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cf-RRBS protocol

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1 Works for me This protocol is published without a DOI.

CMGG

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ABSTRACT

The methylation profile of circulating cell-free DNA (cfDNA) in blood can be exploited to detect and diagnose cancer and other tissue pathologies and is therefore of great diagnostic interest. There is an urgent need for a cost-effective genome-wide methylation profiling method that is simple, robust and automatable and that works on highly fragmented cfDNA. We report on a novel sample preparation method for reduced representation bisulfite sequencing (RRBS), rigorously designed and customized for minute amounts of highly fragmented DNA. Our method works in particular on cfDNA from blood plasma. It is a performant and cost-effective methodology (termed cf-RRBS) which enables clinical cfDNA epigenomics studies.

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MATERIALS TEXT

- Thin-walled Eppendorf (0.2 mL) tubes
- LoBind Eppendorf tubes (1.5 mL)
- Unmethylated lambda DNA (10 ng/µL stock solution)
- Recombinant Shrimp Alkaline Phosphatase (rSAP, NEB) (1U/µL)
- 10X CutSmart buffer
- MspI (NEB) (20U/µL)
- Nuclease-free H2O
- Klenow Fragment (NEB, 3' -> 5' exo-) (5U/µL)
- dNTPs: 40µM dATP, 4µM dCTP and 4µM dGTP
- T4 DNA ligase (2000U/µL) (NEB)
- ATP (10mM) (NEB)
- NEBNext-adapter (NEB) 10 µM (seq: 5´-/Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTC/ideoxyU/ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3´) without phosphorotioate bond at the 3' end.
- Exonuclease I (20U/µL) (NEB)
- Exonuclease III (50U/µL) (NEB)
- Exonuclease VII (10U/µL) (NEB)
- Antarctic Thermolabile UDG (1U/µL) (NEB)
- Endonuclease VIII (10U/µL) (NEB)
- EZ DNA Methylation-Lightning™ Kit (ZymoResearch)
- KAPA HiFi HotStart Uracil + ReadyMix PCR Kit (KAPA Biosystems): 2X Polymerase mix
- NEBNext universal primer (IDT)
- NEBNext index primer (IDT)
- Magnetic bead cleanup kit (CleanNA – GC biotech).

BEFORE STARTING

Input DNA:

The protocol is tested at 10 ng input DNA, but lower is possible. The DNA should be eluted in maximum 10.6 or 11.1 µL. If the volume is too high, concentrate the sample using a SpeedVac at the lowest possible temperature. The protocol was tested with the SpeedVac at 30°C.

The sample should beforehand be evaluated on the Femto Pulse (Advanced Analytical) for the presence of cfDNA.

The adaptor should be aliquotted per 10-20 samples from the stock solution, heated to 95°C and slowly cooled off (-0.1°C/s).

Preparation of the dNTP mix

Start of by using a stock concentration of 100 mM of dATP, dGTP and dCTP (or adjust calculations accordingly).
Dilute 10X (=> 10 mM of dATP, dGTP and dCTP).
Combine:

- 40 µL of the 10 mM dATP solution together
- 4 µL of the 10 mM dCTP solution
- 4 µL of the 10 mM dGTP solution (= total volume 48 µL).

Add 52 µL of H2O to this mixture
Result: 100 µL working solution of the dNTP mixture.

Prepare input DNA

1 Label 1 thin-walled Eppendorf (0.2 µL) tube per sample (work in strips of 8).

2 Collect DNA samples. The volume should be lower than 10.6 µL*

If the standard amount of lambda spike-in is to be added (1 µL of 0.01 ng/µL):

- Add nuclease-free H2O to a **total volume of 10.6 µL**

If a lower amount of lambda spike-in is to be added (0.5 µL of 0.01 ng/µL), or if the total DNA is lower than 10 ng:

- Add nuclease-free H2O to a **total volume of 11.1 µL**

*If the volume is higher, concentrate the samples using a vacuum centrifuge at 30°C. Don't forget to turn the vacuumpump on.

Lambda spike-in and dephosporylation

3 Add to Eppendorf tube:
- Unmethylated lambda DNA (0.01 ng/µL, 0.1 %w/w): **1 µL or 0.5 µL** depending on the DNA input (prepare by going from 10 ng/µL stock to 1 ng/µL to 0.1 ng/µL to 0.01 ng/µL)
- Recombinant Shrimp Alkaline Phosphatase (rSAP, NEB) (1U/µL): **1 µL**
- 10X CutSmart buffer: **1.4 µL**
- DNA: **10.6 µL or 11.1 µL**

Total volume: 14 µL

Pipet up and down to mix DNA with samples.

Note: all steps with enzymes should be in a cooling block.

4
- Incubate 60 minutes at 37°C.
- Heat kill the phosphatase for 30 minutes at 75°C.

MspI digest

5 Prepare mastermix containing per sample + 1:
- 1 µL mixture containing:
- 10X CutSmart buffer: **0.1 µL**
- MspI (NEB) (20U/µL): **0.5 µL**
- Nuclease-free H20: **0.4 µL**

Add **1 µL mastermix** to each sample.
Total volume: 15 µL

Pipet up and down to mix DNA with samples.

6 Incubate for 30 minutes at 37°C.

Meanwhile, aliquot and melt the adapter (step 9).

End-repair and A-tailing

7 Prepare mastermix containing per sample + 1:
- 10 µL mixture containing:
- Klenow Fragment (NEB, 3' -> 5' exo-) (5U/µL): **0.5 µL**
- dNTP: **0.25 µL** (40µM dATP, 4µM dCTP and 4µM dGTP)
- 10X CutSmart buffer: **1 µL**
- Nuclease-free H2O: **8.25 µL**

Add 10 µL of the mastermix to each sample
Total volume: 25 µL

8 Incubate this 25 µL-mixture:
- 20 minutes at 30°C
- 20 minutes at 37°C
- Heat kill for 20 minutes at 75°C.

Adapter ligation

9 Beforehand, the adapter should be aliquoted and heated to 95°C and afterwards -0.1°C/sec to 4°C. This is to reduce the number of secondary structures. A slow cooling ensures the most stable secondary structure to be formed.

Prepare 2 mastermixes containing per sample + 1

1. Ligase-mixture: **5 µL**
   - T4 DNA ligase (2000U/µL) (NEB): **0.5 µL**
   - 10X CutSmart buffer: **0.5 µL**
   - Nuclease-free H20: **4 µL**
2. Adapter mixture: **10 µL**
   - Adapter* 10 µM: **1 µL**
   - ATP (10mM) (NEB): **4 µL**
   - 10X CutSmart buffer: **1 µL**
   - Nuclease-free H20: **4 µL**

Keep the two mixtures separated for as long as possible (or else the T4 ligase can ligate the adapters).
- Add both mixtures (**ligase: 5µL**, **adapter: 10 µL**) to the Eppendorf tube.

Total volume: 40 µL

*The adapter is identical to the NEBNext-adapter (NEB) (seq: 5´-/Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTC/ideoxyU/ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3´) and was synthesized at IDT, but without phosphorotioate bond at the 3' end. Phosphorothioate bonds are resistant to nuclease degradation, but our cfRRBS protocol is depending on the action of exonucleases (see further).

10 Ligation was done overnight (14 hours) at 16°C and the ligase was heat killed at 65°C for 10 minutes.

Exonuclease

11 Prepare mastermix containing per sample + 1:
- **5 µL** mixture containing:
- Exonuclease I (20U/µL) (NEB): **0.5 µL**
- Exonuclease III (50U/µL) (NEB): **0.5 µL**
- Exonuclease VII (10U/µL) (NEB): **0.5 µL**
- 10X CutSmart buffer: **0.5 µL**
- Nuclease-free H20: **3 µL**

Add mixture to Eppendorf tube
Total volume: 45µL

Note: keep enzymes on ice.

12
- Incubate for 2 hours at 37°C
- Heat kill 20 minutes at 95°C.

The exonucleases will degrade unligated adapter and partially ligated DNA-fragments but leave completely circular DNA-products derived from MspI/MspI-fragments produced from cfDNA untouched.

Endonuclease

13 Prepare mastermix containing per sample +1:
5 µL mixture containing:
- Antarctic Thermolabile UDG (1U/µL) (NEB): **1 µL**
- Endonuclease VIII (10U/µL) (NEB): **0.5 µL**
- 10X CutSmart: **0.5 µL**
- Nuclease-free H2O: **3 µL**

Add mixture to Eppendorf tube
Total volume: 50 µL

UGD cuts a uracyl which results in an abasic site. This abasic site is recognised by endonuclease VIII. This step cuts the hairpin and creates ssDNA (bisulfite requires ssDNA).

14
- Incubate for 1 hour at 37°C
- Heat kill for 10-20 minutes at 75°C.

Bisulfite conversion

15 The MspI/MspI-digested adapter-ligated DNA fragments are bisulfite converted using the EZ DNA Methylation-Lightning™ Kit (ZymoResearch) according to manufacturer's instructions.

More information: https://www.zymoresearch.com/media/amasty/amfile/attach/_D5030T_D5030_D5031_EZ_DNA_Methylation-Lightning_Kit_Instructions_ver_1_0_4_LKN-SW_.pdf

16 Label new thin-walled Eppendorf tubes 0.2 mL: 2 per sample.

As there is 50 µL total sample, two Eppendorf tubes per sample is needed.

17 Add **130 µl** of Lightning Conversion Reagent to **25 µl (differs from the manifactors protocol)** of a DNA sample in a PCR tube.

Mix, then centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.

Note: If the volume of DNA is less than 20 µl, compensate with water.

18 Place the PCR tube in a thermal cycler and perform the following steps:
1. 98°C for 8 minutes
2. 54°C for 60 minutes
3. 4°C storage for up to 20 hours.

Note: Replicate reactions can be cleaned using the same column for each by repeating steps 3-5. The capacity of the collection tube with the column inserted is 800 µl. Empty the collection tube whenever necessary to prevent contamination of the column contents by the flowthrough.

19 Label the columns.

20 Add **600 µl** of **M-Binding Buffer** to a Zymo-SpinTM IC Column and place the column into a provided Collection Tube.

Note: make sure ethanol was added to the M-Wash buffer (step 29).

21 Load the sample (from Step 2) into the Zymo-SpinTM IC Column containing the M-Binding Buffer.

Close the cap and mix by inverting the column several times.

22 Centrifuge at full speed (> 10,000 x g) for 30 seconds.

Discard the flow-through.

*****REPEAT THE PREVIOUS STEP*****

23 Add **100 µl** of **M-Wash Buffer** to the column.

Centrifuge at full speed for 30seconds.

24 Add 200 µl of **L-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes.

During this step, label Eppendorf **Lo-Bind** tubes in which the universal and sample index will be added (see below).

After the incubation, centrifuge at full speed for 30 seconds.

Add **200 µl** of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds.

25

26 ***Repeat this wash step***

27 **Place the column into a LoBind Eppendorf tube (don't forget or you lost your sample)** and add **15 µl of nuclease-free H2O (instead of the Elution buffer according to the protocol)*** directly to the column matrix.

Let stand at room temperature for 3 minutes.

Centrifuge for 30 seconds at full speed to elute the DNA.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long-term storage, store at or below -70°C.

*Elution buffer can inhibit the PCR.

Final amplification step (KAPA HiFi HotStart Uracil+ ReadyMix PCR Kit (KAPA Biosystems)

28 Add mixture to the indices-labelled Eppendorf tubes containing:
- 2X Polymerase mix: **15 µL**
- NEBNext universal primer (IDT): **0.9 µL**
- NEBNext index primer (IDT): **0.9 µL**

Add 14 µL of the bisulfite converted sample to the mixture.

29 PCR protocol:
1. 5 minutes 95°C,
2. 17-19x (20 seconds 98°C, 15 seconds 65°C, 45 seconds 72°C)
3. 5 minutes 72°C

Meanwhile, **take the Ampure beads out of the fridge.**

Magnetic bead purification

30 Meanwhile, the sample is cleaned up by magnetic bead cleanup (Ampure)

Label LoBind Eppendorf tubes:
- 1 Eppendorf tube per sample for magnetic bead purification
- 1 Eppendorf tube containing the final libraries: label carefully.

Vortex the beads very thoroughly.

Make **FRESH** 80% ethanol by adding 2 mL H2O to 8mL ethanol.

31 Add beads 2.5X the volume of the PCR (e.g. 30 µL PCR: add 75 µL beads)

This removes all fragments < 70 bp.

32 Incubate for 5 minutes.

After this step, the beads contain DNA, so don't remove them.

33 Add Eppendorf tubes to magnet and incubate for 2 minutes.

Remove the liquid from the Eppendorf tubes.

34

35 Add **190 µL** washing buffer (= 80% ethanol).

36 **Remove 190 µL with 200 µL** pipet, so no liquid remains.

37 ***Repeat the washing step***

38 Remove any remaining ethanol with a smaller pipet.

Dry the sample for 2-5 minutes at 38°C.

39 Remove the Eppendorf from the magnet.
Add **20 µL** TE buffer (0.1X) and pipet up and down

- Prepare TE buffer by adding 900 µL nuclease-free H2O to 100 µL TE-buffer (1X).

40 Incubate for 3 minutes.

41 DNA is now present in the TE buffer, so the beads can be removed.

Pipet the mixture containing the sample and TE buffer to the final Eppendorf tube.

Repeat the PCR if the libraries are not amplified enough (based on the FA results).

Fragment Analyser

42 Load samples onto the Fragment Analyser. Give additional PCR cycles based on the FA profile if neccesary.

Quantification and sequencing

43

Based on the concentration value obtained the libraries were pooled and were sequenced on a NextSeq500 instrument, performing a SE75 run using 15% PhiX with 7 dark cycles if all the samples in the run are of RRBS origin. Else, 1-5% PhiX and no dark cycles are sufficient.