Enhanced methylation analysis of Plasmodium berghei by recovery of unsequenceable fragments

Gordon R. McInroy, Dario Beraldi, Eun-Ang Raiber, Katarzyna Modrzynska, Pieter van Delft, Oliver Billker, Shankar Balasubramanian

Detailed Experimental Protocol

1) DNA fragmentation

Covaris M220 sonication

Sonicate genomic DNA in 50uL 1X TE buffer (10mM TRIS-HCl pH 8.0, 0.1 mM EDTA) to an appropriate fragment size. E.g.: for 250 bp peak use the following settings: incident power 75 W, duty factor 10%, cycles per burst 200, treatment time 130s.

2) End Repair (NEB Next Kit)

DNA	1ug	(in 85uL water or 10mM Tris-HCl pH7.4)
End repair buffer	10uL	
End repair enzyme	5uL	
Total	100uL	

- *Mix by gently pipetting entire volume up and down 10x*
- *Incubate for 30min at 20°C*
- *Purification (column or ampure beads)*
- *Elution in 44uL water*

3) A-tailing (NEB Next Kit)

DNA	42uL
Buffer	5uL
Enzyme	3uL
Total	50uL

- *Mix by gently pipetting entire volume up and down 10x*
- *Incubate for 30min at 37°C*
- *Purification (column or ampure beads)*
- *Elution in 35uL water*

4) Ligation (Next Next Kit)

DNA	32.5uL
Ligation buffer	10uL
Adapter pair A	2.5uL
Ligase	5uL
Total	50uL

- *Incubate for 20min at 20°C (r.t. is not 20°C)*
- *Add 1.6x ampure beads for removal of small DNA fragments*
- *Wash beads*
- *Elute with 22uL water, recover 20uL (max for oxBS workflow)*

5) Enter BS protocol

Perform bisulfite conversion protocol of choice, following manufacturer instructions. Once converted DNA is purified, proceed to step 6). This protocol has been established using the EZ DNA Methylation-Gold Kit from Zymo Research.

6) Single extension (eg: with VeraSeq ultra or KAPA HiFi Uracil +)

DNA	20uL	
LM primer	5uL	(10uM stock concentration)
dNTP	1uL	(10mM stock concentration)
Buffer	10uL	
Veraseq Ultra	0.5uL	
Water	13.5uL	
Total	50uL	

Denaturation	*95°C for 3 min*
Annealing	*54°C for 45 sec*
Extension	*74°C for 30 min*

- *Purification with 60uL streptavidin coated magnetic beads (Promega Magnasphere; 1 mg/mL)*
    - *Wash 60uL beads with 1X BB (4x 300uL)*
        - *10 mM Tris pH 7.5*
        - *1 mM EDTA*
        - *2M NaCl*
        - *0.1% Tween-20*
    - *Resuspend beads in 50uL 2X BB*
    - *Add 50uL reaction mixture and incubate for 20 minutes at room temperature*
    - *Wash beads with 1X BB (3x 300uL)*

7) End repair of singly adapted library

End repair buffer	10uL
End repair enzyme	5uL
Water	85uL
Total	50uL

- *Incubate for 30min at 20°C*
- *Wash beads with 1X binding buffer (3x400uL)*

8) A-tailing

Buffer	5uL
Enzyme	3uL
Water	42uL
Total	50uL

- *Incubate for 30min at 37°C*
- *Wash with 1X binding buffer (3x400uL)*

9) Ligation of adapter pair B (NEB Next Quick Ligation Module)

- *Oligos 1b and 2b must be annealed to a final concentration of 25uM in 10mM Tris-HCl pH 7.4 and 50mM NaCl (annealing conditions as in Appendix 1b)*

Ligation buffer	10uL
Adapter pair B	2.5uL
Ligase	5uL
Water	32.5uL
Total	50uL

- *Incubate for 20min at 20°C*
- *Wash with 1X BB (3x 300uL)*
- *Elution with 20uL NaOH (50mM, fresh dilution from 1M stock), 65°C for 15min*

10) qPCR with Kapa Master Mix

Quantify the library by qPCR with the KAPA quantification kit before sequencing.

Appendix: Annealing conditions for adapters:

Perform the following thermocycler program, with ramp rate at minimum (generally 0.1 °C/s)

95 °C	15 min
90 °C	3 min
85 °C	3 min
80 °C	3 min
70 °C	5 min
10 °C	10 min
4 °C	hold