SureSelect Target Enrichment System for Illumina Paired-End Sequencing Library

SureSelect Target Enrichment for Illumina Paired-End Multiplexed Sequencing

Protocol

Version 1.0, May 2010

SureSelect platform manufactured with Agilent SurePrint Technology

Research Use Only. Not for use in Diagnostic Procedures.

Agilent Technologies

Notices

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In this Guide...

This guide describes Agilent's recommended operational procedures to capture the genomic regions of interest using Agilent's SureSelect Target Enrichment Kit for Illumina Paired-End Multiplex Sequencing and sample preparation kits for next-generation sequencing. 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, specifically adjusted to provide high performance with SureSelect.

This guide uses an optimized protocol for Illumina paired-end multiplexed library preparation.

1 Before You Begin

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

2 Sample Preparation

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

3 Hybridization

This chapter describes the steps to prepare and hybridize samples.

4 Addition of IndexBarcode Tags by Post-Hybridization Amplification

This chapter describes the steps to amplify, purify, and assess quality of the sample libraries. Samples are pooled by mass prior to sequencing.

5 Reference

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

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 This protocol differs from the Illumina Multiplexed Paired-End sequencing manual and other SureSelect protocols at several steps. Pay close attention to the primers used for each amplification step and the blocking agents used during hybridization.

NOTE Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols 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
Agilent QPCR NGS Library Quantification Kit (Illumina GA)	Agilent p/n G4880A
AMPure Kit (SPRI XP beads) 5 mL 60 mL 450 mL	Agencourt p/n A63880 p/n A63881 p/n A63882
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Applied Biosystems p/n 4389764
Illumina Paired-End Genomic DNA Sample Prep Kit	Illumina Cat # PE-102-1001
Illumina Multiplexing Sample Preparation Oligonucleotide Kit (contains Index Adapter mix, InPE1.0 PCR primer, and Index-specific PCR primers)	Illumina Cat # PE-400-1001
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer 100 assays, 2-1000 ng 500 assays, 2-1000 ng	 Invitrogen p/n Q32850 Invitrogen p/n Q32853
Qubit assay tubes	Invitrogen p/n Q32856
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Herculase II Fusion DNA Polymerase (includes dNTP mix and 5x Buffer) 200 reactions 400 reactions	Stratagene p/n 600677 p/n 600679
70% Ethanol (for SPRI clean-up)	

Table 2 Required Reagents for Hybridization

Description	Vendor and part number
SureSelect Target Enrichment System Kit	
10 reactions, <0.2 to 6 Mb (Level MP0 through MP4)	Agilent p/n G3360A*
25 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360B*
50 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360C*
100 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360D*
250 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360E*
500 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360F*
1000 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360G*
2000 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360H*
5000 reactions, 3 to 6 Mb (Level MP0)	Agilent p/n G3360J*
100 reactions, <0.2 Mb (Level MP1)	Agilent p/n G3360K*
250 reactions, <0.2 Mb (Level MP1)	Agilent p/n G3360L*
500 reactions, <0.2 Mb (Level MP1)	Agilent p/n G3360M*
1000 reactions, <0.2 Mb (Level MP1)	Agilent p/n G3360N*
100 reactions, 0.2 to 0.49 Mb (Level MP2)	Agilent p/n G3360O*
250 reactions, 0.2 to 0.49 Mb (Level MP2)	Agilent p/n G3360P*
500 reactions, 0.2 to 0.49 Mb (Level MP2)	Agilent p/n G3360Q*
1000 reactions, 0.2 to 0.49 Mb (Level MP2)	Agilent p/n G3360R*
100 reactions, 0.5 to 1.49 Mb (Level MP3)	Agilent p/n G3360S*
250 reactions, 0.5 to 1.49 Mb (Level MP3)	Agilent p/n G3360T*
500 reactions, 0.5 to 1.49 Mb (Level MP3)	Agilent p/n G3360U*
1000 reactions, 0.5 to 1.49 Mb (Level MP3)	Agilent p/n G3360V*
100 reactions, 1.5 to 2.99 Mb (Level MP4)	Agilent p/n G3360W*
250 reactions, 1.5 to 2.99 Mb (Level MP4)	Agilent p/n G3360X*
500 reactions, 1.5 to 2.99 Mb (Level MP4)	Agilent p/n G3360Y*
1000 reactions, 1.5 to 2.99 Mb (Level MP4)	Agilent p/n G3360Z*
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Dynabeads MyOne Streptavidin T1	Invitrogen
2 mL	Cat #656-01
10 mL	Cat #656-02
100 mL	Cat #656-03

* Use option 012 for Illumina Genome Analyzer Multiplex Sequencing.

Required Equipment

Table 3 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
Agilent 2100 Bioanalyzer	Agilent p/n G2938C
Thermal cycler	BioRad (MJ Research) DNA Engine PTC-200, Applied Biosystems Veriti Thermal Cycler, 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
Microcentrifuge	Eppendorf Microcentrifuge Model 5417C or equivalent
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Qubit Fluorometer	Invitrogen p/n Q32857
Dynal DynaMag-2 magnetic stand	Invitrogen p/n 123-21D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Mx3005P Real-Time PCR System	Stratagene p/n 401449 or equivalent
Ice bucket	
Powder-free gloves	
PCR tubes, strips, or plates	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Heat block at 37°C	

Table 4 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	Applied Biosystems p/n 4306311 or equivalent
BD Clay Adams Nutator Mixer	BD Diagnostics p/n 421105 or equivalent
Dynal DynaMag-2 magnetic stand	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
PCR tubes, strips, or plates	
Sterile, nuclease-free aerosol barrier pipette tips	
Thermal cycler	BioRad (MJ Research) DNA Engine PTC-200, Applied Biosystems Veriti Thermal Cycler, or equivalent
Timer	
Vortex mixer	
Water bath or heat block set to 65°C	

Optional Equipment

Table 5 Optional Equipment for Hybridization

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

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 Paired-End Library Preparation Kit (p/n PE-102-1001) in combination with the Illumina Multiplexing Sample Preparation Oligonucleotide Kit (PE-400-1001). For each sample to be sequenced, individual library preparations, hybridizations, and captures are performed. The samples are then tagged by PCR with an index (barcode) sequence. Depending on the target size of the SureSelect capture, up to 12 samples can be pooled and sequenced in a single lane using Illumina's multiplex index tags.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of SPRI XP beads for all purification steps, and primers used for PCR.

Refer to the Illumina protocol *Preparing Samples for Mutiplexed Paired-End Sequencing* (p/n 1005361 Rev. C) 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.

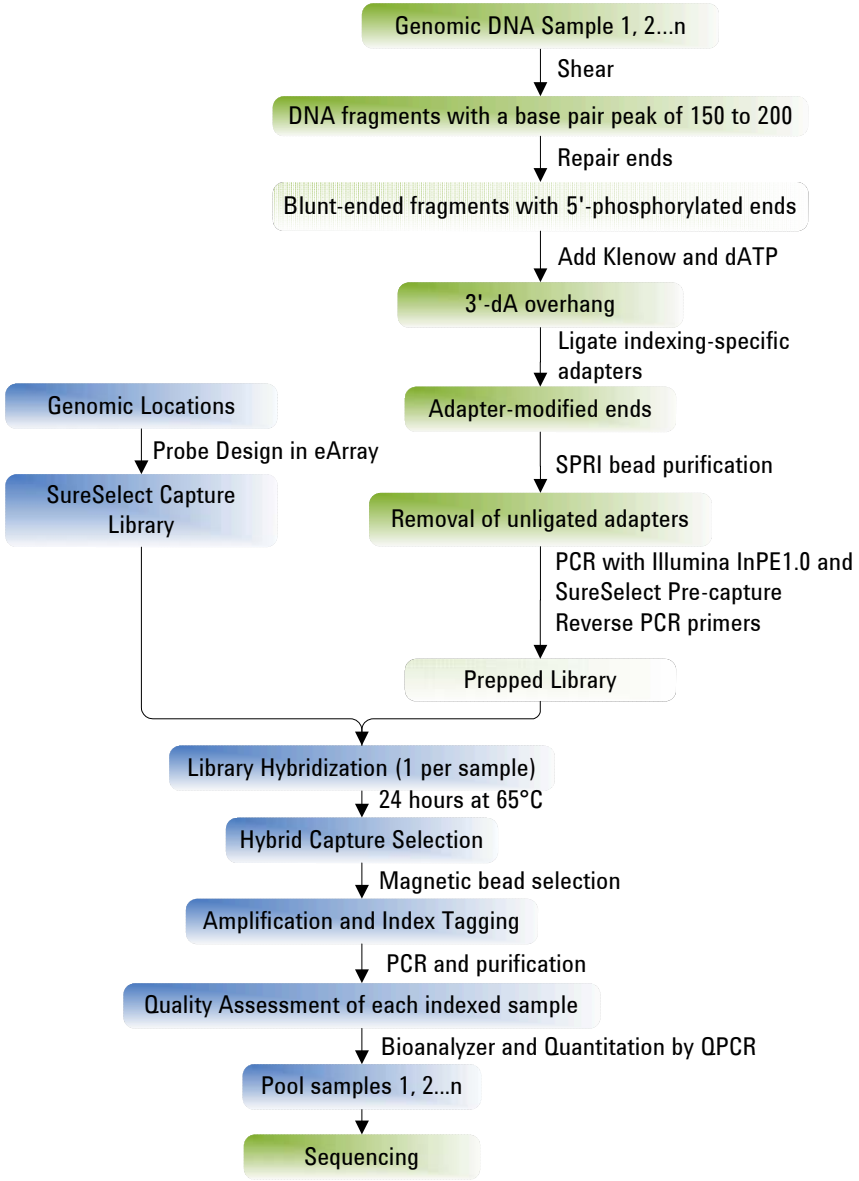

Figure 1 Overall sequencing sample preparation workflow.

Table 7 Overview and time requirements

Step	Time
Illumina Prepped library Production	1 day
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 and QPCR	2 to 3 hours
Pool indexed samples by mass	< 1 hour

Step 1. Shear DNA

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

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 Dilute each 3 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 120 µL.

3 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.

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 120 µL DNA sample through the pre-split septa.

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

6 Secure the microTube in the tube holder and shear the DNA with the settings in Table 8. The target peak for base pair size is 150 to 200 bp.

Table 8 Covaris shear settings

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cyles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4°C

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

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

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

Step 2. Purify the sample using the Agencourt AMPure XP beads

1. Let the SPRI 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 SPRI XP beads to a 1.5-mL LoBind tube, and add the sheared DNA library (~120 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.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol. Repeat this step once.
8. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
9. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
10. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
11. Remove approximately 30 µL of the supernatant to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

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

Step 3. Assess quality with the 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.

   Check that the electropherogram shows a distribution with a peak height between 150 to 200 nucleotides.

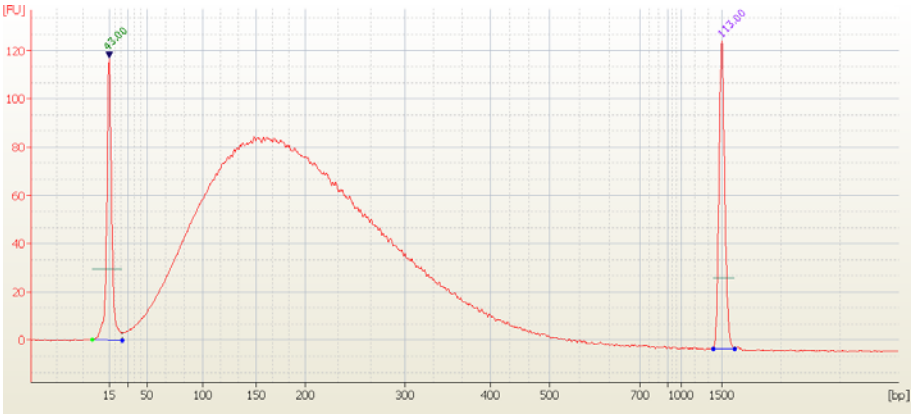

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows a distribution with a peak size between 150 to 200 nucleotides.

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.

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 For 1 library:

- In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 9, on ice. Use the Paired-End Sample Preparation Kit (p/n PE-102-1001). Mix well by gently pipetting up and down.

3 For multiple libraries:

a Prepare the reaction mix in Table 9, on ice. Use the Paired-End Sample Preparation Kit (p/n PE-102-1001). Mix well on a vortex mixer.

b Add 71 µL of the reaction mix to each well or tube.

c Add 29 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 9 End Repair Mix*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	~29 µL	
Nuclease-free water	46 µL	575 µL
T4 DNA ligase buffer with 10mM ATP	10 µL	125 µL
10 mM dNTP mix	4 µL	50 µL
T4 DNA polymerase	5 µL	62.5 µL
Klenow DNA polymerase	1 µL	12.5 µL
T4 PNK	5 µL	62.5 µL
Total Volume	**100 µL**	**888 µL (71 µL/sample)**

* These reagents are included in the Illumina Paired-End Sample Preparation Kit (p/n PE-102-1001).

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

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

1. Let the SPRI 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 SPRI XP beads to a 1.5-mL LoBind tube, and add the repaired DNA library (~100 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol. Repeat this step once.
8. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
9. Add 32 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
10. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
11. Remove the supernatant (~32 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

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

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

1 For 1 library:

- In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 10, on ice. Use the Paired-End Sample Preparation Kit (p/n PE-102-1001). Mix well by gently pipetting up and down.

2 For multiple libraries:

a Prepare the reaction mix in Table 10, on ice. Use the Paired-End Sample Preparation Kit (p/n PE-102-1001). Mix well on a vortex mixer.

b Add 18 µL of the reaction mix to each well or tube.

c Add 32 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 10 Adding "A" Bases*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	~32 µL	
10x Klenow buffer	5 µL	62.5 µL
1 mM dATP	10 µL	125 µL
Klenow Fragment (3′ to 5′ exo minus)	3 µL	37.5 µL
Total Volume	**50 µL**	**225 µL (18 µL/sample)**

* These reagents are included in the Illumina Paired-End Sample Preparation Kit (p/n PE-102-1001).

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

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

Step 7. Purify the sample using the Agencourt AMPure XP beads

1. Let the SPRI 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 SPRI XP beads to a 1.5-mL LoBind tube, and add the A-tailed DNA library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol. Repeat this step once.
8. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
9. Add 15 µL of nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
10. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
11. Remove the supernatant (~15 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.
12. Proceed immediately to the next step, "Step 8. Ligate the indexing-specific paired-end adapter".

Step 8. Ligate the indexing-specific 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 shearing.

1 For 1 library:

- In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 11, on ice. Except where noted, use the Paired-End Sample Preparation Kit (p/n PE-102-1001). Mix well by gently pipetting up and down.

2 For multiple libraries:

a Prepare the reaction mix in Table 11, on ice. Except where noted, use the Paired-End Sample Preparation Kit (p/n PE-102-1001). Mix well on a vortex mixer.

b Add 36 µL of the reaction mix to each well or tube.

c Add 14 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 11 Ligation master mix

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	14 µL	
2X DNA ligase buffer	25 µL	313 µL
Index PE Adapter oligo mix*	6 µL	75 µL
DNA ligase	5 µL	62.5 µL
Total Volume	**50 µL**	**450 µL (36 µL/sample)**

* Included in the Illumina Multiplexing Oligonucleotide kit. *Do not use the adapter from the Paired-End Sample Prep kit.*

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

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

1. Let the SPRI 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 SPRI beads to a 1.5-mL LoBind tube, and add the ligated library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol. Repeat this step once.
8. Dry the samples on the 37°C heat block for 5 minutes until the residual ethanol is completely evaporated.
9. Add 52 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
10. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
11. Remove the supernatant (~52 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

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

Step 10. Amplify adapter-ligated library

This protocol uses half of the adapter-ligated fragments for amplification. The remainder can be saved at 20°C for future use, if needed.

CAUTION

Do not use InPE2.0 from the Illumina Multiplexing Oligonucleotide Kit. Replace with the SureSelect Indexing Pre-Capture PCR (Reverse) Primer.

Do not use the PCR Index Primers from the Illumina Multiplexing Oligonucleotide kit at this time. You will use them after capture.

Do not use the Phusion polymerase from the Illumina Kit. Use the Herculase II polymerase instead.

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 12, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   - **a** Prepare the reaction mix in Table 12, 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 12 Components for PCR mix

Reagent	Vol for 1 reactions	Vol for 12 reactions (includes excess)
Index PE Adapter-ligated library	25 µL	
Nuclease-free water	11.5 µL	144 µL
Illumina InPE1.0 (forward) PCR primer*	1 µL	12.5 µL
SureSelect Indexing Pre-Capture PCR (Reverse) Primer	1 µL	12.5 µL
Herculase II 5X Reaction Buffer†	10 µL	125 µL
dNTP mix†	0.5 µL	6.25 µL
Herculase II polymerase	1 µL	12.5 µL
Total	**50 µL**	**313 µL (25 µL/reaction)**

* Included in the Illumina Multiplexing Sample Preparation Oligonucleotide Kit (p/n PE-400-1001).

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

3 Run the program in Table 13 in a thermal cycler.

Table 13

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 4 to 6 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, 5 cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low or non-specific high molecular weight products are observed, adjust the number of cycles accordingly with the remaining extra library template.

As an alternative, you can prepare three small-scale 10 µL PCR reactions and run for 4, 5 or 6 cycles. Use the optimal cycle number to repeat PCR at the 50 µL reaction scale.

Step 11. Purify the sample with the Agencourt AMPure SPRI XP beads

1. Let the SPRI 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 SPRI XP beads to a 1.5-mL LoBind tube, and add the 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.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol. Repeat this step once.
8. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.
9. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
10. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
11. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

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

Step 12. 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. Check that the electropherogram shows a distribution with a peak size approximately 250 to 275bp. Measure the concentration of the library by integrating under the peak.

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

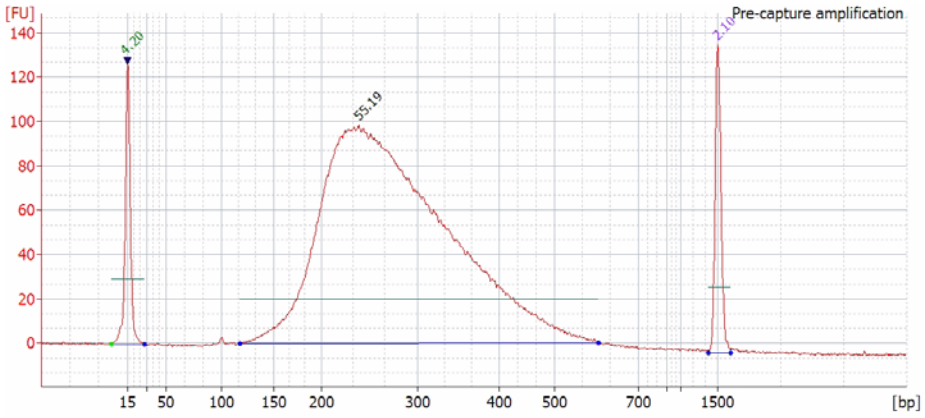

Figure 3 Analysis of amplified prepped library DNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 250 to 275 bp.

3 Hybridization

This chapter describes the steps to combine the prepped library with the blocking agents and the SureSelect capture library. Each DNA library sample must be hybridized and captured individually prior to addition of the indexing tag by PCR.

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

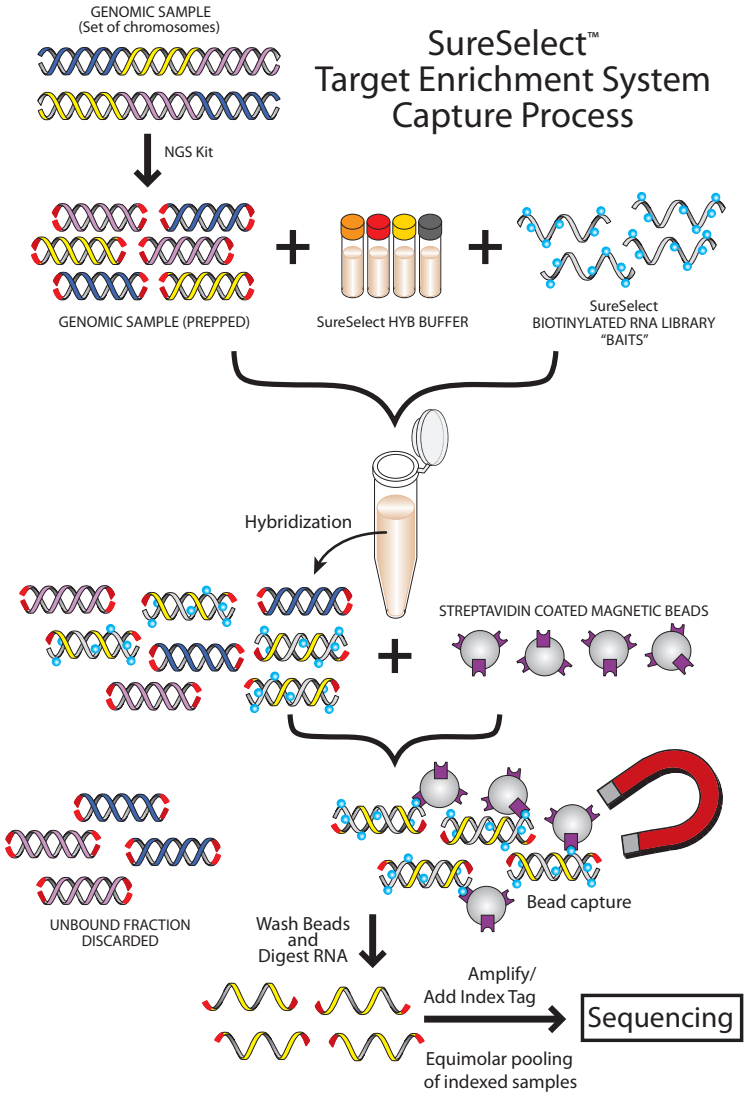

Figure 4 SureSelect Target Enrichment System Capture Process

Table 14 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 Indexing 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 Indexing Block #3	tube with brown cap	tube with brown cap	-20°C
SureSelect RNase Block	tube with purple cap	tube with purple cap	-20°C
SureSelect Indexing Pre-Capture PCR (Reverse) Primer	tube with clear cap	tube with clear cap	-20°C
SureSelect Indexing Post-Capture PCR (Forward) Primer	tube with orange cap	tube with orange cap	-20°C
SureSelect Oligo Capture Library	tube with red cap	tube with red cap	-80°C

The SureSelect reagents are packaged in separate kits. See Table 15.

Table 15 Part number for reagent kits

Reagent Kit	50 reactions	250 reactions
Room temperature reagents	5190-1953	5190-1959
Cold reagents, -20°C	5190-2332	5190-2333

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 62.

Step 1. Hybridize the library

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

1 If the prepped library concentration is below 147 ng/µL, use a vacuum concentrator to concentrate the sample at ≤ 45°C.

   a Add the entire 50 µL of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

   You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.

   b Completely lyophilize. Use a vacuum concentrator on low heat (less than 45°C) to dehydrate.

   c Reconstitute with nuclease-free water to bring the final concentration to 147 ng/µL (or greater if sample recovery is of concern). Pipette up and down along the sides of the tube for optimal recovery.

   d Mix well on a vortex mixer and spin in a microfuge for 1 minute.

2 *(Optional)* To test recovery after lyophilization, reconstitute the sample to greater than 147 ng/µL and check the concentration on an Agilent Bioanalyzer DNA 1000 chip. See "Step 12. Assess quality and quantity with Agilent 2100 Bioanalyzer" on page 32. After quantitation, adjust the sample to 147 ng/µL.

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

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

Table 16 Hybridization Buffer

Reagent	Volume for 1 capture (µL), includes excess	Volume for 12 captures (µL), includes excess
SureSelect Hyb # 1	25	250
SureSelect Hyb # 2 (red cap)	1	10
SureSelect Hyb # 3 (yellow cap)	10	100
SureSelect Hyb # 4	13	130
Total	**49**	**490 (40 µL/sample)**

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

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

- **a** Keep tubes on ice until step 10.
- **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 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 RNase Block 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	1:9 (10%)	5 µL
≥ 3.0 Mb	5 µL	1:3 (25%)	2 µL

6 Mix the contents in Table 18 to make the correct amount of SureSelect Block mix for the number of samples used.

Table 18 SureSelect Block Mix

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

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

a Add 3.4 µL of 147 ng/µL prepped library to the "B" row in the PCR plate. Put each sample into a separate well.

b Add 5.6 µL of the SureSelect Block Mix to each well in row B.

c Mix by pipetting up and down.

d 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.

e Run the following thermal cycler program in Table 19.

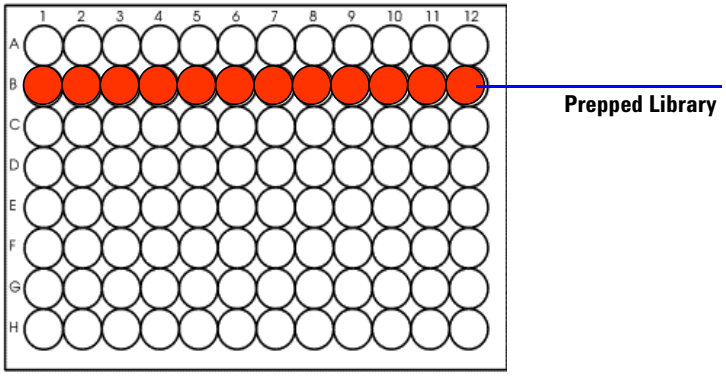

Figure 5 Prepped library shown in red

Table 19 PCR program

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

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

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

9 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.

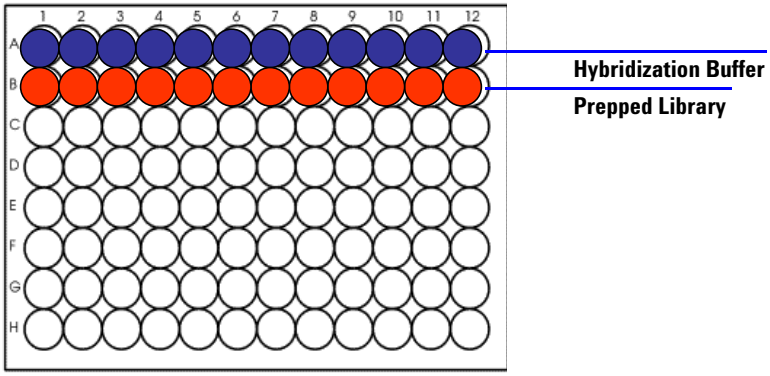

Figure 6 Hybridization buffer shown in blue

Make sure that the plate is at 65°C for a minimum of 5 minutes before you go to step 10.

10 Add the capture library mix from step 5 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.

11 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.)

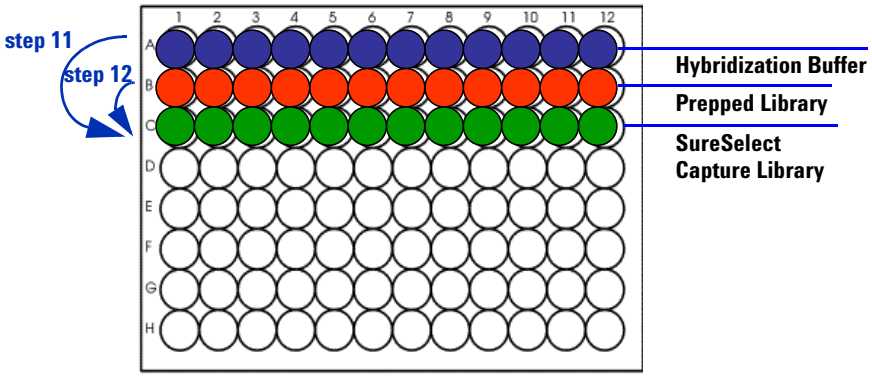

Figure 7 SureSelect Capture Library, or "Baits", shown in Green

12 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.

13 Seal the wells with strip caps or 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.

14 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 or heat block for use in "Step 3. Select hybrid capture with SureSelect".
2. Vigorously resuspend the Dynal MyOne Streptavidin T1 (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 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 (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.

   Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 25 on page 63 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 Dynal magnetic separator and remove the supernatant.
6. Resuspend the beads in 500 µL of SureSelect Wash Buffer #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 Dynal magnetic separator and remove the supernatant.
10. Wash the beads:
    a. 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.
    b. Incubate the samples for 10 minutes at 65°C. Occasionally mix on a vortex mixer.
    c. Briefly spin in a centrifuge.
    d. Separate the beads and buffer on a Dynal 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 Incubate the samples for 10 minutes at room temperature. Occasionally mix on a vortex mixer.

13 Briefly spin in a centrifuge.

14 Separate the beads and buffer on a Dynal 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 the Agencourt AMPure XP beads

1. Let the SPRI 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 SPRI 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.
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.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

4 Addition of IndexBarcode Tags by Post-Hybridization Amplification

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

Step 1. Amplify the captured library to add indexbarcode tags

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 20, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:

a Prepare the reaction mix in Table 20, on ice. Mix well on a vortex mixer.

b Add 35 µL of the reaction mix to each well or tube.

c Add 1 µL of the appropriate SureSelect Indexing Primer from the Illumina Multiplexing Sample Preparation Oligonucleotide Kit to each well and mix by pipetting.

Use a different index primer for each sample to be sequenced in the same lane. See Table 21 for the optimal number of indexes per sequencing lane. For low-level multiplexing of fewer than 12 samples, refer to the Illumina Multiplexed Sequencing manual to select the optimal index tag set.

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

Table 20 Herculase II Master Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Captured DNA	14 µL	
Nuclease-free water	22.5 µL	281.25 µL
5X Herculase II Reaction Buffer *	10 µL	125 µL
dNTP mix (25 mM each) *	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase	1 µL	12.5 µL
SureSelect Indexing Post-Capture PCR (Forward) Primer†	1 µL	12.5 µL
Index PCR (reverse) primer‡	1 µL	
Total	**50 µL**	**437.5 µL (35 µL/reaction)**

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

† Included with the SureSelect Target Enrichment System Kit for Illumina Genome Analyzer Multiplex Sequencing.

‡ Use one of the 12 primers included in the Illumina Multiplex Sample Preparation Oligonucleotide Kit (PE-400-1001).

Table 21 Optimal number of indexes per sequencing lane

Capture Size	Optimal number of indexes per sequencing lane*
<0.2 Mb	12
0.2 to 0.49 Mb	10
0.5 to 1.49 Mb	6
1.5 to 2.99 Mb	4
3.0 to 6.0 Mb	2

* Values are approximate and depend on sequencing efficiency and flow cell capacity. These values refer to the GAII instrument with v2 cluster generation reagents, generating approximately 1 Gb of high-quality sequencing data per lane. More samples can be sequenced per lane when higher cluster densities are obtained.

Some sequencing experiments require the use of fewer than 12 index sequences in a lane with a high cluster density. In such cases, select indexes carefully to ensure optimum cluster discrimination by having different bases at each cycle of the index read. Illumina recommends the following sets of indexes for low-level pooling experiments.

Pool of 2 samples:

- Index #6 GCCAAT
- Index #12 CTTGTA

Pool of 3 samples:

- Index #4 TGACCA
- Index #6 GCCAAT
- Index #12 CTTGTA

Pool of 6 samples:

- Index #2 CGATGT
- Index #4 TGACCA
- Index #5 ACAGT
- Index #6 GCCAAT
- Index #7 CAGATC
- Index #12 CTTGTA

3 Put the tubes in a thermal cycler and run the program in Table 22.

Table 22 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 16 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

As with the pre-capture PCR amplification, try to enrich the captured DNA with a minimum of PCR cycles. The use of only half of the captured DNA for amplification lets you adjust the number of cycles by repeating the PCR if needed.

As an alternative, you can prepare three small-scale 10 µL PCR reactions and cycle for 10or 12 cycles. Use the optimal cycle number to repeat PCR at the 50 µL reaction scale. See Table 23 for approximate number of cycles for a given library size. Results may vary based on library content.

Table 23 Cycle times

Capture Size	Cycles
<0.5 Mb	16 cycles
0.5 to 1.49 Mb	14 cycles
> 1.5 Mb	12 cycles

Step 2. Purify the sample using the Agencourt AMPure XP beads

1. Let the SPRI 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 SPRI 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.
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.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

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

Step 3. Assess quality with the Agilent 2100 Bioanalyzer High Sensitivity DNA assay

Use a Bioanalyzer High Sensitivity DNA Assay to assess the quality and size range. You may need to dilute your 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.

   Determine the concentration of the sample by integration under the peak. Use QPCR for the most accurate concentration determination and most even pooling.

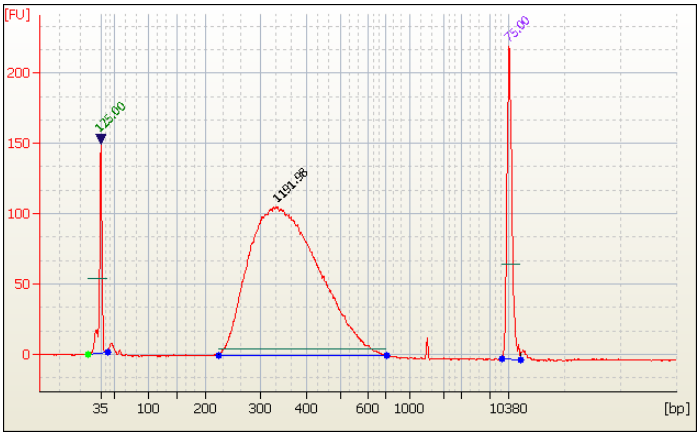

Figure 8 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows a peak in the size range of approximately 300 to 325 nucleotides.

1 Prepare 10 nM (10 fmol/µL) dilutions of the amplified capture, based on the Bioanalyzer quantitation.

Step 4. Assess the quantity of each index-tagged library by QPCR

Refer to the protocol that is included with the Agilent QPCR NGS Library Quantification Kit (p/n G4880A) for more details to do this step.

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

   Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.
4. Prepare the QPCR master mix with Illumina adapter-specific PCR primers according to instructions provided in the kit.
5. Add an aliquot of the master mix to PCR tubes and add template.
6. On a QPCR system, such as the MX3005p, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.
7. Use the standard curve to determine the concentration of each unknown index-tagged library, in nM.

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

Step 5. Pool samples for Multiplexed Sequencing

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

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

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

C(f) is the desired final concentration of all the DNA in the pool, for example, 10 nM for the standard Illumina protocol

is the number of indexbarcode tags, and

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

Table 24 shows an example of the amount of 4 index-tagged (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 24 Example of index volume calculation for a total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

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

4 Proceed to template denaturation and flow cell preparation. Refer to the appropriate Illumina protocol.

Exact library pool dilution and processing can vary based on the flow cell capacity and analysis pipeline versions being used. Refer to the appropriate Illumina user guide for instructions. This protocol has been validated with 36-base paired-end reads. However, read length can be adjusted to achieve the desired research goals.

5 Reference

This chapter contains reference information.

Alternative Capture Equipment Combinations

Table 25 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 25 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

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In This Book

This guide contains information to run the SureSelect Target Enrichment System for Illumina Paired-End Sequencing protocol with the SureSelect Target Enrichment for Illumina Paired-End Multiplexed Sequencing kit.

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