Guideline for Whole Genome Analysis using NOMe-Seq

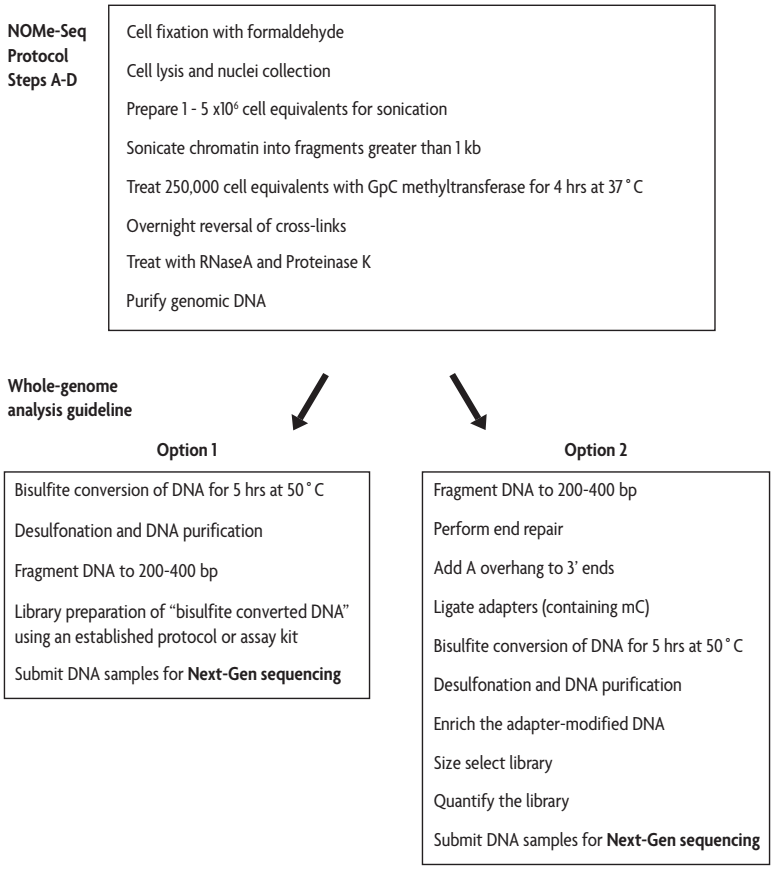
Catalog No.: 54000
Name: NOMe-Seq
Format: 10 rxns

The following guideline is designed to be used with Active Motif's NOMe-Seq Assay and consists of recommendations for the preparation of whole-genome sequencing libraries utilizing NOMe-Seq sample material. There are two options for preparing the samples for whole-genome sequencing and the protocol varies depending on the option selected. Please select the option that best suits your needs and follow the guideline for your selection.

Option 1: Follow the instructions in the NOMe-Seq Assay manual for Steps A – F to prepare samples. This will net bisulfite converted DNA. Sonicate the bisulfite converted DNA to create DNA fragments between 200-400 bp (refer to Step A below for sonication recommendations). Use an established protocol (*e.g.* Epicentre EpiGnome™ Methyl-Seq Kit, Catalog No EGMK81312) for "post-bisulfite conversion" library construction. Follow the recommendations of the manufacturer to generate the sequencing library.

Option 2: Follow the instructions in the NOMe-Seq Assay manual for Steps A – D to prepare samples. This will net artificially methylated genomic DNA. Follow the entire protocol below to add methylated adapters to the fragmented NOMe-Seq DNA. The adapted DNA should then undergo bisulfite conversion, amplification and size selection to generate the library. Samples can be analyzed in Next-Generation Sequencing methods, such as Illumina® Genome Analyzer.

This protocol is designed to serve as a guideline to enable whole-genome analysis of NOMe-Seq DNA. Active Motif is not responsible for the analysis of whole-genome data generated using this guideline. Bioinformatics programs can be found online to assist with the analysis of the data (*e.g.* BisSNP, http://epigenome.usc.edu/publicationdata/bissnp2011/), but these programs are beyond the scope of Active Motif and the NOMe-Seq Assay.



Additional materials required but not provided include:

- Sonicator (*e.g.* Active Motif's EpiShear™ Probe sonicator, Cat. Nos. 53051 & 53052)
- Enzymes needed to perform end repair, adapter ligation and PCR amplification of bisulfite converted DNA
- Qiagen MinElute Kit (Cat. No. 28004)
- Qiagen Gel Extraction Kit (Cat. No. 28704
- Thermocycler and PCR tubes
- Adapters and PCR amplification primers compatible with downstream sequencing platform (*e.g.* Illumina). Utilize adapters that contain methylated cytosines since bisulfite conversion will occur after adapter ligation.
- Razor blades for gel extraction
- Picogreen or Nanodrop to determine DNA concentration
- Agarose gel and electrophoresis apparatus

Step A: Fragment NOMe-Seq DNA

1. Quantify the DNA from Step D.15 of the NOMe-Seq Assay manual using Picogreen or Nanodrop.
2. Add 1-5 µg of NOMe-Seq DNA to a microcentrifuge tube and adjust the final volume to 300 µl by addition of 10 mM Tris-HCl pH 8.5. Use a probe sonicator (EpiShear™Probe Sonicator) at an amplitude of 30% with 20 seconds "On" and 20 seconds "Off" for 15 pulses (total elapsed time of 10 minutes) in order to fragment the DNA to a size range of 200-400 bp.
3. Prepare a 2% agarose TAE gel with 400 ng/ml ethidium bromide.

   Note: Ethidium bromide is a mutagen. Use appropriate precaution when handling.
4. Add 3 µl loading buffer (50 mM Tris pH8.0, 40 mM EDTA, 40% (w/v) sucrose) to 100 ng fragmented DNA. Load DNA and ladder on a 2% agarose TAE gel and run the gel at 120V until the tracking dye is 2/3 down the length of the gel. Confirm the DNA fragments are between 200-400 bp.
5. Purify the fragmented DNA using Qiagen MinElute kit (Cat. No. 28004) to concentrate the DNA. Elute the DNA using 40 µl Buffer EB.

Step B: Perform End Repair

1. Prepare a fresh 1:5 dilution of the DNA Polymerase I Klenow just before use. Add 0.5 µl DNA Polymerase I Klenow (5 U/µl) to 2 µl sterile dH_2O. Pipet up and down to mix. Discard any unused enzyme dilution.
2. Using 200 µl PCR tubes, prepare end repair reactions on the fragmented DNA.

Reagents	**One rxn**
Fragmented NOMe-Seq DNA (1- 5 µg)	X µl
dH_2O	Up to 40 µl
10X T4 DNA ligase buffer	5 µl
10 mM dNTP mix	2 µl
T4 DNA polymerase (3 U/µl)	1 µl
Diluted DNA Polymerase I Klenow (from Step A.1)	1 µl
T4 Polynucleotide Kinase (10 U/µl)	1 µl
Total Volume	**50 µl**

3. Incubate the reactions in a thermal cycler for 30 minutes at 20°C.
4. Purify the reactions using Qiagen MinElute kit (Cat. No. 28004). Elute the DNA using 36 µl Buffer EB.

Step C: Add 'A' Overhang to 3´ Ends of DNA Fragments

1. Using 200 µl PCR tubes, prepare reactions to add an 'A' overhang to the 3´ ends of the DNA fragments.

Reagents	One rxn
DNA from Step B	34 µl
10X NEB Buffer 2	5 µl
1 mM dATP mix	10 µl
Klenow DNA polymerase 3´ to 5´ exo- (5 U/µl)	1 µl
Total Volume	**50 µl**

2. Incubate the reactions in a thermal cycler for 30 minutes at 37°C.
3. Purify the reactions using Qiagen MinElute kit (Cat. No. 28004). Elute the DNA using 14 µl Buffer EB.

Step D: Ligate Adapters to DNA Fragments

1. Prepare adapters as recommended by the manufacturer. Choose adapters that are compatible with the sequencing platform to be used. (*e.g.* We have validated Illumina PE Adapters and TruSeq Index Adapters with this protocol.)

   Note: Since bisulfite conversion occurs after the adapters are ligated to the sample, **use adapters that contain methylated cytosines** in order to preserve the presence of the cytosine following bisulfite conversion.

2. Set up adapter ligation reactions as follows:

Reagents	One rxn
DNA from Step C	12.5 µl
2X Quick Ligation Buffer	15 µl
5 µM Adapter oligo mix (from Step #1 above)	1 µl
Quick T4 DNA Ligase (2,000,000 U/µl)	1.5 µl
Total Volume	**30 µl**

3. Incubate for 20 minutes at room temperature (approximately 24°C).
4. Purify the reactions using Qiagen MinElute kit (Cat. No. 28004). Elute the DNA using 14 µl Buffer EB.
5. Quantify the DNA using Picogreen or Nanodrop.

Step E: Perform Bisulfite Conversion of Adapter-ligated DNA

1. Follow the instructions in the NOMe-Seq Assay Manual for Step E: Bisulfite Conversion and Step F: On-Column Desulfonation and DNA Purification. The optimal range for bisulfite conversion is 500 ng - 2 µg per reaction. It may be necessary to set up multiple bisulfite conversion reactions and to pool the contents following the on-column desulfonation DNA purification step.
2. (Optional) If multiple bisulfite conversion reactions were pooled together, concentrate the DNA using Qiagen MinElute kit (Cat. No. 28004). Elute the DNA using 2 x 18 µl Buffer EB.

Step F: Enrich the Adapter-Modified DNA Fragments by PCR

1. Using a 200 µl PCR tube, prepare the following reactions:

Reagents	One rxn
Adapter Ligated DNA	X µl
dH_2O	Up to 36 µl
PCR primer mix (12.5 µM each)	2 µl
5X Hot Start Reaction Buffer	10 µl
10 mM dNTP mix	1.5 µl
Hot Start *Taq* DNA polymerase (2 U/µl)	0.5 µl
Total Volume	**50 µl**

Note: We recommend using a Hot Start *Taq* DNA polymerase that is optimized for use with bisulfite converted DNA. Follow the recommendations of the manufacturer for preparing the PCR reaction.

2. Amplify the reactions in a thermal cycler as follows:

   95°C for 90 seconds
   (98°C for 10 seconds, 65°C for 30 seconds and 72°C for 30 seconds) for 6 cycles
   72°C for 5 minutes
   Hold at 4°C

3. Remove 45 µl of the PCR reaction for use as the library. Due to the limited number of cycles, it may be difficult to see the DNA on a gel for size selection of the library. Therefore, we recommend using the remaining 5 µl of the reaction and continuing the PCR as stated in Step F.2 for an additional 12 cycles. This sample can be run alongside the library sample on the gel for visualization of the appropriate fragment size to isolate.

Step G: Size Select the Library

1. Prepare a 2% agarose TAE gel with 400 ng/ml ethidium bromide. (Use appropriate precaution when handling ethidium bromide.)
2. Split the 6 cycle PCR amplification reacting into two tubes (22.5 µl each). Add 3 µl loading buffer (50 mM Tris pH8.0, 40 mM EDTA, 40% (w/v) sucrose) to each reaction. Add 1 µl loading buffer to the 18 cycle PCR reaction.
3. Run adapter ligated DNA on a 2% agarose gel for size selection of the DNA library. To avoid potential cross contamination of adjacent wells, leave at least one empty lane between DNA ladder, each reaction of the 6 cycle library sample, the 18 cycle library sample and an additional lane of DNA ladder.
4. Run the gel at 120V for approximately 1 hour or until the tracking dye is 2/3 down the length of the gel.
5. Visualize the DNA using a Dark Reader transilluminator to avoid exposure to UV light. If using a UV light, work quickly when excising the band to minimize direct exposure to UV. Prolonged exposure to UV can damage DNA.
6. Using a clean razor blade, excise a gel slice in the 200-300 bp range. You only need to excise the two 6 cycle library samples. The 18 cycle sample is only used as a visual reference for band size identification since the 6 cycle may be difficult to visualize on the gel.
7. Use a Qiagen Gel Extraction Kit (Cat. No. 28704) to purify the DNA from the agarose in the size range of 200-300 bp. Make the following protocol modifications to the Qiagen Gel Extraction Kit protocol:
   a. Incubate gel slice in 3 volumes Buffer QG and 1 volume isopropanol at **37°C for 30 minutes,** or until completely dissolved, instead of the suggested 50°C.
   b. Add the recommended extra 0.5 ml Buffer QG to the QIAquick column.
   c. During the wash steps, incubate 2-5 minutes in Buffer PE before centrifugation.
   d. Elute the DNA in 20 µl Buffer EB.
8. Determine the final concentration of the library using Picogreen or Nanodrop and submit samples for Next-Gen sequencing.