Supplementary Material For:

Technical considerations for reduced representation bisulfite sequencing with multiplexed libraries

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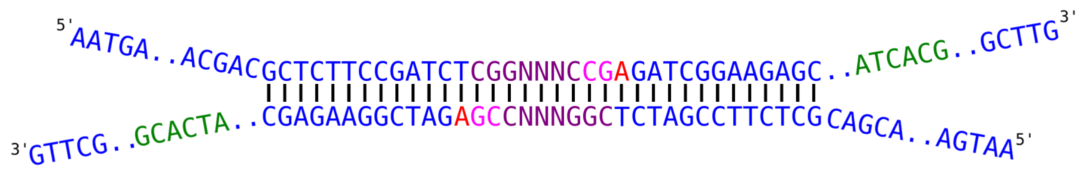

Figure S1. Hypothetical MspI digested fragment ligated with barcoded adaptor. Genomic DNA was digested with MspI (C^CGG). Digested DNA (purple) was end repaired (fuchsia pink) and 3' adenine overhangs (red) were added. DNA fragments were ligated to Illumina TruSeq adaptors (blue) with a unique 6 bp index (green).

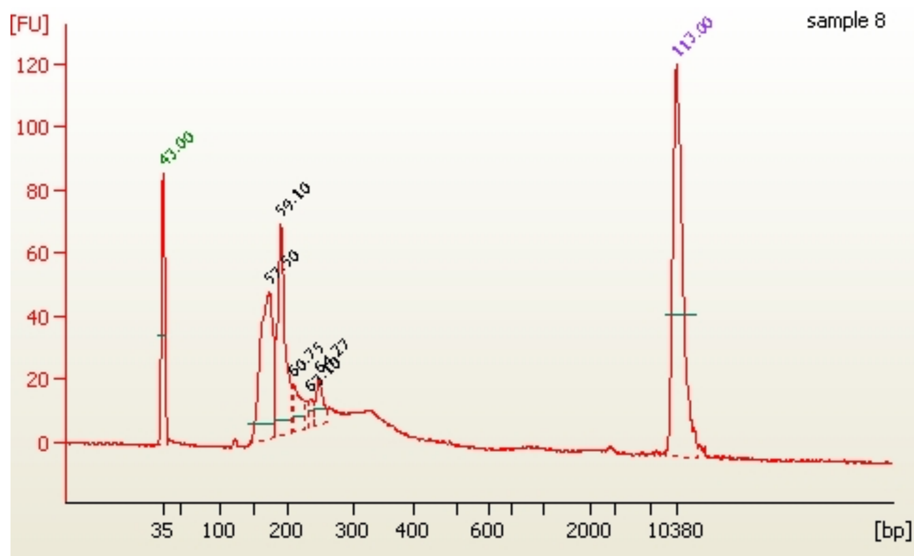

Figure S2. Representative Bioanalyzer electropherogram of a RRBS library.

Each of the RRBS libraries was run on an Agilent 2100 Bioanalyzer using the high sensitivity DNA kit to determine the quality of the samples. The electropherogram displays a data plot of fragment size (bp) versus fluorescence intensity (fluorescence units, FU). Peaks at 35 bp and 10380 bp represent lower and upper markers. The 160-340 bp peaks represent the RRBS library.

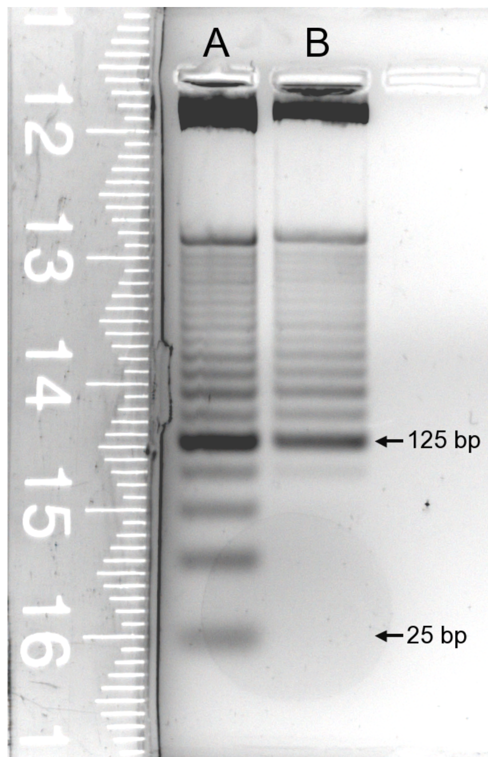

Figure S3. Loss of smaller fragments (<100 bp) in AMPure bead purification. Agencourt AMPure magnetic beads were used to purify RRBS libraries following adaptor ligation. To verify exclusion of <100 bp fragments, 5 µL of 25 bp DNA ladder was either left untreated (A) or purified using AMPure beads (B) and separated on a 3% (w/v) NuSieve agarose gel.

(a)

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```

(b)

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+
;?=?BDDD)@3D9CE91:4?8:):)7(--(-5A37@CEA#####################################
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+
AAAADFFFFHGHHHJJEGGGIJFGIJJIJIFGGIJ4E4*?0)99?FIHIIIIEFDDDBDDDDDBDDDDDDBDBBD
```

Figure S4. Examples of different formats of sequenced reads in different versions of machines. A. Two reads from a run performed on GAII machines. B. Two reads from a run performed on HiSeq2000.

Table S1. Comparison of basic features between Illumina GAII and HiSeq 2000 sequencer

Features	Illumina GAII	Illumina HiSeq2000
Chemistry	Version 2	Version 3
Tiles	120 (0.55 mm^2)	24 (5.5 mm^2)
Flow cells	1	2
Light Intensity and imaging	Remains same throughout and smaller imaging area (TIRF)*.	Increased after 75 cycle for accurate base-calling even at the end of the reads. More than 7x larger imaging area (Epi-illumination*)
Maximum read length (base pairs)	100	150
Alignment output	150 GB	1.2 TB
Base call data	100 GB	660 GB
No of reads per lane	20-30 million	~190 million
Run time	10.5 days for PE 100 bp run	8.5 days for SE
Approx. cost of sequencing one sample**	2500 USD	600 USD

* Total Internal Reflection Fluorescence (TIRF) Microscopy; Epi-illumination involves illuminating and detecting from one side of the sample

**Assuming 20 million reads per sample

Table S2. Comparison of Illumina Paired-End and TruSeq sample preparation

Features	Paired-End kit protocol	TruSeq v2 kit protocol
Input DNA	5 µg	1 µg
Clean-up procedure	Column purification	AMPure XP beads
Adaptor	Non-methylated adaptor; methylated adaptor sold separately	Universal methylated adaptor; 12 indexes available*
Throughput	<10 at a time	Optimized for 96-well plate
Reagents	5 enzymes; 2 substrates; 3 buffers	4 master mixes; 1 buffer
Controls	0	4 In-line controls

* TruSeq v3 kit has 24 indexes available for multiplexing

Table S3. Multiplexed adaptor ligation protocol

Component	Volume (µL)
MspI Digestion	
DNA (175 ng/µL)	14
MspI (20U/µL)	4
10× restriction buffer	4
MilliQ water	18
TOTAL	40
Incubate overnight at 37°C; QIAquick purify, 60 µL elution	
End Repair	
Sample (from above)	60
TruSeq End repair mix	40
TOTAL	100
Incubate for 30 min at 30°C; MinElute purify, 17.5 µL TE elution	
A Addition	
Sample (from above)	17.5
TruSeq A-tailing mix	12.5
TOTAL	30
Incubate for 30 min at 37°C	
Adaptor Ligation	
Sample (from above)	30
TE buffer	2.5
TruSeq indexed adaptor	2.5
TruSeq DNA ligase mix	2.5
Incubate for 10 min at 30°C, add 5 µL TruSeq stop ligase mix; AMPure purify, 18 µL elution	

Table S4. PCR Master mix for bisulfite converted DNA

Component	Volume (μL)*
10× PfuTurbo buffer	1.20
dNTP stock (2.5 mM)	1.44
TruSeq primer cocktail	1.44
BS DNA template	1.44
PfuTurbo Cx	0.58
MilliQ water	5.90

*The volumes given are for a single 12 μL PCR reaction and can be scaled up accordingly