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Methyl-RAD protocol

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We use this protocol and it's working

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Abstract

This protocol outlines a streamlined and cost-effective approach for genome-wide DNA methylation profiling based on the MethylRAD technique, an adaptation of the 2b-RAD method (Wang et al., 2012). The method employs methylation-dependent restriction enzymes (originally LpnP1, now substituted by MspJI) to selectively digest fully methylated CpG sites in genomic DNA, generating uniform 32 bp fragments. Custom-designed adapters (5Metrad and 3Metrad) are ligated to the digested fragments, followed by PCR amplification using indexed Illumina-compatible primers, allowing for multiplexed high-throughput sequencing. The protocol provides detailed instructions for adapter preparation, digestion, ligation, and PCR optimization, including guidance on minimizing star activity and maximizing library yield. This technique offers a scalable solution for comparative methylome analysis across multiple samples and is particularly suited for non-model organisms lacking reference genomes.

Image Attribution

Gel image: The right band, NC = negative control, DNA fully processed but w/o restriction enzyme

Guidelines

Amplification

Prepare a test-scale PCR to determine optimum cycles number and evaluate relative yield across samples.

For each reaction prepare the following master mix. The following recipe is for a single reaction, so multiply by the number of samples plus some small amount for pipetting error.

H_2O 13.2 µl
DNA ligated 5.0 µl
10 µM ILL-Lib1 0.5 µl
10 µM ILL-Lib2 0.5 µl
2 µM ILL-HT 0.6 µl
2 µM ILL-BC 0.6 µl
10X KAPA Taq Buffer A 2.5 µl
25 mM $MgCl_2$ 0.5 µl
10 mM dNTP Mix 0.5 µl
Evagreen 1 µl
Kapa Taq 0.1 µl

60°C 60 sec; (98°C 5 sec, 60°C 20 sec, 72°C 10 sec) X 10 to 17 cycles

Notes: $MgCl_2$ concentration increased to 2mM; don't use a Hot-start DNA polymerase

Analyze amplicons on a 2% agarose gel with a low molecular weight marker to confirm molecular weight of PCR product.

Select the minimum number of cycles required to produce a visible product at 171-173 bp. Note that primer dimers are likely to be visible at ~70-90 and ~130 bp, so be sure to run the gel until these sizes can be resolved.

ILL-Lib1 AATGATACGGCGACCACCGAG
ILL-Lib2 CAAGCAGAAGACGGCATACGA

ILL-HT_i5index AATGATACGGCGACCACCGAGATCTACAC index ACACTCTTTCCCTACACGACGCTCTTCCGATCT
ILL-BC_i7index CAAGCAGAAGACGGCATACGAGAT index GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Preparatory-scale PCRs

- Plan and record barcode assignments. Each sample has to be assigned a unique combination of index i7 and index 2 i5.
- Prepare a mastermix with all components but ILL-HT and ILL-BC primers for all the samples plus a 10% excess.
- Aliquot 18.8 µl master mix into each well of a PCR plate or strip.

- Using one tip per oligo, add 0.6 µl of the appropriate “BC” oligonucleotide (2 µM) to each well.
- Using a new tip for each reaction, add 0.6 µl of the appropriate HT oligonucleotide (2 µM) to each well.
- Using a new tip for each sample, add 5 µl of each ligation product to the appropriate well or tube. Gently mix with a pipette.
- Amplify using the minimum cycles number determined above.
- Run a high resolution agarose gel and eventually cut-off and purify the right band.

Materials

10X TLE Buffer:
- 100 mM Tris HCl, pH 8
- 1 M NaCl
- 1 mM EDTA
(1 ml Tris HCl 1M pH8, 20ul EDTA 0.5M, 2 ml NaCl 5M, H2O to 10 ml, filter in 0.22um micropore-store in 800 ul aliquots at -20°C)

Oligos:
- 5Metrad: CTACACGACGCTCTTCCGATCT
- 3Metrad: CAGACGTGTGCTCTTCCGATCT
- cmetrad: NNNNAGATCGGAAGAGC_spC3

Adaptors:
- 5Metrad: 5' CTACACGACGCTCTTCCGATCT 3', 3' *spC3*CGGAAGGCTAGANNNN 5'
- 3Metrad: 5' CAGACGTGTGCTCTTCCGATCT 3', 3' *spC3*CGGAAGGCTAGANNNN 5'

For Methylation Dependent Restriction:
- DNA (0.1 µg)
- 10X rCutSmart™ Buffer
- Restriction Enzyme LpnP1 5U/µl (or MspJI as alternative)
- 30X Enzyme Activator Solution
- Nuclease-free water

For Adapter Ligation:
- Adapter 5Metrad 2 µM
- Adapter 3Metrad 2 µM
- H2O
- NEB 10X Ligation Buffer w/ATP
- NEB T4 DNA Ligase (M0202L)

For Amplification:
- H2O
- DNA ligated
- 10 µM ILL-Lib1
- 10 µM ILL-Lib2
- 2 µM ILL-HT
- 2 µM ILL-BC
- 10X KAPA Taq Buffer A
- 25 mM MgCl2
- 10 mM dNTP Mix
- Evagreen

- Kapa Taq

Safety warnings

Hold at 35°C for 15′ then cool to 4°C. (Dont′ hold the tube with the fingers close to the solution. The adapters could melt!)

Adaptors preparation

1 To create adapter 5Metrad, combine each oligo 5Metrad with its complementary oligo cMetrad in a 1:1 ratio in TLE (final buffer concentration 1x) for a total annealed adapter concentration of 10 µM (for example, if purchased oligos are resuspended to an initial concentration of 100 µM, use 10 µl oligo 5Metrad, 10 µl oligo cMetrad, 10 µl 10x TLE buffer and 70 µl nuclease-free water). Do the same for oligos 3Metrad.

2 In a thermocycler, incubate at 90°C for 2 minutes, 95° and then 60 cycles -1°/cycle until the solution reaches a temperature of 35°C. **Hold at 35°C for 15' then cool to 4°C. (Don't hold the tube with the fingers close to the solution. The adapters could melt!)**

3 Store aliquots of 80 µl at -20°C.

Methylation Dependent Restriction

4 Set up the following reaction in a sterile microcentrifuge tube (it is important to add the restriction enzyme last):
- Nuclease-free water: to 15 µl (add first)
- 10X rCutSmart™ Buffer: 1.5 µl (add second)
- DNA (0.1 µg): 100 ng (add third)
- 30X Enzyme Activator Solution: 0.45 µl (add fourth)
- Restriction Enzyme LpnP1 5U/µl: 0.8 µl (add fifth; LpnP1 was discontinued, MspJI is the alternative, same protocol)

5 Incubate at 37°C for 4 hours (extended reaction times may increase the yield of the 32-mer but will also increase star activity).

6 Inactivate the enzyme at 50°C for 20 min then transfer samples to ice.

Adapter Ligation

7 Prepare adapter ligation by making a mastermix with the following reagents and amounts:
- Digested DNA: 10 µl
- Adapter 5Metrad 2 µM: 1.5 µl (dilute 1/5 adaptors)
- Adapter 3Metrad 2 µM: 1.5 µl (dilute 1/5 adaptors)
- H2O: 12 µl
- NEB 10X Ligation Buffer w/ATP: 3 µl
- NEB T4 DNA Ligase: 2 µl (800 U, M0202L)

The total volume in the microfuge tube should be 30 µl. Mix the contents by pipetting up and down several times.

8 Incubate in a thermal cycler 6–12 h at 4°C.

Amplification

9 Prepare a test-scale PCR to determine optimum cycles number and evaluate relative yield across samples.

10 For each reaction prepare the following master mix. The following recipe is for a single reaction, so multiply by the number of samples plus some small amount for pipetting error.
H_2O 13.2 µl
DNA ligated 5.0 µl
10 µM ILL-Lib1 0.5 µl
10 µM ILL-Lib2 0.5 µl
2 µM ILL-HT 0.6 µl
2 µM ILL-BC 0.6 µl
10X KAPA Taq Buffer A 2.5 µl
25 mM $MgCl_2$ 0.5 µl
10 mM dNTP Mix 0.5 µl
Evagreen 1 µl
Kapa Taq 0.1 µl

11 PCR cycling conditions: 60°C 60 sec; (98°C 5 sec, 60°C 20 sec, 72°C 10 sec) X 10 to 17 cycles
Notes: $MgCl_2$ concentration increased to 2mM; don't use a Hot-start DNA polymerase

12 Analyze amplicons on a 2% agarose gel with a low molecular weight marker to confirm molecular weight of PCR product.

13 Select the minimum number of cycles required to produce a visible product at 171-173 bp. Note that primer dimers are likely to be visible at ~70-90 and ~130 bp, so be sure to run the gel until these sizes can be resolved.

14 Run the preparatory-scale PCRs.

14.1 Plan and record barcode assignments. Each sample has to be assigned a unique combination of index i7 and index 2 i5.

14.2 Prepare a mastermix with all components but ILL-HT and ILL-BC primers for all the samples plus a 10% excess.

14.3 Aliquot 18.8 µl master mix into each well of a PCR plate or strip.

14.4 Using one tip per oligo, add 0.6 µl of the appropriate “BC” oligonucleotide (2 µM) to each well.

14.5 Using a new tip for each reaction, add 0.6 µl of the appropriate HT oligonucleotide (2 µM) to each well.

14.6 Using a new tip for each sample, add 5 µl of each ligation product to the appropriate well or tube. Gently mix with a pipette.

14.7 Amplify using the minimum cycles number determined above.

14.8 Run a high resolution agarose gel and eventually cut-off and purify the right band.

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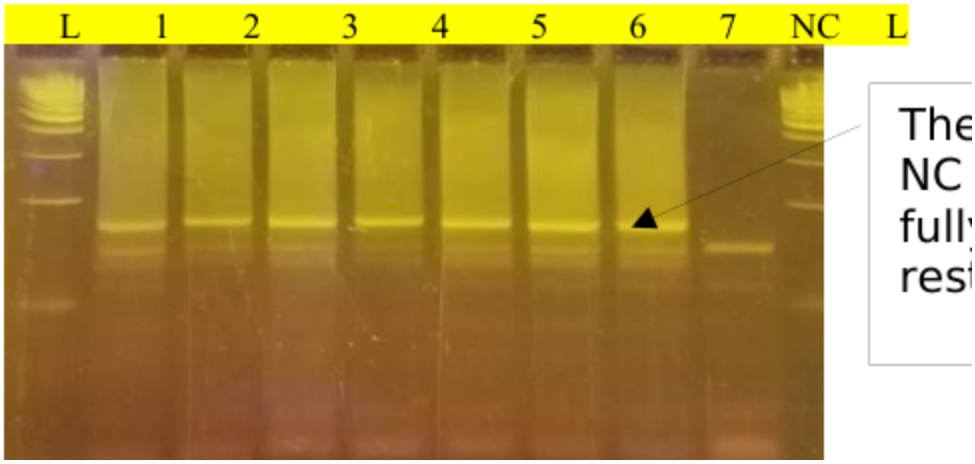

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