Protocol: Amplicon Bisulfite Sequencing

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Reagents:

Reagents	Link
EZ DNA Methylation Kit (Zymo)	https://www.zymoresearch.de/ez-dna-methylation-kit
HotStarTaq (QIAGEN)	https://www.qiagen.com/us/shop/pcr/end-point-pcr-enzymes-and-kits/regular-pcr/hotstartaq-dna-polymerase/#orderinginformation
Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen)	https://www.thermofisher.com/order/catalog/product/P11496
AMPure XP SPRI beads	https://www.beckman.de/reagents/genomic/cleanup-and-size-selection/pcr
Illumina Nextera Indices	https://emea.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-xt-dna.html?langsel=/de/

Workflow:

1.) Bisulfite Conversion

Follow the manufacturer's instructions of the EZ DNA Methylation Kit (Zymo). We have listed a short version of the protocol here:

Prior to protocol:
-add 24 ml EtOH (100%) to 6 ml M-Wash Buffer
-dilute DNA sample to 250 ng in 45 µl H2O

Prepare BS conversion reagent *(each tube contains 10 reactions)*
-add 750 µl H2O and 210 µl M-Dilution Buffer to a tube of CT Conversion reagent
-mix 10 min with a vortexer until completely solubilized

-add 5 µl M-Dilution Buffer to 45 µl DNA sample & mix
-incubate at 37 °C for 15 min
-add 100 µl of CT conversion reagent, mix and spin down
-incubate at 50°C o/n in the dark
-put samples on ice for 10 min
-add 400 µl M-Binding buffer to a Zymo Spin IC column
-add the sample to the column
-close the tube and invert few times
-centrifuge at full speed for 30 sec (discard flow-through)
-add 100 µl M-Wash Buffer, centrifuge full speed for 30 sec
-add 200 µl M-Desulphonation buffer

-incubate at RT for 15-20 min, centrifuge full speed for 30 sec
-add 200 µl M-Wash Buffer, centrifuge full speed for 30 sec
-add 200 µl M-Wash Buffer, centrifuge full speed for 30 sec
-put the column in a clean 1.5ml Eppi
-elute twice in 30 ul M-elution buffer

2.) Primer design for PCR on bisulfite converted DNA

- Primers should not contain CG dinucleotides
- Primer design with https://amplicondesign.dkfz.de
- Amplicons should have a length between 150 and 300 bp
- Add the following adapter to the forward or reverse primer respectively:

Adapter which have to be added to primer:
i5 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[primer_fwd]
i7 5' AGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[primer_rev]

3.) Test the ordered primers in a bisulfite PCR

Reagent	1x [25 µl]	8x
10x PCR buffer	2.5 µl	20 µl
dNTPs (10mM)	0.5 µl	4 µl
Primer fwd (5 uM)	0.5 µl	1 µl of Mix
Primer rev (5 uM)	0.5 µl	
Hot Star Taq	0.125 µl	1 µl
Template DNA	1 ng	8 µl
H2O	19.875	159 µl

Time	Temp	
15 min	95 °C	1x
30 sec	94 °C	45x
30 sec	52 °C	
1 min	72 °C	
10 min	72 °C	1 x

You should try 50, 52 and 55°C as annealing temperatures for the primers and choose the best temperature.

Analyze the PCR products on an agarose gel. Check for the expected sizes and re-design primers with an unspecific amplification.

4.) AmpBS-Seq PCR

With the tested primers we now want to amplify our regions of interest of the samples:

Reagent	1x [25 µl]	1x [12.5 µl]
10x PCR buffer	2.5 µl	1.25 µl
dNTPs (10mM)	0.5 µl	0.25 µl
Primer fwd (5 uM)	0.5 µl	0.5 µl
Primer rev (5 uM)	0.5 µl	0.5 µl
Hot Star Taq	0.125 µl	0.0625 µl
Template DNA	1 ng	0.5 µl
H2O	19.875 µl	9.4375 µl

Time	Temp	
15 min	95 °C	1x
30 sec	94 °C	45x
30 sec	52 °C	
1 min	72 °C	
10 min	72 °C	1 x

5.) Measure the concentration of the PCR product

Measure the concentration of the PCR products and pools all products of one sample at an equimolar ratio.
We use PicoGreen to determine the DNA concentrations according to the manufacturers instructions.
Calculate the molarity of each amplicon:

Total pmol: (((conc.[ng/µl] * 5 µl)/1000)/(660*AmpliconSize[bp]))*10^6

We want to use a maximum of 3µl per PCR product. If the molarity of one amplicon is smaller than the 25% quantile of all amplicons for one patient, then we use 3 µl.

For a lot of samples: Measure 4-5 samples and determine the mean concentration for each PCR product. Pipet according to your measurements.

6.) Bead clean-up

Perform AMPure XP bead clean-up according to the manufacturer's instructions. In short, use 0.8x beads and 1x PCR product. Elute in 25 µl:

- Add 0.8x beads to PCR products
- Incubate for 10 min at RT
- 3x wash with 100 µl EtOH (80%)
- Let pellet dry
- Elute in 25 µl H2O

7.) Barcoding PCR

Reagent	1x [25 µl]
NEBNext High-Fidelity 2x PCR mix	12,5 µl
FWD index primer	0,75 µl
REV index primer	0,75 µl
SYBR-Green (100x)	X
DNA	5 µl
H2O	6 µl

Add SYBR-Green in the PCR Master Mix in a 1:100 dilution.
Run the PCR in a qPCR and stop when you see a nice amplification coming up (mostly just 3-5 cycles)

DO NOT OVER AMPLIFY AS THIS MIGHT CAUSE A PCR BIAS TOWARDS EITHER METHYLATED OR UNMETHYLATED FRAGMENTS!

Cycle:

Temp	Time	Repeat
95 °C	3min	1x
95 °C	20 sec	
62 °C	15 sec	x
72 °C	30 sec	

8.) SPRI clean-up

Add SPRI-beads in a 1:1 ratio and perform the clean-up as stated before.

Pool the samples equimolar and submit the library for sequencing on a MiSeq 150bp paired-end.