Takara Bio USA, Inc.

EpiXplore™ Meth-Seq DNA Enrichment Kit User Manual

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(100317)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

The EpiXplore Meth-Seq DNA Enrichment kit utilizes the MBD2 protein's specificity for binding to methylated DNA to separate out methylated and unmethylated fractions of sheared genomic DNA. This enrichment is followed by a clean-up step and ligation-free library preparation using DNA SMART™ technology, facilitating the generation of Illumina®-compatible sequencing libraries.

In brief, this enrichment protocol (Figure 1) involves the binding of methylated DNA to his-tagged MBD2, followed by the removal of the unmethylated DNA fraction using Capturem™ Nanoprep Columns, which bind the his-tagged MBD2 protein. Since the methylated DNA is bound to the MBD2 protein, the flowthrough and subsequent wash steps remove the unmethylated DNA fraction. The methylated DNA fragments are then eluted from the column using a high-salt buffer. Both the unmethylated and methylated DNA fractions are then purified separately using Agencourt AMPure XP beads. The total amount of DNA in the final eluate from the clean-up steps is quantified with a Qubit fluorometer, and then the percentage yield is calculated. This enriched DNA is used for library preparation using DNA SMART components, generating Illumina–ready sequencing libraries.

DNA SMART ChIP-Seq Components are used to generate Illumina-compatible sequencing libraries (Figure 2) from the DNA enriched in the first part of the protocol.

Preparation of meth-seq libraries is challenging due to the small amount of DNA recovered after the enrichment of methylated DNA. By modifying template switching technology for use with DNA, the DNA SMART ChIP-Seq Components provide a sensitive, ligation-free method for the addition of sequencing adapters, expanding both the amount and type of DNA available for library preparation.

Takara Bio's template switching technology, known as SMART® (**S**witching **M**echanism **a**t 5' End of **R**NA **T**emplate) technology, has been used as a basis for cDNA synthesis for next-generation sequencing (NGS) applications (Chenchik *et al*., 1998). SMART technology allows for single-step adapter addition, and has the inherent sensitivity to accurately amplify picogram quantities of nucleic acids. In the past, SMART technology had been limited to use with RNA samples, but with the development of the DNA SMART ChIP-Seq Kit, this sensitive technology was able to accommodate DNA templates as well. The DNA SMART ChIP-Seq Components included in this kit allow that technology to be applied to your enriched DNA.

To generate meth-seq Illumina libraries with the DNA SMART ChIP-Seq Components, a priming site is first added to the 3' end of the enriched DNA template using the Terminal Deoxynucleotidyl Transferase. This is followed by annealing of a proprietary DNA SMART Poly(dA) Primer, which anneals to the T-tail added by the Terminal Deoxynucleotidyl Transferase. This primer is then used by the SMARTScribe™ Reverse Transcriptase (RT) to copy the DNA strand. When the SMARTScribe RT reaches the 5' end of the DNA template, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3' end of the newly synthesized DNA. The carefully designed DNA SMART Oligonucleotide base-pairs with these additional non-template nucleotides and creates an extended template, enabling the SMARTScribe RT to continue replicating to the end of the oligonucleotide. Sequencing libraries are then generated by PCR-mediated addition of Illumina adapters using primers compatible with regions on the DNA SMART Poly(dA) Primer and the DNA SMART Oligonucleotide.

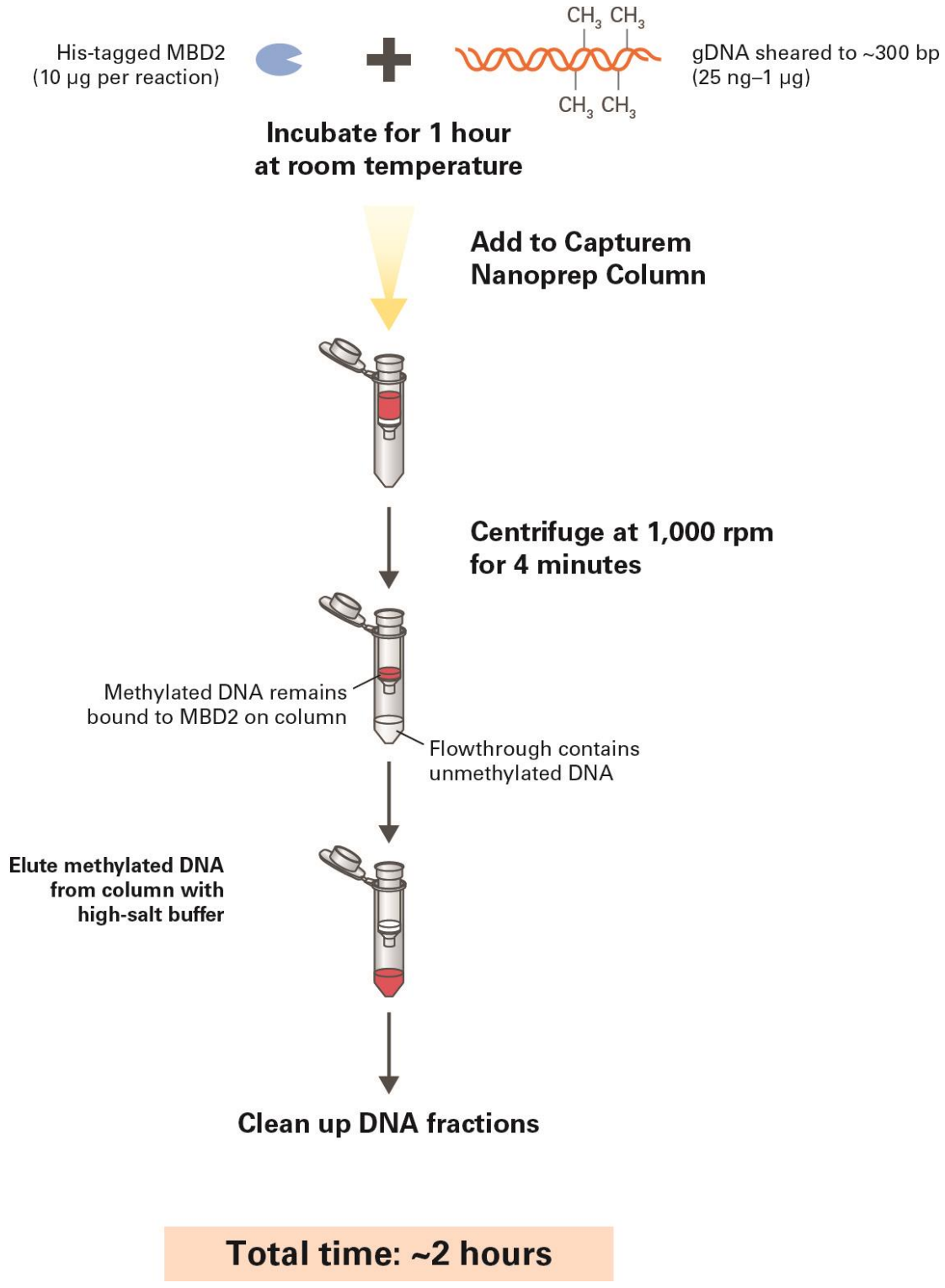

Figure 1. Flowchart for separating methylated and unmethylated fractions of DNA.
In the enrichment portion of the protocol, sheared DNA is incubated with his-tagged MBD2 protein for 1 hr, which allows the methylated DNA fraction to bind to the protein. The solution is then added to the Capturem nanoprep column, which binds the his-tagged protein and thus the methylated DNA. Subsequent washes with the Unmethylated Elution Buffer remove the unmethylated DNA. The methylated DNA is then eluted in a high-salt buffer. After cleanup, this enriched DNA can be used as the input for library preparation.

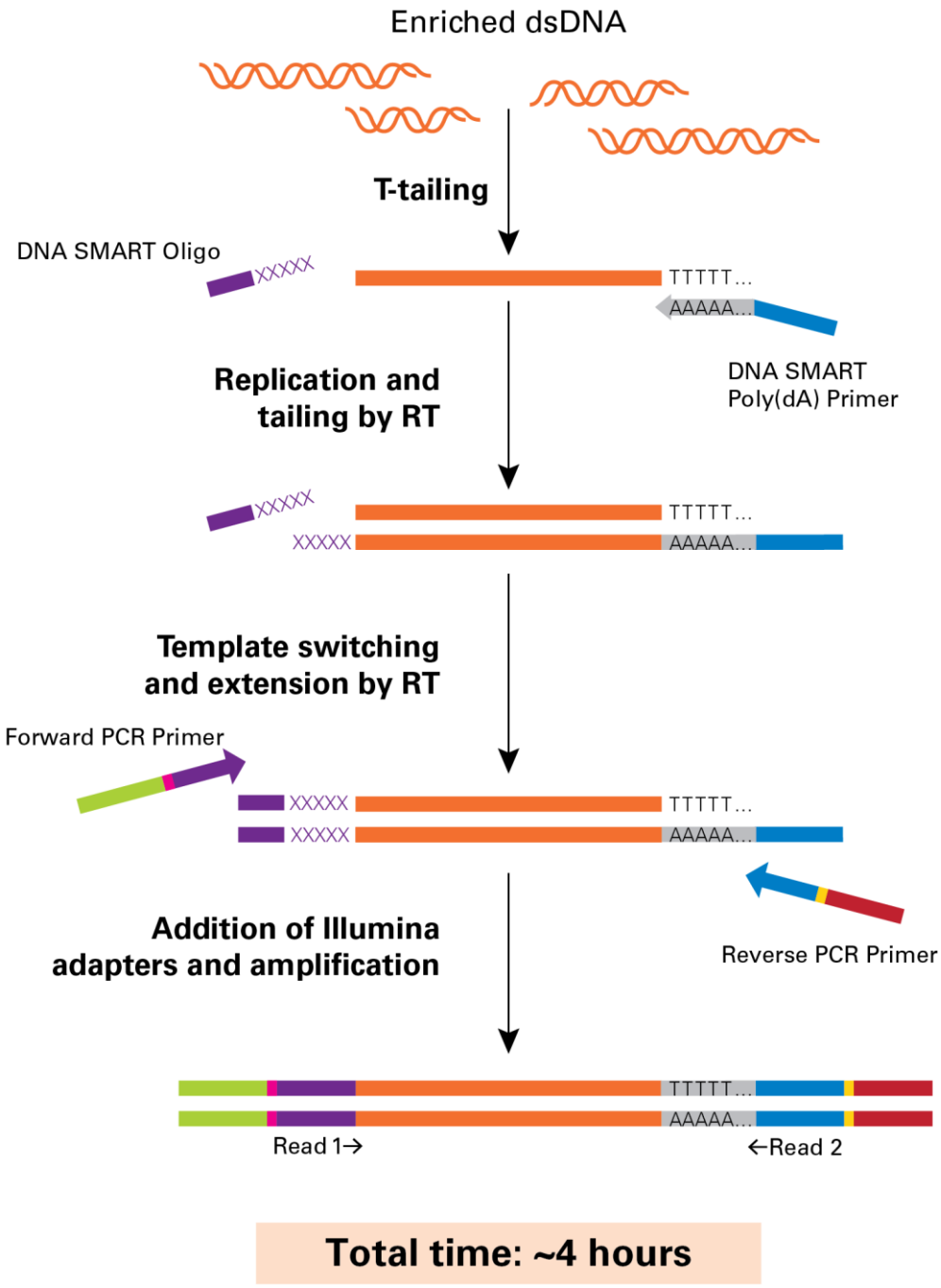

Figure 2. Flowchart for DNA-SMART ChIP-Seq technology.
This single-tube workflow allows users to generate Illumina-compatible libraries for meth-seq experiments. After library size selection and purification, the total time from input DNA to meth-seq library is approximately four hours. DNA SMART technology eliminates the need for an adapter ligation step and associated cleanup, reducing the loss of limited input DNA.

The DNA SMART ChIP-Seq Components facilitate an easy workflow that can be completed in approximately four hours (Figure 3). DNA template switching technology in the DNA SMART ChIP-Seq Components provides a robust and reliable tool for meth-seq applications, particularly at low input levels.

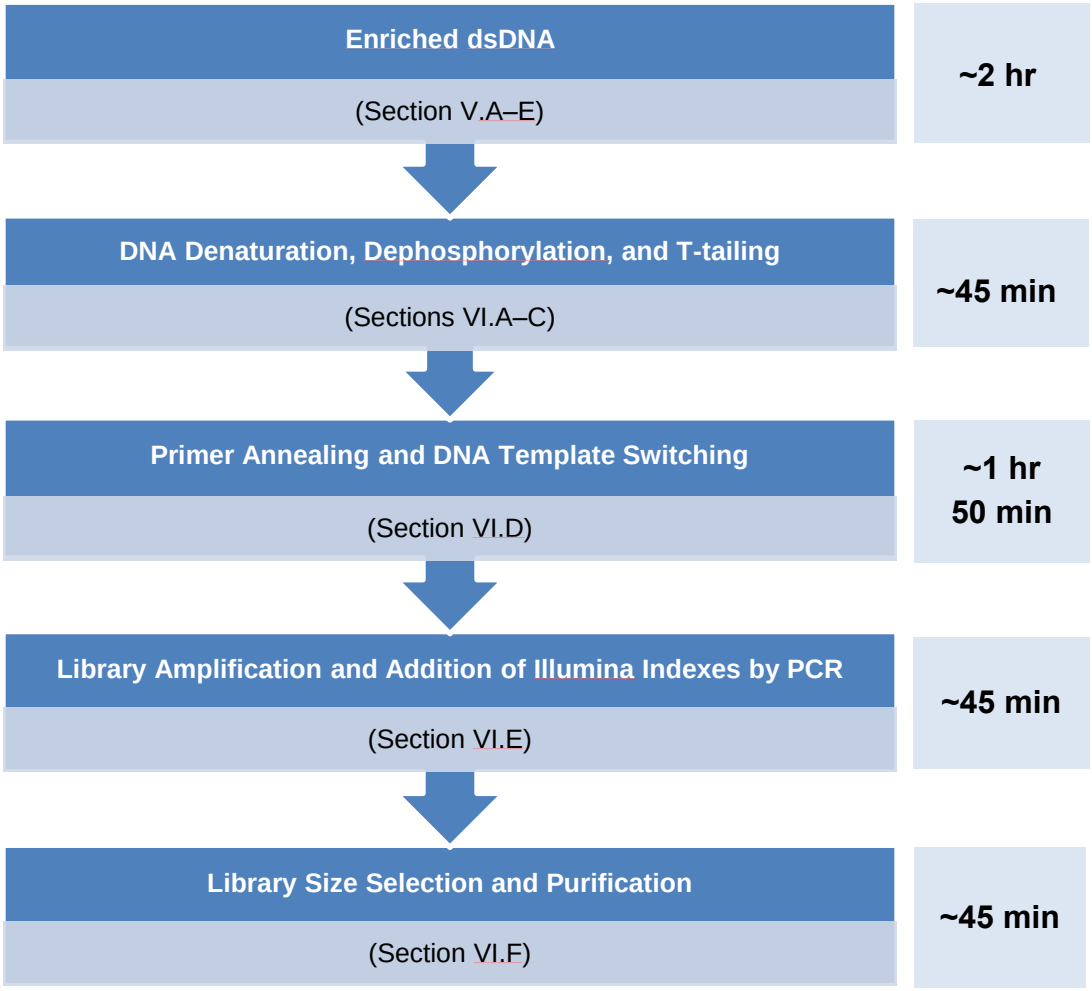

Figure 3. Workflow for meth-seq library preparation using the DNA SMART ChIP-Seq Components.
dsDNA can be enriched for methylated or unmethylated fractions, and then used to generate Illumina-compatible sequencing libraries for meth-seq in a total of approximately 6 hr.

II. List of Components

The EpiXplore Meth-Seq DNA Enrichment Kit consists of components for the enrichment assay for isolating the methylated DNA fraction from sheared genomic DNA (not sold separately), DNA SMART ChIP-Seq Components (not sold separately), the Indexing Primer Set HT for Illumina - 12 (not sold separately), and SeqAmp™ DNA Polymerase. These components have been specifically designed to work together and are optimized for this particular protocol. **Please do not make any substitutions.** Substituting any of the reagents in the kit and/or a modifying the protocol may lead to unexpected results.

Sufficient reagents are included in the DNA SMART ChIP-Seq Components to make 12 libraries from the enriched DNA. If you wish to generate libraries for all methylated and unmethylated fractions, you will need to purchase an additional 12-reaction DNA SMART ChIP-Seq Kit (Cat. No. 634865). There are many reagents included in the DNA SMART ChIP-Seq Components. **Please read the reagent labels carefully to ensure that the correct reagent is used at each step of the protocol**. For convenience, the color of the tube cap for each of the DNA SMART ChIP-Seq Components is indicated in the list of components below.

EpiXplore Meth-Seq DNA Enrichment Kit	
EpiXplore Meth-Seq DNA Enrichment Components (Not sold separately. Storage conditions are listed below for Packages 1, 2, and 3.)	
Package 1 (Store at room temperature.)	
xTractor™ Buffer	2.4 ml
Unmethylated DNA Elution Buffer	600 µl
Capturem Nanoprep Columns	12 rxns
Package 2 (Store at 4°C.)	
4X Incubation Buffer	150 µl
Methylated DNA Elution Buffer	1.2 ml
Package 3 (Store at –20°C.)	
MBD2 Protein (1 mg/ml)	120 µl
Unmethylated Control DNA (1 ng/µl)	50 µl
Methylated Control DNA (1 ng/µl)	50 µl
Nuclease-Free Water	1 ml
SeqAmp DNA Polymerase (Store at –20°C.)	
SeqAmp DNA Polymerase	50 µl
SeqAmp PCR Buffer (2X)	1.25 ml

EpiXplore Meth-Seq DNA Enrichment Kit (continued)		
	DNA SMART ChIP-Seq Components (Not sold separately. Storage conditions are listed below for Packages 1 and 2.)	
Cap Color	**Package 1** (Store at –70°C.)	
Purple	DNA SMART Oligonucleotide Mix[a]	72 µl
	Package 2 (Store at –20°C.)	
Blue	DNA Dilution Buffer (5 mM)	1 ml
Red	DNA SMART Buffer	111 µl
Red	Shrimp Alkaline Phosphatase (1 U/µl)	9 µl
Green	Terminal Deoxynucleotidyl Transferase	12 µl
Green	DNA SMART T-Tailing Mix	12 µl
Yellow	DNA SMART Poly(dA) Primer[b]	24 µl
Purple	SMARTScribe™ Reverse Transcriptase (100 U/µl)	48 µl
Pink	Library Elution Buffer	500 µl
Clear	Control Fragmented Human gDNA (5 ng/µl)	10 µl
Clear	DNA SMART Custom Read2 Seq Primer (100 µM)	40 µl
	[a] Contains the DNA SMART template switching oligonucleotide with Takara Bio's proprietary sequence [b] Takara Bio proprietary sequences	
	Indexing Primer Set HT for Illumina - 12 (Not sold separately. Store at –20°C.)	
	Forward PCR Primer HT Index 2 (F2; 12.5 µM)	2 x 15 µl
	Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
	Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

III. Additional Materials Required

The following reagents are required but not supplied. These materials have been validated to work with this protocol. Of particular importance is a strong magnetic separation device and nuclease-free thin-wall PCR tubes with strong caps.

For Methylated DNA Enrichment:

- Benchtop tube rotator (SilentShake Revolver HYQ-1130A or similar)
- Microcentrifuge (benchtop, for 1.5-ml tubes and 0.2-ml tubes)
- Nuclease-free thin-walled PCR tubes with caps (0.2 ml; USA Scientific, Cat. No.1402-4700)
- Nuclease-free non-sticky 1.5-ml tubes (USA Scientific, Cat. No. 1415-2600)
- Pipettes and filter tips
- Qubit 2.0 Fluorometer (Life Technologies, Cat. No. Q32866) with HS dsDNA assay kit (Cat. No. Q32851/Q32854) and 500-µl thin-walled polypropylene tubes (Cat. No. Q32856)

For PCR Amplification:

- Thermal cycler
- Nuclease-free thin-walled PCR tubes (0.2 ml; GeneMate, Cat. No. T-3035-1; USA Scientific, Cat. No.1402-4700)
- Nuclease-free nonsticky 1.5-ml tubes (Eppendorf, Cat. No. 022431021; USA Scientific; Cat. No. 1415-2600)

For AMPure XP Bead Purification:

- Agencourt AMPure XP PCR Purification Kit (5 ml, Beckman Coulter, Part No. A63880; 60 ml, Beckman Coulter, Part No. A63881)
- 80% ethanol—made fresh for each experiment
- Magnetic Separator—a 1.5-ml tube device (homemade version recommended) and a PCR strip device (Cat. No. 635011; homemade version can also be used)
    - Visit the following link for directions on creating a homemade magnetic separation device: http://www.clontech.com/US/Products/cDNA_Synthesis_and_Library_Construction/NGS_Learning_Resources/SMARTer_RNA-Seq_Tips
    - If you are following the instructions in the video above in order to make a homemade separation device, you will need to use a P1000 tip box rather than a P200 tip box for the 1.5-ml tube device.

For DNA Library Validation:

- Qubit 2.0 Fluorometer (Life Technologies, Cat. No. Q32866)
- Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854)
- 500-µl thin-walled polypropylene tubes for analysis using the Qubit 2.0 Fluorometer (Life Technologies, Cat. No. Q32856)
- Agilent High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)

IV. General Considerations

A. General Recommendations

- Please read the entire protocol before starting.
- Extreme care must be taken to avoid contaminating input samples and reagents with foreign DNA. This kit is extremely sensitive; any environmental contaminants (e.g., skin or bacterial DNA) could be incorporated into the libraries. Similarly, contaminating DNases and RNases may compromise the success of the experiment.
- The assay is very sensitive to variations in volume, etc. Ensure that all pipettes are calibrated for reliable delivery and that nothing is attached to the outside of the tips.
- Do not increase (or decrease) the amount or concentration of any reagent. The amounts and concentrations have been carefully optimized for this protocol.
- We recommend performing the complete library preparation protocol without stopping. However, for convenience, samples can be left overnight at 4°C after the template switching reaction (Step VI.D) or after the PCR step (Step VI.E).
- When performing this protocol for the first time, it is highly recommended to process a positive control and a negative (no DNA) control along with your samples. The methylated and unmethylated control DNA should be run alongside your samples in the enrichment portion of the protocol. For the library preparation portion of the protocol, the Fragmented Human gDNA control should be run alongside your samples. Dilute the provided Fragmented Human gDNA control with the DNA Dilution Buffer and use an input concentration similar to that of your estimated enriched DNA input. For example, if you are starting with 25 ng of genomic DNA, use 1 ng of your input DNA or 1 ng of the Fragmented Human gDNA control and process this side-by-side with your samples (including using the same number of PCR cycles). Processing a negative control is particularly important any time you are using 17 or more PCR cycles.

B. Sample Preparation

Input DNA must be free of contamination.

- During the clean-up steps in the methylated DNA enrichment part of the protocol, eluting the DNA in 24 µl of the DNA Dilution Buffer (5 mM Tris-HCl pH 8.5; included in the DNA SMART ChIP-Seq Components, blue cap) will provide the correct input volume (20 µl) for the DNA SMART library preparation.
- Evaluating the amount of DNA obtained from the methylated DNA enrichment part of this workflow can be challenging. If enrichment verification is to be performed by using the included kit controls, we strongly recommend the use of a Qubit 2.0 Fluorometer (Life Technologies, Cat. No. Q32866) along with the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854).

NOTE: Even using these sensitive methods, quantification may still be inaccurate. To determine the number of PCR cycles for library amplification (Step VI.E), we recommend performing a pilot library preparation using one or two samples. If the input DNA concentration is too low to be quantified, or quantification is uncertain, use 16 or more PCR cycles.

C. Sample Requirements

- This kit can enrich for methylated and unmethylated DNA from 25 ng to 1 µg of genomic DNA sheared to ~300 bp fragments. However, for optimal recovery and ample input for the library preparation steps, we recommend using a minimum input of 100–200 ng.
- We recommend physical fragmentation of DNA to ~300-bp fragments using acoustic shearing/sonication. We do not recommend enzymatic shearing due to the associated biases that may be introduced.
- The starting material should be in 1–20 µl of an alkaline, low-ionic buffer such as the DNA Dilution Buffer (5 mM Tris-HCl, pH 8.5) provided, or water. The optimal pH range of samples is 7.5–8.5. Avoid using EDTA-containing buffers.

D. Recommendations for a Smooth Workflow

- **Master mixes:** Always prepare enough master mix for the number of samples, plus an extra 10% to allow for pipetting errors. Prepare and keep all master mixes on ice. While adding the master mix to the samples, keep samples on ice, or place them back on ice immediately after adding the master mix.
- **Mixing:** Adequate mixing is required after the addition of components at each step. We recommend a quick, **gentle** vortexing (avoiding the creation of bubbles as much as possible) or flicking the tubes with your fingers for mixing. Although gentle pipetting up and down can be used for mixing, we found that it can lead to a lower yield. However, **we do not recommend vortexing the samples when adding the AMPure XP beads**.
- **Controls:** To assess the efficiency of the enrichment protocol, DNA controls are included in this kit. Both controls are 90 bp in length.The Unmethylated Control DNA is completely unmethylated (22 unmethylated cytosine residues) and the Methylated Control DNA contains 11 methylated and 11 unmethylated cytosine residues. By using these controls in reactions alongside your samples you will be able to calculate the efficiency of the enrichment process. We typically see enrichment of 60% or higher for both the methylated and unmethylated controls (>60% yield of the methylated control in the methylated fraction and >60% yield of the unmethylated control in the unmethylated fraction), with minimal non-specific binding.
- **Thermal cycling:** Pre-program the thermal cycler with all five programs used in this protocol before starting:
  - DNA-94 (page 12; to use in Section VI.A and Section VI.D)
  - DNA-B (page 12; to use in Section VI.B)
  - DNA-C (page 13; to use in Section VI.C)
  - DNA-D (page 14; to use in Section VI.D)
  - DNA-PCR (page 15; to use in Section VI.E)
- **AMPure XP Beads:** Aliquot beads into 1- to 2-ml aliquots. Before use, beads should be brought to room temperature for 30 min and mixed well to disperse.

E. Sequencing Analysis Guidelines

- The first three bases of the first sequencing read (Read 1) are derived from the DNA SMART template switching oligo. Consequently, these three bases need to be trimmed prior to mapping.
- If you are performing paired-end sequencing, we highly recommend using the DNA SMART Custom Read2 sequencing primer for the second sequencing read (Read 2). This custom primer will allow sequencing to start right after the priming site of the DNA SMART Poly(dA) Primer, and avoid reading through the A/T tail created on the 3' end of ssDNA molecules during Step VI.C. Sequencing through the stretch of adenine bases may result in low sequencing performance due to low complexity.

V. Methylated DNA Enrichment

A. PROTOCOL: Setting up the DNA: MBD2 Protein Binding Reaction

For this step you will need the following components: 4X Incubation Buffer, MBD2 Protein, Methylated Control DNA (optional), Unmethylated Control DNA (optional), sheared gDNA samples, and Nuclease-Free Water.

1. Thaw the Methylated Control DNA, Unmethylated Control DNA, and sheared gDNA samples on ice. Gently vortex each to mix, then spin down briefly. Store on ice. Store the MBD2 protein on ice.
2. For each reaction, set up the following in a 0.2-ml PCR tube:

Reagent	Control Reactions	Experimental Reactions
Incubation Buffer	12.5 µl	12.5 µl
MBD2 Protein	10 µl	10 µl
Control DNA (Methylated or Unmethylated; 1 ng/µl)	25 µl	—
Sample DNA	—	25 ng—1 µg
Nuclease-Free Water	2.5 µl	Up to 50 µl
Total volume per reaction	**50** µl	**50** µl

3. Mix gently, then spin down briefly.
4. Securely tape the tubes onto a benchtop tube rotator and turn it on. Incubate at room temperature for 1 hr to allow binding of the methylated DNA to the MBD2 protein.

B. PROTOCOL: Isolation of the Unmethylated DNA Fraction

For this step you will need the following components: Capturem Nanoprep Columns, xTractor buffer, Unmethylated DNA Elution Buffer.

1. Place the required number of Capturem Nanoprep Columns in a tube rack and add 200 µl xTractor buffer to each membrane (be careful not to touch the membrane with the pipette tip).
2. Centrifuge at 11,000 rpm for 1 min at room temperature to equilibrate the column.
3. Discard the flowthrough and the 1.5-ml collection tube. Place the Nanoprep Column into a new 1.5-ml collection tube.
4. Remove the 0.2-ml tubes that have been incubating for 1 hr from the rotator, and spin down briefly. Transfer the 50 µl of the DNA-MBD2 protein reaction to a Capturem Nanoprep Column. Pipette the 50 µl directly onto the membrane but do not touch the membrane with the pipette tip.

5. Centrifuge at **1000 rpm for 4 minutes**.

NOTE: This SLOW spin is critical here, as it allows the his-tagged MBD2 protein to bind to the Capturem Nanoprep Column.

6. **DO NOT discard the flowthrough**; leave it in the collection tube.
7. Add 50 µl of Unmethylated DNA Elution Buffer to the column. Centrifuge at 11,000 rpm for 1 min.
8. The combined flowthrough in the tube from Steps 6 and 7 contains the unmethylated (unbound) DNA fraction. Cap the collection tube containing the unmethylated DNA fraction.

C. PROTOCOL: Elution of the Methylated DNA Fraction

For this step you will need the following components: Clean 1.5-ml tubes, Methylated DNA Elution Buffer.

1. Transfer the Capturem Nanoprep Columns to a clean 1.5-ml tube for the collection of the methylated DNA fraction.
2. To elute the methylated DNA from the MBD2 protein that is bound to the column, add 50 µl Methylated DNA Elution Buffer directly to the membrane of the Capturem Nanoprep Column, taking care not to touch the membrane with the pipette tip. Centrifuge at 11,000 rpm for 1 min at room temperature. DO NOT discard the flowthrough.
3. Repeat Step 2 by adding another 50 µl of Methylated DNA Elution Buffer to the Capturem Nanoprep Column, again taking care not to touch the membrane. Centrifuge at 11,000 rpm for 1 min. The 100 µl of flowthrough contains the methylated DNA fraction. Cap the collection tube containing the methylated DNA fraction and discard the Capturem Nanoprep Column.

D. PROTOCOL: Cleanup of Both Enriched DNA Fractions

For this step you will need the following components: Agencourt AMPure XP beads, freshly made 80% ethanol, DNA Dilution Buffer (DNA SMART ChIP-Seq Components, blue cap).

You will now have two tubes for each initial sample: one containing the unmethylated DNA fraction and one containing the methylated DNA fraction. Both fractions require cleanup before quantification.

1. Add AMPure XP beads to each sample tube as described below, then mix by pipetting the entire volume up and down at least 10 times.
    a. For the tubes that contain your sample DNA, add 150 µl of beads to each tube. (The sample volume is 100 µl, thus this is a 1.5:1 ratio, which will bind almost all DNA present over 100 bp. Since the sample was sheared to ~300 bp, this will capture all DNA.)
    b. For the tubes that contain the supplied Control DNA, add 300 µl of beads to each sample tube. (The sample volume is 100 µl, thus this is a 3:1 ratio, which will bind almost all DNA present. The larger bead-to-sample ratio is required to ensure binding of all of the 90-bp fragments of the Control DNA.)

NOTE: The beads are viscous; suck the entire volume up and push it out slowly. DO NOT VORTEX. Vortexing will generate bubbles, making subsequent handling of the beads difficult.

2. Incubate at room temperature for 8 min to allow the DNA to bind to the beads

3. Place the reaction tubes on the magnetic stand for 20 min or until the solution is completely clear. The time for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear before moving to the next step. There is no disadvantage in separating the samples for more than 20 min.

4. While the tubes are sitting on the magnetic stand, remove the supernatant. (The DNA is bound to the beads.)
5. Keep the tubes on the magnetic stand. Add 500 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully remove the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 one more time.
7. Perform a brief spin on the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for 12 min or until the pellet appears dry. You may see a tiny crack in the pellet.

   NOTE: Be sure to sufficiently dry the pellet.
   - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your DNA recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
   - If you over-dry the pellet it may take longer to rehydrate.

9. Once the pellet is dry, remove the tubes from the magnetic stand and add 24 µl of DNA Dilution Buffer (blue cap) to cover the beads. Mix thoroughly by pipetting the beads up and down to ensure complete dispersion.

   NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube.

10. Incubate at room temperature for at least 7 min to rehydrate.
11. Place the tubes on the magnetic stand for 5 min or longer, until the solution is completely clear.
12. **OPTIONAL:** Transfer the clear supernatant from each tube to a new tube. Place the tubes on the magnetic stand for 2–3 min, until the solutions are completely clear

    NOTE: The second removal of magnetic beads ensures that no beads are left in the sample. Beads carried over in your sample may affect downstream reactions.

13. Transfer the clear supernatant containing the purified DNA from each tube to a labeled nuclease-free nonstiky tube.

    NOTE: If desired, the reactions can be left overnight at 4°C or frozen at –20°C for one week.

E. PROTOCOL: Assessment of DNA Enrichment

See the user manual for the complete Qubit dsDNA HS kit instructions (available on the Qubit webpage: https://www.thermofisher.com/order/catalog/product/Q32851)

1. Use 1–2 µl of the enriched DNA after quantification using the Qubit 2.0 Fluorometer (Life Technologies) with the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854). See the Qubit dsDNA HS Assay Kits User Manual for instructions.
2. Evaluate the concentration in ng/µl of enriched DNA.
3. To assess the yield of the assay, calculate the total output from the enrichment reaction. For each sample, multiply the concentration in ng/µl by 24 (the elution volume) to give the total amount eluted. Then divide the total output by the input amount and multiply by 100 to give an overall percentage yield.

Eluted concentration (ng/µl) x 24 = Total amount eluted (ng)

$$\frac{\text{Total amount eluted (ng)}}{\text{Input amount (ng)}} \times 100 = \%\ \text{Yield}$$

NOTE: If desired, the reactions can be left overnight at 4°C or frozen at –20°C for one week before moving to the library preparation part of the protocol.

VI. Library Preparation Using DNA SMART Technology

A. PROTOCOL: Denaturation of DNA

During this step, dsDNA is converted to ssDNA. We recommend performing the positive control reaction using the provided Fragmented Human gDNA the first time you use this kit.

For this step you will need the following components: DNA Dilution Buffer (blue cap).

1. Aliquot appropriate amounts of DNA in a maximum volume of 20 µl in PCR tubes. We recommend using 1–5 ng of your enriched DNA. However, if quantification reveals less than this amount in your elution, use all 20 µl. If necessary, add DNA Dilution Buffer (5 mM Tris-HCl, pH 8.5) to bring the total volume to 20 µl.

1–20 µl	Enriched dsDNA (1–5 ng)
0–19 µl	DNA Dilution Buffer (blue cap)
20 µl	Total volume

2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler (DNA-94) for exactly 2 min.

DNA-94	94°C	hold

3. Immediately remove the samples and place on ice for at least 2 min.
4. Spin the tubes briefly to bring down any condensation.

NOTE: Proceed immediately to the next step.

B. PROTOCOL: Dephosphorylation of 3' Ends

The 3' end of ssDNA is dephosphorylated by Shrimp Alkaline Phosphatase in preparation for T-tailing.

For this step you will need the following components: DNA SMART Buffer (red cap), Shrimp Alkaline Phosphatase (red cap)

1. Prepare enough Dephosphorylation Master Mix for all reactions, plus 10%, by combining the following reagents on ice:

3.25 µl	DNA SMART Buffer (red cap)
0.75 µl	Shrimp Alkaline Phosphatase (red cap)
4 µl	Total volume per reaction

NOTE: Add the Shrimp Alkaline Phosphatase to the buffer immediately prior to use. Mix well by gently vortexing, then spin the tubes briefly in a microcentrifuge. **Keep the DNA SMART Buffer on ice** until its next use in Step VI.D.

2. Add 4 µl of the Dephosphorylation Master Mix to each reaction tube from Step VI.A. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
3. Place the tubes in a preheated thermal cycler and run program DNA-B. Leave the tubes at 4°C until the next step.

DNA-B	37°C	10 min
	65°C	5 min
	4°C	hold

NOTE: During the 65°C incubation step, prepare the T-Tailing Master Mix in Step VI.C.1 below.

C. PROTOCOL: T-Tailing

A poly(T) tail is added to the ssDNA, providing a priming site for the DNA SMART Poly(dA) Primer.

For this step you will need the following components: DNA SMART T-Tailing Mix (green cap) and Terminal Deoxynucleotidyl Transferase (green cap)

1. Prepare enough T-Tailing Master Mix for all reactions, plus 10%, by combining the following reagents on ice:

1 µl	DNA SMART T-Tailing Mix (green cap)
1 µl	Terminal Deoxynucleotidyl Transferase (green cap)
2 µl	Total volume per reaction

2. Add 2 µl of the T-Tailing Master Mix to each reaction tube. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
3. Place the tubes in a preheated thermal cycler and run program DNA-C. Leave the tubes at 4°C until the next step.

DNA-C	37°C	20 min
	70°C	10 min
	4°C	hold

D. PROTOCOL: Primer Annealing and DNA SMART Template Switching Reaction

The DNA SMART Poly(dA) Primer anneals to the ssDNA template followed by DNA replication and DNA SMART template switching. At the end of this step, the DNA is double stranded with partial adapters on both ends.

For this step you will need the following components: DNA SMART Poly(dA) Primer (yellow cap), DNA SMART Buffer (red cap), DNA SMART Oligonucleotide Mix (purple cap), and SMARTScribe Reverse Transcriptase (100 U/µl) (purple cap)

NOTE: Prepare the DNA SMART Template Switching Master Mix (Step VI.D.5) prior to starting the primer annealing step below.

1. **Primer Annealing:** Add 2 µl of the DNA SMART Poly(dA) Primer (yellow cap) to each reaction tube. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler (DNA-94) for exactly 1 min.

DNA-94	94°C	hold

3. Immediately remove the samples and place on ice for at least 2 min.
4. Spin the tubes briefly to bring down any condensation.

NOTE: Proceed immediately to the next step.

5. Prepare enough DNA SMART Template Switching Master Mix for all reactions, plus 10%, by combining the following reagents on ice:

6 µl	DNA SMART Buffer (red cap)
6 µl	DNA SMART Oligonucleotide Mix (purple cap)
4 µl	SMARTScribe Reverse Transcriptase (100 U/µl)* (purple cap)
16 µl	Total volume per reaction

* DO NOT add the SMARTScribe Reverse Transcriptase to the buffer until immediately prior to use (Step VI.D.6). Mix well by gently vortexing, then spin the tubes briefly in a microcentrifuge.

NOTE: The DNA SMART Buffer is also used in Step VI.B.1.

6. **DNA Replication and DNA SMART Template Switching:** Add the SMARTScribe Reverse Transcriptase (purple cap) to the DNA SMART Template Switching Master Mix prepared in Step VI.D.5, then add 16 µl of the DNA SMART Template Switching Master Mix to each reaction tube. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
7. Place the tubes in a preheated thermal cycler and run program DNA-D. Leave the tubes at 4°C until the next step.

DNA-D	42°C	90 min
	70°C	15 min
	4°C	hold

NOTE: If desired, the reactions can be left overnight at 4°C.

E. PROTOCOL: Meth-Seq Library Amplification by PCR

The dsDNA produced in the Primer Annealing and DNA SMART Template Switching Reaction (Step VI.D) is directly amplified into meth-seq libraries using SeqAmp DNA Polymerase and the Forward and Reverse Primers from the Indexing Primer Set HT for Illumina. No pre-PCR purification or size selection is necessary.

For this step you will need the following components: SeqAmp DNA Polymerase, SeqAmp PCR Buffer (2X), Indexing Primer Set HT for Illumina - 12

IMPORTANT:

- **Optimal cycling parameters may vary with different templates and thermal cyclers.** The guidelines in Table 1 (in Step 5, below) are optimized for DNA fragments with an average size between 200–400 bp. To determine the optimal number of cycles for your samples and conditions, we strongly recommend that you perform a range of cycles.

1. Prepare enough PCR Master Mix for all reactions, plus 10%. Combine the following reagents *in the order shown*, then mix well and spin the tube briefly in a microcentrifuge.

50 µl	SeqAmp PCR Buffer (2X)
2 µl	Forward PCR Primer (12.5 µM)
2 µl	Reverse PCR Primer (12.5 µM)
2 µl	SeqAmp DNA Polymerase
56 µl	Total volume per reaction

NOTE: Separate PCR Master Mixes should be prepared for each combination of Forward and Reverse Primers. If each sample will receive a different index/set of primers, prepare a master mix excluding the forward and reverse primers (total volume per reaction will be 52 µl), and add 2 µl each of forward and reverse primers directly to each reaction tube. **Not all indexes can be pooled for multiplexing! Consult Appendix A and Illumina literature for more information.**

2. Add 56 µl (52 µl if forward and reverse primers are excluded) of PCR Master Mix to each reaction tube.
3. **OPTIONAL:** If the forward and reverse PCR primers were not added to the PCR Master Mix, add 2 µl of the primers to each reaction tube.
4. Mix the contents of the tubes by gently vortexing, then spin the tubes briefly.
5. Place the tubes in a preheated thermal cycler and run program DNA-PCR. Leave the tubes at 4°C until the next step.

DNA-PCR	94°C		1 min
	X[a] cycles:		
		98°C	15 sec
		55°C	15 sec
		68°C	30 sec
	4°C		hold

[a] The number of cycles depends on the amount of input DNA. See Table 1 (below) for guidelines.

Table 1. Cycling Guidelines Based on Amount of Starting Material.

Amount of Input DNA (ng)*	Typical Number of PCR Cycles
5–10	12
1	15
All eluted DNA (undetectable quantification)	18

* Amount of DNA used for library preparation after enrichment. Quantification of input DNA amount may be inaccurate; when in doubt, add additional PCR cycles.

NOTE: If desired, the reactions can be left overnight at 4°C or frozen at –20°C for one week.

F. PROTOCOL: Library Size Selection and Purification

The meth-seq library is size-selected and purified using AMPure XP beads. The protocol involves a single size selection that removes primers and primer dimers. The prepared library is added to the beads so that fragments of the desired size are immobilized. The beads are then washed with 80% ethanol and eluted in Library Elution Buffer.

Single Size Selection Protocol

This protocol will remove primers, primer dimers, and PCR products with very small inserts.

1. Add 90 µl of AMPure XP beads to each reaction tube. Mix by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; suck the entire volume up, and push it out slowly. DO NOT VORTEX, as this will generate bubbles, making subsequent handling of the beads difficult.

2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Place the reaction tubes on the Magnetic Separator for 20 min or until the solution is completely clear. The time for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear as any carryover of beads into the next step will decrease the efficiency of size selection. There is no disadvantage in separating the samples for more than 20 min.

4. While the tubes are sitting on the Magnetic Separator, remove the supernatant. (The library is bound to the beads.)
5. Keep the tubes on the Magnetic Separator. Add 200 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully remove the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 one more time.
7. **OPTIONAL:** Briefly spin the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the Magnetic Separator for 30 sec, then remove all the remaining ethanol with a pipette.

8. Let the sample tubes rest open at room temperature for 12 min or until the pellet appears dry. You may see a tiny crack in the pellet.

   NOTE: Be sure to dry the pellet sufficiently.
   - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your library recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
   - If you over-dry the pellet it may take longer to rehydrate

9. Once the pellet has dried, remove the tubes from the magnetic stand and add 20 µl of Library Elution Buffer (pink cap) to cover the beads. Mix thoroughly by pipetting the beads up and down to ensure complete dispersion.

   NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube.

10. Incubate at room temperature for at least 7 min to rehydrate.
11. Place the tubes on the Magnetic Separator for 5 min or longer, until the solution is completely clear.
12. **OPTIONAL:** Transfer the clear supernatant from each tube to a new PCR strip tube. Place the PCR strip tubes on the Magnetic Separator for 2–3 min, until the solutions are completely clear.

    NOTE: The second removal of magnetic beads ensures that no beads are left in the sample. Beads carried over in your sample may affect the profile obtained with the Agilent 2100 Bioanalyzer.

13. Transfer the clear supernatant containing the purified meth-seq library from each tube to a labeled nuclease-free nonsticky tube.

G. PROTOCOL: Library Validation

The meth-seq library is quantified using a Qubit 2.0 Fluorometer, and the quality of the library is determined using an Agilent 2100 Bioanalyzer.

1. Use 1–2 µl of the amplified meth-seq libraries for quantification using the Qubit 2.0 Fluorometer (Life Technologies) with the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. Nos. Q32851 and Q32854). See the Qubit dsDNA HS Assay Kits User Manual for instructions.
2. Evaluate the library concentration in ng/µl. If the library is more than 2 ng/µl, dilute in water or Library Elution Buffer so that the concentration is between 1–2 ng/µl.
3. Use 1 µl of the diluted sample for validation with the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). See the Agilent High Sensitivity DNA Kit Guide for instructions.
4. Compare the results for your sample and controls to determine whether the sample is suitable for sequencing.

VII. References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. (1998) Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis.* Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319.

Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**:R25.

Zhang, Y., Liu, T. Meyer, C. Eeckhoute, J,. Johnson, D., Bernstein, B., Nusbaum, C., Myers, R., Brown, M. & Liu, S. (2008) Model-based Analysis of ChIP-Seq (MACS). *Genome Biol*ogy **9**:R137.

VIII. Example Results

A successful DNA SMART assay and amplification should yield a distinct Bioanalyzer electropherogram spanning 175–900 bp, with a peak around 300–500 bp (Figure 4, Panels A, B, and C). No product should be seen in the "no MBD2" negative control, prepared using the methylated DNA fraction (Figure 4, Panel D). Without the MBD2 protein present, none of the input methylated DNA is captured. A small amount of PCR product may be visible between 125–250 bp in the "no DNA" control if using 18 cycles of PCR. This background will increase in this control if more PCR cycles are performed. The yield of a meth-seq library generated with this kit should be >10 nM. Both the yield and size distribution will depend on the input DNA (amount and size), number of PCR cycles, and size selection.

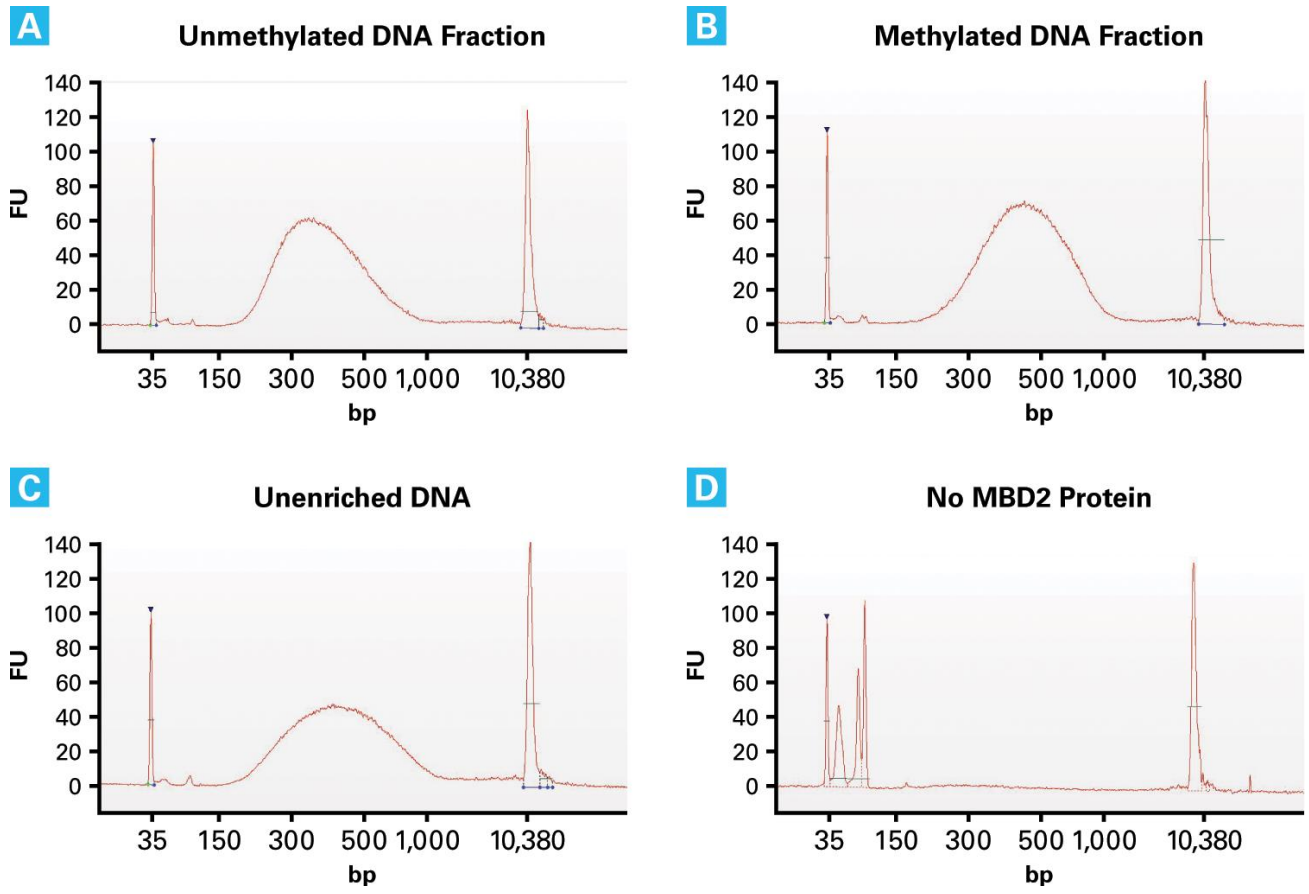

Figure 4. Bioanalyzer electrophoregrams of unmethylated and methylated DNA libraries.
Panel A. Library prepared from the unmethylated DNA fraction. Initial input was 500 ng of genomic DNA sheared to ~300 bp fragments. 1 ng of enriched, eluted DNA was used for library preparation with 15 cycles of PCR. **Panel B.** Library prepared from the methylated DNA fraction. Initial input was 500 ng of genomic DNA sheared to ~300 bp fragments. 1 ng of enriched, eluted DNA was used for library preparation with 15 cycles of PCR. **Panel C.** Library prepared from 1 ng of input DNA (unenriched) with 15 cycles of PCR. This library was prepared from total DNA which was not subject to the enrichment part of the protocol, and thus contains both methylated and unmethylated fractions. **Panel D.** The enrichment protocol and library preparation were performed in the absence of the MBD2 protein. This serves as a negative control, indicating that no nonspecific enrichment of the methylated fraction is taking place. The library was prepared from the entire eluted methylated fraction with 15 cycles of PCR.

Example Sequencing Data

Sequencing of the libraries above (MiSeq®, 1 x 75 bp) identified methylated and unmethylated regions in line with published data. The input sample, which contains both methylated and unmethylated DNA fractions, can be used as the baseline for peak calling. This data (Figure 5) was analyzed by alignment to the reference genome using Bowtie (Langmead *et al.*, 2009), and peaks were called using MACS (Zhang *et al.*, 2008).

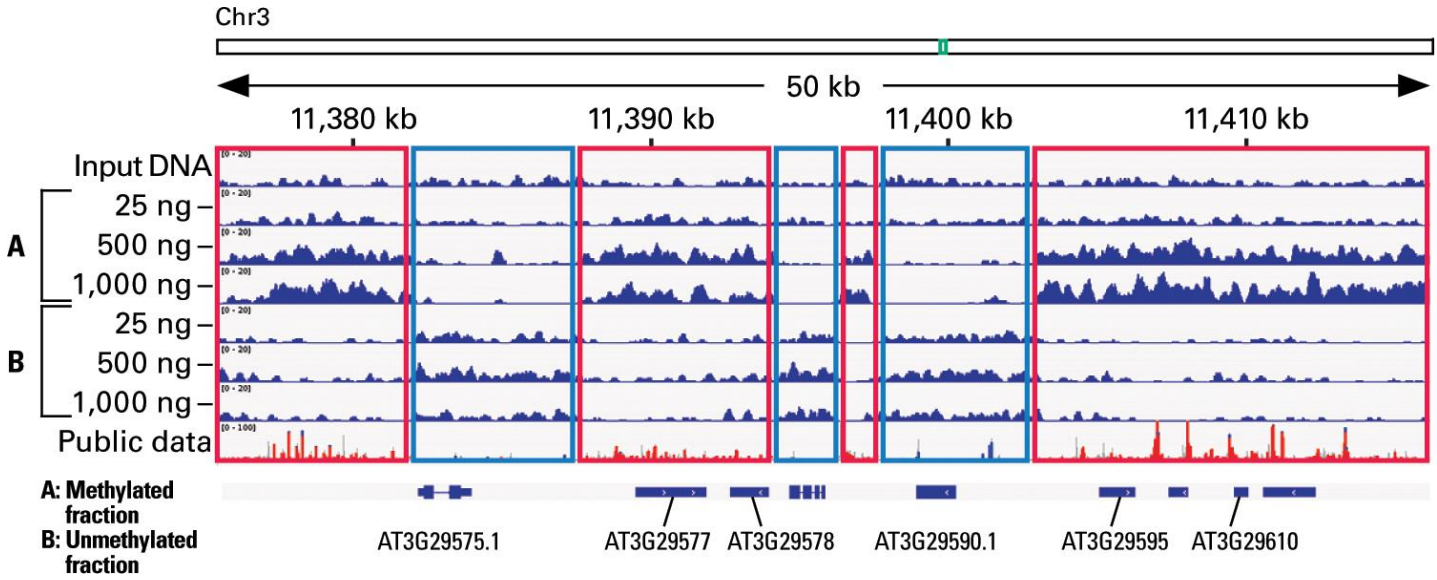

Figure 5. Distinct methylation patterns observed in sequencing results of libraries generated from methylated and unmethylated DNA fractions.
A representative region (outlined in the green box) of *Arabidopsis* chromosome 3, showing the peaks identified in the DNA enriched for methylated an unmethylated fractions. The methylated regions are outlined in the red boxes and the unmethylated regions are outlined in the blue boxes. The amount of initial input DNA is shown along the left-hand side of the alignments. The data show good concordance with the publicly available bisulfite sequencing data. Data were visualized using the Integrative Genomics Viewer (Broad Institute https://www.broadinstitute.org/igv/). Public data was obtained from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM980986.

Appendix A: Illumina HT Indexes

Appropriate combinations of Illumina indexes are necessary to ensure sufficient nucleotide diversity when sequencing a pool of two or more libraries. Consult the Illumina literature (such as the TruSeq® DNA Sample Preparation Guide) for appropriate pooling guideline information. Compare barcode sequences with Illumina barcodes when in doubt about compatibility.

Indexing Primer Set HT for Illumina sequences:

Index (tube label)	Barcode	Index (tube label)	Barcode
F1	TATAGCCT	**R1**	ATTACTCG
F2	ATAGAGGC	**R2**	TCCGGAGA
F3	CCTATCCT	**R3**	CGCTCATT
F4	GGCTCTGA	**R4**	GAGATTCC
F5	AGGCGAAG	**R5**	ATTCAGAA
F6	TAATCTTA	**R6**	GAATTCGT
F7	CAGGACGT	**R7**	CTGAAGCT
F8	GTACTGAC	**R8**	TAATGCGC
		R9	CGGCTATG
		R10	TCCGCGAA
		R11	TCTCGCGC
		R12	AGCGATAG

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