SureSelect Target Enrichment System

Illumina Paired-End Sequencing Platform Library Prep

Protocol

Version 1.0, September 2009

SureSelect platform manufactured with Agilent SurePrint Technology

Research Use Only. Not for use in Diagnostic Procedures.

Agilent Technologies

Notices

Manual Part Number

G3360-90000

Edition

Version 1.0, September 2009

Printed in USA

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

Acknowledgement

Technical Support

Technical product support may be obtained by contacting your local Agilent Support Services representative. Agilent's worldwide sales and support center telephone numbers can be obtained at the following Web site:
www.agilent.com/chem/contactus

or send an email to:
SureSelect.Support@agilent.com

Notice to Purchaser

Research Use Only. Not for use in diagnostic procedures.

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This guide describes Agilent's recommended operational procedures to capture genomic regions of interest using Agilent's SureSelect Paired-End Target Enrichment System Kit and sample preparation kits for next-generation 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.

This guide uses the Illumina paired-end sequencing platform for library preparation.

If you have comments about this protocol, send an e-mail to feedback_genomics@agilent.com.

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.

Content

1 Before You Begin

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 Target Enrichment Kit 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
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
Nuclease-free Water (not DEPC-treated)	Ambion, Cat # AM9930
Illumina Paired-End Genomic DNA Sample Prep Kit	Illumina, Cat # PE-102-1001
Sybr-Gold	Invitrogen p/n S-11494
Trackit Cyan/Orange Loading Dye (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose)	Invitrogen p/n 10482-028
Trackit 50 BP DNA ladder	Invitrogen p/n 10488-043
NuSeive GTG Agarose	Lonza p/n 50080
QIAquick PCR Purification Kit	Qiagen, Cat # 28104
QIAquick or MinElute Gel Extraction Kit	Qiagen p/n 28704 or Qiagen p/n 28604
MinElute PCR Purification Kit	Qiagen, Cat # 28004
Isopropanol	Sigma-Aldrich p/n I9516
Herculase II Fusion DNA Polymerase	Stratagene p/n 600677
50x TAE buffer	
Distilled water	
100% Ethanol, molecular biology grade	
Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	

Table 2 Required Reagents for Hybridization

Description	Vendor and part number
SureSelect Target Enrichment System, Paired-End	
10 reactions	Agilent p/n G3360A*
25 reactions	Agilent p/n G3360B*
50 reactions	Agilent p/n G3360C*
100 reactions	Agilent p/n G3360D*
250 reactions	Agilent p/n G3360E*
500 reactions	Agilent p/n G3360F*
1000 reactions	Agilent p/n G3360G*
2000 reactions	Agilent p/n G3360H*
5000 reactions	Agilent p/n G3360J*
Nuclease-free Water (not DEPC-treated)	Ambion, Cat # AM9930
Dynabeads® M-280 Streptavidin	Invitrogen, Cat # 112-05D
MinElute PCR Purification Kit (50)	Qiagen p/n 28004
MinElute PCR Purification Kit (250)	Qiagen p/n 28006

* Use option 001 for Illumina Genome Analyzer sequencer and option 011 for the paired-end sequencing protocol.

Optional Reagents

Table 3 Optional Reagents

Description	Vendor and part number
SureSelect Target Enrichment, Demo	Agilent p/n G4459A*

* Use option 001 for Illumina Genome Analyzer sequencer and option 011 for the paired-end sequencing protocol.

Required Equipment

Table 4 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
Agilent 2100 Bioanalyzer	Agilent p/n G2938C
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent
Dark Reader transilluminator	Clare Chemical Research, Inc. p/n DR45M
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
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Thermal cycler	BioRad (MJ Research) DNA Engine PTC-200 or Applied Biosystems Veriti Thermal Cycler
Disposable scalpels or razor blades	
Electrophoresis unit	
Electrophoresis power supply	
Gel trays and tank	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Vacuum concentrator	

Table 5 Required Equipment for Hybridization

Description	Vendor and part number
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425
BD Clay Adams Nutator Mixer	BD Diagnostics p/n 421105 or equivalent
Dynal DynaMag-2	Invitrogen 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	
Timer	
Vortex mixer	
Water bath	

Optional Equipment

Table 6 Optional Equipment for Hybridization

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

Table 7 Optional Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
Agilent 2100 Bioanalyzer	Agilent p/n G2938C

2 Sample Preparation

This section contains instructions for prepped library production specific to the Illumina paired-read sequencing platform. It is intended for use with the Illumina prep kit (p/n PE-102-1001). The steps in this section differ from the Illumina protocol in the shear size, the use of the Covaris sample preparation system for gDNA shearing, and the gel purification. Other methods of gDNA shearing have not been validated.

Refer to the Illumina protocol *Preparing Samples for Paired-End Sequencing* (p/n 1005063 Rev. A) for more information.

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

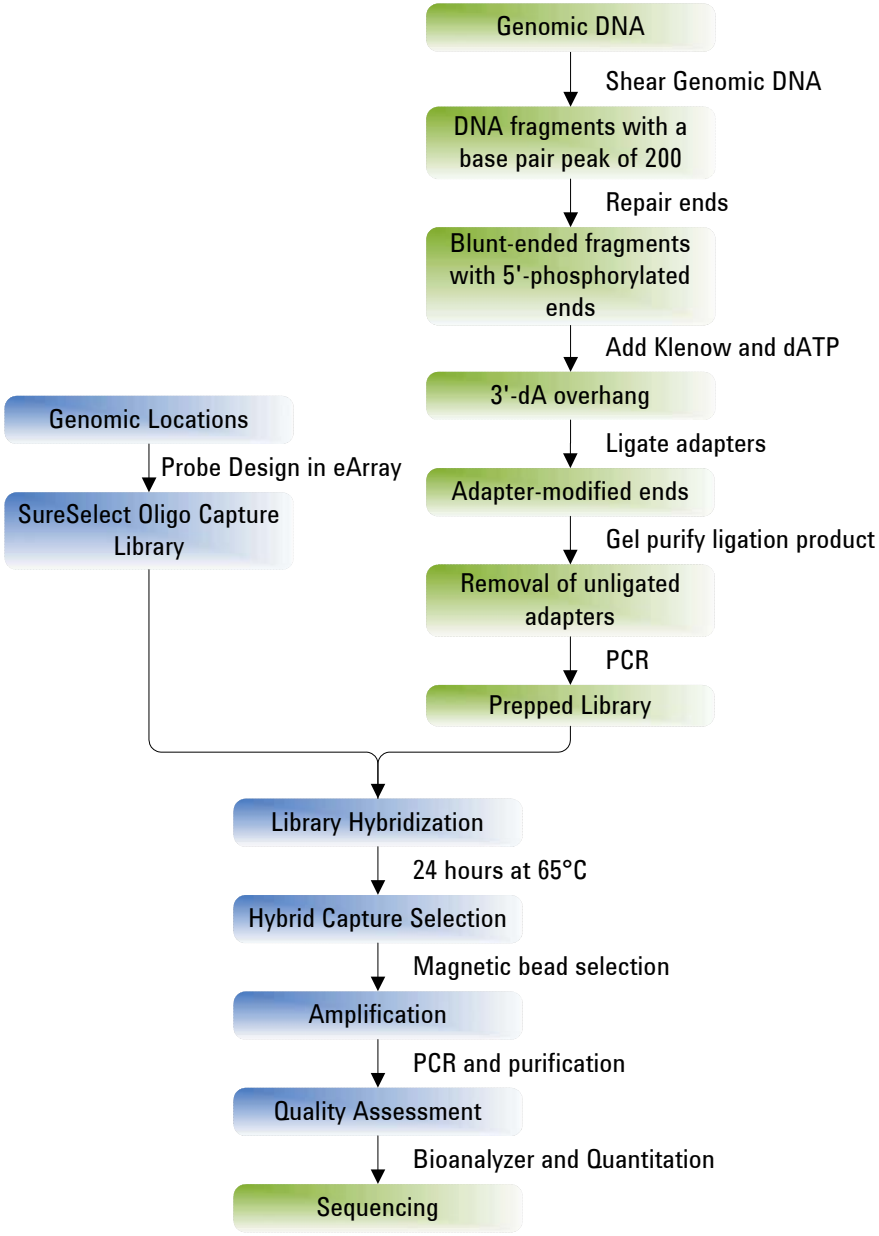

Figure 1 Overall sequencing sample preparation workflow.

Table 8 Overview and time requirements

Step	Time
Illumina Prepped library Production	2 days
Library Hybridization	25 hours (optional 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

1. For every 3 µg DNA, add enough 1X Low TE Buffer in a LoBind tube for a total volume of 100 µL to dilute the genomic DNA.
2. Put a Covaris microTube into the loading station.

   Keep the cap on the tube.
3. Set up the Covaris system.

   Refer to the Covaris instrument user guide.
4. Use a tapered pipette tip to slowly transfer the 100 µL DNA sample through the pre-split septa.

   Be careful not to introduce a bubble into the bottom of the tube.
5. Shear the DNA with the settings in Table 9. The target peak for base pair size is 200.

Table 9 Covaris shear settings

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	180 seconds
Set Mode	Frequency sweeping
Temperature	4°C

Step 2. Purify the sample with the QIAquick PCR Purification Kit

1. If you haven't already done so, add the pH indicator to the Buffer PB.
2. Add 500 µL of 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 QIAquick spin column in a 2 mL collection tube.
5. Transfer the 600 µL sample to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (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 17,900 x g (13,000 rpm). Discard the flow-through.
7. Put the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Transfer the QIAquick 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 30 µL of buffer EB (10 mM Tris·Cl, pH 8.5) directly onto the QIAquick filter membrane.
11. Wait 60 seconds, then centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
12. Collect the eluate.

Step 3. Assess quality with Agilent 2100 Bioanalyzer

Use a Bioanalyzer DNA 1000 chip and reagent kit.

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.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
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.

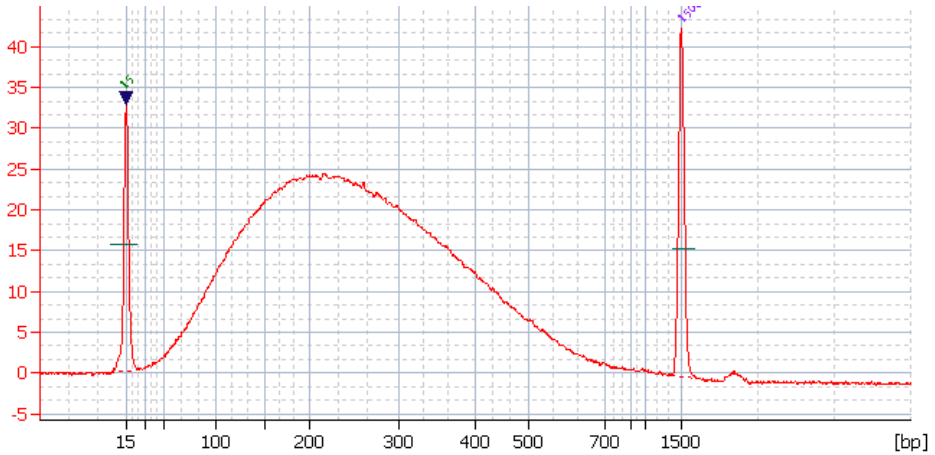

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows a distribution with a peak size of 200 nucleotides.

Step 4. Repair the ends

1 If T4 DNA ligase buffer with 10 mM ATP shows a visible precipitate after thawing, warm up to 37°C for 5 minutes and thoroughly mix on a vortex mixer.

2 In PCR tubes, strip tubes, or plates, prepare the reaction mix in Table 10 for each prepped library, on ice. Mix well by gently pipetting up and down.

Table 10 End Repair

Reagent	Volume for 1 Library
DNA sample	29 µL
Nuclease-free water	46 µL
T4 DNA ligase buffer with 10mM ATP	10 µL
dNTP mix	4 µL
T4 DNA polymerase	5 µL
Klenow enzyme	1 µL
T4 PNK	5 µL
Total Volume	100 µL

3 Incubate in a thermal cycler for 30 minutes at 20°C. Do not use a heated lid.

Step 5. Purify the repaired DNA with the QIAquick PCR Purification Kit

1. If you haven't already done so, add the pH indicator to the Buffer PB.
2. Add 500 µL of 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 QIAquick spin column in a 2 mL collection tube.
5. Transfer the 600 µL sample to the QIAquick column Spin the sample in a centrifuge for 60 seconds at 17,900 x g (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 17,900 x g (13,000 rpm). Discard the flow-through.
7. Put the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Transfer the QIAquick 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 32 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
11. Collect the eluate.

Step 6. Add 'A' Bases to the 3' end of the DNA fragments

1 In PCR tubes, strip tubes, or plates, prepare the reaction mix in Table 11 for each prepped library, on ice. Mix well by gently pipetting up and down.

Table 11 Adding "A" Bases

Reagent	Volume for 1 Library
DNA sample	32 µL
Klenow buffer	5 µL
dATP	10 µL
Klenow exo (3′ to 5′ exo minus)	3 µL
Total Volume	**50 µL**

2 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 with a Qiagen MinElute PCR Purification Column

1. Allow the MinElute columns (stored at 4°C) to come to room temperature.
2. If you haven't already done so, add the pH indicator to the Buffer PB.
3. Add 250 µL of PB per sample and mix well by pipetting.
4. 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.
5. Put a MinElute spin column in a 2 mL collection tube.
6. Transfer the 300 µL sample to the MinElute column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
7. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
8. Put the MinElute column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
9. Transfer the MinElute column to a new 1.5-mL collection tube to elute the cleaned sample.
10. Let sit for 2 minutes to completely evaporate residual ethanol.

    All traces of ethanol must be removed.
11. Add 10 µL buffer EB directly onto the MinElute filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
12. Collect the eluate, which can be stored at 4°C.

Step 8. Ligate the paired-end adapter

This step uses a 10:1 molar ratio of adapter to genomic DNA insert, based on a starting quantity of 3 µg of DNA before fragmentation.

1 Prepare the reaction mix in Table 12 on ice. Mix well by gently pipetting up and down.

Table 12 Ligation master mix

Reagent	Volume for 1 Library
DNA sample	10 µL
Nuclease-free water	4 µL
DNA ligase buffer	25 µL
Paired-End Adapter oligo mix	6 µL
DNA ligase	5 µL
Total Volume	**50 µL**

2 Incubate for 15 minutes at 20°C on a thermal cycler. Do not use a heated lid.

Step 9. Purify the sample with the QIAquick PCR Purification Kit

1. If you haven't already done so, add the pH indicator to the Buffer PB.
2. Add 250 µL of 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 QIAquick spin column in a 2 mL collection tube.
5. Transfer the 300 µL sample to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (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 17,900 x g (13,000 rpm). Discard the flow-through.
7. Put the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Transfer the QIAquick 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 30 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
11. Collect the eluate.

Step 10. Select size using a gel

WARNING **Prolonged exposure to UV light can damage your DNA.**

Excise as narrow a band as possible from the gel during gel purification.

CAUTION Do not purify multiple samples on a single gel. Cross-contamination between libraries can occur.

1. Prepare a 4% NuSeive agarose gel with distilled water and TAE. Final concentration of TAE should be 1X. Use a large well comb to prevent overloading.

   Typical gel dimensions are 12 cm x 14 cm, using 100 mL gel volume.
2. Add 10 µL of Trackit 50 BP ladder to two lanes on opposite sides of the gel.
3. Add 5 µL Trackit Loading Buffer to 30 µL of the DNA from the purified ligation reaction. Mix on a vortex mixer.
4. Load the entire sample in another lane of the gel, leaving at least a gap of one empty lane between the ladders and sample.
5. Run the gel at 25 V for 17 hours.
6. Incubate the gel in SYBR Gold solution for 60 minutes.
7. View the gel on a Dark Reader transilluminator, which is a safer alternative to a UV transilluminator.
8. Put a clean ruler across the gel at the 300 bp marker. Use a fresh scalpel or razor blade to excise a 2 mm slice of the sample DNA even with the 300 bp marker.

Step 11. Purify the gel

Use a Qiagen Gel Extraction Kit (Qiagen, p/n 28704) to purify the DNA from the agarose slices.

1. Weigh the gel slice.
2. Add 6 volumes of Buffer QG to 1 volume of gel (100 mg = 100 µL).

   You may need to do this in a 15 mL conical tube or in two tubes.
3. Incubate at room temperature for 10 minutes (or until the gel slice has completely dissolved). To help dissolve gel, mix the tube in a vortex mixer every 2 to 3 minutes during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.

   For example, if the agarose gel slice is 100 mg, add 100 µL isopropanol.

   Do not spin the sample in a centrifuge at this time.
6. Put a QIAquick spin column in a provided 2 mL collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and spin in a centrifuge for 1 minute at 17,900 x g (13,000 rpm).

   The maximum volume of the column reservoir is 800 µL. For sample volumes of more than 800 µL, simply load and spin again.
8. Discard flow-through and put the QIAquick column back in the same collection tube.
9. To wash, add 750 µL of Buffer PE to QIAquick column and spin in a centrifuge for 2 minutes at 17,900 x g (13,000 rpm).
10. Discard the flow-through and spin the QIAquick column in a centrifuge for an additional 1 minute at 17,900 x g (13,000 rpm).
11. Put the QIAquick column into a clean 1.5-mL microcentrifuge tube.
12. Let sit for 2 minutes to completely evaporate residual ethanol.

    All traces of ethanol must be removed.
13. To elute DNA, add 30 µL of Buffer EB to the center of the QIAquick membrane. Wait 60 seconds, then spin the column in a centrifuge for 1 minute.

Step 12. Amplify prepped library

This step uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends, and to amplify the amount of DNA in the library. The PCR is done with two primers that anneal to the ends of the adapters. Six to eight cycles of PCR are used.

CAUTION

This protocol was optimized to minimize PCR-based bias in the library preparation. While most library preparations yield enough DNA (500 ng) for at least a single hybridization, poor quality DNA samples or other factors can affect yield.

If yield is consistently below 500 ng, you can hybridize with less library. Do not add less than 250 ng of DNA to the hybridization, or sequencing results can be affected.

To determine the number of cycles needed, do a trial amplification with 6 cycles. If you do not get enough yield, repeat with 8 cycles.

1 Prepare the PCR reaction mix in Table 13, on ice. Mix well by gently pipetting up and down.

Table 13 PCR Components

Reagent	Volume for 1 Library
DNA	23 µL
PCR primer PE 1.0	1 µL
PCR primer PE 2.0	1 µL
PCR Polymerase (p/n 1000524 from Illumina Kit)	25 µL
Total Volume	**50 µL**

2 Amplify using the following PCR program:

Table 14 PCR protocol

Step	Temperature	Time
Step 1	98°C	30 seconds
Step 2	98°C	10 seconds
Step 3	65°C	30 seconds
Step 4	72°C	30 seconds
Step 5		Repeat Step 2 through Step 4 for a total of 6 to 8 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

Step 13. Purify the sample with the QIAquick PCR Purification Kit

1. If you haven't already done so, add the pH indicator to the Buffer PB.
2. Add 250 µL of PB per sample and mix well by pipetting.
3. Put a QIAquick spin column in a 2 mL collection tub.
4. Transfer the 300 µL sample to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
5. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
6. Put the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
7. Transfer the QIAquick column to a new 1.5-mL collection tube to elute the cleaned sample.
8. Let sit for 2 minutes to completely evaporate residual ethanol.

   All traces of ethanol must be removed.
9. Add 50 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
10. Collect the eluate, which can be stored at 4°C.

Step 14. Assess quality and quantity with Agilent 2100 Bioanalyzer

Use the Bioanalyzer DNA 1000 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.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
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. Use the manual integration function to determine the library concentration under the 350 bp peak.

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

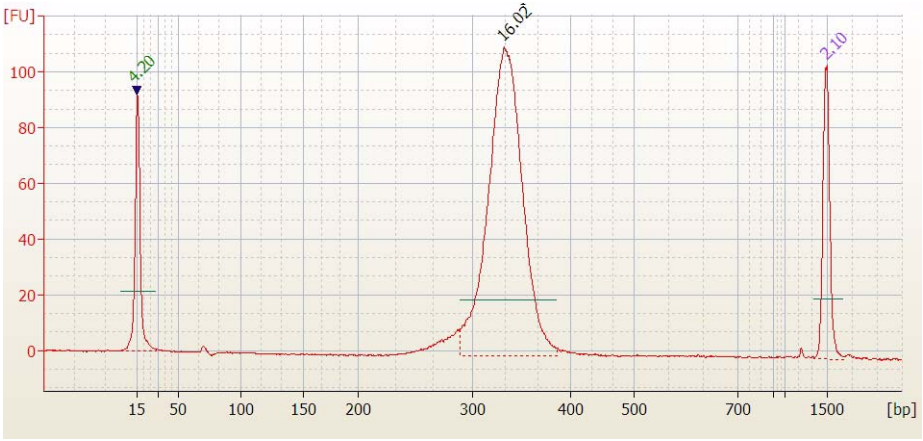

Figure 3 Analysis of amplified prepped library DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows a single peak in the size range of approximately 350 +/- 10% nucleotides.

3 Hybridization

This chapter describes the steps to combine the prepped library with the blocking agents and the SureSelect Oligo Capture Library.

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

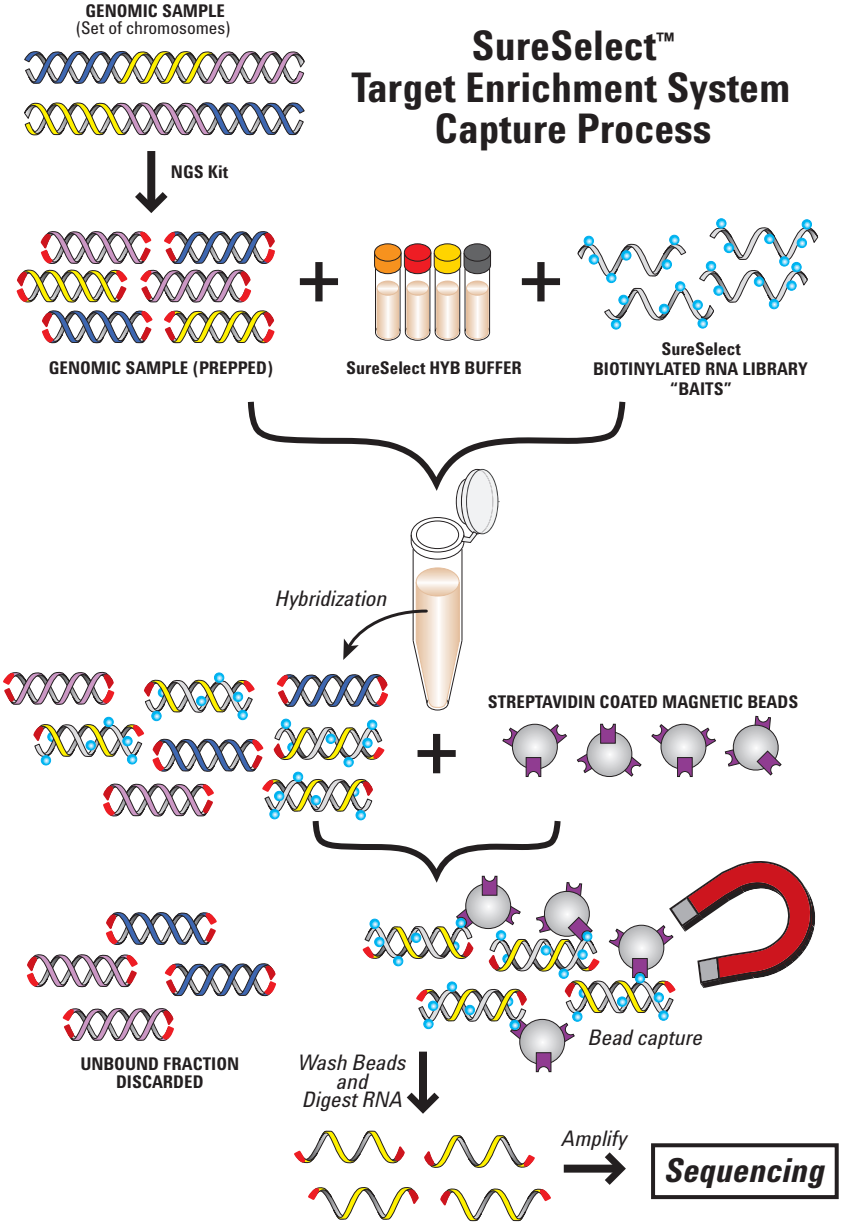

Figure 4 SureSelect Target Enrichment System Capture Process

Table 15 SureSelect Reagent Kit Components

Kit Component	250 RXN Kit	50 RXN Kit	Storage
SureSelect Hyb # 1	bottle	tube with orange cap	Room Temperature
SureSelect Hyb # 2	tube with red cap	tube with red cap	Room Temperature
SureSelect Hyb # 4	bottle	tube with black cap	Room Temperature
3M Sodium Acetate	tube with clear cap	tube with clear cap	Room Temperature
SureSelect Binding buffer	bottle	bottle	Room Temperature
SureSelect Wash Buffer #1	bottle	bottle	Room Temperature
SureSelect Wash Buffer #2	bottle	bottle	Room Temperature
SureSelect Elution Buffer	bottle	bottle	Room Temperature
SureSelect Neutralization Buffer	bottle	bottle	Room Temperature
SureSelect Hyb # 3	tube with yellow cap	tube with yellow cap	-20°C
SureSelect Block #1	tube with green cap	tube with green cap	-20°C
SureSelect Block #2	tube with blue cap	tube with blue cap	-20°C
SureSelect PE Block #3	tube with brown cap	tube with brown cap	-20°C
RNase Block	tube with purple cap	tube with purple cap	-20°C
SureSelect GA PCR Primers	tube with clear cap	tube with clear cap	-20°C

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 Buffers (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 50.

Step 1. Hybridize the library

1 If the prepped library concentration is below 147 ng/µL, use a vacuum concentrator to concentrate the sample.

   a Add the entire 50 µL 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/ul and check the concentration on an Agilent Bioanalyzer DNA 1000 chip. See "Step 14. Assess quality and quantity with Agilent 2100 Bioanalyzer" on page 30. After quantification, adjust the sample to 147 ng/µL.

3 Mix the components in Table 16 at room temperature to prepare the hybridization buffer.

Table 16 Hybridization Buffer

Reagent	Volume for 1 capture (µL)	Volume for 5 captures (µL)	Volume for 12 captures (µL)
SureSelect Hyb # 1	25	125	275
SureSelect Hyb # 2 (red cap)	1	5	11
SureSelect Hyb # 3 (yellow cap)	10	50	110
SureSelect Hyb # 4	13	65	143
Total	**49**	**245**	**539**

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

5 In a PCR plate, strip tubes, or tubes, prepare the SureSelect Oligo Capture Library Mix for target enrichment:

- **a** Add 5 µL of the SureSelect Oligo Capture Library.
- **b** Add 1 µL of nuclease-free water to the SureSelect Oligo Capture Library.
- **c** Use nuclease-free water to prepare a 1:1 dilution of the RNase Block (purple cap).
- **d** Add 1 µL of diluted RNase Block to each Capture Library, and mix by pipetting.
- **e** Keep tubes on ice until step 9.

6 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 "B" row in the PCR plate. Put each sample into a separate well.
- **b** Add 2.5 µL of SureSelect Block #1 (green cap) to row B.
- **c** Add 2.5 µL of SureSelect Block #2 (blue cap) to row B.
- **d** Add 0.6 µL of SureSelect PE Block #3 (brown cap) to row B.
- **e** Mix by pipetting.
- **f** 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.
- **g** Run the following thermal cycler program in Table 17.

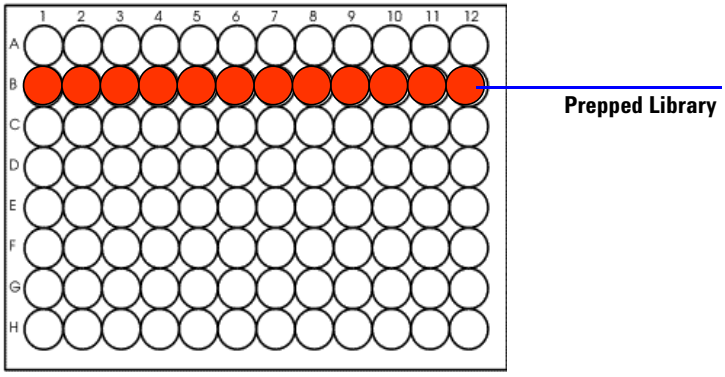

Figure 5 Prepped library shown in red

Table 17 PCR program

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

7 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.

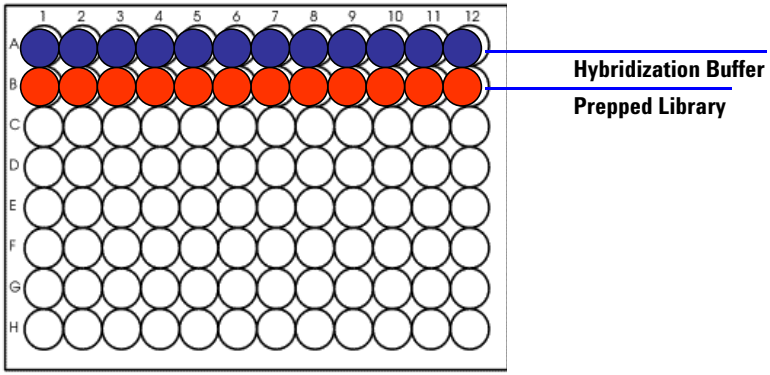

Figure 6 Hybridization buffer shown in blue

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.

Make sure that the plate is at 65°C for a minimum of 5 minutes before you go to step 9.

9 Add the Capture Library mix from step 5 to the PCR plate:

a Add the Capture Library mix 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 (see Figure 7).

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.

10 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.

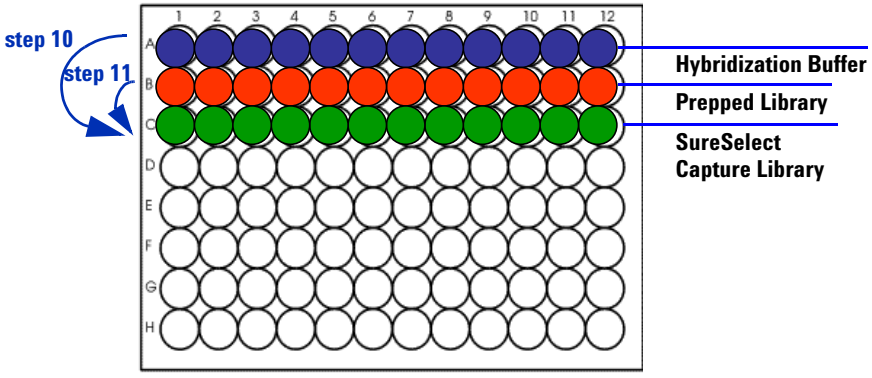

Figure 7 SureSelect Capture Library, or "Baits", shown in Green

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

12 Seal the wells with double adhesive film. Make sure all wells are completely sealed.

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.

The hybridization mixture is now 27 µL.

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

Samples may be hybridized for up to 72 hours, but when you hybridize at longer periods, check that there is no extensive evaporation.

Step 2. Prepare magnetic beads

1. Prewarm SureSelect Wash Buffer #2 at 65°C in a circulating water bath for use in "Step 3. Select hybrid capture with SureSelect".
2. Vigorously resuspend the Dynal (Invitrogen) magnetic beads on a vortex mixer. Dynal beads settle during storage.
3. For each hybridization, add 50 µL Dynal magnetic beads to a 1.5-mL microfuge tube.
4. Wash the beads:
   a. Add 200 µL 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 (Invitrogen).
   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

1. Estimate and record the volume of hybridization that remained after 24 hour incubation.
2. Add the hybridization mixture directly from the thermal cycler to the bead solution, and invert the tube to mix 3 to 5 times.
3. Incubate the hybrid-capture/bead solution on a Nutator 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 Dynal magnetic separator and remove the supernatant.
6. Resuspend the beads in 500 µL SureSelect Wash Buffer #1 by mixing on a vortex mixer for 5 seconds.
7. Incubate the samples for 15 minutes at room temperature.
8. Wash the beads:
   a. Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.
   b. Resuspend the beads in 500 µL of prewarmed SureSelect Wash Buffer #2 and mix on a vortex mixer for 5 seconds to resuspend the beads.
   c. Incubate the samples for 10 minutes at 65°C.
   d. Invert the tube to mix. The beads may have settled.
   e. Repeat step a through step d for a total of 3 washes.

   Make sure all of the wash buffer has been removed.
9. Mix the beads in 50 µL SureSelect Elution Buffer on a vortex mixer for 5 seconds to resuspend the beads.
10. Incubate the samples for 10 minutes at room temperature.
11. Separate the beads and buffer on a Dynal magnetic separator.
12. Use a pipette to transfer the supernatant to a new 1.5-mL microcentrifuge tube.

    The supernatant now contains the captured DNA. The beads can now be discarded.
13. Add 50 µL of SureSelect Neutralization Buffer to the captured DNA.

Step 4. Desalt the capture solution

In this step you desalt the capture solution with a Qiagen MinElute PCR purification column.

1. Allow the MinElute columns (stored at 4°C) to come to room temperature.
2. Add 500 µL of PB to the 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 600 µL sample to the MinElute column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g. Discard the flow-through.
6. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g. 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 17,900 x g.
8. Transfer the MinElute column to a new 1.5-mL collection tube to elute the cleaned sample.
9. Let the column sit for 2 minutes to completely evaporate residual ethanol.

   All traces of ethanol must be removed.
10. Add 15 µL buffer EB directly onto the MinElute filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
11. Collect the eluate (captured library), which can be stored at -20°C.

4 Post-Hybridization Amplification

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

Step 1. Amplify the sample

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

CAUTION This protocol was optimized to minimize PCR-based bias in the library preparation.

To determine the number of cycles needed, do a trial amplification with 12 cycles. If you do not get enough yield for Illumina sequencing, repeat with 14 cycles.

1 For each hybrid capture, prepare only 1 amplification reaction. Mix the components in Table 18 on ice. Mix well by gently pipetting up and down.

Table 18 Herculase Master Mix

Reagent	Volume for 2 reactions (including excess)	Volume for 10 reactions (including excess)	Volume for 24 reactions (including excess)
Nuclease-free water	76.25 µL	335.5 µL	762.5 µL
5X Herculase II Reaction Buffer	25 µL	110 µL	250 µL
dNTP mix (25 mM each, 100 mM total)	1.25 µL	5.5 µL	12.5 µL
SureSelect GA PCR Primers	2.5 µL	11 µL	25 µL
Herculase II Fusion DNA Polymerase	2.5 µL	11 µL	25 µL
Total	**107.5 µL**	**473 µL**	**1,075 µL**

2 Add 43 µL of the mixture into a PCR plate or tubes for each capture.

3 Add 7 µL of the Captured library to each Master Mix.

4 Put the tubes in a thermal cycler and run the program in Table 19.

Table 19 PCR program

Step	Temperature	Time
Step 1	98°C	30 seconds
Step 2	98°C	10 seconds
Step 3	57°C	30 seconds
Step 4	72°C	30 seconds
Step 5		Repeat Step 2 through Step 4 for a total of 12 to 14 times.
Step 6	72°C	7 minutes
Step 7	4°C	Hold

Step 2. Purify PCR with the QIAquick PCR Purification Kit

1. If you haven't already done so, add the pH indicator to the Buffer PB.
2. Add 250 µL of PB to the 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 QIAquick spin column in a 2 mL collection tube.
5. Transfer the 300 µL sample to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (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 17,900 x g (13,000 rpm). Discard the flow-through.
7. Put the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Transfer the QIAquick 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 30 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
11. Collect the eluate, which can be stored at -20°C.

Step 3. Assess quality with Agilent 2100 Bioanalyzer

Use a Bioanalyzer High Sensitivity DNA Kit to assess the quality and size range. Note that the concentration of each sample loaded on the chip must be within the linear range of the assay to accurately quantify (5 pg to 500 pg). You may need to dilute sample accordingly.

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.

   You can use the High Sensitivity Kit to quantify the amount of sample to be used for Illumina sequencing.

   The linear range of the High Sensitivity kit is 5 pg to 500 pg. If the reading far exceeds 500 pg, dilute and run the Bioanalyzer chip again. If the yield is too low or too high, adjust the PCR cycles accordingly. The goal is to minimize cycles, while you produce enough library for the quantification needed for application to the flow cell.
8. Continue to sequencing.

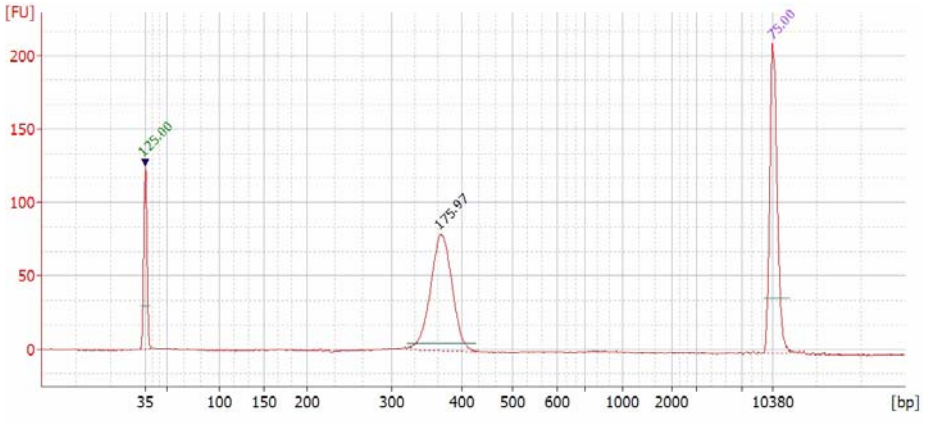

Figure 8 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows a single peak in the size range of approximately 350 nucleotides.

5 Reference

This chapter contains reference information.

Alternative Capture Equipment Combinations

Table 20 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 20 Tested options that show minimal evaporation

PCR Machine	Plate/Strips	Cover	Comments
Stratagene Mx3005P QPCR	Mx3000P Strip Tubes (401428)	MX3000P Optical Strip Caps (401425)	Heated Lid
Stratagene 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
ABI Veriti (4375786)	Stratagene strip tubes 410022 (Mx4000)	Stratagene Strip cap domed 410096 (Robocycler)	Heated Lid
ABI Veriti (4375786)	Stratagene strip tubes 410022 (Mx4000)	Stratagene Optical cap 401425 (Mx3000/3005)	Heated Lid
BioRad (MJ Research) PTC-200	Stratagene strip tubes 410022 (Mx4000)	Stratagene Optical cap 410024 (Mx4000)	Heated Lid
BioRad (MJ Research) PTC-200	Stratagene strip tubes 410022 (Mx4000)	Stratagene Optical cap 401425 (Mx3000/3005)	Heated Lid
BioRad (MJ Research) PTC-200	Stratagene 96-well Plate 410088 (Mx3000/3005)	Stratagene Optical cap 401425 (Mx3000/3005)	Heated Lid
BioRad (MJ Research) PTC-200	Stratagene 96-well Plate 410088 (Mx3000/3005)	Stratagene Plate sealers 400774-15	Heated Lid 2 layers of plate sealer

www.agilent.com

In This Book

This guide contains information to run the SureSelect Target Enrichment protocol with the Illumina Paired-End Sequencing Platform.

Printed in USA
Version 1.0, September 2009

G3360-90000

Agilent Technologies