SureSelect Target Enrichment System for Roche 454 GS FLX and GS Junior Sequencing Platforms

Protocol

Version 1.4, February 2012

SureSelect platform manufactured with Agilent SurePrint Technology

Research Use Only. Not for use in Diagnostic Procedures.

Agilent Technologies

Notices

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Acknowledgement

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CAUTION

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In this Guide...

This guide describes the recommended operational procedures to use the Agilent SureSelect Target Enrichment Kits with Roche 454 Rapid Libraries on the Roche 454 GS FLX and GS Junior Systems using Titanium chemistry. This protocol is specifically developed and optimized to use biotinylated RNA oligomer libraries to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus.

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 sample for target enrichment.

3 Hybridization

This chapter describes the steps to prepare and hybridize samples.

4 Post-Hybridization Amplification

This chapter describes the steps to amplify, purify, and assess quality of the sample library.

5 Reference

This chapter contains information on alternative equipment that can be used with this protocol.

What's New in 1.4

- New product configuration and product numbers for SureSelect reagent kits and capture libraries, up to 24 Mb.
- Support for the optional use of the Agilent 2200 TapeStation for DNA quantitation and qualification.
- Support for SureSelect Human All Exon v4 and All Exon v4+UTRs capture libraries.

What's New in 1.3

- Corrections to volume in "Step 2. Purify the sample with the QIAGEN MinElute PCR Purification Kit."
- Corrections to "Step 9. Purify the sample with the Agencourt AMPure XP beads."

What's New in 1.2

- Support for SureSelect Human Kinome Plus kits.

What's New in 1.1

- Support for SureSelect Mouse All Exon Kits.

Content

1 Before You Begin

Make sure you have the most current protocol. Go to the SureSelect Related Literature page on genomics.agilent.com and search for manual number G3360-90003.

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

- Prolonged exposure of SureSelect Elution Buffer to air can decrease product performance by altering the pH of the solution. Keep the Elution Buffer container tightly sealed when not in use.
- 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
DNA 7500 Kit	Agilent p/n 5067-1506
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Herculase II Fusion DNA Polymerase (includes dNTP mix and 5x Buffer) 200 reactions 400 reactions	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 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
MinElute PCR Purification Kit (250)	Qiagen p/n 28006
GS FLX Titanium Rapid Library Preparation Kit	Roche p/n 05608228001
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Distilled water	

Table 2 SureSelect Reagent Kit

Reagent Kits	16 Reactions	96 Reactions	480 Reactions
SureSelect TE Reagent kit, RCH	G9607A	G9607B	G9607C

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} DNA Kinome	5190-4646	5190-4647	5190-4649
SureSelect^{XT} X-chromosome	5190-4651	5190-4652	5190-4653
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

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

Required Equipment

Table 5 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2938C
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent
Thermal cycler	Agilent SureCycler, Life Technologies Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Covaris S-series Single Tube Sample Preparation System, Model S2	Covaris
Covaris microTUBE with AFA fiber and snap cap	Covaris p/n 520045
Nuclease-free 0.2 mL PCR tubes, thin-walled	Eppendorf p/n 951010006 or equivalent
Microcentrifuge	Eppendorf Microcentrifuge Model 5417C or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Heat block at 37°C	

Table 6 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 7 Optional Equipment for Hybridization

Description	Vendor and part number
Tube-strip capping tool	Agilent p/n 410099
Qubit Quantitation Starter Kit	Life Technologies p/n Q32860

Table 8 Optional Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
2100 Bioanalyzer System	Agilent p/n G2938C
2200 TapeStation System	Agilent p/n G2964AA or G2965AA
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

2 Sample Preparation

This section contains instructions for prepped library production specific to the Roche 454 Rapid Library preparation. It is intended for use with Roche 454 Titanium Chemistry.

The steps in this section differ from the Roche protocol in the use of the Covaris system for gDNA shearing, and implementation of DNA library amplification after adaptor ligation.

Refer to the *Roche Rapid Library Preparation Method Manual (GS FLX Titanium Series)* for more information.

CAUTION

Use a non-absorbance-based quantitation system, such as the Qubit system, to quantify genomic DNA before library preparation.

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

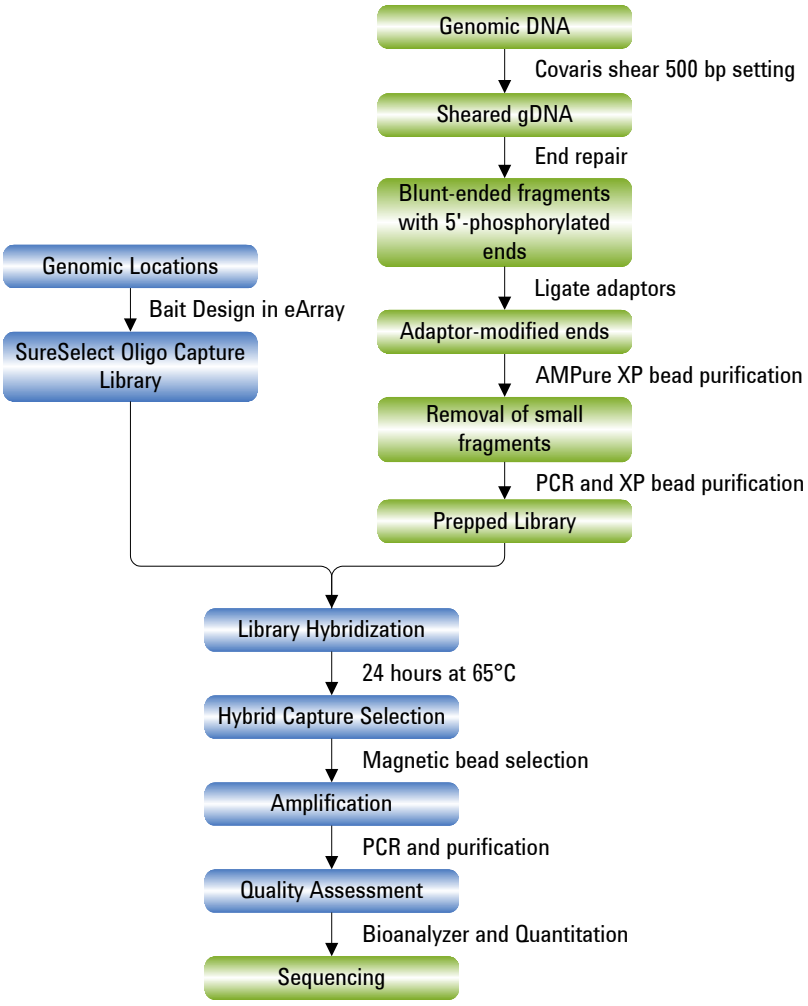

Figure 1 Overall sequencing sample preparation workflow.

Table 9 Overview and time requirements

Step	Time
Roche 454 Sequencing Library Production	1 day
Library Hybridization	24 hours (optional up to 72 hours)
Bead preparation	30 minutes
Capture Selection and Washing	2 hours
DNA purification	30 minutes
Post-Hybridization Amplification	1 hour
PCR purification	30 minutes
Bioanalyzer QC	1 hour

Step 1. Shear DNA

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

CAUTION Use a non-absorbance-based quantitation system, or the library complexity and capture efficiency can be severely compromised.

1. Use the Qubit dsDNA BR 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 500 ng 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 recommended settings in Table 10 to get a 500 bp target peak size, which results in an actual peak size of 800 bp.

Table 10 Covaris shear settings

Setting	Value
Duty Cycle	5%
Intensity	3
Cycles per Burst	200
Time	3 cycles of 30 seconds
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.

1

Step 2. Purify the sample with the QIAGEN MinElute PCR Purification Kit

1. If you haven't already done so, add the pH indicator to the Buffer PB.
2. Add 650 µL of Buffer PB per sample and mix well by pipetting.
3. Check for the yellow color to make sure buffer PB pH is correct.

   For more information on how to check buffer pH, refer to the Qiagen MinElute Handbook. If needed, use 5 µL of the 3M Sodium Acetate (included in the kit) to adjust the pH to the proper range.
4. Put a MinElute spin column in a 2 mL collection tube.
5. Transfer the 780 µL sample to the MinElute column. Spin the sample in a centrifuge for 60 seconds at 17,900g (13,000 rpm). Discard the flow-through.
6. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900g (13,000 rpm). Discard the flow-through.
7. Put the MinElute column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900g (13,000 rpm).
8. Transfer the MinElute column to a new 1.5-mL collection tube to elute the cleaned sample.
9. Let sit for 2 minutes to completely evaporate residual ethanol.

   All traces of ethanol must be removed.
10. Add 17 µL of buffer EB (10 mM Tris·Cl, pH 8.5) directly onto the MinElute filter membrane.
11. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900g (13,000 rpm).
12. Collect the eluate and transfer 17 µL to PCR tubes.

Step 3. Assess quality with the 2100 Bioanalyzer

NOTE As an alternative, you can use the D1K ScreenTape (Agilent p/n 5067-5361) and D1K Reagents (Agilent p/n 5067-5362). For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

Use a Bioanalyzer High Sensitivity DNA chip and reagent kit. See 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), 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.

   Check that the electropherogram shows a distribution with a peak height of 800 bp (±100 bp) as shown in Figure 2.

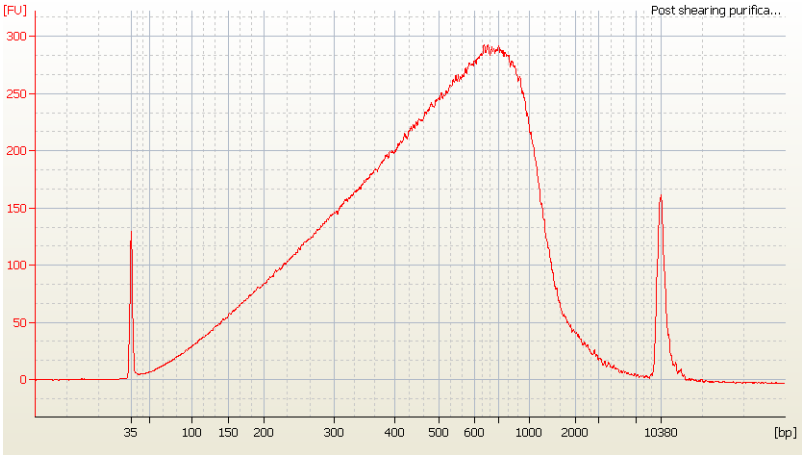

Figure 2 Analysis of sheared DNA using a Bioanalyzer High Sensitivity DNA assay.

Step 4. Repair the ends

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 12 samples (including excess) are shown in each table as an example.

Use the GS FLX Titanium Rapid Library Preparation Kit (Roche p/n 05608228001).

Prepare the master mix on ice.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 11. Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 11.
   b Add 9 µL of the reaction mix to each well or tube.
   c Add 16 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 11 Fragment End Repair Mix*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	16 µL	
RL ATP	2.5 µL	31.25 µL
RL 10 x Buffer	2.5 µL	31.25 µL
RL dNTP Mix	1 µL	12.5 µL
RL T4 Polymerase	1 µL	12.5 µL
RL PNK	1 µL	12.5 µL
RL Taq DNA Polymerase	1 µL	12.5 µL
Total Volume	**25 µL**	**112.5 µL (9 µL/reaction)**

* These reagents are included in the GS FLX Titanium Rapid Library Preparation Kit (Roche p/n 05608228001).

3 Run the End Repair program (Table 12) on a thermal cycler with a heated lid:

Table 12 End Repair program

Step	Temperature	Time
Step 1	25°C	20 minutes
Step 2	72°C	20 minutes
Step 3	4°C	Hold

Step 5. Prepare the Agencourt AMPure XP beads

The Sizing Solution and TE Buffer are part of the Roche 454 Rapid Library Preparation Kit.

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 125 µL of homogenous AMPure XP beads to a 200-µL PCR tube.
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. Add 125 µL of Sizing Solution to the beads. Mix on a vortex mixer for 5 seconds, then spin briefly on a centrifuge.
7. Keep the tube on ice until the ligation step is completed.

Step 6. Ligate the adaptor

1. Add 1 µL of the RL to the reaction tube.
2. Add 1 µL of the RL Ligase to the reaction tube.
3. Mix on a vortex mixer for 5 seconds, then spin briefly in a centrifuge.
4. Incubate for 10 minutes at 25°C on a thermal cycler. Do not use a heated lid.

Step 7. Remove small fragments with AMPure XP beads

1. Add the sample to the AMPure beads that you prepared in "Step 5. Prepare the Agencourt AMPure XP beads" on page 25.
2. Incubate at room temperature for 5 minutes.
3. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
4. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
5. Add 25 µL of TE Buffer. Mix on a vortex mixer for 5 seconds.
6. Add 125 µL of Sizing Solution. Mix on a vortex mixer for 5 seconds.
7. Incubate at room temperature for 5 minutes.
8. Put the 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 discard the cleared solution from the tubes.
10. Repeat step 5 through step 9 once.
11. Continue to keep the tube on the magnetic stand while you dispense 175 µL of 70% ethanol in each tube.

    Use fresh 70% ethanol for optimal result.
12. Let the tube sit for 1 minute to let any disturbed beads settle, and remove the ethanol.
13. Repeat step 11 through step 12 once.
14. Dry the samples on a 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.
15. Add 28 µL of water. Mix on a vortex mixer for 5 seconds and spin briefly.
16. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
17. Remove 25 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 8. Amplify adaptor-ligated library

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

1 For 1 library:
   - In a PCR 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 well on a vortex mixer.
   - **b** Add 25 µL of the reaction mix to each well or tube.
   - **c** Add 25 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 13 Components for PCR mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Adaptor-ligated library	25 µL	
Nuclease-free water	12.5 µL	156.25 µL
SureSelect RCH PCR Primer (clear cap)*	1.0 µL	12.5 µL
5X 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**	**312.5 µL (25 µL/reaction)**

* Included in the SureSelect Target Enrichment Kit RCH Hyb Module Box #2.

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

3 Run the program in Table 14 in a thermal cycler.

Table 14

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	20 seconds
Step 3	60°C	45 seconds
Step 4	72°C	1 minute and 30 seconds
Step 5		Repeat Step 2 through Step 4 for a total of 12 to 14 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

NOTE

Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, 12 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.

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

The Sizing Solution and TE Buffer are part of the GS FLX Titanium Rapid Library Preparation Kit (Roche p/n 05608228001).

1. Prepare a new batch of Agencourt AMPure XP beads and let it 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 125 µL of homogenous AMPure XP beads to a 200-µL PCR tube.
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. Add 125 µL of Sizing Solution to the beads. Mix on a vortex mixer for 5 seconds, then spin briefly on a centrifuge.
7. Add the amplified library to the AMPure beads.
8. Incubate at room temperature for 5 minutes.
9. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
10. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
11. Add 25 µL of TE Buffer. Mix on a vortex mixer for 5 seconds.
12. Add 125 µL of Sizing Solution. Mix on a vortex mixer for 5 seconds.
13. Incubate at room temperature for 5 minutes.
14. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
15. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
16. Repeat step 11 through step 15 once.
17. Continue to keep the tube on the magnetic stand while you dispense 175 µL of 70% ethanol in each tube.

    Use fresh 70% ethanol for optimal result.

18 Let the tube sit for 1 minute to let any disturbed beads settle, and remove the ethanol.

19 Repeat step 17 through step 18 once.

20 Dry the samples on a 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.

21 Add 28 µL of water. Mix on a vortex mixer for 5 seconds and spin briefly.

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

23 Remove 25 µL of the supernatant to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Make sure no beads remain in the sample.

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

Step 10. Assess quality and quantity with 2100 Bioanalyzer

NOTE As an alternative, you can use the D1K ScreenTape (Agilent p/n 5067-5361) and D1K Reagents (Agilent p/n 5067-5362). For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

Use the Bioanalyzer DNA 7500 chip and reagent kit to assess the quantity, quality and size distribution of the PCR products.

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), 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. Check that the electropherogram shows a distribution with a peak size between 600 to 800 bp. Measure the concentration of the library by integrating under the peak.

NOTE A minimum of 500 ng of library is required for hybridization.

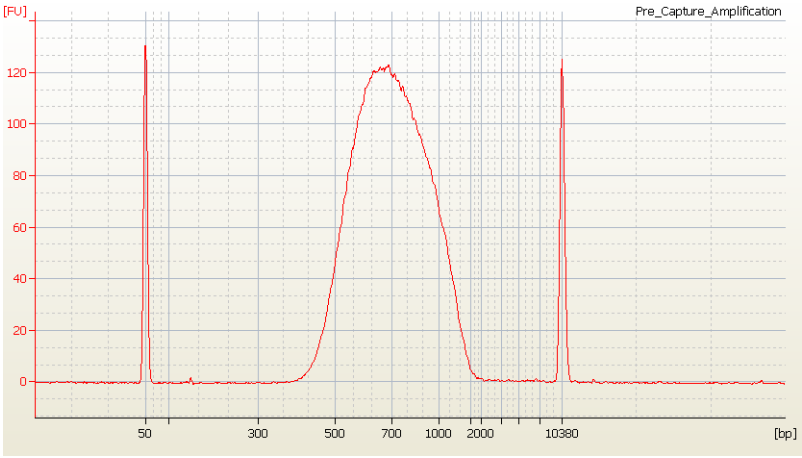

Figure 3 Analysis of amplified prepped library DNA using a Bioanalyzer DNA 7500 assay. The electropherogram shows fragment sizes that range between 400 bp and 2 kb, with a peak height of approximately 700 bp ± 100 bp.

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.

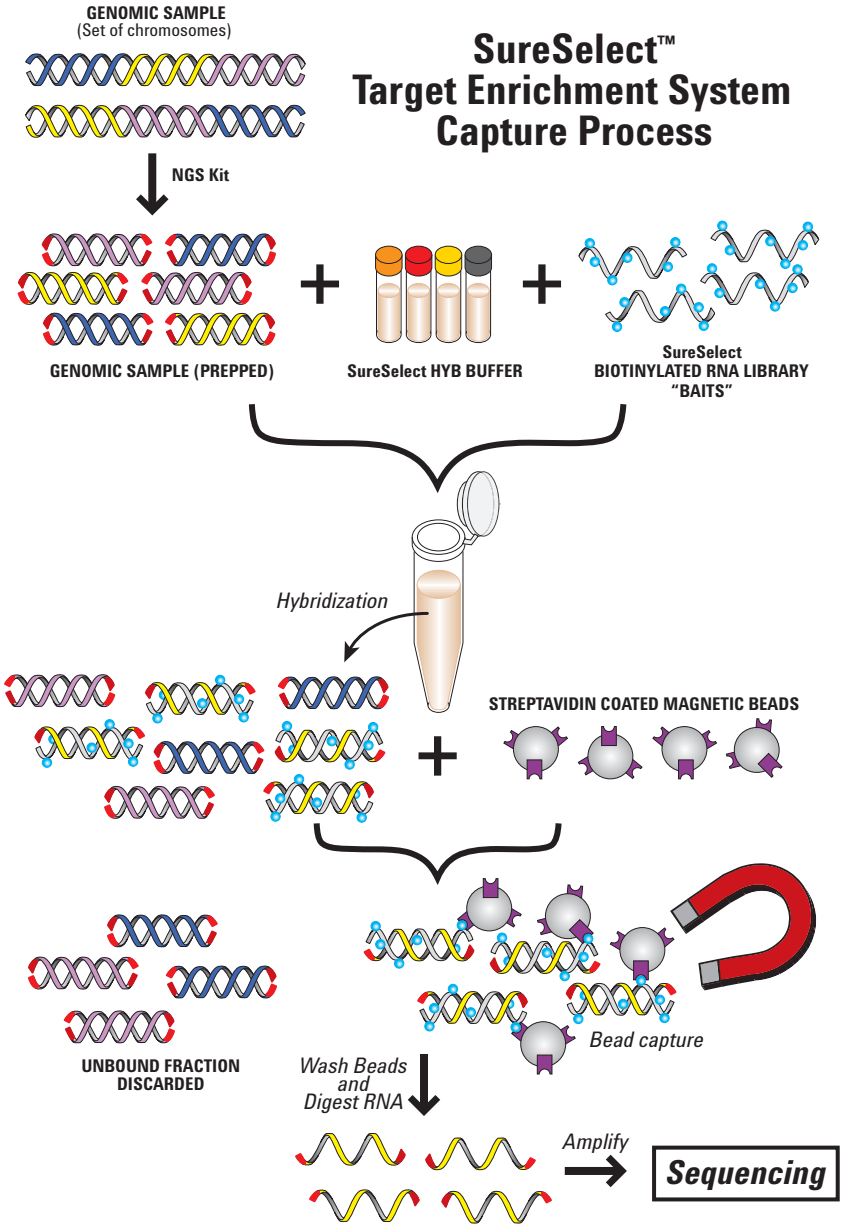

Figure 4 SureSelect Target Enrichment System Capture Process

Refer to "SureSelect Reagent Kit Content" on page 54 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 27 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 58.

Step 1. Hybridize the library

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

1. Add 500 ng of the amplified library to a 1.5-mL LoBind tube.
2. Mix the contents in Table 15 to make the correct amount of SureSelect Block mix for the number of samples used.

Table 15 SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
SureSelect RCH Block #1 (green cap)	10 µL	125 µL
SureSelect Block #2 (blue cap)	2.5 µL	31.25 µL
SureSelect RCH Block #3 (brown cap)	1.8 µL	22.5 µL
Total	**14.3 µL**	**178.75 µL**

3. Add 14.3 µL of Blocker Mix to the amplified library.
4. Dry sample down to 9 µL at 45°C in a vacuum concentrator. To process multiple libraries of varying concentration, add water to equalize the total volumes of all samples so that sample drying time variance is minimized.
5. Mix the components in Table 16 at room temperature to prepare the hybridization buffer.

Table 16 Hybridization Buffer

Reagent	Volume for 1 capture (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	125	250
SureSelect Hyb #2 (red cap)	1	5	10
SureSelect Hyb #3 (yellow cap)	10	50	100
SureSelect Hyb #4 (black cap, or bottle)	13	65	130
Total	**49 (40 µL needed)**	**245 (40 µL/sample)**	**490 (40 µL/sample)**

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

7 In a PCR plate, strip tubes, or tubes, prepare the SureSelect capture library mix for target enrichment:

- **a** Keep tubes on ice until step 11.
- **b** For each sample, add the amount of SureSelect capture library as listed in Table 17, 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 17.

  Prepare enough RNase Block dilution for all samples, plus excess.
- **d** Add the amount of diluted SureSelect RNase Block (purple cap) listed in Table 17 to each capture library, and mix by pipetting.

Table 17 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	1:2	5 µL
≥ 3.0 Mb	5 µL	1:9	2 µL

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

a Add 9 µL of amplified library and blocker mix to the "B" row in the PCR plate. Put each sample into a separate well.

b Seal the wells of row "B" 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.

c Start the thermal cycler program in Table 18.

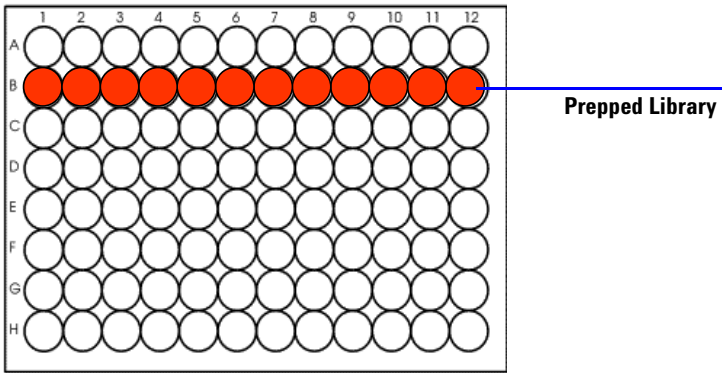

Figure 5 Prepped library shown in red

Table 18 PCR program

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

9 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.

10 Maintain the plate at 65°C while you load 40 µL of hybridization buffer per well into the "A" row of the PCR plate. The number of wells filled in Row A is the number of libraries prepared.

The example in Figure 6 is for 12 captures.

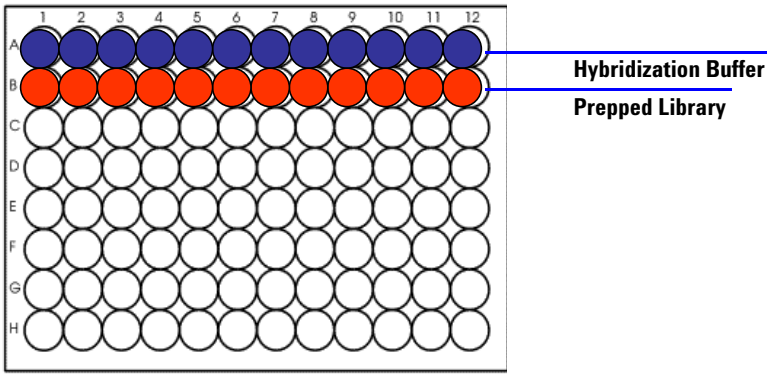

Figure 6 Hybridization buffer shown in blue

Make sure that the plate is at 65°C for a minimum of 5 minutes before you go to step 11.

11 Add the capture library mix from step 7 to the PCR plate:

a Add the capture library mix (7 µL) to the "C" row in the PCR plate.

For multiple samples, use a multi-channel pipette to load the capture library mix into the "C" row in the PCR plate.

Keep the plate at 65°C during this time.

b Seal the wells with strip caps. Use a capping tool to make sure the fit is tight.

c Incubate the samples at 65°C for 2 minutes.

12 Maintain the plate at 65°C while you use a multi-channel pipette to take 13 µL of Hybridization Buffer from the "A" row and add it to the SureSelect capture library mix contained in row "C" of the PCR plate for each sample. (See Figure 7.)

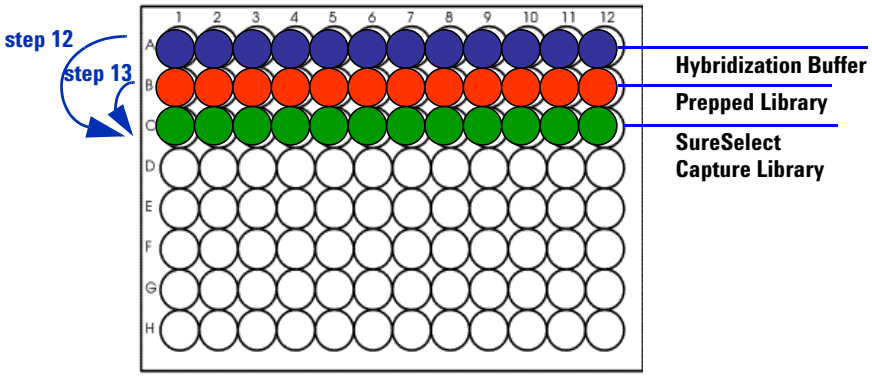

Figure 7 SureSelect Capture Library, or "Baits", shown in Green

13 Maintain the plate at 65°C while you use a multi-channel pipette to transfer the entire contents of each prepped library mix in row "B" to the hybridization solution in row "C". (See Figure 7.) Mix well by slowly pipetting up and down 8 to 10 times.

The hybridization mixture is now 27 to 29 µL, depending on degree of evaporation during the preincubations.

14 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 previous incubation steps.

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

If you use strip tubes, test for evaporation before you do the first experiment to make sure the tube/capping method is appropriate for the thermal cycler. Check that no more than 3 to 4 µL is lost to evaporation.

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

Step 2. Prepare magnetic beads

Use these reagents from the SureSelect Target Enrichment Kit Box #1:

- SureSelect Binding Buffer
- SureSelect Wash 2

1. Prewarm SureSelect Wash 2 at 65°C in a circulating water bath for use in "Step 3. 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 microfuge 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. Hybrid capture with SureSelect

Use these reagents from the SureSelect Target Enrichment Kit Box #1:

- SureSelect Wash 1
- SureSelect Wash 2
- SureSelect Elution Buffer
- SureSelect Neutralization Buffer

CAUTION Keep the Elution Buffer container tightly sealed when not in use. Prolonged exposure of SureSelect Elution Buffer to air can decrease product performance by altering the pH of the solution.

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 29 on page 58 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** Briefly spin in a centrifuge.
- **d** Separate the beads and buffer on a magnetic separator and remove the supernatant.
- **e** Repeat step a through step d for a total of 3 washes.

  Make sure all of the wash buffer has been removed.

11 Mix the beads in 50 µL of SureSelect Elution Buffer on a vortex mixer for 5 seconds to resuspend the beads.

12 Occasionally mix on a vortex mixer.

13 Briefly spin in a centrifuge.

14 Separate the beads and buffer on a magnetic separator.

15 Use a pipette to transfer the supernatant to a new 1.5-mL microfuge tube.

The supernatant contains the captured DNA. The beads can now be discarded.

16 Add 50 µL of SureSelect Neutralization Buffer to the captured DNA.

17 Briefly mix on a vortex mixer.

Step 4. 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 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add 100 µL of captured DNA library. 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 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 33 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.

4 Post-Hybridization Amplification

This chapter describes the steps to amplify, purify, assess quality of the library, and dilute the sample appropriately for cluster amplification.

Step 1. Amplify the captured library

Use reagents from:

- Herculase II Fusion DNA Polymerase (Agilent)
- SureSelect Target Enrichment Kit RCH Hyb Module Box #2

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.

1 For 1 library:
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 19, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   a Prepare the reaction mix in Table 19, on ice. Mix well 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 to avoid cross-contamination.

Table 19 Herculase II Master Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Captured DNA	15 µL	
Nuclease-free water	22.5 µL	281.25 µL
5X 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 RCH PCR Primer (clear cap)†	1 µL	12.5 µL
Total	**50 µL**	**437.5 µL (35 µL/reaction)**

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

† Included in the SureSelect Target Enrichment Kit RCH Hyb Module Box #2

3 Put the tubes in a thermal cycler and run the program in Table 20.

Table 20 PCR program

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	20 seconds
Step 3	60°C	45 seconds
Step 4	72°C	1 minute and 30 seconds
Step 5		• Repeat Step 2 through Step 4 for a total of 12 to 14 times.
Step 6	72°C	5 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 90 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 33 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 3. Assess quality and quantity with the 2100 Bioanalyzer High Sensitivity DNA assay

NOTE As an alternative, you can use the High Sensitivity D1K ScreenTape (Agilent p/n 5067-5363) and High Sensitivity D1K Reagents (Agilent p/n 5067-5364). For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

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

   Determine the concentration of the sample by integration under the peak.

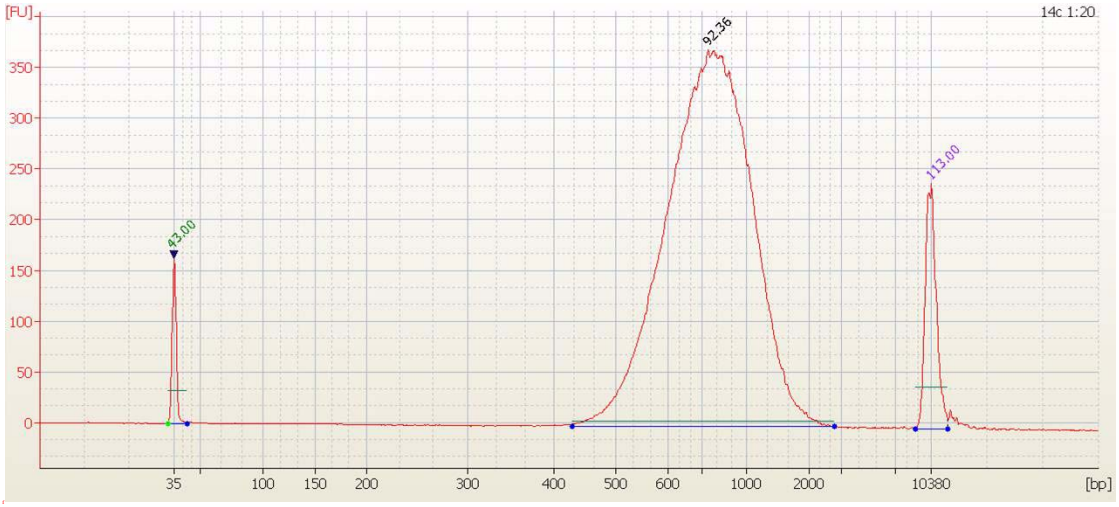

Figure 8 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows fragment sizes ranging from 400 bp to 2 kb and a peak height of approximately 700 bp ± 100 bp.

Step 4. Perform emPCR and sequencing with Roche 454 Titanium chemistry

- Run samples on a GS FLX or GS Junior System. Use the instrument that is suited to the size of the capture library and level of coverage needed.

  See Table 21.

Table 21

Capture Library Size (Mb)	Instrument	Run Type
≥ 3	GS FLX	Full
1.5 up to 2.9	GS FLX	2-pad
0.5 up to 1.4	GS FLX	4-pad
≤ 0.4	GS Junior	Full

5 Reference

This chapter contains reference information.

SureSelect Reagent Kit Content

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

Table 22 SureSelect Reagent Kit Contents

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect Target Enrichment Kit Box #1	Room Temperature	5190-4393	5190-4394	5190-4395
SureSelect Target Enrichment Kit RCH Hyb Module Box #2	-20°C	5190-4449	5190-4450	5190-4451

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

Table 23 SureSelect Target Enrichment Kit Box #1

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
SureSelect Elution Buffer
SureSelect Neutralization Buffer

Table 24 SureSelect Target Enrichment Kit RCH Hyb Module Box #2

Kit Component
SureSelect Hyb #3 (yellow cap)
SureSelect RCH Block #1 (green cap)
SureSelect Block #2 (blue cap)
SureSelect RCH Block #3 (brown cap)
SureSelect RNase Block (purple cap)
SureSelect RCH PCR Primer (clear cap)

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 25 Herculase II Fusion DNA Polymerase (Agilent)

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

Table 26 D1K Reagents (Agilent p/n 5067-5362)

Components
D1K ladder
D1K sample buffer

Table 27 High Sensitivity D1K Reagents (Agilent p/n 5067-5364)

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

Table 28 GS FLX Titanium Rapid Library Preparation Kit (Roche p/n 05608228001)

Components
TE Buffer
Sizing Solution
RL 10 x Buffer
RL dNTP Mix
RL T4 Polymerase
RL ATP
RL PNK
RL *Taq* DNA Polymerase

Alternative Capture Equipment Combinations

Table 29 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 29 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 Target Enrichment System for Roche 454 GS FLX and GS Junior Sequencing Platforms protocol with the Roche 454 Rapid Libraries with Titanium Sequencing Chemistry.

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