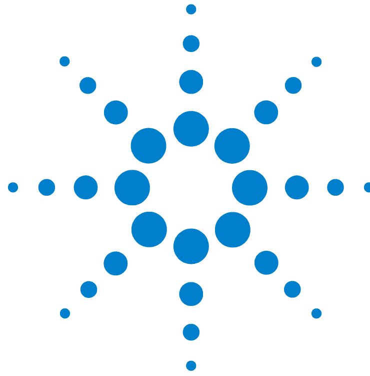

SureSelect RNA Target Enrichment for Illumina Paired-End Multiplexed Sequencing

Protocol

Version 2.2.1, February 2012

SureSelect platform manufactured with Agilent SurePrint Technology

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CAUTION

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In this Guide...

This guide describes the recommended operational procedures to capture transcripts of interest using the Agilent SureSelect RNA Capture 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 transcriptome.

This guide uses the Illumina paired-end multiplex sequencing platform for 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 RNA sample for target enrichment.

3 Hybridization

This chapter describes the steps to prepare and hybridize samples.

4 Addition of Index Tags by Post-Hybridization Amplification

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

What's New in 2.2

- New product configuration and product numbers for SureSelect reagent kits and capture libraries.
- Support for the optional use of the Agilent 2200 TapeStation for RNA quantitation and qualification.
- Support for custom SureSelect RNA capture libraries.

What's New in 2.1

- SureSelect RNA Primer Kit for Illumina replaces SureSelect Library Prep Kit.
- Dynabeads MyOne Streptavidin T1 beads replaces Agilent LodeStars 2.7 Streptavidin beads.

What's New in 2.0

- Agilent LodeStars 2.7 Streptavidin beads replaces Dynabeads MyOne Streptavidin T1 beads.
- NEBNext mRNA Sample Prep Reagent Set 1 replaces the Illumina mRNA-Seq Prep Kit.
- QIAquick PCR Purification Kit replaces Agencourt AMPure XP beads for RNA purification.
- Agarose gel purification step is added in sample preparation.

What's New in 1.1

- Reagent cap colors are listed where available.
- More details given for the reagent kits to use for each step.
- Update to cluster generation reagents and procedure.

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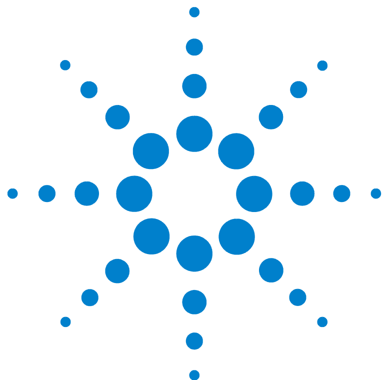

1
Before You Begin

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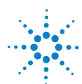
Make sure you have the most current protocol. Go to the SureSelect Related Literature page on genomics.agilent.com and search for manual number G7580-90010.

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

NOTE

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



Procedural Notes

- Prolonged exposure of SureSelect Elution Buffer to air can decrease product performance by altering the pH of the solution. Keep the Elution Buffer container tightly sealed when not in use.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing RNA 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 RNA solutions.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Herculase II Fusion DNA Polymerase (includes dNTP mix and 5x Buffer) 200 reactions 400 reactions	Agilent p/n 600677 p/n 600679
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 4389764
E-Gel SizeSelect 2% Agarose Gel	Life Technologies p/n G6610-02
SuperScript II Reverse Transcriptase 2,000 units 10,000 units 4×10,000 units	Life Technologies p/n 18064-022 p/n 18064-014 p/n 18064-071
NEBNext mRNA Sample Prep Reagent Set 1	New England BioLabs p/n E6100S
Buffer EB (10mM Tris-Cl, ph 8.5)	Qiagen p/n 19086
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
3 M NaOAc, pH 5.2	
Distilled water	

Table 2 Required Reagents for Cluster Generation and Sequencing

Description	Vendor and part number
Illumina Cluster Generation Kit (depending on your instrument and setup)	
TruSeq PE Cluster Kit v5–CS–GA	Illumina p/n PE-203-5001
TruSeq PE Cluster Kit v2–cBot–HS	Illumina p/n PE-401-2001
TruSeq PE Cluster Kit v2.5–cBot–HS	Illumina p/n PE-401-2510
PhiX Control Kit V2 (for HiSeq 2000)	Illumina p/n CT-901-2001
Illumina Sequencing Kit (depending on your instrument and setup)	
TruSeq SBS Kit v5–GA (36-cycle)	Illumina p/n FC-104-5001
TruSeq SBS Kit–HS (50 cycle)	Illumina p/n FC-401-1002

Table 3 SureSelect Reagent Kit

Reagent Kits	16 Reactions	96 Reactions	480 Reactions
SureSelect TE RNA Reagent Kit, HSQ	G9601A	G9601B	G9601C

Table 4 SureSelect Capture Library (select one)

Capture Libraries	16 Reactions	96 Reactions	480 Reactions
SureSelect RNA Kinome	5190-4801	5190-4802	5190-4803
SureSelect RNA Capture 1 kb up to 499 Kb	5190-4934	5190-4935	5190-4937
(reorder)	5190-4939	5190-4940	5190-4942
SureSelect RNA Capture 0.5 Mb up to 2.9 Mb	5190-4944	5190-4945	5190-4947
(reorder)	5190-4949	5190-4950	5190-4952
SureSelect RNA Capture 3 Mb up to 5.9 Mb	5190-4954	5190-4955	5190-4957
(reorder)	5190-4959	5190-4960	5190-4962

Table 5 Required Reagents for Hybridization

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

Optional Reagents

Table 6 Optional Reagents

Description	Vendor and part number
Ethylene glycol	American Bioanalytical p/n AB00455
RNeasy MinElute Cleanup Kit	Qiagen p/n 74204

Required Equipment

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

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

Table 8 Required Equipment for Hybridization

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

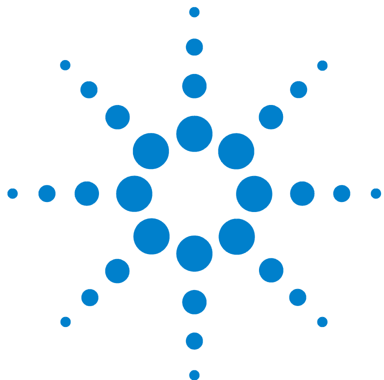
Optional Equipment

Table 9 Optional Equipment for Hybridization

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

Table 10 Optional Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
2200 TapeStation System	Agilent p/n G2964AA or G2965AA
D1K ScreenTape	Agilent p/n 5067-5361
D1K Reagents	Agilent p/n 5067-5362
High Sensitivity D1K ScreenTape	Agilent p/n 5067-5363
High Sensitivity D1K Reagents	Agilent p/n 5067-5364



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This section contains instructions for prepped library production specific to the Illumina -read sequencing platform. It is intended for use with the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

Refer to the *NEBNext mRNA Sample Prep Reagent Set 1 Instruction Manual* for more information.

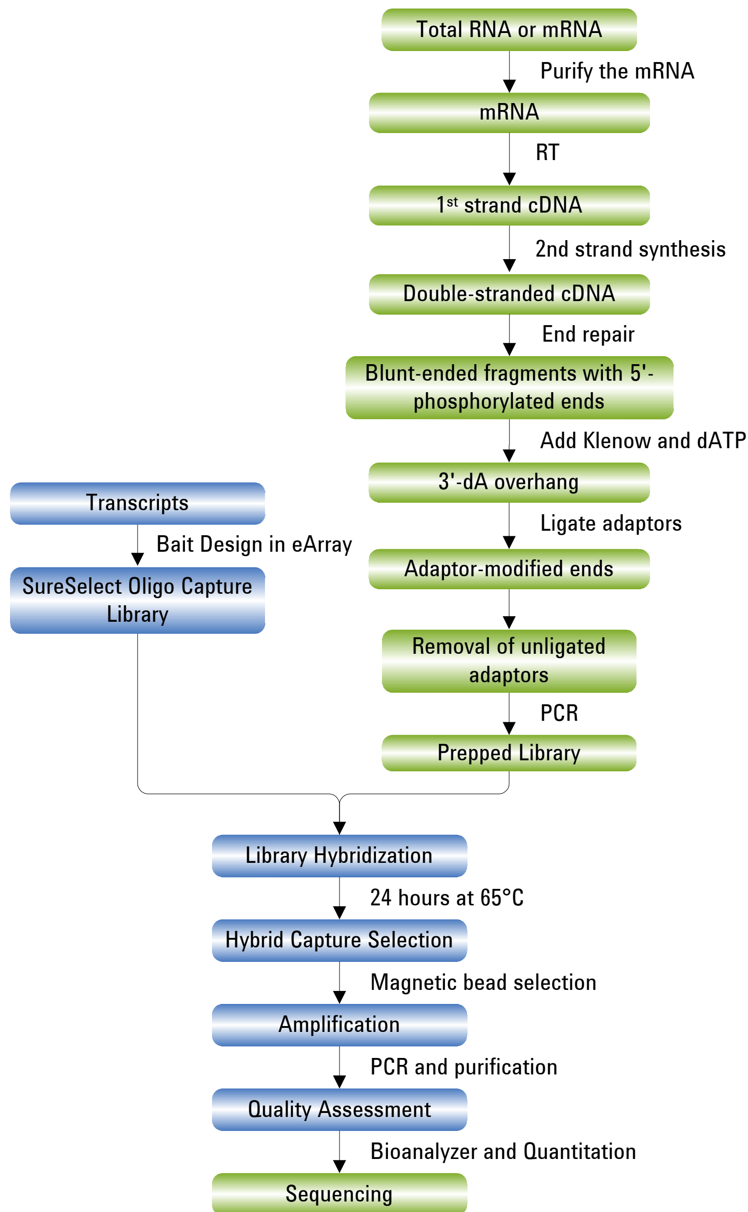


Figure 1 Overall sequencing sample preparation workflow.

Table 11 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. Purify the mRNA

- Purify 1 to 10 µg of total RNA. Use the purification kit of your choice.

Step 2. Fragment the RNA

Use reagents from the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S):

- NEBNext RNA Fragmentation Buffer (10X)
- NEBNext RNA Fragmentation Stop Solution (10X)

1 Preheat a PCR thermal cycler to 94°C.

2 Add the components in Table 12 to a 200 µL thin wall PCR tube.

Table 12 Fragmentation Buffer Mix

Component	Volume
NEBNext RNA Fragmentation Buffer (10X)*	2 µL
Purified mRNA	1 to 18 µL
Nuclease-free Water	enough to bring total volume to 20 µL
Total	**20 µL**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

3 Incubate the tube in a preheated PCR thermal cycler at 94°C for exactly 5 minutes.

4 Add 2 µL of NEBNext RNA Fragmentation Stop Solution (10X).

5 Put the tube on ice.

Step 3. Clean up the fragmented mRNA

In this step, you precipitate the fragmented mRNA, using reagents from the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

As an alternative, you can use the RNeasy MinElute Cleanup Kit. Elute the RNA in 13.5 µL of Nuclease-Free Water or elution buffer.

1 Transfer the solution to a 1.5 mL RNase-free non-sticky tube.
2 Add the components in Table 13 to the tube and incubate at -80°C for 30 minutes or overnight as desired.

Table 13

Component	Volume
3 M NaOAC, pH 5.2	2 µL
Linear Acrylamide (10 mg/mL)*	1 to 2 µL
100% ethanol	60 µL

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

Stopping Point You can safely stop the protocol here. Store the samples at -15°C to -25°C. Do not stop the protocol at any other point while the sample is RNA.

3 Spin the tube in a microcentrifuge at 14,000 rpm (20,200 relative centrifugal force) for 25 minutes at 4°C.
4 Carefully pipette off the ethanol without dislodging the RNA pellet.

   The RNA pellets are small and almost colorless. To avoid dislodging the pellets, remove the ethanol in several steps. Remove 90% at each step and switch to smaller pipette tips for each step.
5 Without disturbing the pellet, wash the pellet with 300 µL of 70% ethanol.
6 Spin the pellet in a centrifuge and carefully pipette out the 70% ethanol.
7 Air dry the pellet for 10 minutes at room temperature.
8 Resuspend the RNA in 13.5 µL of Nuclease-free water.

Step 4. Synthesize first strand cDNA

Use reagents from the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S) and the SuperScript II Reverse Transcriptase.

1 Add the components in Table 14 to a 200 µL thin wall PCR tube:

Table 14 Random Primer Mix

Component	Volume
Random Primers (3 µg/µL)*	1 µL
mRNA	13.5 µL
Total	**14.5 µL**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S)

2 Incubate the sample in a PCR thermal cycler at 65°C. Use a heated lid at 105°C.

3 Spin the sample briefly, and then put the tube on ice.

4 Set the PCR thermal cycler to 25°C.

5 Mix the reagents in Table 15 in the order listed in a separate tube. Prepare 10% extra reagent mix if you are preparing multiple samples.

Table 15 Random Primer Mix

Component	Volume for 1 reaction	Volume for 10 reactions (with excess)
NEBNext First Strand Synthesis Reaction Buffer (5X)*	4 µL	44 µL
Murine RNase Inhibitor*	0.5 µL	5.5 µL
Total	**4.5 µL**	**49.5 µL**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

6 Add 4.5 µL of mixture to the PCR tube and mix well.

7 Heat the sample in the preheated PCR thermal cycler at 25°C for 2 minutes. Do not use a heated lid.

8 Add 1 µL of SuperScript II Reverse Transcriptase to the sample.

9 Run the program listed in Table 16 in a thermal cycler to incubate the sample. Use a heated lid at 105°C.

Table 16 PCR program

Temperature	Time
25°C	10 minutes
42°C	50 minutes
70°C	15 minutes
4°C	Hold

10 Put the tube on ice.

Step 5. Synthesize second strand cDNA

Use reagents from NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

1 Preheat a PCR thermal cycler to 16°C.
2 Add 48 µL of Nuclease-Free Water to the first strand cDNA synthesis mix.
3 Add the reagents the in Table 17 to the mix:

Table 17

Component	Volume
NEBNext Second Strand Synthesis Reaction Buffer (10X)*	8 µL
NEBNext Second Strand Synthesis Enzyme Mix*	4 µL

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

4 Mix well by gentle pipetting.
5 Incubate the sample at 16°C for 2.5 hours in a thermal cycler. Use a heated lid at 50°C.

Step 6. Purify the sample with the QIAquick PCR Purification Kit

Use the reagents from the QIAquick PCR Purification Kit (Qiagen p/n 28104).

1 If you haven't already done so, add the pH Indicator I to the Buffer PB.

2 Add 400 µL of Buffer PB to 80 µL of 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 QIAquick 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 480 µL sample to the QIAquick spin column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.

6 Add 750 µL of Buffer PE (concentrate) to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.

7 Put the QIAquick spin column back in the collection tube (2 mL) and spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).

8 Transfer the QIAquick spin column to a new 1.5 mL microcentrifuge 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 50 µL of Buffer EB directly onto the QIAquick filter membrane.

11 Wait 60 seconds, then centrifuge for 60 seconds at 17,900 × g (13,000 rpm).

12 Collect the eluate.

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

Step 7. Repair the ends

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

Prepare the master mix on ice.

Use the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

1 Preheat one heat block to 20°C and the other heat block to 37°C.
2 For 1 library (prepare on ice):
   - In a sterile microcentrifuge tube, prepare the reaction mix in Table 18. Mix well by gently pipetting up and down.
3 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 18. Mix well on a vortex mixer.
   b Add 50 µL of the reaction mix to each 1.5 mL microcentrifuge tube.
   c Add 50 µL of each DNA sample to each tube. Mix by pipetting. Change pipette tips between samples.
4 Incubate in a thermal cycler for 30 minutes at 20°C. Do not use a heated lid.

Table 18 End Repair Mix[*]

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
Purified double-stranded cDNA sample	50 µL	
Nuclease-Free Water	25 µL	342.5 µL
Phosphorylation Reaction Buffer (10X)	10 µL	125 µL
Deoxynucleotide Solution Mix (10 mM each dNTP)	4 µL	20 µL
T4 DNA Polymerase	5 µL	62.5 µL
DNA Polymerase I, Large (Klenow) Fragment	1 µL	12.5 µL
T4 Polynucleotide Kinase	5 µL	62.5 µL
Total Volume	**100 µL**	**625 µL (50 µL/sample)**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

Step 8. Purify the sample with the QIAquick PCR Purification Kit

Use the reagents from the QIAquick PCR Purification Kit (Qiagen p/n 28104).

1. If you haven't already done so, add the pH Indicator I to the Buffer PB.
2. Add 500 µL of Buffer PB to 100 µL of 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 QIAquick 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 of sample to the QIAquick spin column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
6. Add 750 µL of Buffer PE (concentrate) to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
7. Put the QIAquick spin column back in the collection tube (2 mL) and spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
8. Transfer the QIAquick spin column to a new 1.5 mL microcentrifuge 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.
11. Wait 60 seconds, then centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
12. Collect the eluate.

Step 9. Add ‘dA’ Bases to the 3' end of the cDNA fragments

Use the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

1 For 1 library (prepare on ice):

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

2 For multiple libraries (prepare on ice):

a Prepare the reaction mix in Table 19. 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 19 Adding “A” Bases*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
Purified end-repaired cDNA sample	32 µL	
NEBuffer 2 for Klenow Fragment (3′ → 5′ exo$^-$) (10X)	5 µL	62.5 µL
Deoxyadenosine 5′- Triphosphate (dATP) (1.0 mM)	10 µL	125 µL
Klenow Fragment (3′ → 5′ exo$^-$)	3 µL	37.5 µL
Total Volume	**50 µL**	**225 µL (18 µL/sample)**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

3 Incubate in a thermal cycler for 30 minutes at 37°C. Do not use a heated lid.

Step 10. Purify the sample with the QIAquick PCR Purification Kit

Use the reagents from the QIAquick PCR Purification Kit (Qiagen p/n 28104).

1. If you haven't already done so, add the pH Indicator I to the Buffer PB.
2. Add 250 µL of Buffer PB to 50 µL of 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 QIAquick 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 of sample to the QIAquick spin column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
6. Add 750 µL of Buffer PE (concentrate) to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
7. Put the QIAquick spin column back in the collection tube (2 mL) and spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
8. Transfer the QIAquick spin column to a new 1.5 mL microcentrifuge 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 23 µL of Buffer EB directly onto the QIAquick filter membrane.
11. Wait 60 seconds, then centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
12. Collect the eluate.

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

Step 11. Ligate the paired-end adaptor

Use the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S) and the SureSelect RNA Primer Kit.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 20.
2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 20.
   b Add 27 µL of the reaction mix to each well or tube.
   c Add 23 µL of each cDNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 20 Ligation master mix

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
Purified dA-Tailed DNA sample	23 µL	
Quick Ligation Reaction Buffer (2X)*	25 µL	312.5 µL
Quick T4 DNA Ligase	1.0 µL	12.5 µL
SureSelect Adaptor Oligo Mix (brown cap)†	1.0 µL	12.5 µL
Total Volume	**50 µL**	**337.5 µL (27 µL/sample)**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S).

† Included in the SureSelect RNA Primer Kit.

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

Step 12. Purify the sample with the QIAquick PCR Purification Kit

Use the reagents from the QIAquick PCR Purification Kit (Qiagen p/n 28104).

1 If you haven't already done so, add the pH Indicator I to the Buffer PB.
2 Add 250 µL of Buffer PB to 50 µL of 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 QIAquick 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 of sample to the QIAquick spin column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
6 Add 750 µL of Buffer PE (concentrate) to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
7 Put the QIAquick spin column back in the collection tube (2 mL) and spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
8 Transfer the QIAquick spin column to a new 1.5 mL microcentrifuge 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 10 µL of Buffer EB directly onto the QIAquick filter membrane.
11 Wait 60 seconds, then centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
12 Collect the eluate.

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

Step 13. Size-select the DNA fragments with a E-Gel SizeSelect 2% Agarose gel

Use the E-Gel SizeSelect 2% Agarose Gel.

1 Remove a E-Gel SizeSelect 2% Agarose Gel from its package. Remove the combs from the top sample-loading wells and the middle collection wells. Set the E-Gel on the E-Gel iBase linked with the E-Gel Safe Imager.

2 Load the E-Gel as follows:

   a Load 20 µL of the ligated, purified DNA into a well in the *top row*. Do not use the center well or outermost wells (to avoid edge effects). Do not load more than 1 µg of DNA.

   If the sample volume < 20 µL, add nuclease-free water to the well for a total volume of 20 µL.

   b Load 2 µL 50-bp ladder at 0.1 µg/µL to the center top well. Add 15 µL of water to fill the well.

   c Fill empty wells in the top row with 20 µL of nuclease-free water.

   d Fill each of the collection wells in the *middle* of the gel with 25 µL of nuclease-free water. Add 20 µL of nuclease-free water to the *middle* center well.

3 Run the gel:

   - iBase program: **Run E-Gel DC**
   - Approximate run time: **13:45** (13 minutes and 45 seconds)

   Monitor the E-Gel in real-time with the E-Gel® Safe Imager.

4 If needed during the run, fill the middle collection wells with nuclease-free water.

5 When the 200-bp band from the marker lane is in the center of the collection well, stop the run if the run has not already stopped (see Figure 2).

6 Collect the solution from the sample well.

7 Wash each collection well with 25 µL of nuclease-free water, pipette up and down, then retrieve the wash solution and combine with the respective sample solution collected in step 6 for a total of 50 µL.

See Table 21 for expected lengths of the insert and PCR according to the excised cDNA length.

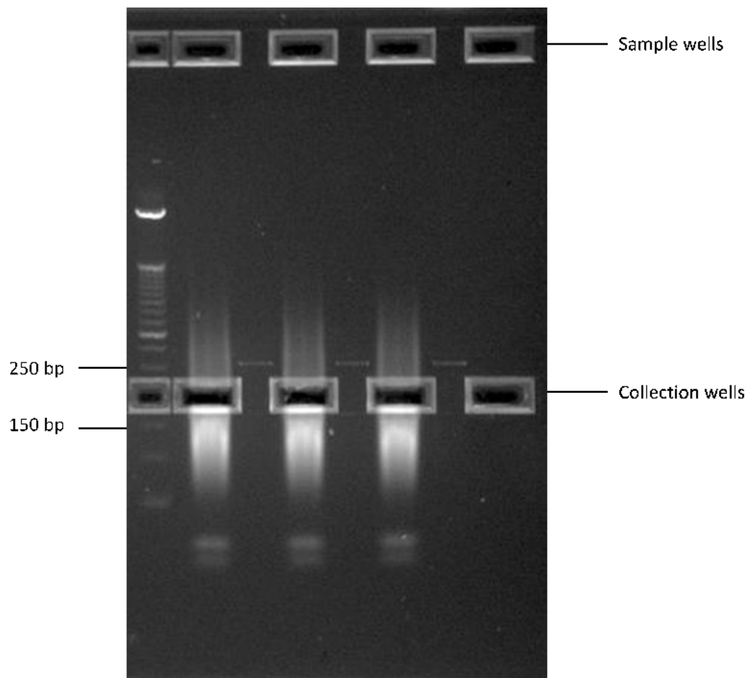


Figure 2 Elution of an approximately 200 bp region. This image shows three samples on the same gel.

Table 21 Expected lengths of the insert and PCR according to the excised cDNA length

Excised cDNA length (nt)	Insert length (bp)	PCR product length (bp)
50	~0	~100
100	~50	~150
150	~100	~200
200	~150	~250
250	~200	~300

Step 14. Purify the sample with the QIAquick PCR Purification Kit

Use the reagents from the QIAquick PCR Purification Kit (Qiagen p/n 28104).

1 If you haven't already done so, add the pH Indicator I to the Buffer PB.
2 Add 250 µL of Buffer PB to 50 µL of 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 QIAquick 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 spin column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
6 Add 750 µL of Buffer PE (concentrate) to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
7 Put the QIAquick spin column back in the collection tube (2 mL) and spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
8 Transfer the QIAquick spin column to a new 1.5 mL microcentrifuge 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 29 µL of Buffer EB directly onto the QIAquick filter membrane.
11 Wait 60 seconds, then centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
12 Collect the eluate.

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

Step 15. Amplify adaptor-ligated library

This step uses PCR to selectively enrich those cDNA fragments that have adaptor molecules on both ends, and to amplify the amount of cDNA in the library. The PCR is done with two primers that anneal to the ends of the adaptors. Ten to fourteen 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 cDNA (100 ng) for at least a single hybridization, poor quality RNA samples or other factors can affect yield.

Use reagents in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S), the SureSelect RNA Primer Kit.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 22. Mix well by gently pipetting up and down.
2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 22. Mix well on a vortex mixer.
   b Add 21 µL of the reaction mix to each well or tube.
   c Add 29 µL of each cDNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 22 PCR Components*

Reagent	Volume for 1 Library	Volume for 12 Libraries
Size-selected cDNA	29 µL	
PhusionR HF Buffer (5X) (manufactured by Finnzymes Oy)	10 µL	125 µL
SureSelect Primer (brown cap)†	1 µL	12.5 µL
SureSelect ILM Indexing Pre Capture PCR Reverse Primer (clear cap)†	1 µL	12.5 µL
Deoxynucleotide Solution Mix (10 mM each dNTP)	1.5 µL	18.75 µL
Nuclease-Free Water	7 µL	87.5 µL
PhusionR High-Fidelity DNA Polymerase (manufactured by Finnzymes Oy)	0.5 µL	6.25 µL
Total Volume	**50 µL**	**262.5 µL (21 µL/reaction)**

* Included in the NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S), except where indicated.

† Included in the SureSelect RNA Primer Kit.

3 Run the program listed in Table 23 in a thermal cycler to amplify the sample. Use a heated lid at 105°C.

4 Amplify using the following PCR program:

Table 23 PCR program

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 15 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

Step 16. Purify the sample with the QIAquick PCR Purification Kit

Use the reagents from the QIAquick PCR Purification Kit (Qiagen p/n 28104).

1 If you haven't already done so, add the pH Indicator I to the Buffer PB.
2 Add 250 µL of Buffer PB to 50 µL of 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 QIAquick 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 of sample to the QIAquick spin column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
6 Add 750 µL of Buffer PE (concentrate) to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm). Discard the flow-through.
7 Put the QIAquick spin column back in the collection tube (2 mL) and spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
8 Transfer the QIAquick spin column to a new 1.5 mL microcentrifuge 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.
11 Wait 60 seconds, then centrifuge for 60 seconds at 17,900 × g (13,000 rpm).
12 Collect the eluate.

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

Step 17. Assess quality and quantity with 2100 Bioanalyzer

NOTE

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

Use the Bioanalyzer DNA 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 200 to 250 bp. Measure the concentration of the library by integrating under the peak.

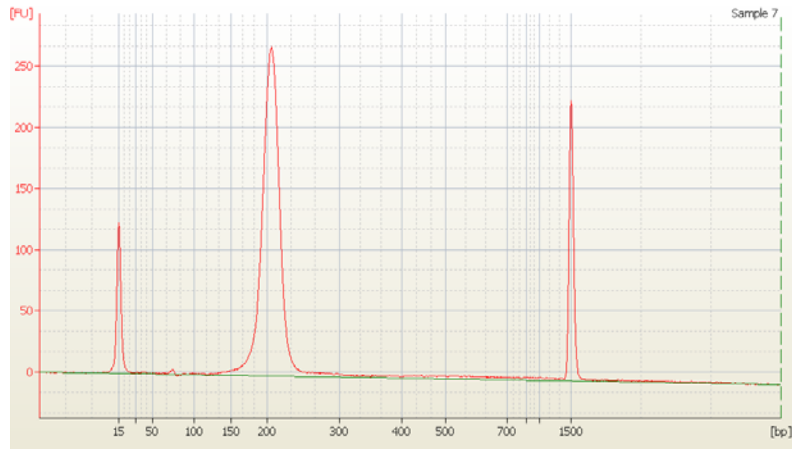
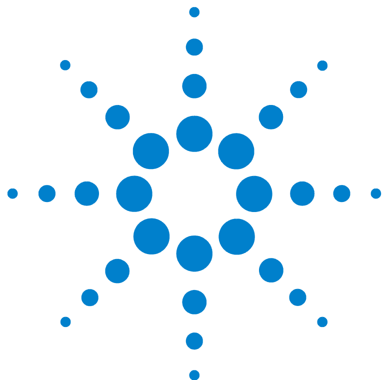


Figure 3 Analysis of amplified prepped library DNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 200 to 250 bp.



3 Hybridization

Step 1. Hybridize the library 44
Step 2. Prepare magnetic beads 50
Step 3. Select hybrid capture with SureSelect 51
Step 4. Purify the sample using Agencourt AMPure XP beads 53

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

CAUTION

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

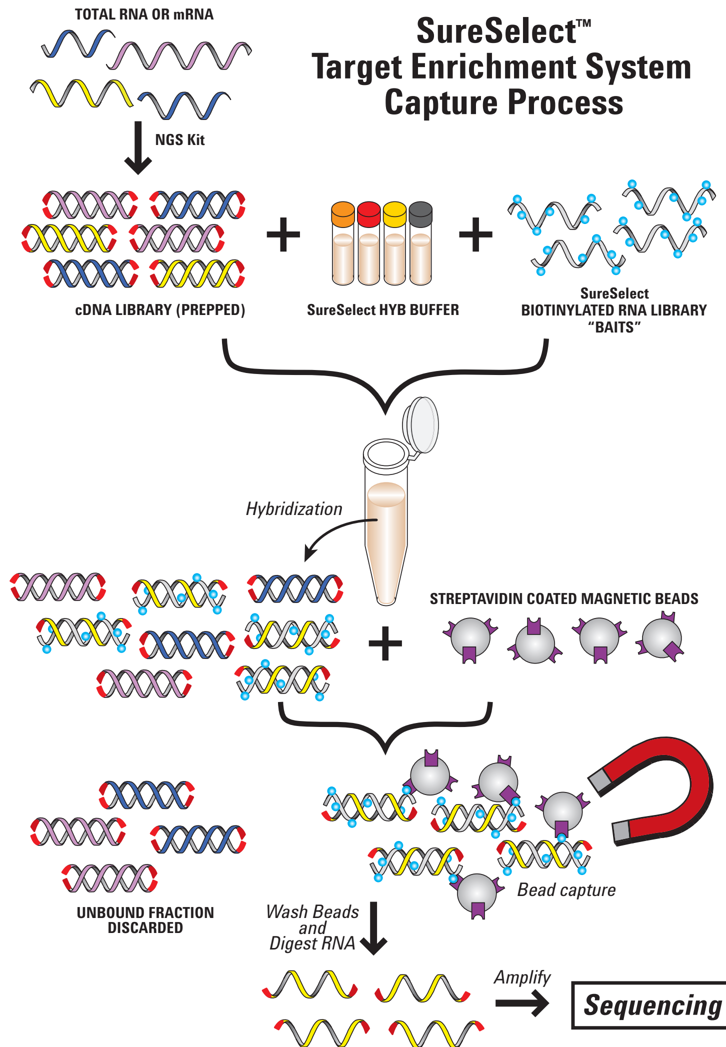


Figure 4 SureSelect RNA Capture Process

Refer to "SureSelect Reagent Kit Content" on page 68 for a complete content listing of each SureSelect RNA Capture kit.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 24 hour or greater incubation.

If you want to use a different combination of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape), first test the conditions. Incubate 27 µL of SureSelect Hybridization Buffer (without DNA) at 65°C for 24 hours (or longer, if applicable) as a test. Include buffer in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 3 to 4 µL.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 74.

Step 1. Hybridize the library

The hybridization reaction requires 100 ng of cDNA with a maximum volume of 3.4 µL.

1 If the prepped library concentration is below 30 ng/µL, use a vacuum concentrator to concentrate the sample at ≤ 45°C.

a Add the entire volume 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 30 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 30 ng/µL and check the concentration on a Bioanalyzer DNA 1000 chip. See "Step 17. Assess quality and quantity with 2100 Bioanalyzer" on page 38. After quantitation, adjust the sample to 30 ng/µL.

Alternatively, concentrate a 100 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 24 at room temperature to prepare the hybridization buffer.

Table 24 Hybridization Buffer

Reagent	Volume for 1 capture (µL), includes excess	Volume for 6 captures (µL), includes excess	Volume for 12 captures (µL), includes excess
SureSelect Hyb #1 (orange cap, or bottle)	25	125	250
SureSelect Hyb #2 (red cap)	1	5	10
SureSelect Hyb #3 (yellow cap)	10	50	100
SureSelect Hyb #4 (black cap, or bottle)	13	65	130
Total	**49 (40 µL needed)**	**245 (40 µL/sample)**	**490 (40 µL/sample)**

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 capture library mix for target enrichment:

  a Keep tubes on ice until step 10.

  b For each sample, add 5 µL of SureSelect capture library.

  c For 1 library, combine 1 µL SureSelect RNase Block (purple cap) with 2 µL nuclease-free water. For multiple libraries, use 1 part SureSelect RNase Block (purple cap) to 2 parts nuclease-free water to make enough mix for 2 µL per capture library, plus excess.

  d Add 2 µL of diluted SureSelect RNase Block (purple cap) to each capture library, and mix by pipetting.

6 Mix the contents in Table 25 to make the correct amount of SureSelect Block mix for the number of samples used.

Table 25 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 30 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 Start the thermal cycler program in Table 26.

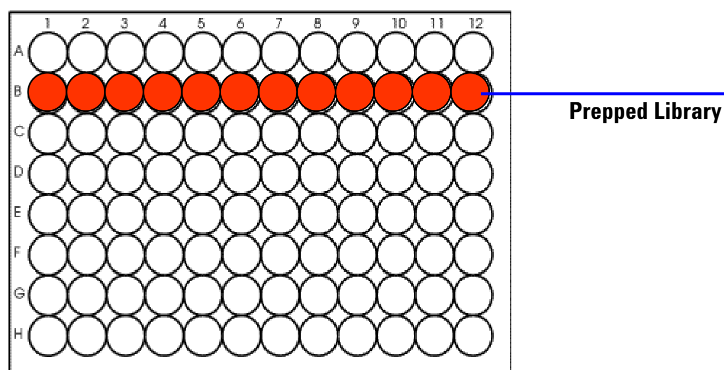


Figure 5 Prepped library shown in red

Table 26 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.

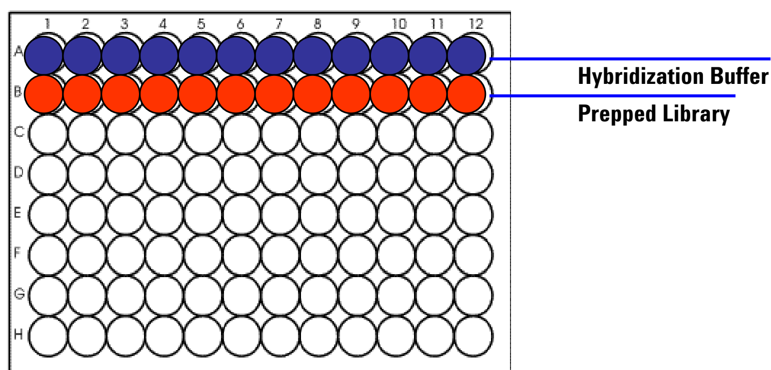


Figure 6 Hybridization buffer shown in blue

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.)

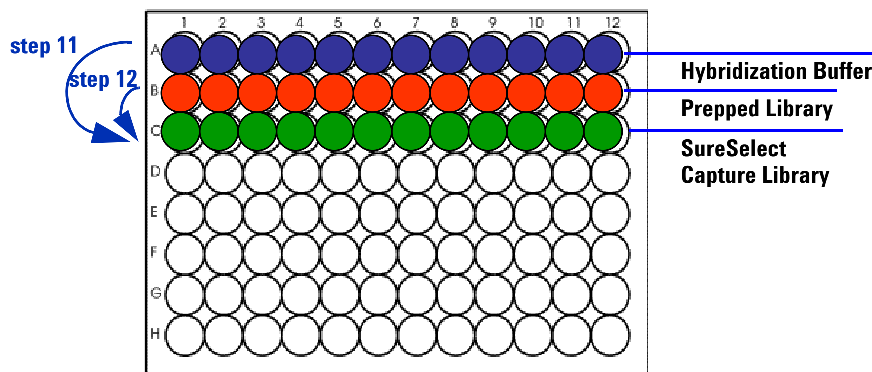


Figure 7 SureSelect Capture Library, or “Baits”, shown in Green

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.

Use new adhesive seals or strip caps. The structural integrity of the seals and caps can be compromised during the previous incubation steps.

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

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

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

Step 2. Prepare magnetic beads

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

- SureSelect Binding Buffer
- SureSelect Wash 2

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

Step 3. Select hybrid capture with SureSelect

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

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

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

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

   Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 43 on page 74 for tips to minimize evaporation.
3. Incubate the hybrid-capture/bead solution on a Nutator or equivalent for 30 minutes at room temperature.

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

9 Wash the beads:

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

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

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

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

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

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

  Make sure all of the wash buffer has been removed.

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

11 Incubate the samples for 10 minutes at room temperature.

12 Separate the beads and buffer on a magnetic separator.

13 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.

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

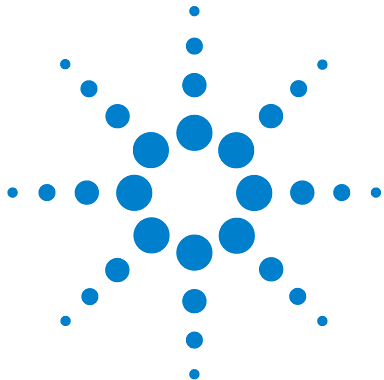
Step 4. Purify the sample using Agencourt AMPure XP beads

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

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

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 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.



4
Addition of Index Tags by Post-Hybridization Amplification

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Step 6. Prepare sample for cluster amplification 65

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

Step 1. Amplify the sample to add index tags

Use reagents from:

- Herculase II Fusion DNA Polymerase (Agilent)
- SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2
- SureSelect RNA Primer Kit

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.

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

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

To see the nucleotide sequence in each of the index included in SureSelect reagent kits, see "SureSelect^{XT} Indexes for Illumina" on page 73.

1 For 1 library:

- In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 27, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:

a Prepare the reaction mix in Table 27, on ice. Mix well on a vortex mixer.

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

c Add 1 µL of the appropriate index PCR Primer Index 1 through Index 16 (clear caps) from the SureSelect RNA Primer Kit to each well and mix by pipetting.

Use a different index primer for each sample to be sequenced in the same lane.

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 27 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 Rxn Buffer (clear cap) *	10 µL	125 µL
100 mM dNTP Mix (green cap)*	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase (red cap)*	1 µL	12.5 µL
SureSelect ILM Indexing Post Capture Forward PCR Primer (orange cap)†	1 µL	12.5 µL
PCR Primer Index 1 through Index 16 (clear caps)‡	1 µL	
Total	**50 µL**	**437.5 µL (35 µL/reaction)**

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

† Included in the SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2.

‡ Use one of the 16 primers included in the SureSelect RNA Primer Kit.

3 Put the samples in a thermal cycler with a heated lid at 105°C. Run the program listed in Table 28.

Table 28 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, minimize the number of PCR cycles used to enrich the captured DNA. The use of only half of the captured DNA for amplification lets you adjust the number of cycles by repeating the PCR if needed.

Use the optimal cycle number to repeat PCR at the 50 µL reaction scale. See Table 29 for approximate number of cycles for a given library size. Results may vary based on library content.

Table 29 Cycle times

Capture Size	Cycles
1 kb up to 0.5 Mb	16 cycles
0.5 Mb up to 1.49 Mb	14 cycles
> 1.5 Mb	12 cycles

Step 2. Purify the sample using Agencourt AMPure XP beads

1 Let the AMPure XP beads come to room temperature for at least 30 minutes.

2 Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.

3 Add 90 µL of homogenous AMPure beads to a 1.5-mL LoBind tube, and add sample library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.

4 Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).

5 Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.

6 Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.

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

8 Repeat step 6 and step 7 once.

9 Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

10 Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.

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

12 Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at 4°C for up to a week, or at -20°C for longer periods.

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

NOTE

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

Use a Bioanalyzer High Sensitivity DNA Assay to assess the quality and size range. 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 your sample accordingly. Refer to the *Agilent High Sensitivity DNA Kit Guide* at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=59504.

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

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

   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 non-specific peaks are observed in the electropherogram, repeat the PCR with more or fewer cycles. 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.

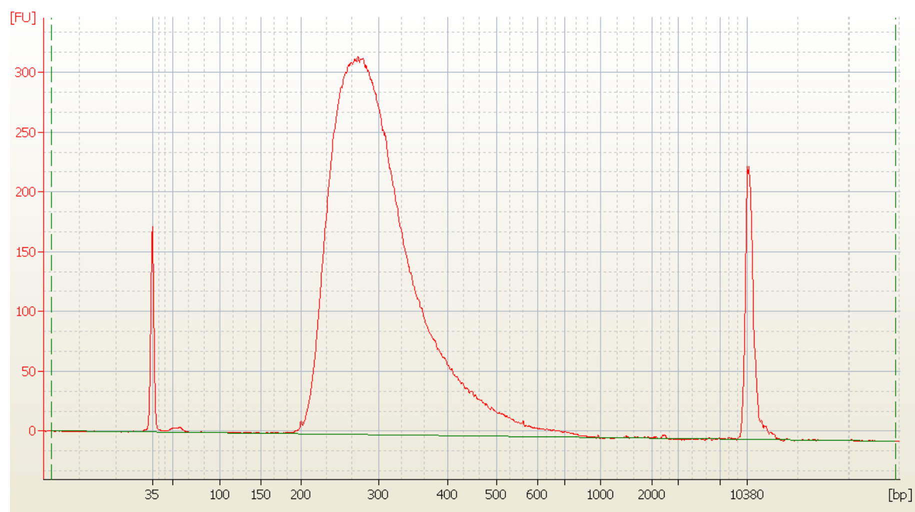


Figure 8 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows a single peak in the size range of approximately 250 to 300 ± 20% nucleotides.

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

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

1 Use the QPCR NGS Library Quantification Kit (Illumina GA) 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 adaptor-specific PCR primers according to instructions provided in the kit.

5 Add an aliquot of the master mix to PCR tubes and add template.

6 On a QPCR system, such as the MX3005P, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.

7 Use the standard curve to determine the concentration of each unknown 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 index sample to use.

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

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

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

is the number of index, and

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

Table 30 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 30 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 20 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.

Step 6. Prepare sample for cluster amplification

In this step you set up cluster amplification.

Conditions are optimized to provide 700K to 900K clusters/mm^2 on the GAIIx and 400K to 600K clusters/mm^2 on a HiSeq instrument.

Genome Analyzer IIx

Use reagents from the TruSeq Cluster Generation Kit appropriate for your instrument:

- HT1 (Hybridization Buffer)
- HP3 (2 N NaOH)

1 Dilute 30 fmol (3µL) of the 10 nM multiplexed sample pool with 16 µL of Buffer EB (10mM Tris-Cl, ph 8.5) for a total volume of 19 µL.
2 Add 1 µL of HP3 (2 N NaOH).
3 Mix the sample briefly on a vortex mixer and pulse centrifuge.
4 Incubate for 5 minutes at room temperature to denature the DNA.
5 Place the sample on ice until you are ready to proceed to final dilution.
6 Dilute 8 µL of denatured DNA with 992 µL of pre-chilled HT1 (Hybridization Buffer) for a final concentration of 12 pM.
7 Mix the sample briefly on a vortex mixer and pulse centrifuge.
8 Continue with cluster generation. Use the TruSeq SBS Kit v5–GA (36-cycle) and the appropriate Illumina multiplexed sequencing protocol.

HiSeq2000 with PhiX spike-in controls

Use reagents from the appropriate for your instrument:

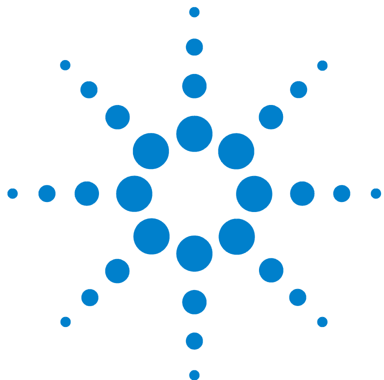
- HT1 (Hybridization Buffer)
- HP3 (2 N NaOH)

Use the PhiX Control Kit V2 (Illumina CT-901-2001) for:

- PhiX Control

1. Prepare a 1:20 dilution of HP3 (2 N NaOH) down to 0.1N NaOH.
2. Prepare 10 nM (10 fmol/µL) dilutions of the amplified capture, based on the Bioanalyzer quantitation.
3. Add 20 fmol (2 µL) of the 10 nM multiplexed sample pool into 8 µL of Buffer EB (10mM Tris-Cl, ph 8.5) to make a 2 nM solution.
4. Add 10 µL of 0.1 N NaOH.
5. Mix the sample briefly on a vortex mixer and pulse centrifuge.
6. Incubate for 5 minutes at room temperature to denature the DNA.
7. Add 980 µL of HT1 (Hybridization Buffer) to the denatured DNA to make 20 pM template solution.
8. Mix the sample briefly on a vortex mixer and pulse centrifuge.
9. Prepare 4 pM template by mixing 200 µL of 20 pM solution with 800 µL of Pre-Chilled HT1 (Hybridization Buffer).

   If densities higher than 400K-600K clusters/mm^2 are desired, prepare a more concentrated sample from the 20 pM solution.
10. Mix the sample briefly on a vortex mixer and pulse centrifuge.
11. Remove 10 µL from solution (1000 µL) to get 990 µL.
12. Add 10 µL of PhiX Control.
13. Mix the sample briefly on a vortex mixer and pulse centrifuge.
14. Dispense 120 µL of diluted denatured sample DNA template and PhiX Control into a strip tube.
15. Place on ice until ready to use.
16. Continue with cluster generation. Use TruSeq SBS Kit–HS (50 cycle) and the appropriate Illumina multiplexed sequencing protocol.



5
Reference

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Alternative Capture Equipment Combinations 74

This chapter contains reference information.

SureSelect Reagent Kit Content

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

Table 31 SureSelect Reagent Kit Contents

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect Target Enrichment Kit Box #1	Room Temperature	5190-4393	5190-4394	5190-4395
SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2	-20°C	5190-4455	5190-4456	5190-4457
SureSelect RNA Primer Kit	-20°C	5190-5337	5500-5338	

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

Table 32 SureSelect Target Enrichment Kit Box #1

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

Table 33 SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2

Kit Component
SureSelect Hyb #3 (yellow cap)
SureSelect Indexing Block #1 (green cap)
SureSelect Block #2 (blue cap)
SureSelect Indexing Block #3 (brown cap)
SureSelect RNase Block (purple cap)
SureSelect ILM Indexing Pre Capture PCR Reverse Primer (clear cap)
SureSelect ILM Indexing Post Capture Forward PCR Primer (orange cap)

Table 34 SureSelect RNA Primer Kit

Kit Component
SureSelect Adaptor Oligo Mix (brown cap)
SureSelect Primer (brown cap)
PCR Primer Index 1 through Index 16 (clear caps)

Other Reagent Kits Content

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

Table 35 Herculase II Fusion DNA Polymerase (Agilent)

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

Table 36 D1K Reagents (Agilent p/n 5067-5362)

Components
ladder
D1K sample buffer

Table 37 High Sensitivity D1K Reagents (Agilent p/n 5067-5364)

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

Table 38 TruSeq Cluster Generation Kit*

Components
HT1 (Hybridization Buffer)
HP3 (2 N NaOH)

* Use the Illumina Cluster Generation Kit that is appropriate for your instrument and setup. See Table 5 on page 11.

Table 39 PhiX Control Kit V2 (Illumina CT-901-2001)

Components
PhiX Control

Table 40 NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S)

Component
NEBNext RNA Fragmentation Buffer (10X)
NEBNext RNA Fragmentation Stop Solution (10X)
Linear Acrylamide (10 mg/mL)
Random Primers (3 µg/µL)
Murine RNase Inhibitor
NEBNext First Strand Synthesis Reaction Buffer (5X)
NEBNext Second Strand Synthesis Enzyme Mix
NEBNext Second Strand Synthesis Reaction Buffer (10X)
Phosphorylation Reaction Buffer (10X)
Deoxynucleotide Solution Mix (10 mM each dNTP)
T4 DNA Polymerase
DNA Polymerase I, Large (Klenow) Fragment
T4 Polynucleotide Kinase
Deoxyadenosine 5′- Triphosphate (dATP) (1.0 mM)
Klenow Fragment (3′ → 5′ exo$^-$)
NEBuffer 2 for Klenow Fragment (3′ → 5′ exo$^-$) (10X)
Quick T4 DNA Ligase
Quick Ligation Reaction Buffer (2X)
Nuclease-free water

Table 40 NEBNext mRNA Sample Prep Reagent Set 1 (New England Bioscience p/n E6100S) (continued)

Component
PhusionR High-Fidelity DNA Polymerase (manufactured by Finnzymes Oy)
PhusionR HF Buffer (5X) (manufactured by Finnzymes Oy)

Table 41 QIAquick PCR Purification Kit (Qiagen p/n 28104)

Components
QIAquick spin column
Buffer PB*
Buffer PE (concentrate)
Buffer EB
pH Indicator I
collection tube (2 mL)
loading dye

* Contain chaotropic salts which are irritants. Take appropriate laboratory safety measures and wear gloves when handling.

SureSelect^{XT} Indexes for Illumina

The nucleotide sequence of each of the SureSelect^{XT} index is listed in Table 42.

Table 42 SureSelect^{XT} Indexes 1-16

Index Number	Sequence
1	ATCACG
2	CGATGT
3	TTAGGC
4	TGACCA
5	ACAGTG
6	GCCAAT
7	CAGATC
8	ACTTGA
9	GATCAG
10	TAGCTT
11	GGCTAC
12	CTTGTA
13	AAACAT
14	CAAAAG
15	GAAACC
16	AAAGCA

Alternative Capture Equipment Combinations

Table 43 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 43 Tested options that show minimal evaporation

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

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In This Book

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

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