SureSelect^{XT} Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing

Protocol

Version A.1, April 2012

SureSelect platform manufactured with Agilent SurePrint Technology

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Agilent Technologies

Notices

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CAUTION

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In this Guide...

This guide describes an optimized protocol for DNA methylation analysis using the Agilent SureSelect target enrichment system to prepare bisulfite-sequencing samples for the Illumina paired-end multiplexed sequencing platform.

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 DNA samples for target enrichment.

3 Hybridization

This chapter describes the steps to hybridize and capture the gDNA library using the SureSelect^{XT} Human Methyl-Seq Capture Library.

4 Bisulfite Conversion

This chapter describes the steps for bisulfite treatment of the captured DNA library to differentiate methylated and unmethylated DNA segments.

5 Indexing and Sample Pooling for Multiplexed Sequencing

This chapter describes the steps to index the captured DNA libraries that were modified by bisulfite conversion and to pool the indexed samples for multiplexed sequence analysis.

6 Reference

This chapter contains reference information, including component kit contents and index sequences.

What's New in Version A.1

- Corrections to index nucleotide sequence information (Table 22)
- Updates to shearing protocol (page 15 to page 16)
- Updates to sequencing sample preparation guidelines (page 56)

Content

1 Before You Begin

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

NOTE

Agilent cannot guarantee the SureSelect^{XT} Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols or instruments 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 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. Possible stopping points, where gDNA samples may be stored overnight at 4°C, are marked in the protocol. When storing samples for >24 hours, store the samples at –20°C, but do not subject the samples to multiple freeze/thaw cycles.
- When preparing 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 vials used during an experiment on ice or in a cold block.
  4. Library Preparation Master Mixes should not be frozen and thawed more than five times. If you plan to use the reagents in more than five experiments, aliquot to multiple vials to minimize freeze/thaw cycles for each vial.
- 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 SureSelect^{XT} Methyl-Seq Target Enrichment

Description	Vendor and part number
SureSelect^{XT} Methyl-Seq Reagent Kit	Agilent
16 reactions	p/n G9651A
96 reactions	p/n G9651B
480reactions	p/n G9651C
SureSelect^{XT} Human Methyl-Seq Capture Library	Agilent
16 reactions	p/n 5190-4661
96 reactions	p/n 5190-4662
480reactions	p/n 5190-4663
EZ-DNA Methylation-Gold Kit	Zymo Research
50 reactions	p/n D5005
200 reactions	p/n D5006
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Applied Biosystems p/n 4389764
Agencourt AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1 2 mL 10 mL 100 mL	Life Technologies Cat #65601 Cat #65602 Cat #65603
Qiagen MinElute PCR Purification Columns	Qiagen p/n 28004
Agilent QPCR NGS Library Quantification Kit (Illumina)	Agilent p/n G4880A
Quant-iT dsDNA BR Assay Kit 100 assays, 2-1000 ng 500 assays, 2-1000 ng	 Life Technologies Cat #Q32850 Cat #Q32853
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

Required Equipment

Table 2 Required Equipment for SureSelect^{XT} Methyl-Seq Target Enrichment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible 96-well plates	Agilent p/n 410088
Optical strip caps	Agilent p/n 401425
Agilent 2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
Agilent 2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
Covaris Sample Preparation System, E-series or S-series	Covaris
Covaris sample holders	
96 microTUBE plate (E-series instruments only)	Covaris p/n 520078
microTUBE for individual sample processing	Covaris p/n 520045
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857 or equivalent
Qubit assay tubes	Life Technologies p/n Q32856
Magnetic separator	DynaMag-2 magnet, Life Technologies p/n 123-21D or equivalent
Nutator plate mixer	BD Diagnostics p/n 421105 or equivalent
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent

Table 2 Required Equipment for SureSelect^{XT} Methyl-Seq Target Enrichment

Description	Vendor and part number
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Water bath or heat block suitable for incubation temperatures up to 65°C	

2 Sample Preparation

This section contains instructions for gDNA library preparation for target enrichment for methyl-C sequence analysis using the Illumina platform. For each sample to be sequenced, an individual methylated adaptor-ligated library is prepared.

See Figure 1 for a summary of the overall SureSelect^{XT} Methyl-Seq workflow.

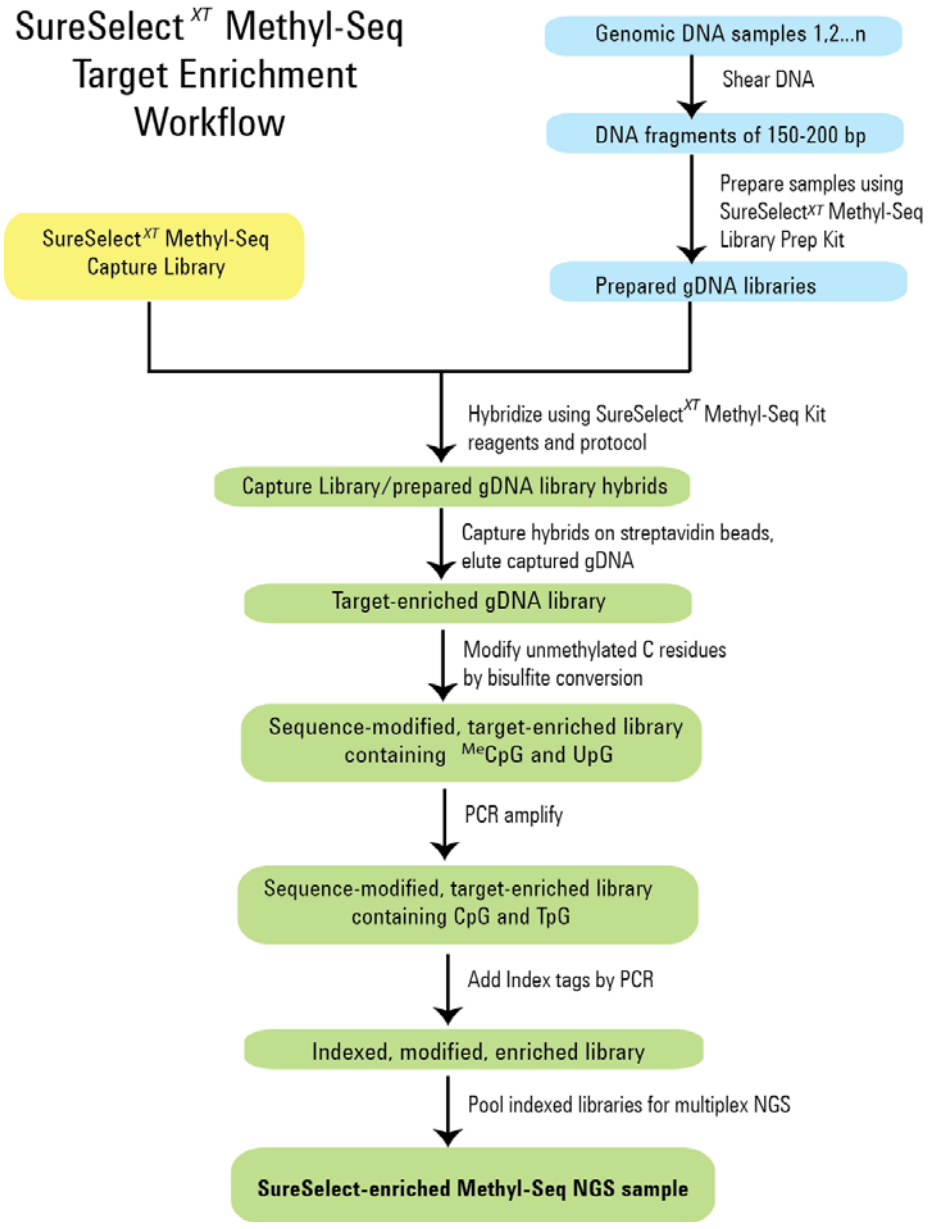

Figure 1 Overall sequencing sample preparation workflow.

Step 1. Shear DNA

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

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

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.

   Follow the instructions for the instrument.

2. Dilute 3 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 50 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 50-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 3 or Table 4, depending on the Covaris instrument SonoLab software version used.

The target DNA fragment size is 140 to 180 bp.

Table 3 Shear settings for Covaris instruments using SonoLab software version 7 or newer

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

Table 4 Shear settings for Covaris instruments using SonoLab software prior to version 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

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

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

9 Transfer the 50-µL sheared DNA sample to a fresh 1.5 mL LoBind tube.

Step 2. Repair the DNA ends

Hold samples on ice during step 1 below.

1 To each 50-µL sheared DNA sample, add 50 µL of XT2 End-Repair Master Mix.

Mix well by pipetting up and down or by gentle vortexing.

CAUTION SureSelect master mix solutions are viscous and thorough mixing is required to combine these mixtures with other solutions.

2 Incubate in a thermal cycler for 30 minutes at 20°C.

Do not use a heated lid.

Step 3. Purify sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to each 100-µL DNA sample in the tube. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the tube into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the tube in the magnetic stand while you add 200 µL of 70% ethanol into the tube

   Use fresh 70% ethanol for optimal results.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Dry the samples on a thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.

    Do not overdry the bead pellet. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
11. Add 22 µL nuclease-free water to each sample.
12. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.
13. Incubate for 2 minutes at room temperature.
14. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
15. Remove the cleared supernatant (approximately 22 µL) to a fresh LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, samples may be stored at –20°C overnight

Step 4. Assess quality with the Agilent 2100 Bioanalyzer DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit. See the *Agilent DNA 1000 Kit Guide*.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
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 DNA 1000 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 an average DNA fragment size of 150 to 200 bp. If the fragment size peak is >300 bp, repeat Step 1 (DNA shearing) to Step 4 (Bioanalyzer analysis).

   A sample electropherogram is shown in Figure 2.

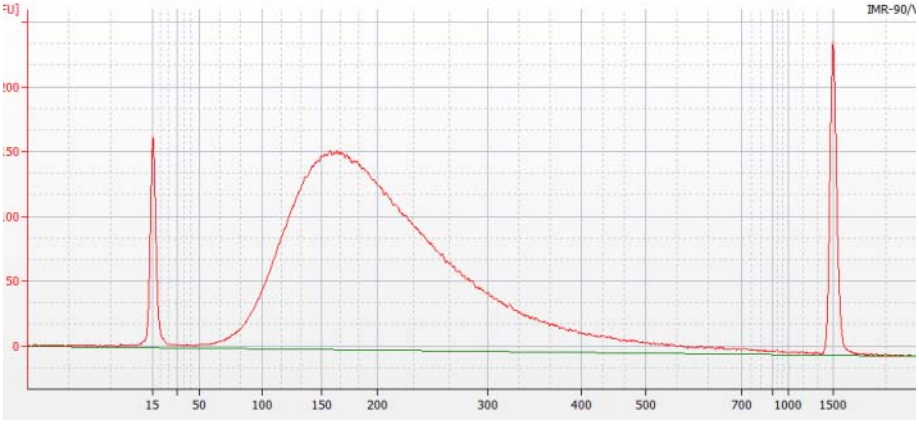

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows an average DNA fragment size between 150 to 200 bp.

Step 5. Adenylate the 3' end of the DNA fragments

1 To each end-repaired, purified DNA sample (approximately 20 µL), add 20 µL of XT2 dA-Tailing Master Mix.

   Mix well by pipetting up and down or by gentle vortexing.

2 Incubate in the SureCycler thermal cycler for 30 minutes at 37°C.

   Do not use a heated lid.

Step 6. Ligate the methylated adaptor

1. To each A-tailed DNA sample held on ice, add 5 µL of XT2 Ligation Master Mix and 5 µL of SureSelect Methyl-Seq Methylated Adaptor.

   Mix well by pipetting up and down or by gentle vortexing.

2. Incubate in the SureCycler thermal cycler for 15 minutes at 20°C.

   Do not use a heated lid.

   Do not exceed the 15 minute incubation time. Proceed immediately to free adaptor removal in Step 7. Purify the adaptor-ligated DNA using AMPure XP beads.

Step 7. Purify the adaptor-ligated DNA using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 60 µL of homogenous AMPure XP beads to each 50-µL adaptor-ligated DNA sample in the tube. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the tube into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the tube in the magnetic stand while you add 200 µL of 70% ethanol into the tube.

   Use fresh 70% ethanol for optimal results.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Dry the samples on a thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.

    Do not overdry the bead pellet. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
11. Add 22 µL nuclease-free water to each sample.
12. Mix well on a vortex mixer and briefly spin the tube to collect the liquid.
13. Incubate for 2 minutes at room temperature.
14. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
15. Remove the cleared supernatant (approximately 22 µL) to a fresh LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, samples may be stored at –20°C overnight.

Step 8. Assess quality and quantity with the Agilent 2100 Bioanalyzer DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit to analyze samples of adaptor-ligated DNA. See the *Agilent DNA 1000 Kit Guide* for more details to do this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
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 DNA 1000 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.
   a. Check that the electropherogram shows a single peak with an average DNA fragment size of approximately 200 to 250 bp.
   b. Integrate under the peak to determine the yield of adaptor-ligated DNA. If the yield is <500 ng, prepare additional adaptor-ligated DNA by repeating Step 1 (DNA shearing) to Step 8 (Bioanalyzer analysis).

A sample electropherogram is shown in Figure 3.

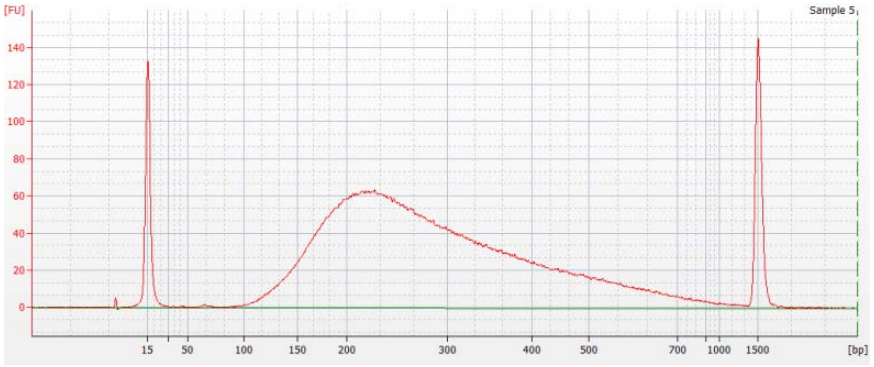

Figure 3 Analysis of adaptor-ligated DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows an average DNA fragment size of 200 to 250 bp.

3 Hybridization

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

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

Step 1: Hybridize the library

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.

The hybridization reaction requires at least 500 ng of adaptor-ligated DNA in a maximum volume of 3.4 µL, for a final concentration of ≥147 ng/µL.

If you have more than 500 ng DNA, use all of the adaptor-ligated DNA in the hybridization reaction to increase capture performance.

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 prepped library to an Eppendorf tube (up to the entire 20 µL sample volume). 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** Dehydrate using a vacuum concentrator on low heat (less than 45°C).
   - **c** Reconstitute with nuclease-free water to a final volume of 3.4 µL. Pipette up and down along the sides of the tube for optimal recovery.
   - **d** Mix well on a vortex mixer and spin in a centrifuge for 1 minute.

2 Mix the components in Table 5 at room temperature to prepare the hybridization buffer.

Table 5 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	25	125	250
SureSelect Hyb #2	1	5	10
SureSelect Hyb #3	10	50	100
SureSelect Hyb #4	13	65	130
Total	**49 (40 µL needed)**	**245 (40 µL/sample)**	**490 (40 µL/sample)**

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

4 Prepare the SureSelect capture library mix for target enrichment:

a Prepare the appropriate dilution of SureSelect RNAse block, based on the size of your capture library, according to Table 6. Using nuclease-free water, prepare the amount required for the number of hybridization reactions in the run, plus excess.

Table 6

Capture Library Size	RNase Block dilution (parts RNase Block:parts water)	Volume of dilute RNase Block Required per hybridization reaction
<3.0 Mb	10% (1:9)	5 µL
>3.0 Mb	25% (1:3)	2 µL

b In a PCR plate (kept on ice), for each hybridization reaction well, combine the indicated volumes of SureSelect Capture Library and

dilute RNase Block, according to Table 7. Mix well by pipetting.

Table 7

Capture Library Size	Volume of Capture Library per hybridization reaction	Volume of dilute RNase Block per hybridization reaction
<3.0 Mb	2 µL	5 µL of 10% solution
>3.0 Mb	5 µL	2 µL of 25% solution

5 Mix the components in Table 8 to prepare the SureSelect Block mix.

Table 8 SureSelect Methyl-Seq Block Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
SureSelect Indexing Block #1	2.5 µL	31.25 µL
SureSelect Block #2	2.5 µL	31.25 µL
SureSelect Methyl-Seq Block #3	0.6 µL	7.5 µL
Total	**5.6 µL**	**70 µL**

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

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

   b Add 5.6 µL of the SureSelect Methyl-Seq Block Mix (prepared in step 5) 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; heat only the prepped library with blockers.

   e Run the following thermal cycler program in Table 9.

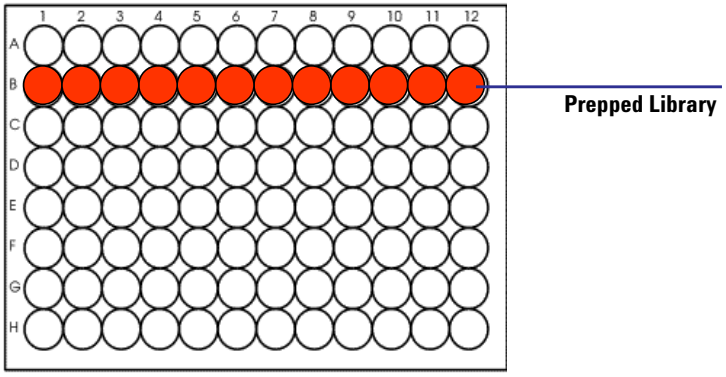

Figure 4 Prepped library shown in red

Table 9 Thermal cycler program

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

7 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.

8 Maintain the plate at 65°C while you load 40 µL of hybridization buffer (prepared in step 2) 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 5 is for 12 captures.

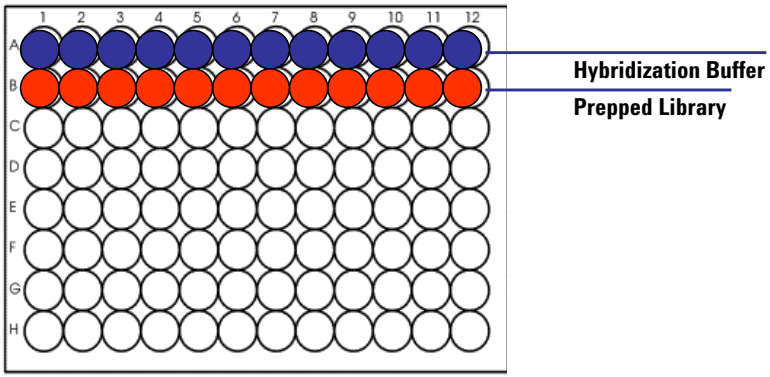

Figure 5 Hybridization buffer shown in blue

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

9 Add the capture library mix from step 4 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.

10 Maintain the plate at 65°C while you use a multi-channel pipette to take 13 µL of Hybridization Buffer from the "A" row and add it to the SureSelect capture library mix contained in row "C" of the PCR plate for each sample. (See Figure 6.)

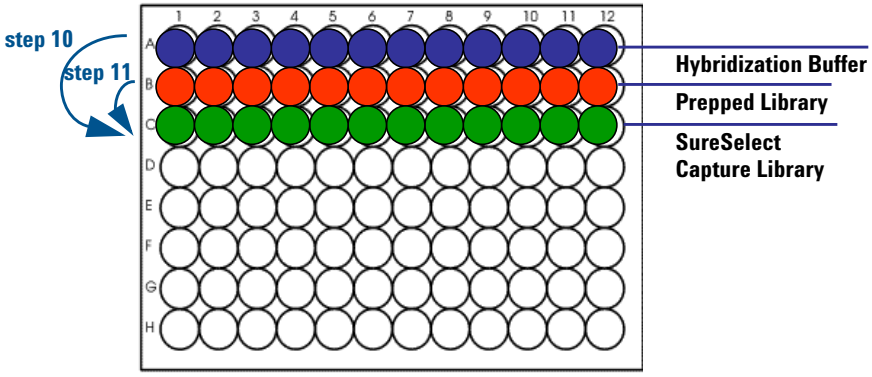

Figure 6 SureSelect Capture Library, or "Baits", shown in Green

11 Maintain the plate at 65°C while you use a multi-channel pipette to transfer the entire contents of each prepped library mix in row "B" to the hybridization solution in row "C". (See Figure 6.) 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.

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

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.

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

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

Step 2. Prepare streptavidin beads

1. Prewarm SureSelect Wash Buffer #2 at 65°C in a circulating water bath for use in "Step 3. Capture hybrids using streptavidin beads".
2. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. Magnetic 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 and allow the solution to clear.
   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. Capture hybrids using streptavidin beads

1. Estimate and record the volume of hybridization solution that remains after the 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.
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.
8. Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.
9. Wash the beads:
   a. Resuspend the beads in 500 µL of 65°C-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 in a recirculating water bath, heat block or equivalent.

      Do not use a tissue incubator. It cannot properly maintain temperature.
   c. Briefly spin in a centrifuge.
   d. Separate the beads and buffer on a 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.
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 Dynal 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. Desalt the capture solution with a Qiagen MinElute column

1. Allow the MinElute column (stored at 4°C) to come to room temperature.
2. Add 500 µL of PB to the 100-µL neutralized DNA sample and mix well by pipetting.
3. Check for the yellow color to make sure buffer PB pH is correct.

   For more information on how to check buffer pH, refer to the Qiagen MinElute Handbook.
4. Put the MinElute spin column in a 2 mL collection tube.
5. Transfer the 600 µL sample to the MinElute column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g. Discard the flow-through, then place the column back in the same collection tube.
6. Wash the column by adding 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 × g. Discard the flow-through.
7. Put the MinElute column back in the 2 mL collection tube and spin in a centrifuge for an additional 60 seconds at 17,900 × g.
8. Air-dry the column for 2 minutes to completely evaporate residual ethanol.

   All traces of ethanol must be removed.
9. Transfer the MinElute column to a new 1.5-mL collection tube to elute the cleaned sample.
10. Add 20 µL Buffer EB directly onto the center of the MinElute filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 × g (13,000 rpm).

Stopping Point If you do not continue to the next step, store the purified DNAsample in Elution Buffer (EB) at -20°C.

4 Bisulfite Conversion

This chapter describes the steps for bisulfite treatment of the captured DNA library to differentiate methylated and unmethylated DNA segments. When treated with bisulfite, unmethylated cytosine residues in the library are converted to uracil residues. Methylated cytosine residues remain unmodified, resulting in a primary sequence difference that may be detected and quantified in subsequent NGS analysis.

After desulphonation, the treated DNA is precisely quantified by QPCR. Treated DNA is then amplified by PCR, converting uracil residues in the sample to thymidine, using a limited number of PCR cycles to minimize PCR-based bias.

Step 1. Modify captured DNA by bisulfite conversion

In this step, you use reagents from Zymo Research's EZ DNA Methylation-Gold Kit to modify unmethylated cytosine residues in the captured DNA library to uracil residues by bisulfite conversion. The treated DNA is then desulphonated using a Zymo-Spin IC column and additional reagents from the EZ DNA Methylation-Gold Kit.

1. Resuspend one vial of solid CT Conversion Reagent by adding 900 µL of nuclease-free water, 300 µL of M-Dilution Buffer, and 50 µL of M-Dissolving Buffer to the vial.
2. Mix for 10 minutes with frequent vortexing at room temperature.
3. To each 20-µL captured library sample, add 130 µL of the CT Conversion Reagent from step 2. Mix by brief vortexing, then briefly spin in a centrifuge.
4. Transfer 75 µL of the mixture to each of two PCR tubes.
5. Place the tubes in a thermal cycler and run the program in Table 10.

   This program includes both denaturation and bisulfite conversion steps.

NOTE After completing the bisulfite conversion step at 64°C, proceed immediately to desulphonation in step 6. Do not maintain the sample at 4°C for an extended period.

Table 10 Thermal cycler program for bisulfite conversion

Step	Temperature	Time
Step 1	98°C	10 minutes
Step 2	64°C	2.5 hours
Step 3	4°C	Hold

6. Desulphonate the sample using a Zymo-Spin IC column. Use one column for each 150-µL DNA sample, after recombining the two 75-µL bisulfite conversion reactions for each DNA library.

Before starting the desulphonation procedure, make sure that the M-Wash buffer provided with the EZ DNA Methylation-Gold Kit has been prepared to contain 80% ethanol, according to the kit instructions.

a Add 600 µL of M-Binding Buffer to a Zymo-Spin IC column and place the column in a collection tube.

b Load the 150-µL bisulfite-converted DNA sample onto the column.

c Cap the column and mix well by inverting the column five times. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through, then place the column back in the same collection tube.

d Wash the column by adding 100 µL of prepared M-Wash Buffer. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through, then place the column back in the same collection tube.

e Add 200 µL of M-Desulphonation Buffer to the column. Incubate at room temperature for 20 minutes.

f Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through, then place the column back in the same collection tube.

g Add 200 µL of prepared M-Wash Buffer to the column. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through, then place the column back in the same collection tube.

h Add another 200 µL of prepared M-Wash Buffer to the column. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through, then place the column back in the same collection tube.

i Allow the column to sit at room temperature for 2 minutes.

j Place the column in a fresh 1.5-mL tube.

k Add 10 µL of M-Elution Buffer to the column and incubate at room temperature for 3 minutes.

l Centrifuge for 60 seconds at 13,000 rpm.

m While retaining the flow-through, add an additional 10 µL of M-Elution Buffer to the column. Incubate at room temperature for 3 minutes.

n Centrifuge for 60 seconds at 13,000 rpm. Retain the combined 20-µL flow-through for further processing.

Step 2. Quantify bisulfite-treated DNA samples by QPCR

Determine the DNA concentration in each bisulfite-treated library using the Agilent QPCR NGS Library Quantification Kit (for Illumina, p/n G4880A). Refer to the QPCR NGS Library Quantification Kit protocol for more details to do this step.

1 Prepare a standard curve, including five 2-fold serial dilutions of the quantification standard included in the kit, covering the range of 10 pM to 0.63 pM.

   Dilute the DNA standard from the kit using 1× Dilution Buffer according to the instructions provided in the user guide.

2 Prepare a 100-fold dilution of the bisulfite-converted DNA by mixing 1 µL of the DNA sample with 99 µL of 1× Dilution Buffer from the QPCR NGS Library Quantification Kit.

3 Prepare the QPCR master mix with Methyl-Seq adaptor-specific PCR primers according to Table 11. Prepare enough master mix to run each standard and each library sample in duplicate.

Table 11 QPCR Master Mix

Reagent	Volume for 1 reaction	Volume for 20 reactions (includes excess)
Nuclease-free water	5.3 µL	111.3 µL
2× Brilliant III Master Mix*	10 µL	210 µL
10× GC Additive	2 µL	42 µL
Methyl-Seq PCR1 Primer F	0.2 µL	4.2 µL
Methyl-Seq PCR1 Primer R	0.2 µL	4.2 µL
Diluted Reference Dye†	0.3 µL	6.3 µL
Total	**18 µL**	**378 µL**

* The master mix contains SYBR Green dye, which is light-sensitive. Keep solutions containing the master mix protected from light whenever possible.

† Dilute the reference dye in PCR-grade water using a dilution factor appropriate for your QPCR platform. For Agilent's Mx3000P or Mx3005P QPCR platform, dilute the Reference Dye 1:500 before use. Keep all solutions containing reference dye protected from light.

4 After vortexing the master mix briefly, dispense 18 µL of the master mix into the wells of a PCR plate or tubes.

5 Add 2 µL of template DNA to the master mix in each well or tube.

Run duplicate samples of all standard dilutions and library dilutions, along with the appropriate controls.

6 Place the plate or tubes in a QPCR instrument, such as the Mx3005p, run the thermal profile in Table 12. Use the SYBR Green instrument setting. Note that a dissociation step following amplification (segment 2) is not necessary.

Table 12 QPCR Thermal Profile

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	30	95°C	30 seconds
		60°C	30 seconds
		72°C	30 seconds

7 Use the standard curve to determine the concentration of each unknown captured library pool, in pM.

NOTE The thermal profile in Table 12 was optimized using Agilent's Mx3000P/Mx3005P system. Some optimization of cycling parameter may be required when using another instrument. Library quantification results are satisfactory when the standard curve yields an R-squared value >0.98 and an amplification efficiency of 90-110%.

The concentration determined by QPCR is used to determine the appropriate cycle number for post-capture amplification.

NOTE If the final concentration of bisulfite-treated library is less than 200 pM, the sample may not have enough complexity to produce high-quality sequencing data. Consider optimizing the library preparation and hybridization steps before proceeding.

Step 3. PCR amplify the bisulfite-treated libraries

In this step, the SureSelect-enriched and bisulfite-converted libraries are PCR amplified using cycling conditions designed to prepare the required amount of DNA library using the minimal number of PCR cycles. Plan your experiments for amplification of libraries that require the same cycle number on the same plate. See Table 15 for cycle number recommendations based on the starting concentration of your bisulfite-converted library (determined by QPCR, as described in "Step 2. Quantify bisulfite-treated DNA samples by QPCR" on page 42).

Prepare 1 amplification reaction for each bisulfite-treated library.

1 Prepare the appropriate volume of PCR reaction mixture, according to Table 13. Mix well using a vortex mixer and keep on ice.

Table 13 Preparation of Post-Capture PCR Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 10 Reactions (includes excess)	Volume for 24 Reactions (includes excess)
Nuclease-free water	30 µL	330 µL	750 µL
SureSelect Methyl-Seq 2× Taq2000 Master Mix	50 µL	550 µL	1250 µL
Methyl-Seq PCR1 Primer F	1 µL	11 µL	25 µL
Methyl-Seq PCR1 Primer R	1 µL	11 µL	25 µL
Total Volume	**82 µL**	**902 µL**	**2050 µL**

2 For each amplification reaction, place 82 µL of the PCR reaction mixture from step 1 in the wells of a PCR plate.

3 Add 18 µL of each bisulfite-converted library to the appropriate PCR reaction mixture well. Mix thoroughly by pipetting.

4 Place the plate in a thermal cycler and run the PCR amplification program shown in Table 14 using the cycle number specified in Table 15.

Table 14 Bisulfite-converted library amplification PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	7-9 see Table 15	95°C	30 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	7 minutes
4	1	4°C	Hold

Table 15 Recommended cycle number based on initial library concentration

Concentration of Bisulfite-Converted Library	Cycles
>800 pM	7 cycles
400 to 800 pM	8 cycles
200 to 400 pM	9 cycles

NOTE

Samples prepared at ≤200 pM may not have enough complexity to produce high-quality sequencing data. Consider optimizing the library preparation and hybridization steps before proceeding in order to increase the library complexity in the final sequencing samples.

Step 4. Purify the amplified bisulfite-treated libraries using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to each 100-µL amplified library in a 1.5-mL tube. Mix by pipetting up and down.
4. Incubate for 5 minutes at room temperature.
5. Put the tube into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution. Do not touch the beads while removing the solution.
7. Continue to keep the tube in the magnetic stand while you add 500 µL of 70% ethanol.

   Use fresh 70% ethanol for optimal results.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 7 minutes or until the residual ethanol completely evaporates.
11. Add 26 µL nuclease-free water to each sample.
12. Mix well on a vortex mixer. Briefly spin the tube in a centrifuge to collect the liquid.
13. Incubate for 2 minutes at room temperature.
14. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
15. Remove the cleared supernatant (approximately 26 µL) to a fresh LoBind tube. You can discard the beads at this time.
16. Reserve 2 µL for Bioanalyzer analysis after sample indexing on page 51.

5 Indexing and Sample Pooling for Multiplexed Sequencing

This chapter describes the steps to index the captured DNA libraries that were modified by bisulfite conversion and to pool the indexed samples for multiplexed sequence analysis.

Step 1. Index the modified libraries by PCR amplification

CAUTION To avoid cross-contamination of 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 indexing amplification reaction for each amplified bisulfite-converted library.

1 Determine the appropriate index assignments for the samples. See Table 22 for nucleotide sequence information for the index portion of SureSelect PCR Primer Indexes 1–16.

For the Illumina HiSeq system used with the TruSeq PE Cluster Kit (v. 3.0) the optimal number of indexes per lane is two. Refer to the appropriate Illumina protocol to determine optimal index density for other systems.

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.

Table 16

Number of Pooled Samples	Recommended indexes
2	Index #6 GCCAAT Index #12 CTTGTA
3	Index #4 TGACCA Index #6 GCCAAT Index #12 CTTGTA
6	Index #2 CGATGT Index #4 TGACCA Index #5 ACAGTG Index #6 GCCAAT Index #7 CAGATC Index #12 CTTGTA

2 Prepare the appropriate volume of PCR reaction mixture, according to Table 17. Mix well using a vortex mixer and keep on ice.

Table 17 Preparation of PCR Indexing Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 10 Reactions (includes excess)	Volume for 24 Reactions (includes excess)
SureSelect Methyl-Seq 2× Taq2000 Master Mix	25 µL	275 µL	525 µL
SureSelect Methyl-Seq Indexing Primer Common	0.5 µL	5.5 µL	12.5 µL
Total Volume	**25.5 µL**	**280.5 µL**	**537.5 µL**

3 For each amplification reaction, place 25.5 µL of the PCR indexing reaction mixture from step 2 in the wells of a PCR plate.

4 Add 0.5 µL of the appropriate PCR Primer Index 1 through 16 to the appropriate PCR reaction mixture well. See Table 16 for index selection guidelines.

5 Add 24 µL of amplified, bisulfite-converted library to the appropriate PCR indexing reaction mixture well. Mix thoroughly by pipetting.

6 Place the plate in a thermal cycler and run the PCR amplification program shown in Table 18.

Table 18 PCR indexing cycling program

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	4	95°C	30 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	7 minutes
4	1	4°C	Hold

Step 2. Purify the indexed libraries using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to each 50-µL amplified library in a 1.5-mL tube. Mix by pipetting up and down.
4. Incubate for 5 minutes at room temperature.
5. Put the tube into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the tube in the magnetic stand. Carefully remove and discard the cleared solution. Do not touch the beads while removing the solution.
7. Continue to keep the tube in the magnetic stand while you add 500 µL of 70% ethanol.

   Use fresh 70% ethanol for optimal results.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.
11. Add 24 µL nuclease-free water to each sample.
12. Mix well on a vortex mixer. Briefly spin the tube in a centrifuge to collect the liquid.
13. Incubate for 2 minutes at room temperature.
14. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
15. Remove the cleared supernatant (approximately 24 µL) to a fresh 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 Agilent 2100 Bioanalyzer High Sensitivity DNA assay

Use a Bioanalyzer High Sensitivity DNA Assay to assess the quality and quantity of the library samples after indexing (using sample from page 50) compared to before indexing (using the sample retained for analysis on page 46).

For more information to do this step, 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, using 1 µL of each sample for the analysis.
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 High Sensitivity DNA 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 fragment size peak between approximately 220 to 280 bp. A sample electropherogram is shown in Figure 7.

   If a significant primer-dimer peak is observed in one or more of the indexed library samples, an additional round of purification is required after samples are pooled for sequencing.
8. Determine the concentration of each library before and after indexing by integration under the peaks in the electropherograms.

   Verify that the total amount of indexed library DNA is at least three-fold greater than the amount in the pre-indexing sample to ensure quantitative index tagging of the library.

Use the quantities of indexed libraries determined at this step to pool samples for Illumina sequencing. The final concentration of each indexed library should be approximately 2 nM.

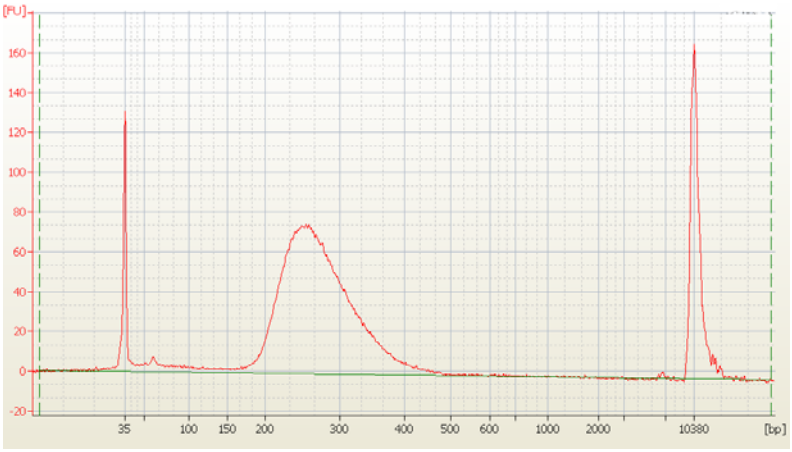

Figure 7 Analysis of indexed DNA sample using a DNA 1000 Bioanalyzer assay. The electropherogram shows an average DNA fragment size of 220 to 280 bp.

Step 4. Pool indexed libraries for sequencing

1 Combine the indexed DNA samples such that each index-tagged sample is present in equimolar amounts in the final sequencing sample pool. For each final pool, use the formula below to determine the amount of each capture pool to use.

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

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

C(f) is the desired final concentration of all the DNA in the pool, for example, 2 nM for the Methyl-Seq sequencing protocol,

is the number of capture pool samples to be combined, and

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

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.

Table 19 shows an example of the amount of 2 capture pool samples and Low TE needed for a final volume of 25 µL at 2 nM final DNA concentration.

Table 19 Example of indexed Methyl-Seq sample volume calculations for a 25-µL final sequencing sample pool containing 2 nM DNA

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	25 µL	2.0 nM	2 nM	2	12.5
Sample 2	25 µL	2.2 nM	2 nM	2	11.4
Low TE					1.1

3 If a significant primer-dimer peak was observed for any of the indexed libraries added to the pool, repeat "Step 2. Purify the indexed libraries using AMPure XP beads" on page 50, using 45 µL of AMPure XP bead suspension for each 25-µL sequencing sample. Elute the purified DNA in 25 µL of nuclease-free water.

Step 5. Analyze the indexed DNA pool with the Agilent 2100 Bioanalyzer High Sensitivity DNA assay

Use a Bioanalyzer High Sensitivity DNA Assay 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.02.07 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, using 1 µL of indexed library pool for the analysis.
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 High Sensitivity DNA 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 single peak between approximately 220 to 280 bp. A sample electropherogram is shown in Figure 8.
8. Determine the concentration of the indexed library pool by integration under the peak in the electropherogram.

   The final concentration of the indexed DNA pool should be 2 nM.
9. Proceed to template denaturation and flow cell preparation. Refer to the appropriate Illumina protocol.

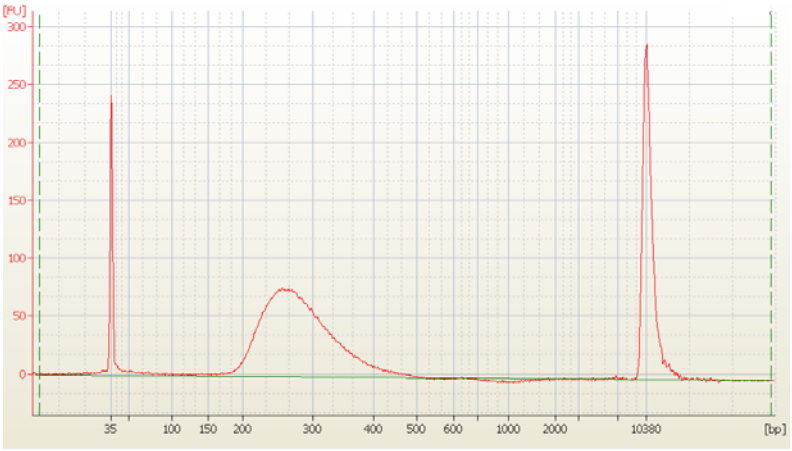

Figure 8 Analysis of indexed DNA pool using the High Sensitivity Bioanalyzer DNA Kit. The electropherogram shows a single peak in the size range of 220 to 280 bp.

Specific 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.

NOTE

Use the following sequencing guidelines for sequencing the Methyl-Seq samples:

- Optimize the seed concentration between 10 and 30 pM. Start with 10 pM and increase the concentration if the cluster density is lower than expected. For example, denature DNA by mixing 10 µL of 2 nM indexed library pool with 10 µL of 0.1 N NaOH. Afterward, dilute 10 µL of the 1 nM denatured DNA with 990 µL of HT1 buffer (hybridization buffer) for a final concentration of 10 pM.
- The base composition of the Methyl-Seq samples is biased. Run a control lane using a sample with unbiased base composition, such as PhiX DNA, and designate this control lane in the HiSeq Control Software.

6 Reference

This chapter contains component kit contents reference information.

Kit Contents

Each SureSelect^{XT} Methyl-Seq Kit contains the following component kits:

Table 20 SureSelect^{XT} Methyl-Seq Kit Contents

Product	Storage Condition
SureSelect Methyl-Seq Library Prep Kit*	-20°C
SureSelect Methyl-Seq Target Enrichment Box #1*	Room Temperature
SureSelect Methyl-Seq Hybridization Kit Box #2*	-20°C
SureSelect Human Methyl-Seq Capture Library	-80°C

* See Table 21 for list of included reagents.

The contents of the component kits listed in Table 20 are detailed in the table below.

Table 21 SureSelect^{XT} Methyl-Seq Component Kit Contents

Component Kit	Included Reagents
SureSelect Methyl-Seq Library Prep Kit (p/n 5500-0107 / 5500-0108)	XT2 End-Repair Master Mix
	XT2 dA-Tailing Master Mix
	XT2 Ligation Master Mix
	SureSelect Methyl-Seq 2× Taq2000 Master Mix
	SureSelect Methyl-Seq Methylated Adapter
	SureSelect Methyl-Seq PCR1 Primer F
	SureSelect Methyl-Seq PCR1 Primer R
	SureSelect Methyl-Seq Indexing Primer Common
	PCR Primer Indexes 1–16
SureSelect Methyl-Seq Target Enrichment Box #1 (p/n 5190-5000 / 5190-5002)	SureSelect Hyb # 1
	SureSelect Hyb # 2
	SureSelect Hyb # 4
	SureSelect Binding Buffer
	SureSelect Wash Buffer #1
	SureSelect Wash Buffer #2
	SureSelect Elution Buffer
	SureSelect Neutralization Buffer
SureSelect Methyl-Seq Hybridization Kit Box #2 (p/n 5190-5001/ 5190-5003)	SureSelect Indexing Block #1
	SureSelect Block #2
	SureSelect Methyl-Seq Block #3
	SureSelect Hyb #3
	SureSelect RNase Block

Nucleotide Sequences of SureSelect Indexes

The nucleotide sequence of each SureSelect^{XT} Index provided with SureSelect^{XT} Methyl-Seq kits is provided in the table below.

Table 22 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

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In This Book

This guide contains information to run the SureSelect^{XT} Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing protocol.

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