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

Protocol

Version E0, April 2018

SureSelect platform manufactured with Agilent SurePrint Technology

For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies

Notices

Manual Part Number

G7530-90002

Edition

Version E0, April 2018

Printed in USA

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In this Guide...

This guide describes an optimized protocol for DNA methylation analysis using the 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 (3 µg DNA Samples)

This chapter describes the steps to prepare libraries for target enrichment from 3-µg gDNA samples.

3 Sample Preparation (1 µg DNA Samples)

This chapter describes the steps to prepare libraries for target enrichment from 1-µg gDNA samples.

4 Hybridization

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

5 Bisulfite Conversion

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

6 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.

7 Reference

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

What's New in Version E0

- Support for use of freshly-prepared 0.1 M NaOH, instead of SureSelect Elution Buffer, for elution of captured library samples from Streptavidin T1 magnetic beads. See concentrated NaOH supplier information in Table 1 on page 10, instructions for preparation and use of 0.1 M NaOH on page 53 and note on page 9, and revised kit configuration details in Table 34 on page 78.
- Information on use of non-supported Capture Libraries (see page 10 and page 49).
- Support for Agilent 4200 TapeStation (see Table 2 on page 12 and revised instructions on page 21, page 27, page 37, page 44, page 68)
- Updates to Agilent 2100 Bioanalyzer system ordering information and use instructions (see Table 2 on page 12 and revised instructions on page 20, page 26, page 36, page 43, page 66).
- Updates to product guarantee and support statement (see *Note* on page 7).
- Updates to description of dA-tailing step (see page 22 and page 38). This is a description-only update with no changes to library preparation materials or conditions.
- Updates to ordering information for materials purchased from Thermo Fisher Scientific (see Table 1 on page 10 and Table 2 on page 11)
- Updates to Reference chapter to remove information in on kits containing discontinued indexing primers 1–16 (typically received before January, 2015; provided in clear-capped tubes). To obtain sequence information or other support for the discontinued components, contact ngs.support@agilent.com.
- Updates to Technical Support contact information (see page 2).

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 guarantees performance and provides technical support for the SureSelect reagents required for this workflow only when used as directed in this Protocol.

Overview of the Workflow

The SureSelect^{XT} Methyl-Seq target enrichment workflow is summarized in Figure 1.

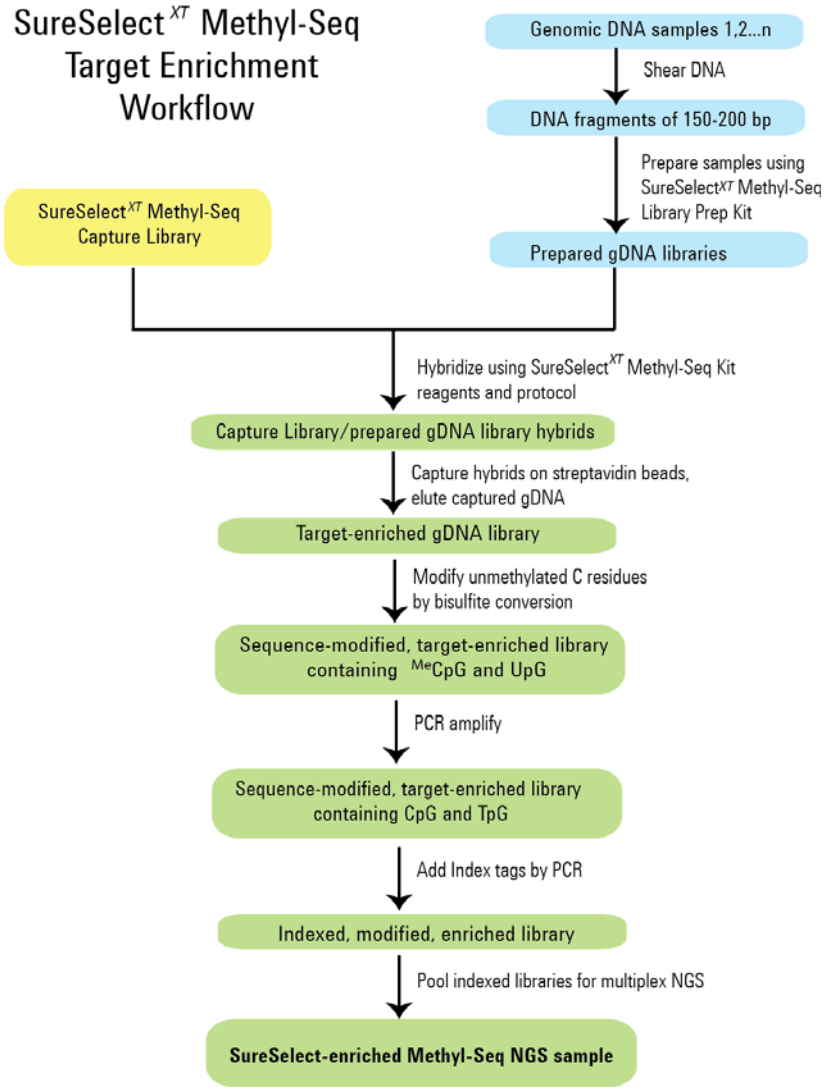

Figure 1 Overall target-enriched sequencing sample preparation workflow.

Procedural Notes

- Prolonged exposure of solutions of 0.1 M NaOH to air can decrease product performance by altering the pH of the solution. Prepare a fresh solution of 0.1 M NaOH for each run and keep containers of NaOH 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.
- 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.
- 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
480 reactions	p/n G9651C
SureSelect^{XT} Human Methyl-Seq Capture Library*	Agilent
16 reactions	p/n 5190-4661
96 reactions	p/n 5190-4662
480 reactions	p/n 5190-4663
EZ-DNA Methylation-Gold Kit	Zymo Research
50 reactions	p/n D5005
200 reactions	p/n D5006
10 M NaOH, molecular biology grade	Sigma, p/n 72068
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090015, or equivalent
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 mL	p/n 65601
10 mL	p/n 65602
50 mL	p/n 65604D
Qubit BR dsDNA Assay Kit	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

* Use of other SureSelect Capture Libraries is not supported by this protocol and requires optimization of hybridization and post-capture PCR conditions.

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 plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
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	Thermo Fisher Scientific p/n Q33226 or equivalent
Qubit assay tubes	Thermo Fisher Scientific p/n Q32856
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, 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
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent*
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
DNA Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200 TapeStation†	Agilent p/n G2991AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585

* Select a magnetic separator configured to collect magnetic particles on one side of each well. Do not use a magnetic separator configured to collect the particles in a ring formation.

† DNA samples may also be analyzed using the Agilent 2200 TapeStation, p/n G2964AA or G2965AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.

2 Sample Preparation (3 µg DNA Samples)

CAUTION This section contains instructions for the preparation of gDNA libraries from 3 µg DNA samples. **For lower input (1 µg) DNA samples, see the library preparation protocol on page 29.**

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 adapter-ligated library is prepared.

Step 1. Shear DNA

Before you begin, you can use the 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 E-series or S-series instrument.

   a. Check that the Covaris water tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate type in use.

   b. Check that the water covers the visible glass part of the tube.

   c. On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes, or according to the manufacturer's recommendations.

   d. Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 4°C.

   e. *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide.

4. Put a Covaris microTube into the loading and unloading station.

   Keep the cap on the tube.

NOTE When using a Covaris E-series instrument to prepare multiple gDNA samples in the same experiment, you can also use the 96 microTube plate (see Table 2 on page 11) for the DNA shearing step.

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.

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 48 µL of each sheared DNA sample to a separate well of a 96-well plate or strip tube.

10 **Optional:** Assess sample quality and quantity using the 2100 Bioanalyzer system and DNA 1000 Assay, as described on page 20, or using the 4200 TapeStation, as described on page 21.

Verify that the electropherogram shows a DNA fragment size peak between 100–175 bp.

Step 2. Repair the DNA ends

Use the SureSelect Methyl-Seq Library Prep Kit for this step.

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 16 samples (including excess) are shown in each table as an example.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of End Repair master mix, as described in Table 5, on ice. Mix well on a vortex mixer.

Table 5 Preparation of End Repair master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	35.2 µL	580.8 µL
10× End Repair Buffer (clear cap)	10 µL	165 µL
dNTP Mix (green cap)	1.6 µL	26.4 µL
T4 DNA Polymerase (purple cap)	1 µL	16.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	33 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	36.3 µL
Total	**52 µL**	**858 µL**

2 Add 52 µL of the master mix to each sample well containing 48 µL of sheared DNA. Mix by vortexing for 5 seconds then spin the samples briefly to collect the liquid.

3 Incubate the samples in the thermal cycler and run the program in Table 6. Do not use a heated lid.

Table 6 End Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Step 3. Purify sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 1.2 mL of fresh 70% ethanol per sample.

3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 180 µL of homogeneous AMPure XP beads to each sample well containing 100 µL of end-repaired DNA. Pipette up and down 10 times to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 7 to 10 minutes).
7. Keep the plate or tube strip in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or tube strip in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 to step 9 step once.
11. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or tube strip to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.
13. Add 44 µL nuclease-free water to each sample well.

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the samples to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 42 µL) to a fresh well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells and store at –20°C.

Step 4. Assess quality

Quality assessment can be done with either the 2100 Bioanalyzer instrument or the 4200 TapeStation instrument.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

Use a DNA 1000 chip and reagent kit for 2100 Bioanalyzer analysis of the end-repaired DNA samples. For more information to do this step, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Check that the electropherogram shows a DNA fragment size peak between 125–175 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.

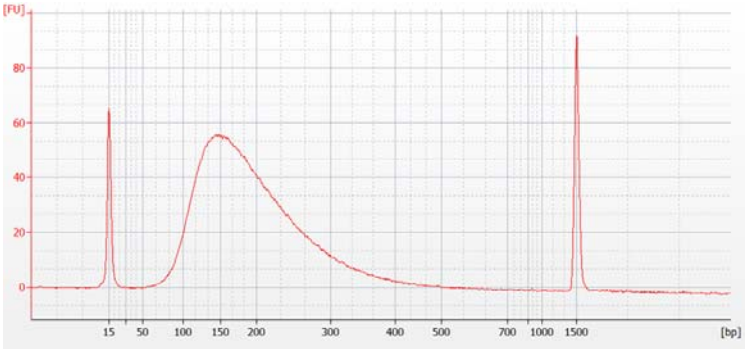

Figure 2 Analysis of end-repaired DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 4200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and D1000 Reagents for analysis of the end-repaired DNA samples using the 4200 TapeStation. For more information to do this step, see the TapeStation instrument user manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 4200 TapeStation as instructed in the instrument user manual. Start the run.

3 Verify that the electropherogram shows a DNA fragment size peak between 125–175 bp. A sample electropherogram is shown in Figure 3.

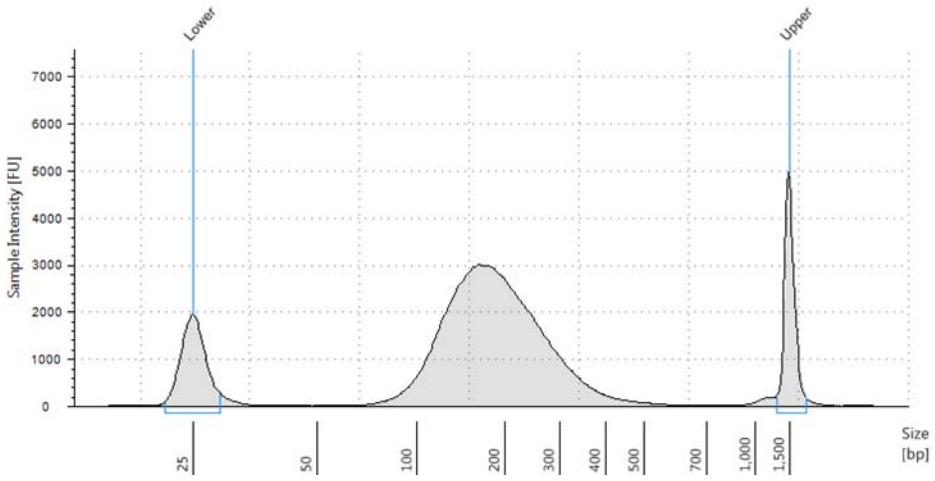

Figure 3 Analysis of end-repaired DNA using a D1000 ScreenTape.

Step 5. dA-Tail the 3' end of the DNA fragments

Use the SureSelect Methyl-Seq Library Prep Kit for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of dA-Tailing master mix, as described in Table 7, on ice. Mix well on a vortex mixer.

Table 7 Preparation of dA-Tailing master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
10× Klenow Polymerase Buffer (blue cap)	5 µL	82.5 µL
dATP (green cap)	1 µL	16.5 µL
Exo(–) Klenow (red cap)	3 µL	49.5 µL
Total	**9 µL**	**148.5 µL**

2 Dispense 9 µL of the dA-Tailing master mix into each sample well containing end-repaired, purified DNA (approximately 41 µL).

3 Mix by vortexing for 5 seconds then spin the samples briefly to collect the liquid.

4 Incubate the samples in the thermal cycler and run the program in Table 8. Do not use a heated lid.

Table 8 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 6. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL dA-tailed DNA sample well. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate or tube strip 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 samples in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate or tube strip to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 35 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or tube strip to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove 33.5 µL of the cleared supernatant to a fresh well. You can discard the beads at this time.
17. Proceed immediately to the next step, "Step 7. Ligate the methylated adapter" on page 24.

Step 7. Ligate the methylated adapter

Use the SureSelect Methyl-Seq Library Prep Kit for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Ligation master mix, as described in Table 9, on ice. Mix well on a vortex mixer.

Table 9 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect Methyl-Seq Methylated Adapter (green cap)	5 µL	82.5µL
5× T4 DNA Ligase Buffer (green cap)	10 µL	165 µL
T4 DNA Ligase (red cap)	1.5 µL	24.75 µL
Total	**16.5 µL**	**272.25 µL**

2 Dispense 16.5 µL of the Ligation master mix into each sample well containing dA-tailed, purified DNA (approximately 33.5 µL).

3 Mix by vortexing for 5 seconds then spin the samples briefly to collect the liquid.

4 Incubate the samples in the thermal cycler and run the program in Table 10. Do not use a heated lid.

Table 10 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Do not exceed the 15 minute incubation time. Proceed immediately to free adapter removal in Step 8. Purify the adapter-ligated DNA using AMPure XP beads.

Step 8. Purify the adapter-ligated DNA using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL adapter-ligated DNA sample well. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate or tube strip 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 samples in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
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. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or tube strip to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 22 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the samples to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 22 µL) to a fresh well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells and store at –20°C.

Step 9. Assess quality and quantity

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

See the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1 Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.

2 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.

3 Verify the results.

   a Check that the electropherogram shows a single peak with the DNA fragment size peak between 200–300 bp.

   b Integrate under the peak to determine the yield of adapter-ligated DNA. If the yield is <350 ng, prepare additional adapter-ligated DNA by repeating Step 1 (DNA shearing) to Step 9 (sample analysis).

   A sample electropherogram is shown in Figure 4.

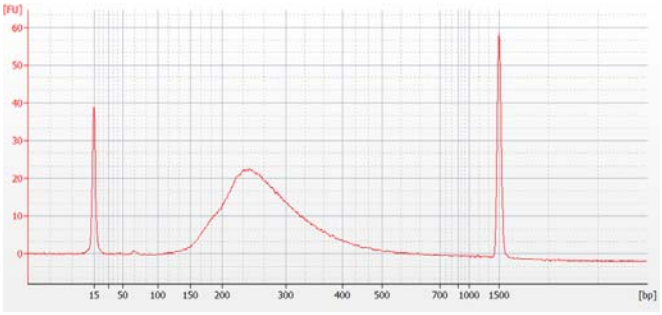

Figure 4 Analysis of adapter-ligated DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 4200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and D1000 Reagents for analysis of the end-repaired DNA samples using the 4200 TapeStation. For more information to do this step, see the TapeStation instrument user manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 4200 TapeStation as instructed in the instrument user manual. Start the run.

3 Verify the results.

a Check that the electropherogram shows a single peak with the DNA fragment size peak between 200–300 bp.

b Integrate under the peak to determine the yield of adapter-ligated DNA. If the yield is <350 ng, prepare additional adapter-ligated DNA by repeating Step 1 (DNA shearing) to Step 9 (sample analysis).

A sample electropherogram is shown in Figure 5.

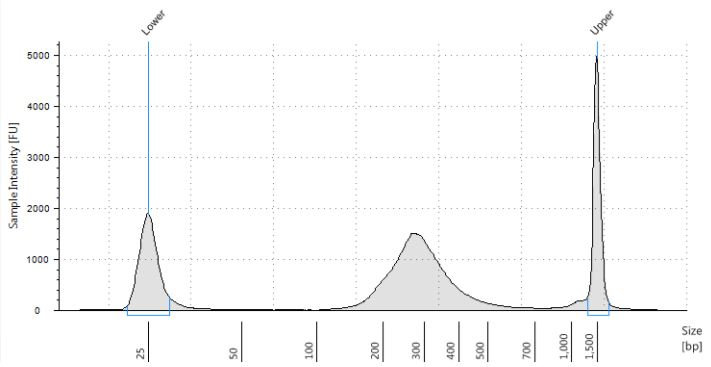

Figure 5 Analysis of adapter-ligated DNA using a D1000 ScreenTape.

3 Sample Preparation (1 µg DNA Samples)

CAUTION This section contains instructions for the preparation of gDNA libraries from 1 µg DNA samples. **For higher input (3 µg) DNA samples, see the library preparation protocol on page 13.**

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 adapter-ligated library is prepared.

Step 1. Shear DNA

Before you begin, you can use the 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 1 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 E-series or S-series instrument.

   a. Check that the Covaris water tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate type in use.

   b. Check that the water covers the visible glass part of the tube.

   c. On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes, or according to the manufacturer's recommendations.

   d. Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 4°C.

   e. *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide.

4. Put a Covaris microTube into the loading and unloading station.

   Keep the cap on the tube.

NOTE When using a Covaris E-series instrument to prepare multiple gDNA samples in the same experiment, you can also use the 96 microTube plate (see Table 2 on page 11) for the DNA shearing step.

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 11 or Table 12, depending on the Covaris instrument SonoLab software version used.

Table 11 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 12 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 48 µL of each sheared DNA sample to a separate well of a 96-well plate or strip tube.

10 **Optional:** Assess sample quality and quantity using the 2100 Bioanalyzer system and DNA 1000 Assay, as described on page 36, or using the 4200 TapeStation, as described on page 37.

Verify that the electropherogram shows a DNA fragment size peak between 100–175 bp.

Step 2. Repair the DNA ends

Use the SureSelect Methyl-Seq Library Prep Kit for this step.

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 16 samples (including excess) are shown in each table as an example.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of End Repair master mix, as described in Table 13, on ice. Mix well on a vortex mixer.

Table 13 Preparation of End Repair master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	35.2 µL	580.8 µL
10× End Repair Buffer (clear cap)	10 µL	165 µL
dNTP Mix (green cap)	1.6 µL	26.4 µL
T4 DNA Polymerase (purple cap)	1 µL	16.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	33 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	36.3 µL
Total	**52 µL**	**858 µL**

2 Add 52 µL of the master mix to each sample well containing 48 µL of sheared DNA. Mix by vortexing for 5 seconds then spin the samples briefly to collect the liquid.

3 Incubate the samples in the thermal cycler and run the program in Table 14. Do not use a heated lid.

Table 14 End Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Step 3. Purify sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 1.2 mL of fresh 70% ethanol per sample.

3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 180 µL of homogeneous AMPure XP beads to each sample well containing 100 µL of end-repaired DNA. Pipette up and down 10 times to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 7 to 10 minutes).
7. Keep the plate or tube strip in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or tube strip in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 to step 9 step once.
11. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or tube strip to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.
13. Add 44 µL nuclease-free water to each sample well.

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the samples to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 42 µL) to a fresh well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells and store at –20°C.

Step 4. Assess quality

Quality assessment can be done with either the 2100 Bioanalyzer instrument or the 4200 TapeStation instrument.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

Use a DNA 1000 chip and reagent kit for 2100 Bioanalyzer analysis of the end-repaired DNA samples. For more information to do this step, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Check that the electropherogram shows a DNA fragment size peak between 125–175 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 6.

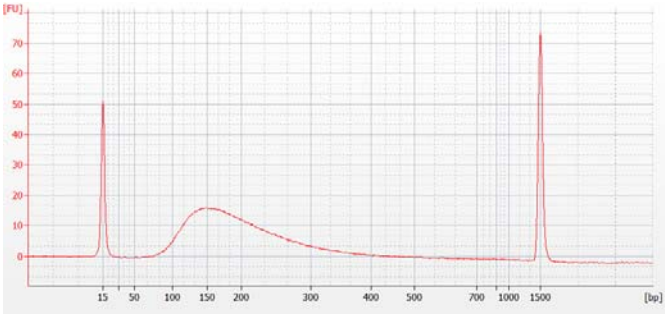

Figure 6 Analysis of end-repaired DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 4200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and D1000 Reagents for analysis of the end-repaired DNA samples using the 4200 TapeStation. For more information to do this step, see the TapeStation instrument user manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 4200 TapeStation as instructed in the instrument user manual. Start the run.

3 Verify that the electropherogram shows a DNA fragment size peak between 125–175 bp. A sample electropherogram is shown in Figure 7.

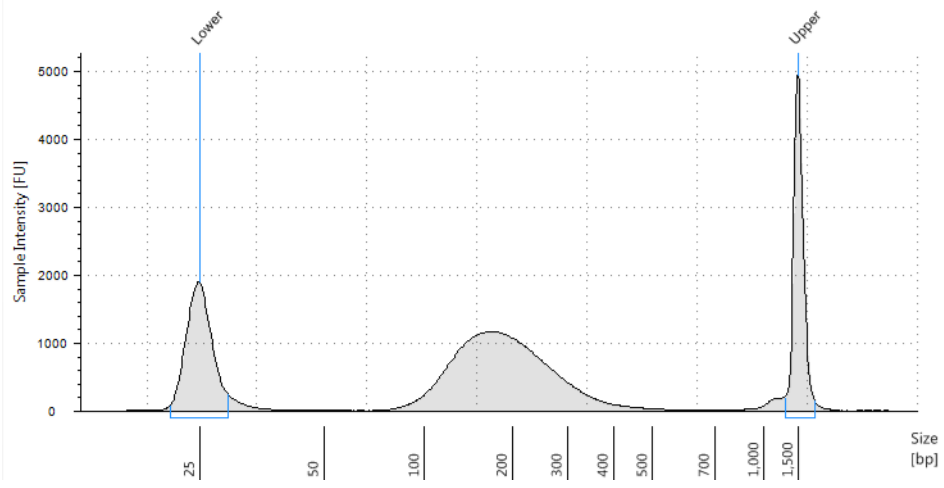

Figure 7 Analysis of end-repaired DNA using a D1000 ScreenTape.

Step 5. dA-Tail the 3' end of the DNA fragments

Use the SureSelect Methyl-Seq Library Prep Kit for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of dA-Tailing master mix, as described in Table 15, on ice. Mix well on a vortex mixer.

Table 15 Preparation of dA-Tailing master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
10× Klenow Polymerase Buffer (blue cap)	5 µL	82.5 µL
dATP (green cap)	1 µL	16.5 µL
Exo(–) Klenow (red cap)	3 µL	49.5 µL
Total	**9 µL**	**148.5 µL**

2 Dispense 9 µL of the dA-Tailing master mix into each sample well containing end-repaired, purified DNA (approximately 41 µL).

3 Mix by vortexing for 5 seconds then spin the samples briefly to collect the liquid.

4 Incubate the samples in the thermal cycler and run the program in Table 16. Do not use a heated lid.

Table 16 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 6. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL dA-tailed DNA sample well. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate or tube strip 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 samples in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate or tube strip to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 35 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or tube strip to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove 33.5 µL of the cleared supernatant to a fresh well. You can discard the beads at this time.
17. Proceed immediately to the next step, "Step 7. Ligate the methylated adapter" on page 40.

Step 7. Ligate the methylated adapter

Use the SureSelect Methyl-Seq Library Prep Kit for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Ligation master mix, as described in Table 17, on ice. Mix well on a vortex mixer.

Table 17 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	2.5 µL	41.25 µL
SureSelect Methyl-Seq Methylated Adapter (green cap)	2.5 µL	41.25µL
5× T4 DNA Ligase Buffer (green cap)	10 µL	165 µL
T4 DNA Ligase (red cap)	1.5 µL	24.75 µL
Total	**16.5 µL**	**272.25 µL**

2 Dispense 16.5 µL of the Ligation master mix into each sample well containing dA-tailed, purified DNA (approximately 33.5 µL).

3 Mix by vortexing for 5 seconds then spin the samples briefly to collect the liquid.

4 Incubate the samples in the thermal cycler and run the program in Table 18. Do not use a heated lid.

Table 18 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Do not exceed the 15 minute incubation time. Proceed immediately to free adapter removal in Step 8. Purify the adapter-ligated DNA using AMPure XP beads.

Step 8. Purify the adapter-ligated DNA using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL adapter-ligated DNA sample well. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate or tube strip 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 samples in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
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. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or tube strip to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 22 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the samples to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 22 µL) to a fresh well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells and store at –20°C.

Step 9. Assess quality and quantity

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

See the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1 Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.

2 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.

3 Verify the results.

   a Check that the electropherogram shows a single peak with the DNA fragment size peak between 200–300 bp.

   b Integrate under the peak to determine the yield of adapter-ligated DNA. If the yield is <350 ng, prepare additional adapter-ligated DNA by repeating Step 1 (DNA shearing) to Step 9 (sample analysis).

   A sample electropherogram is shown in Figure 8.

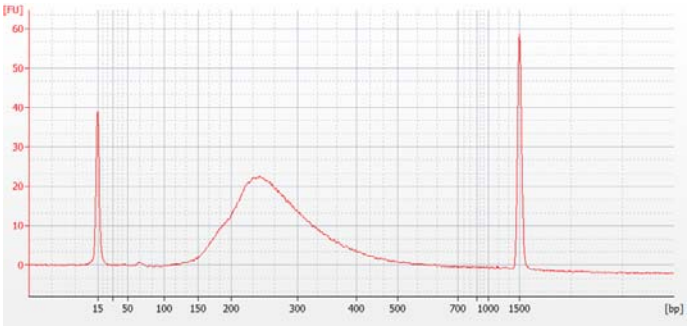

Figure 8 Analysis of adapter-ligated DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 4200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and D1000 Reagents for analysis of the end-repaired DNA samples using the 4200 TapeStation. For more information to do this step, see the TapeStation instrument user manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 4200 TapeStation as instructed in the instrument user manual. Start the run.

3 Verify the results.

a Check that the electropherogram shows a single peak with the DNA fragment size peak between 200–300 bp.

b Integrate under the peak to determine the yield of adapter-ligated DNA. If the yield is <350 ng, prepare additional adapter-ligated DNA by repeating Step 1 (DNA shearing) to Step 9 (sample analysis).

A sample electropherogram is shown in Figure 9.

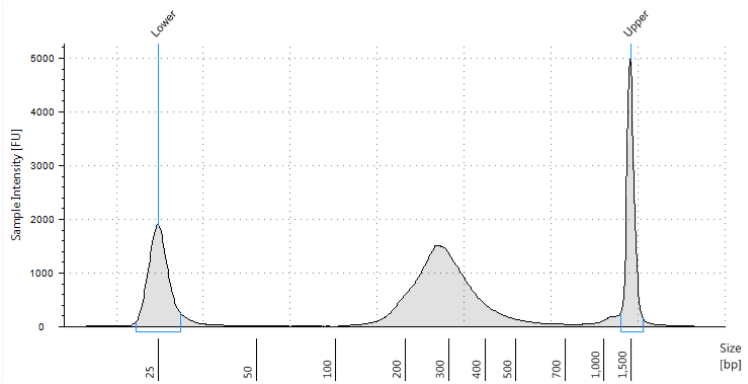

Figure 9 Analysis of adapter-ligated DNA using a D1000 ScreenTape.

4 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.

Protocol steps from hybridization through bisulfite conversion (pages 46 through 57) must be completed without stopping points. Plan your experiments accordingly.

Step 1: Hybridize the library

CAUTION

You must avoid evaporation from the small volumes of the capture during the 16 hour 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 16 hours 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.

Use all of the methylated adapter-ligated DNA in the hybridization reaction for optimal capture performance. The hybridization reaction requires **at least** 350 ng of adaptor-ligated DNA in a volume of 3.4 µL, for a final concentration of ≥102.9 ng/µL.

If you have recovered less than 350 ng of adaptor-ligated DNA, do another round of sample preparation before continuing the protocol.

1. Use a vacuum concentrator to concentrate the samples at ≤ 45°C. Reduce the volume of each sample from the starting volume of approximately 20 µL to a final volume ≤3.4 µL.

   Do not completely dry the samples in the wells.
2. Reconstitute each sample with nuclease-free water to a final volume of 3.4 µL.
3. Mix each sample thoroughly using a vortex mixer and then spin the plate or strip tube in a centrifuge for 1 minute to collect the liquid in each well.

4 Prepare the Hybridization Buffer by mixing the components in Table 19 at room temperature.

If a precipitate forms, warm the Hybridization Buffer at 65°C for 5 minutes.

Keep the prepared Hybridization Buffer at room temperature until it is used in step 9.

Table 19 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction*	Volume for 16 reactions (includes excess)
SureSelect Hyb 1 (orange cap or bottle)	6.63 µL	116 µL
SureSelect Hyb 2 (red cap)	0.27 µL	4.7 µL
SureSelect Hyb 3 (yellow cap or bottle)	2.65 µL	46.4 µL
SureSelect Hyb 4 (black cap or bottle)	3.45 µL	60.4 µL
Total	**13 µL**	**227.5**

* Prepare Hybridization Buffer for at least 5 reaction equivalents per run to allow accurate pipetting volumes.

5 Prepare the SureSelect Block Mix by mixing the components in Table 20. Keep the mixture on ice until it is used in step 6.

Table 20 Preparation of SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect Indexing Block 1 (green cap)	2.5 µL	42.5 µL
SureSelect Block 2 (blue cap)	2.5 µL	42.5 µL
SureSelect Methyl-Seq Block 3 (brown cap)	0.6 µL	10.2 µL
Total	**5.6 µL**	**95.2 µL**

CAUTION For each protocol step that requires removal of tube cap strips, make sure to reseal the tubes with a fresh strip of caps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during incubations.

6 To each DNA library sample well prepared in step 3 on page 46, add 5.6 µL of the SureSelect Block Mix prepared in Table 20. Pipette up and down to mix.

7 Cap the wells, then transfer the sealed plate or strip tube to the thermal cycler and run the following program shown in Table 21.

Use a heated lid, set at 105°C, to hold the temperature at 65°C.

Make sure that the DNA + Block Mix samples are held at 65°C for at least 2 minutes before adding the remaining hybridization reaction components in step 10 below.

Table 21 Thermal cycler program for DNA + Block Mix prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	2 minutes
Step 3	65°C	Hold

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

8 Prepare the required volume of a 1:3 dilution of SureSelect RNase Block (for a final concentration of 25%), on ice, as shown in Table 22.

Table 22 Preparation of 25% RNase Block solution

Component	Volume for 1 reaction	Volume for 16 reactions (includes excess)
RNase Block (purple cap)	0.5 µL	8.5 µL
Nuclease-free water	1.5 µL	25.5 µL
Total	**2 µL**	**34 µL**

NOTE

Prepare the Capture Library mixture described in step 9, below, near the end of the 65°C step of 2 minute duration described in Table 21.

Keep the mixture at room temperature briefly, until adding the mixture to sample wells in step 10. Do not keep solutions containing the SureSelect Capture Library at room temperature for extended periods.

9 Prepare the Methyl-Seq Capture Library Hybridization Mix according to Table 23.

Mix well by vortexing at high speed for 5 seconds then spin down briefly. Proceed immediately to step 10.

Table 23 Preparation of Capture Library Hybridization Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 4	13 µL	221 µL
25% RNase Block solution from step 8	2 µL	34 µL
SureSelect Human Methyl-Seq Capture Library	5 µL	85 µL
Total	**20 µL**	**340 µL**

NOTE

Use of other SureSelect Capture Libraries is not supported by this protocol and requires optimization of amount of capture library added to the hybridization mix. Begin optimization of hybridization mixture components by using amounts shown in Table 23 for Capture Libraries ≥3 Mb, and by using 2 µL of Capture Libraries <3 Mb (brought to 5 µL using nuclease-free water).

Additional protocol steps, including post-capture PCR cycling conditions may also require optimization. See the Methyl-Seq Application Note for guidelines.

10 Maintain the DNA library + Block Mix plate or strip tube at 65°C while you add 20 µL of the Capture Library Hybridization Mix from step 9 to each sample well. Mix well by pipetting up and down 8 to 10 times.

The hybridization reaction wells now contain approximately 27 to 29 µL, depending on the degree of evaporation during the thermal cycler incubation.

11 Seal the wells with strip caps. Make sure that all wells are completely sealed.

CAUTION Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted. When using the SureCycler 8800 thermal cycler and sealing with strip caps, make sure to use domed strip caps and to place a compression mat over the PCR plate or strip tubes in the thermal cycler.

12 Incubate the hybridization mixture for 16 hours at 65°C with a heated lid at 105°C.

Step 2. Prepare streptavidin beads

1. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. Magnetic beads settle during storage.
2. For each hybridization, add 50 µL of the magnetic bead suspension to wells of a 96-well plate or 8-well tube strip.
3. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix the beads by pipetting up and down 10 times.
   c. Put the plate or tube strip into a magnetic separation device and allow the solution to clear (approximately 5 minutes).
   d. Remove and discard the supernatant.
   e. Repeat step a through step d for a total of 3 washes.
4. 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 16 hour incubation.

NOTE Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance.

2 Maintain the hybridization plate or strip tube at 65°C while you use a multichannel pipette to transfer the entire volume (approximately 25 to 29 µL) of each hybridization mixture to the plate or strip tube wells containing 200 µL of washed streptavidin beads.

   Mix well by slowly pipetting up and down until beads are fully resuspended.

3 Cap the wells, then incubate the capture plate or strip tube on a Nutator mixer or equivalent for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells.

4 During the 30-minute incubation for capture, prewarm Wash Buffer 2 at 65°C as described below.

   a Place 200-µL aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 3 wells of buffer for each DNA sample in the run.

   b Cap the wells with fresh domed caps and then incubate in the thermal cycler, with heated lid ON, held at 65°C until used in step 11.

5 After the 30-minute incubation initiated in step 3, briefly spin the capture reaction plate or strip tube in a centrifuge or mini-plate spinner.

6 Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear, then remove and discard the supernatant.

7 Resuspend the beads in 200 µL of SureSelect Wash Buffer 1. Mix by pipetting up and down until beads are fully resuspended.

8 Incubate the samples for 15 minutes at room temperature.

9 Briefly spin in a centrifuge or mini-plate spinner.

10 Put the plate or strip tube in the magnetic separator. Wait for the solution to clear, then remove and discard the supernatant.

CAUTION

It is important to maintain bead suspensions at 65°C during the washing procedure below to ensure specificity of capture.

Make sure that the SureSelect Wash Buffer 2 is pre-warmed to 65°C before use.

Do not use a tissue incubator, or other devices with significant temperature fluctuations, for the incubation steps.

11 Wash the beads with SureSelect Wash Buffer 2:

- **a** Resuspend the beads in 200 µL of 65°C prewarmed Wash Buffer 2. Pipette up and down until beads are fully resuspended.
- **b** Cap the wells, then incubate the sample plate or strip tube for 10 minutes at 65°C on the thermal cycler.
- **c** Put the plate or strip tube in the magnetic separator. Wait for the solution to clear, then remove and discard the supernatant.
- **d** Repeat step a through step c for a total of 3 washes.

  Make sure all of the wash buffer has been removed during the final wash.

12 Prepare **fresh** 0.1 M NaOH for elution of the captured libraries from the beads.

Prepare 20 µl per sample (plus excess), of the 0.1 M NaOH solution by diluting the high-quality 10 M NaOH stock solution with nuclease-free water. For example, for runs of up to 48 samples, dilute 10 µL of 10 M NaOH with 990 µL of nuclease-free water.

CAUTION

Using high-quality NaOH is critical for optimal DNA sample quality.

- Do not use stock NaOH solutions that were stored at concentrations below 10 M to prepare the 0.1 M NaOH solution.
- Keep the 0.1 M NaOH solution container sealed when not in use, especially when processing large numbers of samples per run.

13 Add 20 µL of the freshly-prepared 0.1 M NaOH solution to the bead-bound samples from step 11 and mix on a vortex mixer for 5 seconds to resuspend the beads.

14 Incubate the samples for 20 minutes at room temperature.

During the 20-minute incubation, prepare the EZ DNA Methylation-Gold Kit CT Conversion Reagent as described in step 1 and step 2 on page 56.

15 Collect the beads from the elution mixture on a magnetic separator.

16 Use a pipette to transfer the supernatant from each well to wells of a fresh plate or strip tube.

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

Proceed immediately to "Bisulfite Conversion" on page 55.

5 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 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.

   Prepare the appropriate number of vials for the number of samples in the run. One vial is sufficient for 10 samples.

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 wells of a PCR plate or strip tube.
5. Place the tubes in a thermal cycler and incubate the bisulfite conversion reactions at 64°C for 2.5 hours.

NOTE

The bisulfite conversion protocol provided with the Zymo Research kit includes an initial 98°C incubation step. This step can be omitted in the SureSelect Methyl-Seq protocol, as shown in Table 24.

For precise control of the reaction time, include the 4°C hold step shown in Table 24. After completing the bisulfite conversion step at 64°C, however, proceed immediately to desulphonation in step 6. Do not maintain the sample at 4°C for an extended period.

Table 24 Thermal cycler program for bisulfite conversion

Step	Temperature	Time
Step 1	64°C	2.5 hours
Step 2	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.

i Place the column in a fresh 1.5-mL tube. Allow the column to sit at room temperature for 2 minutes.

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

k Centrifuge for 60 seconds at 13,000 rpm.

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

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

Step 2. 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 a minimal number of PCR cycles.

Prepare 1 amplification reaction for each bisulfite-treated library.

1 Prepare the appropriate volume of PCR reaction mixture, according to Table 25. Mix well using a vortex mixer and keep on ice.

Table 25 Preparation of Post-Capture PCR Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
Nuclease-free water	30 µL	495 µL
SureSelect Methyl-Seq PCR Master Mix	50 µL	825 µL
Methyl-Seq PCR1 Primer F	1 µL	16.5 µL
Methyl-Seq PCR1 Primer R	1 µL	16.5 µL
Total Volume	**82 µL**	**1353 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 26.

Table 26 Bisulfite-converted library amplification PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	8	95°C	30 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	7 minutes
4	1	4°C	Hold

Step 3. Purify the libraries using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 180 µL of homogeneous AMPure XP beads to each sample well containing amplified library DNA. Pipette up and down 10 times to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or tube strip into a magnetic separation device. Wait for the solution to clear (approximately 7 to 10 minutes).
7. Keep the plate or tube strip in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or tube strip in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 to step 9 step once.
11. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or tube strip to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or tube strip on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.
13. Add 21 µL nuclease-free water to each sample well.
14. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the samples to collect the liquid.
15. Incubate for 2 minutes at room temperature.
16. Put the plate or tube strip in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
17. Remove the cleared supernatant (approximately 19.5 µL) to a fresh well. You can discard the beads at this time.

6 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 37 on page 80 for nucleotide sequence information for the index portion of the SureSelect 8 bp Indexes A01 through H12.

   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.

2 Prepare the appropriate volume of PCR reaction mixture, according to Table 27. Mix well using a vortex mixer and keep on ice.

Table 27 Preparation of PCR Indexing Reaction Mix

Reagent	Volume for 1 Reaction	Volume for 16 Reactions (includes excess)
SureSelect Methyl-Seq PCR Master Mix	25 µL	412.5 µL
SureSelect Methyl-Seq Indexing Primer Common	0.5 µL	8.25 µL
Total Volume	**25.5 µL**	**420.75 µL**

3 Dispense 25.5 µL of the PCR Indexing Reaction Mix from step 2 into each sample well containing 19.5 µL of amplified, bisulfite-converted library DNA.

4 Add 5 µL of the appropriate Indexing Primer A01–H12 to the appropriate PCR reaction mixture well. Mix thoroughly by pipetting.

5 Place the plate in a thermal cycler and run the PCR amplification program shown in Table 28.

Table 28 PCR indexing cycling program

Segment	Number of Cycles	Temperature	Time
1	1	95°C	2 minutes
2	6	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. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 90 µL of homogeneous AMPure XP beads to each 50-µL amplified DNA sample well. Pipette up and down 10 times to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
7. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 to step 9 step once.
11. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
13. Add 24 µL nuclease-free water to each sample well.
14. Seal the wells then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
15. Incubate for 2 minutes at room temperature.
16. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
17. Remove the cleared supernatant (approximately 24 µL) to a fresh well. 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

Quality assessment and quantification of indexed sample DNA can be done with either the 2100 Bioanalyzer instrument or the 4200 TapeStation instrument using the High Sensitivity DNA Assay appropriate for each platform.

Option 1: Analysis using the 2100 Bioanalyzer and High Sensitivity DNA Assay

See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis.
3. Load the prepared chip into the instrument and start the run within five minutes after preparation.
4. Verify the results. Check that the electropherogram shows a distribution with a fragment size peak between approximately 250 to 300 bp. A sample electropherogram is shown in Figure 10.

   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.
5. Determine the concentration of each library by integration under the peak in each electropherogram.

   Use the quantities of indexed libraries determined at this step to pool samples for Illumina sequencing.

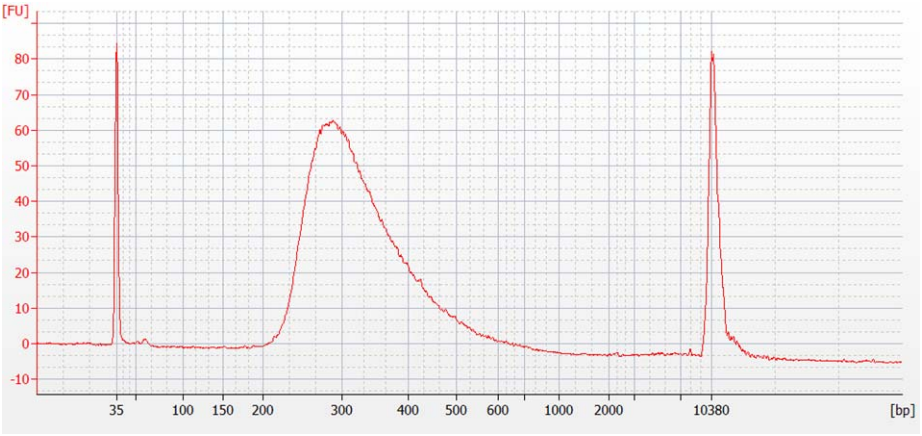

Figure 10 Analysis of indexed DNA sample using a High Sensitivity DNA Bioanalyzer assay.

Option 2: Analysis using the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape and reagent kit to analyze the amplified indexed DNA. For more information to do this step, see the TapeStation instrument user manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 2 µL of each indexed DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. Start the run.

3 Verify the results. Check that the electropherogram shows a distribution with a fragment size peak between approximately 250 to 300 bp. A sample electropherogram is shown in Figure 11.

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.

4 Determine the concentration of each library by integration under the peak in each electropherogram.

Use the quantities of indexed libraries determined at this step to pool samples for Illumina sequencing.

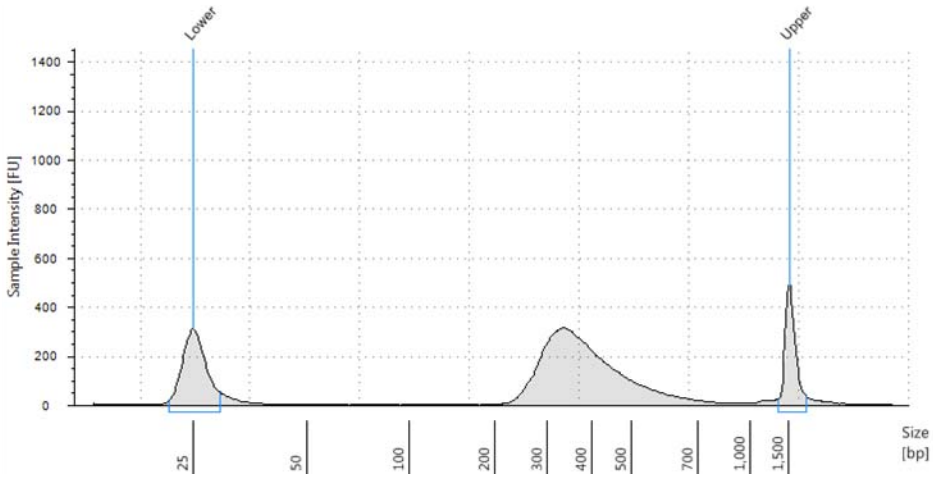

Figure 11 Analysis of indexed DNA sample using a High Sensitivity D1000 ScreenTape.

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, 10 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 29 shows an example of the amount of 2 capture pool samples and Low TE needed for a final volume of 25 µL at 10 nM final DNA concentration.

Table 29 Example of indexed Methyl-Seq sample volume calculations for a 25-µL final sequencing sample pool containing 10 nM DNA

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	25 µL	10 nM	10 nM	2	12.5
Sample 2	25 µL	12.5 nM	10 nM	2	10
Low TE					2.5

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 64, 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

1. Analyze the final indexed DNA pool using either a Bioanalyzer High Sensitivity DNA Assay kit (see page 66 for instructions) or a High Sensitivity D1000 ScreenTape (see page 68 for instructions).
2. Check that the electropherogram shows a single peak between approximately 250 to 300 bp.
3. 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 approximately 10 nM.
4. Proceed to template denaturation and flow cell preparation. Refer to the appropriate Illumina protocol and to "Guidelines for sequencing sample preparation and run setup" on page 73.

Guidelines for sequencing sample preparation and run setup

Use the appropriate Illumina Paired-End Cluster Generation Kit to do cluster amplification. Refer to the instructions that are included with the Illumina Paired-End Cluster Generation Kit.

The optimal seeding concentration for SureSelect^{XT} Methyl-Seq libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 30 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Include PhiX control DNA as a low-concentration spike-in for improved sequencing quality control.

Table 30 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Chemistry	Seeding Concentration	Final PhiX Concentration
HiSeq 2500	Rapid Run	v2	10–13 pM	5%
HiSeq 2500	High Output	v4	12–14 pM	5%
HiSeq 3000/4000	All Runs	v1	210 pM	10%

Sequencing run setup guidelines for 8-bp indexes

For libraries prepared using kits with 8-bp indexes, sequencing runs must be set up to perform an 8-bp index read. For the HiSeq platform, use the *Cycles* settings shown in Table 31. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

For complete 8-bp index sequence information, see Table 37 on page 80.

Table 31 HiSeq platform Run Configuration screen Cycle Number settings

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	0
Read 2	100

7 Reference

This chapter contains component kit contents reference information.

Kit Contents

The SureSelect^{XT} Methyl-Seq system includes the following component kits:

Table 32 SureSelect^{XT} Methyl-Seq Reagent Kit Contents

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect Methyl-Seq Library Prep Kit	–20°C	5500-0128	5500-0129	5 x 5500-0129
SureSelect Methyl-Seq Target Enrichment Box 1	Room Temperature	5190-5000	5190-5002	5 x 5190-5002
SureSelect Methyl-Seq Hybridization Kit Box 2	–20°C	5190-5001	5190-5003	5 x 5190-5003
SureSelect XT Human Methyl-Seq Capture Library	–80°C	5190-4661	5190-4662	5190-4663

The contents of each of the component kits listed in Table 32 are described in the tables below.

Table 33 SureSelect Methyl-Seq Library Prep Kit

Kit Component	16 Reactions	96 Reactions
10X End Repair Buffer	tube with clear cap	tube with clear cap
10X Klenow Polymerase Buffer	tube with blue cap	tube with blue cap
5X T4 DNA Ligase Buffer	tube with green cap	tube with green cap
T4 DNA Ligase	tube with red cap	tube with red cap
Exo(–) Klenow	tube with red cap	tube with red cap
T4 DNA Polymerase	tube with purple cap	tube with purple cap
Klenow DNA Polymerase	tube with yellow cap	tube with yellow cap
T4 Polynucleotide Kinase	tube with orange cap	tube with orange cap
dATP	tube with green cap	tube with green cap
dNTP Mix	tube with green cap	tube with green cap
SureSelect Methyl-Seq PCR Master Mix	tube with clear cap	bottle
SureSelect Methyl-Seq Methylated Adapter	tube with green cap	tube with green cap
SureSelect Methyl-Seq PCR1 Primer F	tube with brown cap	tube with brown cap
SureSelect Methyl-Seq PCR1 Primer R	tube with brown cap	tube with brown cap
SureSelect Methyl-Seq Indexing Primer Common	tube with blue cap	tube with blue cap
SureSelect^{XT} Indexes, 8 bp reverse primers*	SSEL 8 bp Indexes A01 through H02, provided in 16 white-capped tubes	SSEL 8 bp Indexes A01 through H12, provided in blue 96-well plate†

* See Table 37 on page 80 for index sequences.

† See Table 36 on page 79 for a plate map.

Table 34 SureSelect Methyl-Seq Target Enrichment Box 1 Content*

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 1	tube with orange cap	bottle
SureSelect Hyb 2	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

* Kits received prior to May, 2018 may also contain SureSelect Elution Buffer. For optimal results, discard this component and instead use freshly-prepared 0.1 M NaOH for the elution step, as directed in the protocol on page 53.

Table 35 SureSelect Methyl-Seq Hybridization Kit Box 2 Content

Kit Component	16 Reactions	96 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap
SureSelect Indexing Block 1	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap
SureSelect Methyl-Seq Block 3	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap

Table 36 Plate map for SSEL 8 bp Indexes A01 through H12, provided in blue plate in Library Prep kit p/n 5500-0129

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of SureSelect^{XT} Indexes A01 to H12

Each index is 8 nt in length. See page 73 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 37 SureSelect^{XT} Indexes, for indexing primers provided in blue 96-well plate or white capped tubes

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10	AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10	TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10	AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10	AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10	CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10	TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10	CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10	ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

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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.

Version E0, April 2018

G7530-90002

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