vendor and part number
SureSelect gDNA Extraction Kit	
50 reaction kit	Agilent p/n G7505A
250 reaction kit	Agilent p/n G7505B

Required Equipment

Table 6 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2938C
or	
2200 TapeStation System	Agilent p/n G2964AA or G2965AA
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Thermal cycler	Agilent SureCycler, Life Technologies Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Covaris Sample Preparation System, S-series or E-series model	Covaris
Covaris microTUBE with AFA fiber and snap cap	Covaris p/n 520045
Eppendorf Microcentrifuge Model 5417R	Eppendorf p/n 022621807 (120 V/60 Hz), Eppendorf p/n 022621840 (230 V/50 Hz) or equivalent
Eppendorf fixed-angle rotor with standard lid	Eppendorf p/n 022636006
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857
E-Gel iBase and E-Gel Safe Imager Combo Kit or	Life Technologies p/n G6465
Safe Imager Real-Time Transilluminator *and*	Life Technologies p/n G6500
E-Gel iBase Power System	Life Technologies p/n G6400
Dynal DynaMag-2 magnetic stand	Life Technologies p/n 123-21D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Ice bucket	

Table 6 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Heat block at 37°C	

Table 7 Required Equipment for Hybridization

Description	Vendor and part number
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent
MicroAmp Clear Adhesive Film	Life Technologies p/n 4306311 or equivalent
BD Clay Adams Nutator Mixer	BD Diagnostics p/n 421105 or equivalent
Dynal DynaMag-2 magnetic stand	Life Technologies p/n 123-21D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Pipet-Light Multichannel Pipette, 12 channels	Rainin p/n L12-20 or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	
Thermal cycler	Agilent SureCycler, Life Technologies Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Timer	
Vortex mixer	
Water bath or heat block set to 65°C	

Optional Equipment

Table 8 Optional Equipment for Hybridization

Description	Vendor and part number
Tube-strip capping tool	Agilent p/n 410099

2 Sample Preparation

This chapter contains instructions for prepped library production specific to the Life Technologies SOLiD 5500 System.

Before you begin, you can use the SureSelect gDNA Extraction Kit to extract genomic DNA. Refer to the *gDNA Extraction Kit Protocol* (p/n 5012-8701).

NOTE Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0.

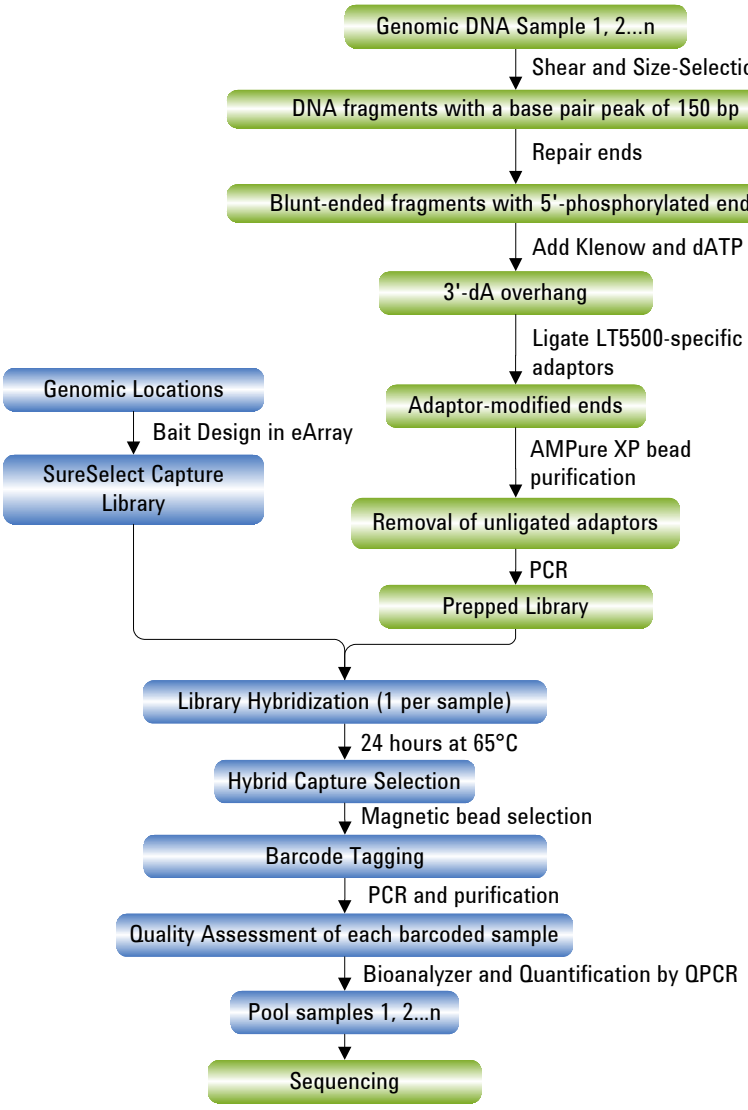

Figure 1 Overall sequencing sample preparation workflow.

Table 10 Overview and time requirements

Step	Time
AB SOLiD 5500 Fragment Library Production	8 hours
Bioanalyzer QC	1 hour
Library Preparation and Hybridization	24.5 hours (optional 72 hours)
Bead Preparation	10 minutes
Capture Selection and Washing	2.5 hours
Post-Hybridization Amplification	1 hour
PCR Purification	30 minutes
Bioanalyzer QC	1 hour

Step 1. Quantify and shear DNA

1. Use the Qubit dsDNA Assay to determine the concentration of your gDNA sample. Make sure the gDNA is of high quality (non-degraded, A_{260}/A_{280} is 1.8 to 2.0).

   Follow the instructions for the instrument.

2. Set up the Covaris instrument.

   a. Check that the water in the Covaris tank is filled with fresh deionized water to fill line level 12 on the graduated fill line label.

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

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

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

   e. On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use.

   Refer to the Covaris instrument user guide.

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

4. Put a Covaris microTube into the loading and unloading station.

   Keep the cap on the tube.

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

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

6. Secure the microTube in the tube holder and shear the DNA with the settings in Table 11 or Table 12, depending on the Covaris instrument SonoLab software version that is used. The target peak for base pair size is 150 bp.

Table 11 Shear settings for Covaris instruments that use SonoLab 7 or newer

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	100
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

Table 12 Shear settings for Covaris instruments that use SonoLab software previous to SonoLab 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	100
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

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

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

9 Transfer the sheared DNA into a new 1.5-mL LoBind tube.

Step 2. Size-select the sample using the Agencourt AMPure XP beads

1. Measure out just enough AMPure XP beads for the number of samples to purify.
2. Let the AMPure XP beads come to room temperature for at least 30 minutes.
3. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
4. Add 143 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the sheared DNA library (~130 µL). Mix well on a vortex mixer and incubate for 5 minutes.
5. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Add 91 µL of homogenous AMPure XP beads to a new 1.5-mL LoBind tube.
7. Keep the original tube in the magnetic stand. Do not touch the beads while you carefully move the cleared solution (approximately 273 µL) from the original tube to the new 1.5-mL LoBind tube. Discard the beads from the original tube. Mix well on a vortex mixer and incubate for 5 minutes.
8. Put the new tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
9. Keep the tube in the magnetic stand. Do not touch the beads while you carefully remove the cleared solution from the tubes.
10. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.

    Use fresh 70% ethanol for optimal result.
11. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
12. Repeat step 10 and step 11 once.
13. Dry the samples on the 37°C heat block for 5 minutes until the residual ethanol is completely evaporated.

    Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
14. Add 50 µL nuclease-free water, mix well on a vortex mixer, and 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 supernatant (~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 3. Assess quality with the 2100 Bioanalyzer

Quality assessment can be done with either the 2100 Bioanalyzer instrument or the 2200 TapeStation.

2100 Bioanalyzer System and DNA 1000 Assay

1. Check that the 2100 Bioanalyzer electrodes have been cleaned and dried as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer instrument and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, then mix on the IKA vortex mixer that is included with the 2100 Bioanalyzer instrument.
4. Load the prepared chip into the 2100 Bioanalyzer instrument and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results. Check that the electropherogram shows a distribution with a peak size around 150 bp.

Stopping Point If you do not continue at the next step, store the purified DNA in RNase-free water at 4°C.

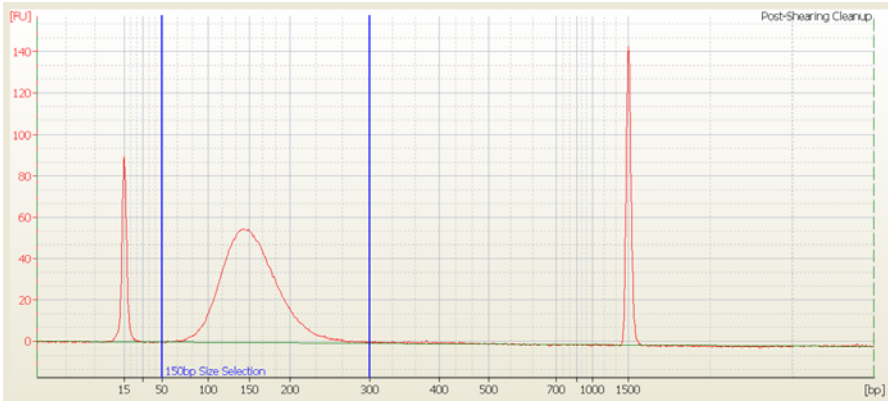

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows a single peak in the size range of 150 bp.

2200 TapeStation and D1K ScreenTape

You can use the 2200 TapeStation for rapid analysis of multiple samples. Use the D1K ScreenTape and D1K Reagents. For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 1 µL of each sheared DNA sample diluted with 3 µL of D1K sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and D1K sample buffer on a vortex mixer for 5 seconds or more for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1K ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows an average DNA fragment size of about 150 bp. A sample electropherogram is shown in Figure 3.

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

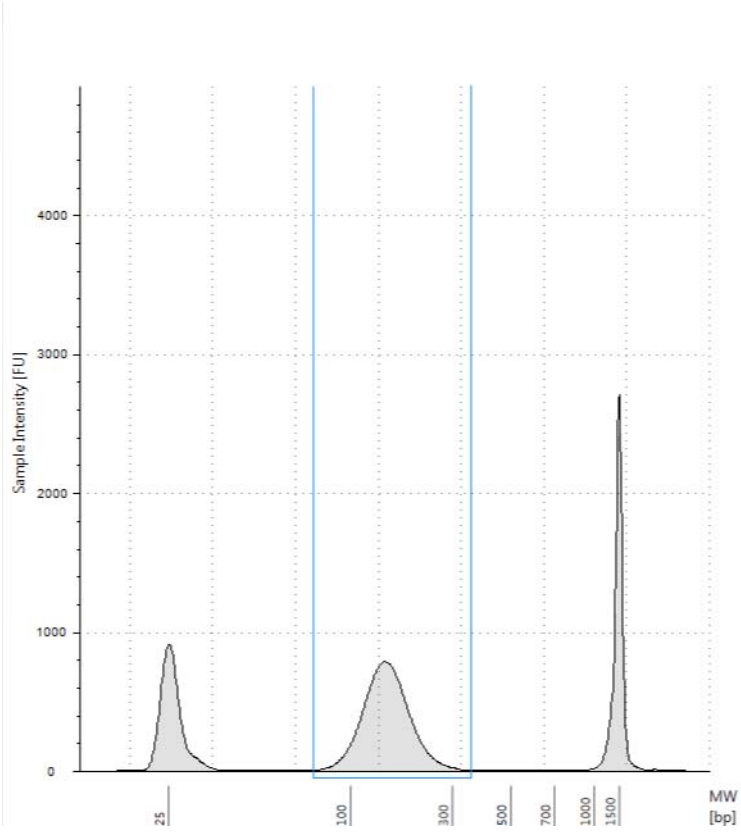

Figure 3 Analysis of sheared DNA using the 2200 TapeStation with a D1K ScreenTape. The electropherogram shows an average DNA fragment size of about 150 bp.

Step 4. Repair the ends

Use reagents from the SureSelect XT Library Prep Kit, S5500.

1 For 1 library:
   - In a 1.5-mL LoBind tube, strip tube, or plate, prepare the reaction mix in Table 13, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   - **a** Prepare the reaction mix in Table 13, on ice. Mix gently on a vortex mixer.
   - **b** Add 52 µL of the reaction mix to each well or tube.
   - **c** Use a pipette to add 48 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 13 End Repair

Reagent	Volume for 1 Library (µL)	Volume for 12 Libraries (µL), includes excess
Sheared DNA	48	
10× End Repair Buffer (clear cap)	10	125
dNTP Mix (green cap)	1.6	20
T4 DNA Polymerase (purple cap)	1	12.5
Klenow DNA Polymerase (yellow cap)	2	25
T4 Polynucleotide Kinase (orange cap)	2.2	27.5
Nuclease-free water	35.2	440
Total Volume	**100**	**650 (52 µL/sample)**

3 Incubate the mixture at 22°C for 30 minutes.

Step 5. Purify the sample using the Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the repaired DNA library (100 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL of nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 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. Add 'A' Bases to the 3' end of the DNA fragments

Use the SureSelect XT Library Prep Kit, S5500.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 14. Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 14. Mix well on a vortex mixer.
   b Add 20 µL of the reaction mix to each well or tube.
   c Add 30 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 14 Adding "A" Bases*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
End-repaired DNA sample	~30 µL	
Nuclease-free water	11 µL	137.5 µL
10× Klenow Polymerase Buffer (blue cap)	5 µL	62.5 µL
dATP (green cap)	1 µL	12.5 µL
Exo(-) Klenow (red cap)	3 µL	37.5 µL
Total Volume	**50 µL**	**250 µL (20 µL/sample)**

* These reagents are included in the SureSelect XT Library Prep Kit, S5500.

3 Incubate in a thermal cycler for 30 minutes at 37°C.

If you use a heated lid, make sure that the lid temperature does not exceed 50°C.

Step 7. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the A-tailed DNA library (~50µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 15 µL of nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~15 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.
13. Proceed immediately to the next step, "Step 8. Ligate the adaptors".

Step 8. Ligate the adaptors

Use reagents from the SureSelect XT Library Prep Kit, S5500 and the SureSelect S5500 Indexing Construction Kit.

1 For 1 library:
   - In a 1.5-mL LoBind tube, strip tube, or plate, prepare the reaction mix in Table 15, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   a Prepare the reaction mix in Table 15, on ice. Mix gently on a vortex mixer.
   b Add 35 µL of the reaction mix to each well or tube.
   c Use a pipette to add 15 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 15 Ligation mix

Component	Volume (µL)	Volume for 12 Libraries (µL) includes excess
A-Tailed DNA	~15	
SureSelect LTI5500 P1 (purple cap)	4.5	56.25
SureSelect LTI5500 IA (blue cap)	4.5	56.25
5× T4 DNA Ligase Buffer	10	125
T4 DNA Ligase (red cap)	1.5	18.75
Nuclease-free water	14.5	181.25
Total	**50**	**437.5 (35 µL/sample)**

3 Incubate at 22 °C for 15 minutes.

Step 9. Purify the sample using the Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the ligated DNA library (50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.

   Use fresh 70% ethanol for optimal result.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 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 10. Amplify adaptor-ligated library

Use reagents from these kits:

- SureSelect XT Library Prep Kit, S5500
- SureSelect S5500 Indexing Construction Kit
- Herculase II Fusion DNA Polymerase (Agilent)

This protocol uses half of the adaptor-ligated fragments 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 For 1 library:
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 16, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   a Prepare the reaction mix in Table 16, on ice. Mix well on a vortex mixer.
   b Add 36 µL of the reaction mix to each well or tube.
   c Add 14 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 16 Components for PCR mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Adaptor-ligated library	14 µL	
Nuclease-free water	20.5 µL	256.25 µL
SureSelect LTI Pre Capture Primer (green cap)*	4 µL	50 µL
5× Herculase II Rxn Buffer (clear cap)†	10 µL	125 µL
100 mM dNTP Mix (green cap)†	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase (red cap)†	1 µL	12.5 µL
Total	**50 µL**	**450 µL (36 µL/reaction)**

* Included in the SureSelect S5500 Indexing Construction Kit.

† Included in the Herculase II Fusion DNA Polymerase (Agilent) kit. *Do not use the buffer or dNTP mix from any other kit.*

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

Table 17

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	30 seconds
Step 3	54°C	10 seconds
Step 4	72°C	1 minute
Step 5		Repeat Step 2 through Step 4 for a total of 4 to 6 times
Step 6	72°C	10 minutes
Step 7	4°C	Hold

NOTE

Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, 5 cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low or non-specific high molecular weight products are observed, adjust the number of cycles accordingly with the remaining extra library template.

As an alternative, you can prepare one PCR master mix as outlined in Table 16. Split the master mix into three small-scale 10 µL PCR reactions and run for 4, 5 or 6 cycles. Clean these PCR reactions using the AMPure XP protocol outlined in "Step 11. Purify the sample using the Agencourt 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 a DNA1000 chip on the Bioanalyzer, as described in "Step 12. Assess quality and quantity with the 2100 Bioanalyzer".

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

Step 11. Purify the sample using the Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. Do not freeze.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the ligated library (50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove approximately 30 µL of the supernatant 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 12. Assess quality and quantity with the 2100 Bioanalyzer

The hybridization protocol in the following section requires 500 ng of each amplified DNA library. Measure the concentration of each library using one of the methods detailed below. Once DNA concentration for each sample is determined, calculate the volume of the library to be used for hybridization using the following formula:

Volume (µL) = 500 ng/concentration (ng/µL)

2100 Bioanalyzer System and DNA 1000 Assay

1. Check that the 2100 Bioanalyzer electrodes have been cleaned and dried as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer instrument and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, then mix on the IKA vortex mixer that is included with the 2100 Bioanalyzer instrument.
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 appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results. Check that the electropherogram shows a distribution with a peak size around 200 bp.

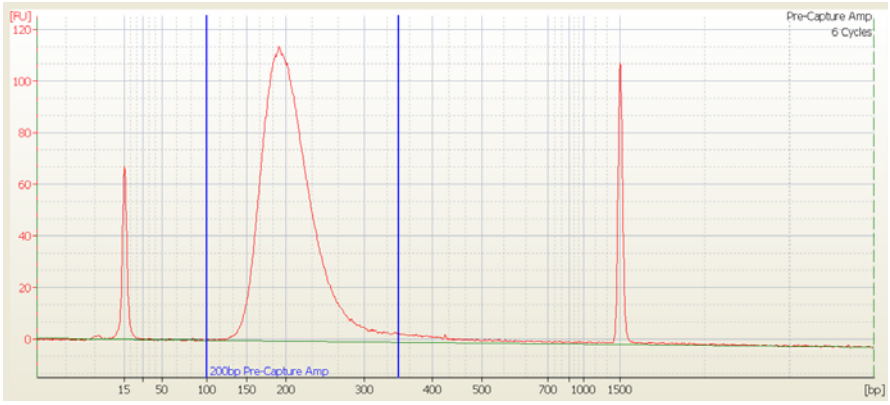

Figure 4 Analysis of amplified prepped library DNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 200 bp.

8 If the concentration of your sample is greater than the high end of the dynamic range of the Bioanalyzer DNA 1000 assay (> 50 ng/µL), use the Qubit Fluorometer to quantitate the library. Dilute your prepped library appropriately, and use the Bioanalyzer DNA 1000 assay to quantitate again. Use the concentration as determined by the Bioanalyzer DNA 1000 assay to calculate the volume of prepped library needed for hybridization (500 ng) in Chapter 3.

2200 TapeStation and D1K ScreenTape

You can use the 2200 TapeStation to analyze the amplified libraries. Use the D1K ScreenTape and D1K Reagents. For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 1 µL of each amplified library DNA sample diluted with 3 µL of D1K sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and D1K sample buffer on a vortex mixer for 5 seconds or more for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1K ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows an average DNA fragment size of about 200 bp. A sample electropherogram is shown in Figure 4.

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

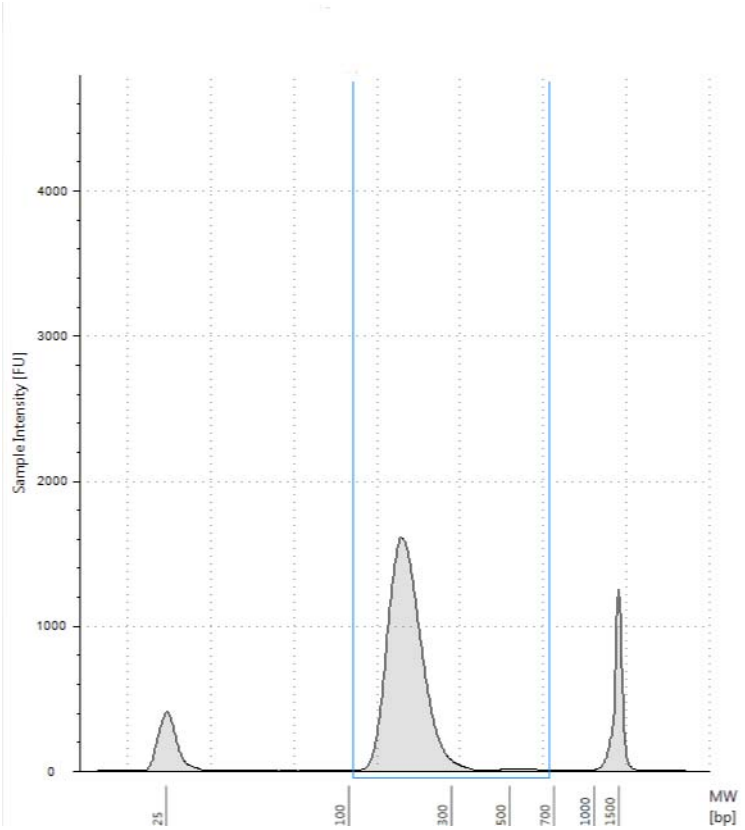

Figure 5 Analysis of amplified library DNA using the 2200 TapeStation with a D1K ScreenTape. The electropherogram shows an average DNA fragment size of about 200 bp.

3 Hybridization

This chapter describes the steps to combine the prepped library with the hybridization reagents, blocking agents and the SureSelect capture library.

CAUTION The ratio of SureSelect capture library to prepped library is critical for successful capture.

Refer to "SureSelect Reagent Kit Content" on page 62 for a complete content listing of each SureSelect Target Enrichment kit.

CAUTION You must avoid evaporation from the small volumes of the capture during the 24 hour or greater 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 29 µL of SureSelect Hybridization Buffer (without DNA) at 65°C for 24 hours (or longer, if applicable) as a test. Include buffer 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 3 to 4 µL.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 71.

Step 1. Hybridize the library

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

The hybridization reaction requires 500 ng of DNA with a maximum volume of 3.4 µL.

1. If the prepped library concentration is below 147 ng/µL, use a vacuum concentrator to concentrate the sample at ≤ 45°C.
   - **a** Add the entire 30 µL of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

     You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.
   - **b** Completely lyophilize. Use a vacuum concentrator on low heat (less than 45°C) to dehydrate.
   - **c** Reconstitute with nuclease-free water to bring the final concentration to 147 ng/µL (or greater if sample recovery is of concern). Pipette up and down along the sides of the tube for optimal recovery.
   - **d** Mix well on a vortex mixer and spin in a microfuge for 1 minute.
2. *Optional.* To test recovery after lyophilization, reconstitute the sample to greater than 147 ng/µL and check the concentration on a Bioanalyzer DNA 1000 chip. See "Step 12. Assess quality and quantity with the 2100 Bioanalyzer" on page 35147

   Alternatively, concentrate a 500 ng aliquot at ≤ 45°C down to 3.4 µL. If the sample dries up completely, resuspend in 3.4 µL of water and mix on a vortex mixer. If processing multiple samples, adjust to equivalent volumes before concentrating.
3. Mix the components in Table 18 at room temperature to prepare the hybridization buffer.

Table 18 Hybridization Buffer

Reagent	Volume for 3 captures (µL), includes excess	Volume for 6 captures (µL), includes excess	Volume for 12 captures (µL), includes excess
SureSelect Hyb #1 (orange cap, or bottle)	25	50	87.5
SureSelect Hyb #2 (red cap)	1	2	3.5
SureSelect Hyb #3 (yellow cap)	10	20	35
SureSelect Hyb #4 (black cap, or bottle)	13	26	45.5
Total	**49 (13 µL/sample)**	**98 (13 µL/sample)**	**171.5 (13 µL/sample)**

4 If precipitate forms, warm the hybridization buffer at 65°C for 5 minutes.

5 In a PCR plate, prepare the SureSelect capture library mix for target enrichment:

- **a** Keep tubes on ice until step 9.
- **b** For each sample, add the amount of SureSelect capture library as listed in Table 19, based on the Mb target size of your design.
- **c** Use nuclease-free water to prepare a dilution of the SureSelect RNase Block (purple cap) as listed in Table 19.

  Prepare enough RNase Block dilution for all samples, plus excess.
- **d** Add the amount of diluted SureSelect RNase Block (purple cap) listed in Table 19 to each capture library, and mix by pipetting.

Table 19 SureSelect Capture Library.

Capture Size	Volume of SureSelect Library	RNase Block Dilution (Parts RNase block: Parts water)	Volume of RNase Block Dilution to Add
< 3.0 Mb	2 µL	1:9 (10%)	5 µL
≥ 3.0 Mb	5 µL	1:3 (25%)	2 µL

6 Mix the contents in Table 20 to make the correct amount of SureSelect Block mix for the number of samples used.

Table 20 SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
SureSelect Indexing Block #1 (green cap)	2.5 µL	31.25 µL
SureSelect Block #2 (blue cap)	2.5 µL	31.25 µL
SureSelect LT Indexing Block #3 (brown cap)	0.6 µL	7.5 µL
Total	**5.6 µL**	**70 µL**

7 In a separate PCR plate, prepare the prepped library for target enrichment.

- **a** Add 3.4 µL of 147 ng/µL prepped library to the "C" row in the PCR plate. Put each sample into a separate well.
- **b** Add 5.6 µL of the SureSelect Block Mix to each well in row C.
- **c** Mix by pipetting up and down.
- **d** Seal the wells of row "C" with caps and put the PCR plate in the thermal cycler. Do not heat the Hybridization Buffer or capture library yet, only the prepped library with blockers.
- **e** Start the thermal cycler program in Table 21.

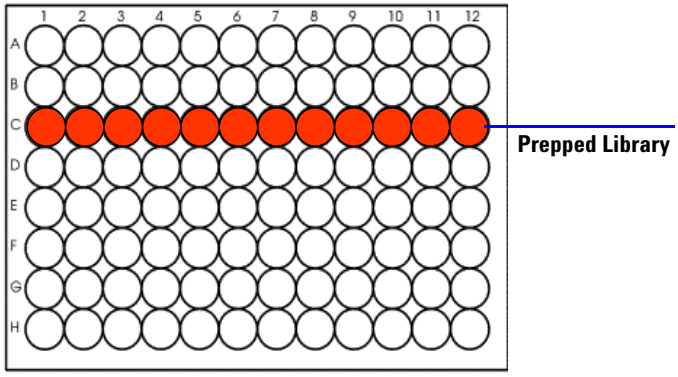

Figure 6 Prepped library shown in red

Table 21 PCR program

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

8 Use a heated lid on the thermal cycler at 105°C to hold the temperature of the plate on the thermal cycler at 65°C.

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

9 While the sample incubates at 95°C, combine 13 µL of hybridization buffer with 7 µL of prepared SureSelect Capture Library mix at room temperature.

10 When the thermal cycler reaches 65°C, maintain the plate at 65°C while you use a multi-channel pipette to add 20 µL of the hybridization buffer and SureSelect Capture Library mix to the prepped library. Slowly pipette up and down 2 to 3 times to mix.

The hybridization mixture is now 27 to 29 µL, depending on the degree of evaporation during the 95°C step.

11 Seal the wells with strip caps or double adhesive film. Make sure all wells are completely sealed. Use new adhesive seals or strip caps. The structural integrity of the seals and caps can be compromised during the 95°C step.

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

Samples can be hybridized for up to 72 hours, but when you hybridize at longer periods, test the sample to make sure that evaporation is not extensive.

Step 2. Prepare magnetic beads

Use these reagents from the SureSelect^{XT} Target Enrichment Box 1 S5500:

- SureSelect Binding Buffer
- SureSelect Wash 2

1. Prewarm SureSelect Wash 2 at 65°C in a circulating water bath for use in "Step 3. Select hybrid capture with SureSelect".
2. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 on a vortex mixer. Magnetic beads settle during storage.
3. For each hybridization, add 50 µL of Dynabeads MyOne Streptavidin T1 to a 1.5-mL LoBind tube.
4. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix the beads on a vortex mixer for 5 seconds.
   c. Put the tubes into a magnetic device, such as the Dynal magnetic separator (Life Technologies).
   d. Remove and discard the supernatant.
   e. Repeat step a through step d for a total of 3 washes.
5. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

Step 3. Select hybrid capture with SureSelect

Use these reagents from the SureSelect^{XT} Target Enrichment Box 1 S5500:

- SureSelect Wash 1
- SureSelect Wash 2

1. Estimate and record the volume of hybridization that remained after 24 hour incubation.
2. Keep the PCR plate or tubes at 65°C in the PCR machine while you add the hybridization mixture directly from the thermal cycler to the bead solution. Invert the tube to mix 3 to 5 times.

   Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 36 on page 71 for tips to minimize evaporation.
3. Incubate the hybrid-capture/bead solution on a Nutator or equivalent for 30 minutes at room temperature.

   Make sure the sample is properly mixing in the tube.
4. Briefly spin in a centrifuge.
5. Separate the beads and buffer on a magnetic separator and remove the supernatant.
6. Resuspend the beads in 500 µL of SureSelect Wash 1 by mixing on a vortex mixer for 5 seconds.
7. Incubate the samples for 15 minutes at room temperature. Occasionally mix on a vortex mixer.
8. Briefly spin in a centrifuge.
9. Separate the beads and buffer on a magnetic separator and remove the supernatant.

10 Wash the beads:

a Resuspend the beads in 500 µL of 65°C prewarmed SureSelect Wash 2 and mix on a vortex mixer for 5 seconds to resuspend the beads.

b Incubate the samples for 10 minutes at 65°C in a recirculating water bath, heat block or equivalent. Occasionally mix on a vortex mixer.

Do not use a tissue incubator. It cannot properly maintain temperature.

c Invert the tube to mix. The beads may have settled.

d Briefly spin in a centrifuge.

e Separate the beads and buffer on a magnetic separator and remove the supernatant.

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

Make sure all of the wash buffer has been removed.

g Mix the beads with 30 µL of nuclease-free water on a vortex mixer for 5 seconds to resuspend the beads.

4 Addition of Barcode Tags by Post-Hybridization Amplification

This chapter describes the steps to add barcode tags by amplification, purify, assess quality and quantity of the libraries, and pool barcoded samples for multiplexed sequencing.

Step 1. Amplify the captured library on the Streptavidin beads to add barcode tags

Use reagents from:

- Herculase II Fusion DNA Polymerase (Agilent)
- SureSelect S5500 Indexing Construction Kit

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

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.

Prepare 1 amplification reaction for each hybrid capture. Include a negative no-template control.

To see the nucleotide sequence in each of the barcode included in SureSelect reagent kits, see "SureSelect^{XT} Barcodes for SOLiD" on page 66.

1 For 1 library:
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 22, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   a Prepare the reaction mix in Table 22, on ice. Mix well on a vortex mixer.
   b Add 34 µL of the reaction mix to each well or tube.
   c Add 2 µL of the appropriate barcode SureSelect LT BC1 through BC16 (clear cap) from the SureSelect S5500 Indexing Construction Kit or BC1 through BC96 from the SureSelect LTI5500 BC1-BC96 plate to each well and mix by pipetting. See Figure 9, "SureSelect LTI5500 BC1-BC96 plate barcode orientation," on page 70 to locate the barcodes on the index plate.

      Use a different barcode primer for each sample to be sequenced in the same lane. Use Table 23 as a guide to determine the number of barcodes to pool per sequencing lane.

Include full sets of 4 barcodes in each lane. A full set of barcodes refers to BC1 through BC4, BC5 through BC8, BC9 through BC12, etc.

If the number of libraries to be combined in a sequencing lane is not a multiple of 4 (based on guides in Table 23), then use multiple barcoding primers, in equal ratios, to amplify a single library. For example for a sequencing sample designed to contain 2 libraries, amplify each library using 2 barcoding primers. For 3 libraries, amplify each library using 4 barcoding primers, for a total of 12 barcodes in the sequencing sample.

d Pipette each DNA sample up and down to make sure that the bead solution is homogenous.

e Use a pipette to add 14 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples to avoid cross-contamination.

Table 22 Herculase II Master Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Captured on-bead DNA	14 µL	
Nuclease-free water	22.5 µL	281.25 µL
5× Herculase II Rxn Buffer (clear cap) *	10 µL	125 µL
100 mM dNTP Mix (green cap)*	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase (red cap)*	1 µL	12.5 µL
SureSelect LT BC1 through BC16 (clear cap)	2 µL	
Total	**50 µL**	**425 µL (34 µL/reaction)**

* Included in the Herculase II Fusion DNA Polymerase (Agilent). *Do not use the buffer or dNTP mix from any other kit.*

Table 23 Sequencing data requirement guidelines

Capture size	Optimal sequencing output per barcode
1 kb up to 0.5 Mb	0.1 to 50 Mb*
0.5 Mb up to 2.9 Mb	50 to 290 Mb*
3 Mb up to 5.9 Mb	300 to 590 Mb*
6 Mb up to 11.9 Mb	600 to 1190 Mb*
12 Mb up to 24 Mb	1.2 to 2.4 Gb*
Human All Exon v4	4 Gb
Human All Exon v4 + UTRs	6 Gb
Human All Exon 50 Mb	5 Gb
Human DNA Kinome	320 Mb
Mouse All Exon	5 Gb

* For custom libraries, Agilent recommends analyzing 100× amount of sequencing data compared to the Capture Library size for each sample. Pool samples according to your expected sequencing output.

3 Put the tubes in a thermal cycler and run the program in Table 24.

Table 24 PCR program

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	30 seconds
Step 3	54°C	10 seconds
Step 4	72°C	1 minute
Step 5		Repeat Step 2 through Step 4, depending on the size of the capture: • 0.2 Mb up to 0.49 Mb: 12 cycles total • 0.5 Mb up to 1.49 Mb: 10 cycles total • 1.5 Mb up to 2.99 Mb: 9 cycles total • 3 Mb or more: 8 cycles total
Step 6	72°C	10 minutes
Step 7	4°C	Hold

Step 2. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 60 µL of homogenous AMPure beads to a 1.5-mL LoBind tube, and add amplified library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 50 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~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 4°C for up to a week, or at -20°C for longer periods.

Step 3. Remove primer-dimers from the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 60 µL of homogenous AMPure beads to a 1.5-mL LoBind tube, and add amplified library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 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 4°C for up to a week, or at -20°C for longer periods.

Step 4. Assess DNA quality

Use a Bioanalyzer High Sensitivity DNA Assay or the 2200 TapeStation to assess the quality and size range.

2100 Bioanalyzer High Sensitivity DNA Assay

You may need to dilute your sample accordingly. Refer to the *Agilent High Sensitivity DNA Kit Guide* at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=59504.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
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 appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results.

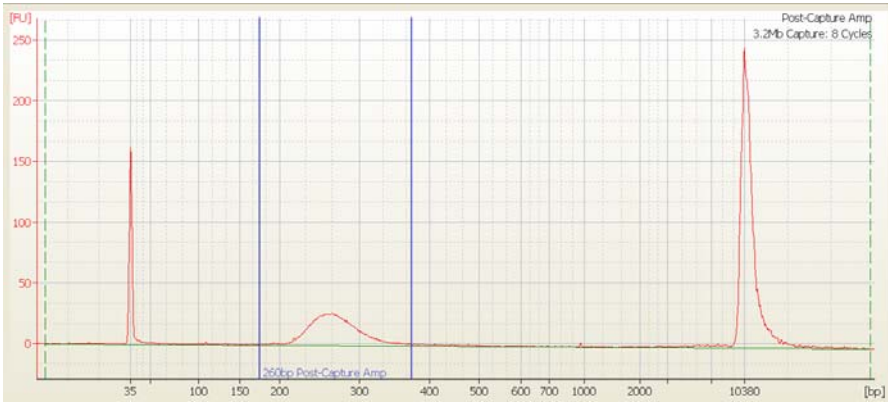

Figure 7 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows a peak in the size range of approximately 260 bp.

2200 TapeStation and High Sensitivity D1K ScreenTape

Use the 2200 TapeStation to analyze the barcoded DNA. Use the High Sensitivity D1K ScreenTape and High Sensitivity D1K Reagents. For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 2 µL of each amplified library DNA sample diluted with 2 µL of High-Sensitivity D1K sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and D1K sample buffer on a vortex mixer for 5 seconds or more for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the High Sensitivity D1K ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows an average DNA fragment size of about 260 bp. A sample electropherogram is shown in Figure 8.

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

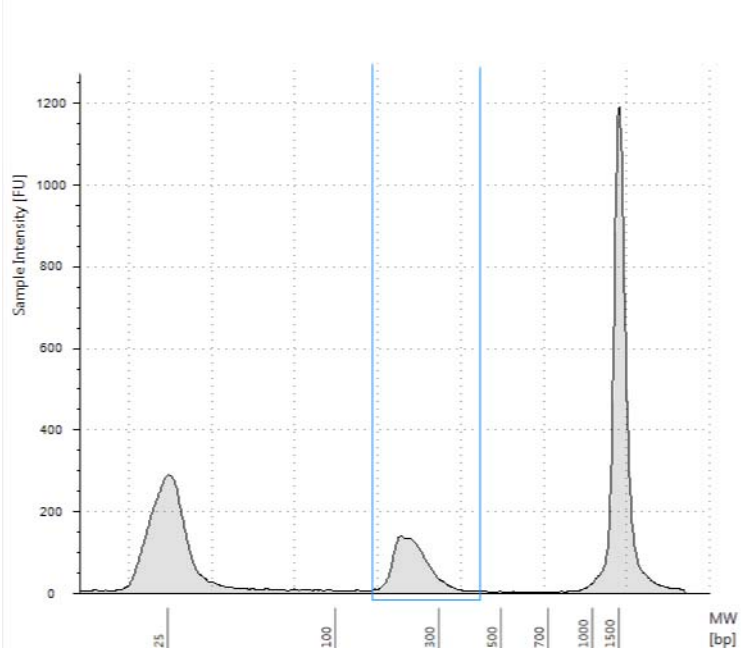

Figure 8 Analysis of amplified library DNA using the 2200 TapeStation with a D1K ScreenTape. The electropherogram shows a peak size of 260 bp.

Step 5. Assess the quantity of each barcode-tagged library by QPCR

Refer to the protocol that is included with the QPCR NGS Library Quantification Kit (SOLiD) for more details to do this step.

1. Use the QPCR NGS Library Quantification Kit (SOLiD) to determine the concentration of each barcode-tagged captured library.
2. Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.
3. Dilute each barcode-tagged captured library such that it falls within the range of the standard curve.

   Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.
4. Prepare the QPCR master mix with SOLiD adaptor-specific PCR primers according to instructions provided in the kit.
5. Add an aliquot of the master mix to PCR tubes and add template.
6. On a QPCR system, such as the MX3005P, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.
7. Use the standard curve to determine the concentration of each unknown barcode-tagged library, in nM.

The concentration will be used to accurately pool samples for multiplexed sequencing.

Step 6. Pool samples for Multiplexed Sequencing

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

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

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

C(f) is the desired final concentration of all the DNA in the pool, for example, 500 pM for the standard SOLiD protocol

is the number of samples to be combined, and

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

See Table 25 for the approximate volume of sample to use.

Table 25 Approximate volume of sample to use

SOLiD Sequencing Capacity	Approximate Sample Volume Needed	Final Concentration Needed
1 Lane	50 µL	500 pM

Table 26 shows an example of the amount of 4 barcoded samples (of different concentrations) and Low TE needed for a final volume of 100 µL at 500 pM.

Table 26 Example of barcode volume calculation for a total volume of 100 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	100 µL	921 pM	500 pM	4	13.6
Sample 2	100 µL	1050 pM	500 pM	4	11.9
Sample 3	100 µL	1352 pM	500 pM	4	9.2
Sample 4	100 µL	684 pM	500 pM	4	18.3
Low TE					47

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.

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

5 Reference

This chapter contains reference information.

SureSelect Reagent Kit Content

NOTE SureSelect capture libraries and reagents must be used within one year of receipt.

Each SureSelect Reagent Kit contains one or more of each of these individual kits:

Table 27 SureSelect Reagent Kit Contents

Product	Storage Condition	16 Reactions	96 Reactions
SureSelect^{XT} Target Enrichment Box 1 S5500	Room Temperature	5190-5931	5190-5932
SureSelect^{XT} Target Enrichment Box 2 S5500	-20°C	5190-5929	5190-5930
SureSelect XT Library Prep Kit, S5500	-20°C	5500-0112	5500-0113
SureSelect S5500 Indexing Construction Kit	-20°C	5190-5455	5190-5456

The content of each of these kits are described in the next tables.

Table 28 SureSelect^{XT} Target Enrichment Box 1 S5500

Kit Component
SureSelect Hyb #1 (orange cap, or bottle)
SureSelect Hyb #2 (red cap)
SureSelect Hyb #4 (black cap, or bottle)
SureSelect Binding Buffer
SureSelect Wash 1
SureSelect Wash 2

Table 29 SureSelect^{XT} Target Enrichment Box 2 S5500

Kit Component
SureSelect Hyb #3 (yellow cap)
SureSelect Indexing Block #1 (green cap)
SureSelect Block #2 (blue cap)
SureSelect LT Indexing Block #3 (brown cap)
SureSelect RNase Block (purple cap)

Table 30 SureSelect XT Library Prep Kit, S5500

Kit Component
10× End Repair Buffer (clear cap)
T4 Polynucleotide Kinase (orange cap)
10× Klenow Polymerase Buffer (blue cap)
T4 DNA Ligase (red cap)
Exo(-) Klenow (red cap)
T4 DNA Polymerase (purple cap)
Klenow DNA Polymerase (yellow cap)
dATP (green cap)
dNTP Mix (green cap)
5× T4 DNA Ligase Buffer

Table 31 SureSelect S5500 Indexing Construction Kit

Component
SureSelect LTI5500 P1 (purple cap)
SureSelect LTI5500 IA (blue cap)
SureSelect LTI Pre Capture Primer (green cap)
SureSelect LT BC1 through BC16 (clear cap) *or* SureSelect LTI5500 BC1-BC96 plate

Other Reagent Kits Content

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

Table 32 Herculase II Fusion DNA Polymerase (Agilent)

Component
DMSO (green cap)
5× Herculase II Rxn Buffer (clear cap)
100 mM dNTP Mix (green cap)
Herculase II Fusion DNA Polymerase (red cap)

Table 33 D1K Reagents (Agilent p/n 5067-5362)

Components
D1K ladder
D1K sample buffer

Table 34 High Sensitivity D1K Reagents (Agilent p/n 5067-5364)

Components
High-Sensitivity D1K ladder
High-Sensitivity D1K sample buffer

SureSelect^{XT} Barcodes for SOLiD

The nucleotide sequence of each of the SureSelect^{XT} barcodes is listed in Table 35. Barcode orientation for the SureSelect LTI5500 BC1-BC96 plate is shown in Figure 9 on page 70.

Table 35 SureSelect^{XT} Barcodes 1 to 96

Barcode Number	**Sequence**
1	GTGTAAGAGG
2	AGGGAGTGGT
3	ATAGGTTATA
4	GGATGCGGTC
5	GTGGTGTAAG
6	GCGAGGGACA
7	GGGTTATGCC
8	GAGCGAGGAT
9	AGGTTGCGAC
10	GCGGTAAGCT
11	GTGCGACACG
12	AAGAGGAAAA
13	GCGGTAAGGC
14	GTGCGGCAGA
15	GAGTTGAATG
16	GGGAGACGTT
17	GGCTCACCGC
18	AGGCGGATGA
19	ATGGTAACTG
20	GTCAAGCTTT

Table 35 SureSelect^{XT} Barcodes 1 to 96 (continued)

Barcode Number	**Sequence**
21	GTGCGGTTCC
22	GAGAAGATGA
23	GCGGTGCTTG
24	GGGTCGGTAT
25	AACATGATGA
26	CGGGAGCCCG
27	CAGCAAACTT
28	AGCTTACTAC
29	GAATCTAGGG
30	GTAGCGAAGA
31	GCTGGTGCGT
32	GGTTGGGTGC
33	CGTTGGATAC
34	TCGTTAAAGG
35	AAGCGTAGGA
36	GTTCTCACAT
37	CTGTTATACC
38	GTCGTCTTAG
39	TATCGTGAGT
40	AAAAGGGTTA
41	TGTGGGATTG
42	GAATGTACTA
43	CGCTAGGGTT
44	AAGGATGATC

Table 35 SureSelect^{XT} Barcodes 1 to 96 (continued)

Barcode Number	**Sequence**
45	GTACTTGGCT
46	GGTCGTCGAA
47	GAGGGATGGC
48	GCCGTAAGTG
49	ATGTCATAAG
50	GAAGGCTTGC
51	AAGCAGGAGT
52	GTAATTGTAA
53	GTCATCAAGT
54	AAAAGGCGGA
55	AGCTTAAGCG
56	GCATGTCACC
57	CTAGTAAGAA
58	TAAAGTGGCG
59	AAGTAATGTC
60	GTGCCTCGGT
61	AAGATTATCG
62	AGGTGAGGGT
63	GCGGGTTCGA
64	GTGCTACACC
65	GGGATCAAGC
66	GATGTAATGT
67	GTCCTTAGGG
68	GCATTGACGA

Table 35 SureSelect^{XT} Barcodes 1 to 96 (continued)

Barcode Number	**Sequence**
69	GATATGCTTT
70	GCCCTACAGA
71	ACAGGGAACG
72	AAGTGAATAC
73	GCAATGACGT
74	AGGACGCTGA
75	GTATCTGGGC
76	AAGTTTTAGG
77	ATCTGGTCTT
78	GGCAATCATC
79	AGTAGAATTA
80	GTTTACGGTG
81	GAACGTCATT
82	GTGAAGGGAG
83	GGATGGCGTA
84	GCGGATGAAC
85	GGAAAGCGTT
86	AGTACCAGGA
87	ATAGCAAAGC
88	GTTGATCATG
89	AGGCTGTCTA
90	GTGACCTACT
91	GCGTATTGGG
92	AAGGGATTAC

Table 35 SureSelect^{XT} Barcodes 1 to 96 (continued)

Barcode Number	**Sequence**
93	GTTACGATGC
94	ATGGGTGTTT
95	GAGTCCGGCA
96	AATCGAAGAG

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC1	BC9	BC17	BC25	BC33	BC41	BC49	BC57	BC65	BC73	BC81	BC89
B	BC2	BC10	BC18	BC26	BC34	BC42	BC50	BC58	BC66	BC74	BC82	BC90
C	BC3	BC11	BC19	BC27	BC35	BC43	BC51	BC59	BC67	BC75	BC83	BC91
D	BC4	BC12	BC20	BC28	BC36	BC44	BC52	BC60	BC68	BC76	BC84	BC92
E	BC5	BC13	BC21	BC29	BC37	BC45	BC53	BC61	BC69	BC77	BC85	BC93
F	BC6	BC14	BC22	BC30	BC38	BC46	BC54	BC62	BC70	BC78	BC86	BC94
G	BC7	BC15	BC23	BC31	BC39	BC47	BC55	BC63	BC71	BC79	BC87	BC95
H	BC8	BC16	BC24	BC32	BC40	BC48	BC56	BC64	BC72	BC80	BC88	BC96

Figure 9 SureSelect LTI5500 BC1-BC96 plate barcode orientation

Alternative Capture Equipment Combinations

Table 36 lists combinations of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape) other than those used in this protocol that have shown minimal evaporation.

Refer to this list for additional of equipment combination options for hybridization. Note that minimal evaporation is needed to ensure good capture results.

Table 36 Tested options that show minimal evaporation

PCR Machine	Plate/Strips	Cover	Comments
Agilent Mx3005P QPCR	Mx3000P Strip Tubes (401428)	MX3000P Optical Strip Caps (401425)	Heated Lid
Agilent Mx3005P QPCR	MicroAmp Optical 96-well reaction plate (N801-0560)	MicroAmp Clear Adhesive Film (4306311)	Heated Lid; ABI compression pad (4312639) Use two layers of film.
ABI GeneAmp 9700	MicroAmp Optical 96-well Reaction Plate (N801-0560)	MicroAmp Caps (8caps/strip) (N801-0535)	Heated Lid
ABI Veriti (4375786)	MicroAmp Optical 96-well Reaction Plate (N801-0560)	MicroAmp Clear Adhesive Film (4306311)	Heated Lid; ABI compression pad (4312639) Use two layers of film.
Eppendorf Mastercycler	Eppendorf 8-Tube PCR Tubes	Attached lids	Lid heating set to 75°C
BioRad (MJ Research) PTC-200	Agilent strip tubes 410022 (Mx4000)	Agilent Optical cap 410024 (Mx4000)	Heated Lid
BioRad (MJ Research) PTC-200	Agilent strip tubes 410022 (Mx4000)	Agilent Optical cap 401425 (Mx3000/3005)	Heated Lid
BioRad (MJ Research) PTC-200	Agilent 96-well Plate 410088 (Mx3000/3005)	Agilent Optical cap 401425 (Mx3000/3005)	Heated Lid

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In This Book

This guide contains information to run the SureSelect^{XT} Target Enrichment System for SOLiD 5500 Multiplexed Sequencing protocol with the SureSelect^{XT} Target Enrichment Kits for AB SOLiD 5500 Multiplexed Sequencing.

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