

Jul 25, 2018

MethylC-seq using EZ DNA Methylation-Gold Bisulfite and Accel-NGS DNA Library Kits

DOI

<https://dx.doi.org/10.17504/protocols.io.p88drzw>

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Protocol Citation: Diep Ganguly, Steven Eichten 2018. MethylC-seq using EZ DNA Methylation-Gold Bisulfite and Accel-NGS DNA Library Kits. protocols.io <https://dx.doi.org/10.17504/protocols.io.p88drzw>

Manuscript citation:

Ganguly D.R., Crisp P.A., Eichten S.R. & Pogson B.J. (2018) Maintenance of pre-existing DNA methylation states through recurring excess-light stress. Plant, cell & environment.

Ganguly D.R., Crisp P.A., Eichten S.R. & Pogson B.J. (2017) The Arabidopsis DNA Methylome Is Stable under Transgenerational Drought Stress. Plant Physiology 175, 1893–1912.

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Protocol status: Working

We use this protocol and it's working

Created: May 19, 2018

Last Modified: July 25, 2018

Protocol Integer ID: 12288

Keywords: MethylC-seq, using ez dna methylation, seq dna library kit, ez dna methylation, ngs dna library kits protocol, sequencing data, methylc, arabidopsi, sequencing, ngs prep with bisulfite, converted dna capability

Abstract

Protocol to use ACCEL-NGS® METHYL-SEQ DNA LIBRARY KIT (NGS Prep with Bisulfite-Converted DNA Capability; Cat. No. DL-ILMMS-12/48) to perform MethylC-sequencing in Arabidopsis.

Code used to analyse raw sequencing data can be found: Please see <https://github.com/dtrain16/NGS-scripts/tree/master/MethylC>.

Attachments



[EZ_bisulfite_convers...](#)

357KB



[covaris_shearing.pdf](#)

961KB



[illumina_low-plex-po...](#)

133KB



[truseq_adapter_pooli...](#)

1.1MB



Materials

MATERIALS

- ✕ 100ml TE Buffer [1X], pH 8.0, Low EDTA (Tris-EDTA; 10mM Tris base, 0.1mM EDTA) **G-Biosciences Catalog #786-150**
- ✕ Qubit **Invitrogen - Thermo Fisher**
- ✕ Homemade SPRI beads solution for DNA clean-up
- ✕ Agencourt AMPure XP SPRI beads **Beckman Coulter Catalog #A63881**
- ✕ Covaris S2/S220 Focused-ultrasonicators **Covaris**
- ✕ EZ DNA Methylation-Gold Kit **Zymo Research Catalog #D5005**
- ✕ Accel-NGS® Methyl-Seq DNA Library Kit **Catalog #30024 30096**
- ✕ Methyl-Seq Set A Indexing Kit (12 indices, 24 rxns) **Catalog #36024**
- ✕ DNeasy Plant Mini Kit **Qiagen Catalog #69104/69106**
- ✕ LabChip GX/GXII **Perkin Elmer**
- ✕ Qubit DS HS assay **Thermo Fisher Scientific Catalog #Q32854**

Before start

Make sure you have fresh nuclease-free water (or low EDTA TE) and 80% ethanol. You will also need working magnetic beads stocks (e.g. AMPure, SPRIselect) and a magnetic stand that holds plates or eppendorf tubes (depending on preference).

Extract high-quality gDNA using method of choice, our lab relies largely on the DNeasy Plant Mini Kit (Qiagen). However, yields from this are quite low despite good quality gDNA.

For all resuspension steps, use low EDTA (0.1 mM) Tris (10mM, pH 8). Alternatively, we also use the elution buffer provided in the DNeasy Plant Mini Kit (Qiagen) - which is probably the same.

Extract and QC gDNA

1. Perform gDNA extraction using method of choice. For example, CTAB can give decent quality gDNA. The DNeasy Plant Mini Kit from Qiagen gives really high-quality plant gDNA, however, yields can be quite low.
2. Run gDNA on a 1% agarose gel to make sure there are no contaminants (e.g. CTAB extraction can leave a large amount of sheared nucleic material) nor degradation.

Normalize and shear gDNA

1. Normalize all gDNA samples to amount of choice (typically 0.5 - 1 µg).
2. Shear aliquoted gDNA with desired input (e.g. 1 µg) and shear using the Covaris ultrasonicator (optimized for obtaining a smear from 200bp - 500bp; peak ~300bp).
 - e.g. covaris settings: 1 × 60 sec cycle; Duty 10%, intensity 5, cycles/burst 200
3. Run on agarose gel (or on LabChip GX) or QC the obtained smear.
4. Use SPRI select beads to clean smaller fragments (Optional - generally not needed). E.g. 0.8X ratio SPRI : sample to remove anything <110bp. Or can also do a right-side cleanup for larger fragments.
 - More info available at:
https://research.fhcrc.org/content/dam/stripe/hahn/methods/mol_biol/SPRIselect%20User%20Guide.pdf
5. Quantify sheared gDNA samples using Qubit.

Bisulfite conversion

1. Conversion is performed using EZ DNA Methylation-Gold Kit (D5005/D5006) as per their manual (see attached PDF).
2. We perform this on an aliquot (20ul) of the sheared gDNA sample (in case anything goes wrong).
3. Make sure to use fresh CT conversion reagent (can be stored at -20C for 1 month).

Generate sequencing library using Accel-NGS Kit

1. Bisulfite converted DNA is used to prepare a next-generation sequencing library as per the Accel-NGS Manual (see PDF attachment) using a normalized input (e.g. 100 - 200 ng converted DNA).
2. The protocol is quite straightforward and reliable. I have had no issues with it. If you are new to library preparations, make sure to read the tips on p. 8.
3. Make sure to plan indexing combinations ahead of time (Methyl-Seq Set A Indexing Kit contains Illumina TruSeq indexing adapters) - prior to PCR amplification (see attachment of low plex pooling) - to facilitate optimal demultiplexing of your samples after the

sequencing run. Particularly important when doing a small number of samples (see PDF attachments).

4. It has been tested that reliably sequence-able libraries have been made using 1/2 reaction volumes from the manual - however, so far I have used full volumes due to a limited number of samples being generated.

5. Depending on input, we try to limit PCR cycling to 6 - 8 cycles, aiming to get a final library molarity of ~ 8nM. Performing a test run on a pooled sample library is definitely worthwhile to optimize the minimal number of samples required to generate a reasonable quantity of library to minimize PCR duplicates.

Pool high-quality libraries equal molar for sequencing

- 5
 1. Final libraries should undergo appropriate quality control. Namely, using a bioanalyser or LabChip GX/GXII to check for fragment size distribution and quantification (we rely on Qubit DS HiSense for quantification, and use the most abundant molecule size to determine library molarity (with the assumption that the average molecular weight of a double-stranded DNA base-pair = 650 g/mol) . This has worked well for us and led to reliable equal molar pooling of samples.
 2. Once all high-quality libraries that are required have been generated, pool all libraries together into an equal-molar sample (make sure to pay attention to molarity and volume required by your sequencing facility, e.g. we aim for 2nM in 15ul using Tris/TE pH 8-8.5). Prepare using aliquots of each individual library, but retain each individual library until data has been generated, screened, and backed-up (consider NCBI or EBI databases which allow for private uploads - ie. data is not accessible publicly).