Multiplexing for oxidative bisulfite sequencing (oxBS-seq)

*Kristina Kirschner[1,2,3], Felix Krueger[6], Anthony R. Green[1,2,3,7], Tamir Chandra[4,5,#, *]*

[1]Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 0XY, United Kingdom
[2] Department of Haematology, University of Cambridge, Cambridge, United Kingdom
[3] Stem Cell Institute, University of Cambridge, Cambridge, United Kingdom
[4]Epigenetics ISP, The Babraham Institute, Cambridge CB22 3AT, United Kingdom
[5]The Wellcome Trust Sanger Institute, Cambridge CB10 1SA, United Kingdom
[6]Bioinformatics, The Babraham Institute, Cambridge CB22 3AT, United Kingdom
[7] Department of Haematology, Addenbrooke's Hospital, Cambridge, United Kingdom
Current address: The Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, EH4 2XU, United Kingdom

*Corresponding author: tamir.chandra@igmm.ed.ac.uk

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Abstract

DNA modifications, especially methylation, is known to play a crucial part in many regulatory processes in the cell. Recently, 5-hydroxymethyl cytosine (5hmC) was discovered, a DNA modification derived as a intermediate of 5mC oxidation. Efforts to gain insights into function of this DNA modification are underway and several methods were recently described to assess 5hmC levels using sequencing approaches. Here we integrate adaptation based multiplexing and high-efficiency library prep into the oxBS-seq workflow reducing the starting amount and cost per sample to identify 5hmC levels genome-wide.

Keywords: oxidative bisulfite sequencing, multiplexing, 5 -hydroxymethyl cytosine, pooling, DNA, methylation

1. Introduction

5-hydroxmethyl cytosine is a recently discovered DNA modification. The role of 5hmC has mainly been studied in brain tissue and embryonic stem (ES) cells where this DNA modification is very prominent and thought to be important in development and differentiation respectively (Fu and He, Current Opinion of Chemical Biology, 2012). Enrichment of 5hmC at promoters and other gene regulatory elements suggests a role in gene regulation, as does its appearance at poised chromatin regions in developmentally regulated ES cell genes (Fu and He, Current Opinion of Chemical Biology, 2012). Moreover, in ES cells the balance between pluripotency and lineage commitment seems to be linked to the balance of 5hmC and 5mC levels (Fu and He, Current Opinion of Chemical Biology, 2012).

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To distinguish 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC), oxidative bisulfite sequencing (oxBS-seq) has been developed [1]. Another technique for the detection of 5hmC, TAB-seq, is described in **Chapter 33** and immunoprecipitation and enrichment methods for 5hmC are described in **Chapter 35**. Conventional bisulfite conversion cannot distinguish between 5mC and 5hmC. The combination of oxBS-seq and BS-seq can distinguish unmodified C, 5mC and 5hmC, because each state shows a unique pattern in BS-seq and oxBS-seq (Table 1), resulting in: 5hmC = Unconverted C in BS-seq – Converted C in oxBS-seq.

Table 1: Conversion scheme

	C	5mC	5hmC
BS	U	C	C
OxBS	U	C	U

Workflow and reagents have been described in a recent protocol [2] and combined in a kit (TrueMethyl^{TM} 6 kit (http://www.cambridge-epigenetix.com/). However, low starting amounts of DNA and cost per sample might limit the use of this method.

Here we report a method to multiplex low input DNA samples with TruSeq Illumina adapters. In short, samples are prepared for adapter ligation, pooled and subjected to oxBS treatment. This approach will reduce costs per sample and allow for low input material to be treated using the TrueMethyl^{TM}6 kit (Figure 1). Pooled samples being chemically converted in single tubes increases the comparability between samples.

However, the success of 5hmC detection genome wide depends on the abundance of the modification and the depth of sequencing. Brain tissue and embryonic stem cells contain high amounts of 5hmC which can therefore be easily detected genome-wide whereas most somatic tissues contain low levels of 5hmC making it hard to detect difference in 5hmC abundance.

2. Materials

2.1 Preparation of the NGS library

1. Covaris® E220.

2. Sonication tubes (Covaris – microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm).

3. NanoDrop (Thermo Fisher Scientific).

4. Quant- it ^{TM}Pico Green® dsDNA assay (Thermo Fisher Scientific).

5. Buffer TE (10mM Tris-CL, 1mM EDTA pH8).

6. Spectrophotometer with plate reader option.

7. NEBNext® Ultra™ DNA library prep kit for Illumina (New England Biolabs).

8. Illumina® TruSeq adapters.

9. Spike in controls from the TruMethyl™ Kit (Cambridge Epigenetix).

10. DNA LoBind® tubes (Eppendorf) or similar.

11. TipOne®RPT (low retention) tips.

12. Acetonitrile, HPLC grade.

13. Magnetic stand for 2 mL tubes.

14. Agencourt® AMPure® XP Beads (Beckman Coulter).

15. Kapa Library Quantification kit for Illumina® platforms (KapaBiosystems).

2.2 oxBS-seq and BS-seq

1. TrueMethyl™ 6 Kit (Cambridge Epigenetix, supplied Biorad® P6 Micro-Bio spin columns need to be stored at 4°C).

2. Thermocycler.

3. PCR tubes or strips (0.2 mL).

4. Minicentrifuge for 0.2 mL PCR strips.

5. Primers for Illumina® TrueSeq indexed adapters.

6. Ethanol, HPLC grade.

7. Agencourt® AMPure® XP Beads (Beckman Coulter).

8. Magnetic stand for 2 mL tubes.

9. Quant- it™ Pico Green® dsDNA assay (Thermo Fisher Scientific).

10. 2100 Bioanalsyer Instrument (Agilent Technologies).

11. High sensitivity DNA Kit (Agilent Technologies).

2.3 Data analysis

1) Seqmonk (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/)

2) Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/)

3. Methods

3.1 Preparation of the NGS library

The ideal input amount into the oxBS-seq workflow is 1 µg. To allow for smaller amounts of input DNA per sample, we pool samples after multiplexing to reach the desired amount (*see* **Note 1**). In this protocol we started with six samples of 150 ng each. Accurate and identical starting amounts are crucial in this workflow (*see* **Note 2**). We used the Covaris® E220 system to sonicate our DNA and quantified it using the Quant- it ™Pico Green® dsDNA assay. For small amounts of DNA to be converted into a NGS library, pipetting steps should be minimalized to avoid loss of sample. Therefore, the end repair and adapter ligation steps are performed using the NEBNext® Ultra™ DNA library prep kit for Illumina® where all reactions take place in the same tube without a clean-up step before the adapter ligation. To allow for multiple samples to be sequenced together multiplexing adapters are used during the ligation step. We are using Illumina® TruSeq adapters for multiplexing in this protocol. For the oxidation reaction to work optimally, ethanol should be avoided during the clean-up procedure, as any residue of ethanol will affect the oxidation step. TrueMethyl™ 6 Kit Ultra pure water is used for elution and 80% acetonitrile is used for washing the beads as an alternative to ethanol since it can be tolerated in the oxidation step.

1. Sonicate samples in 130 µL volume with Covaris® E220 (Settings: Intensity 4, cycles per burst 200, 80 seconds) to approximately 300-400 base pairs (bp) in size.

2. Follow the Quant- it ™Pico Green® dsDNA assay to specify the input amount of DNA for each sample. Use 150 ng in 55 µL (Water or TE) per sample for input into the library generation protocol.

3. Prepare 150 ng sheared DNA in 55 µL per sample as input material in PCR tubes.

4. Add 3 µL End Prep Enzyme Mix and 6.5 µL 10x End repair Reaction Buffer per sample to a total volume of 65 µL.

5. Add 0.5 µL of spike in sequencing control (8 ng/µL) supplied with the TrueMethyl™ 6 Kit directly to the PCR tube (3% w/w; 3.8 µl/µg) (*see* **Note 3**).

6. Mix by pipetting and incubate on thermocycler with heated lid on for 30 min at 20°C and 30 min at 65°C. Hold at 4°C.

7. Add 3.5 µL of TruSeq Illumina® adapters (1:10 dilution in water) for multiplexing. Be careful to not use NEB adapters at this stage.

8. Add 15 µL of blunt/TA Ligase Master Mix and 1 µl ligation enhancer. Mix and spin briefly to collect all sample material.

9. Incubate for 15 minutes at 20°C on a thermal cycler. Note: Heat inactivate ligase for 10 minutes at 65°C if pooling during clean up.

10. The libraries can be pooled in an DNA LoBind® tube at this point. The six pooled libraries should be approximately 500 µL (83*6) (*see* **Note 4**).

11. Add 325 µL (0.65X) of Agencourt® AMPure® XP Beads to the pooled ligation reaction and mix well by pipetting

12. Use a beads ration of 0.65X to remove any Adapter dimers here. However, size selection could also be done after library amplification if starting amounts are extremely low.

13. Incubate for 5 min at room temperature. Put tube on magnetic stand to separate beads from supernatant until the solution is clear. Remove the supernatant without disturbing the beads.

14. Wash the beads with 1 mL 80% Acetronitrile (*see* **Note 5**) per tube whilst on the magnetic stand. Discard supernatant. Perform wash step with 80% Acetronitrile once more and discard the supernatant again. Let the beads air dry for 5 minutes until the beads are look dry and/or start to crack (*see* **Note 5**).

15. Keep tube on the magnetic stand and add 40 µL Ultra pure water to elute the DNA from the beads. Remove the tubes from the magnet and re- suspend the beads by pipetting. Place the tubes onto the magnetic stand until solution has cleared.

16. Remove and keep the supernatant as input to the TrueMethyl ᵀᴹ6 workflow. Purified DNA can be stored up for 24 hours 2-8°C. For longer term storage, freeze at -20°C.

3.2 DNA oxidation

Oxidation of DNA is performed on half of each sample resulting in the 5hmC rate and location of the 5hmC mark for each sample after sequencing. In essence, site- specific oxidation and conversion of 5hmC bases to uracil is performed during the protocol using mild conditions to ensure maximum sample recovery. All centrifugation steps are done at room temperature. Only use Ultra Pure Water supplied with the kit.

1. Separate your sample into two parts for oxBS and BS conversion if pooled material is more than 1 µg. If pooled material is less than 1µg then the sample does not need to be divided for the buffer exchange columns, but must be divided for the oxidation step.

2. Set the thermocycler to 37°C before starting.

3. Resuspend the matrix of the Biorad® P6 Micro-Bio spin column by flicking the column. Centrifuge quickly at 1000 x *g*. Remove the top cap and the bottom tip of the column and place into a new 2.0 mL tube. Centrifuge column at 1000 x *g* for 2 min to pack the column and remove any residual buffer. Discard the flow through.

4. Add 500 µL Ultra Pure Water and centrifuge the column at 1000 x *g* for 1 minute. Discard the flow through. Repeat the wash step three more times, always discarding the flow through. For the final wash, centrifuge the column for 2 min, discard the flow through and place column into a new collection tube.

5. Add the pooled sample DNA to the washed column. Centrifuge at 1000 x *g* for 2 min and keep the eluate, which should be approximately 22 µL (maximum 25 µL). Check by pipetting.

6. Start thawing the Oxidant Solution on ice in the dark. To denature dsDNA take a new 0.2 mL microcentrifuge tube or strips. Add the buffer exchanged dsDNA sample and add reagents according to Table 2, mix and quickly spin. Incubate the DNA denaturing solution at 37°C for 30 min in a thermocycler. Place the tubes or strips on ice after denaturation and proceed immediately to next step as the oxidation reaction does not oxidise dsDNA and re-annealing at a later time point will affect the 5hmC conversion rate.

Table 2: DNA denaturing master mix

Solution	Volume per reaction (µL) (oxBS sample)	Volume per reaction (µL) (BS sample)
DNA (buffer exchanged from step 5)	22.75	
dsDNA		22.75
Denaturing solution	1.25	1.25
Ultra Pure Water	adjust to total volume	adjust to total volume
Total volume	24	24

7. Set thermocycler to 40°C before pipetting. **Split the pooled DNA into two samples here, one for oxidation (oxBS) and one for mock oxidation (BS).** For the oxidation reaction add reagents to the PCR tubes or strips containing the denatured DNA from the previous step according to Table 3. The reaction mix should be orange. Mix the reaction and centrifuge. Incubate the samples at 40 °C for 30 min in a thermocycler. The solution should still be orange after the 30 min incubation. Compare the samples with Appendix 2 in the user manual of the TrueMethyl^TM6 conversion kit as the colour of the oxidation reaction indicates a successful oxidation process.

Table 3: Oxidation master mix

Solution	Volume per reaction (µL) (oxBS sample)	Volume per reaction (µL) (BS sample)

denatured DNA	24	24
Oxidant Solution	1	1
Total volume	25	25

8. Centrifuge samples in a bench-top mini centrifuge for PCR strips at 1177 x *g*, for 10 min to pellet any black precipitate and take the orange supernatant forward into PCR tubes or strips. Be careful not to carry any precipitate over as this can inhibit downstream steps. Proceed immediately to the next step.

3.3 Bisulfite conversion

Bisulfite treatment is performed on both, the oxidised and non-treated sample. Dissolve the Bisulfite Reagent with the Bisulfite Diluent provided and dissolve fully before use (*see* **Note 6**).

1. Bring the oxidised and mock-oxidised samples from the previous step to room temperature. Add 700 µL Bisulfite Diluent to each aliquot of Bisulfite Reagent and incubate at 60 °C for 15 min, vortex until the bisulfite mix is completely dissolved.

2. For the bisulfite conversion add each component to PCR tubes or strips from the DNA oxidation step in the order listed in Table 4. Vortex thoroughly and centrifuge. The bisulfite DNA conversion is performed as shown in Table 5 in a thermocycler. Use the heated lid and set reaction volume to 200 µL or highest possible volume setting available on the thermocycler. The reaction should take approximately 8 hours to complete and is best done overnight.

Table 4: Bisulfite conversion master mix

Solution	Volume per reaction (µL)
DNA	25
Bisulfite Reagent Solution	170
Bisulfite Additive	5
Total volume	200

Table 5: Thermal cylcer conditions for Bisulfite conversion

Step	Time (min)	Temperature (°C)
Denaturing	5	95
Incubation	20	60
Denaturing	5	95
Incubation	40	60
Denaturing	5	95
Incubation	165	60

Denaturing	5	95
Incubation	20	60
Denaturing	5	95
Incubation	40	60
Denaturing	5	95
Incubation	165	60
Store o/n	Hold	20

3. Centrifuge to pellet precipitates once the bisulfite conversion is complete using a bench-top mini centrifuge for PCR strips at 1177 x g for 10 min. Carefully remove 190 µL of the supernatant and transfer to a new 1.5 mL microcentrifuge tube without disturbing the pellet.

4. Add 310 µL Desulfonation Buffer to each sample, mix and centrifuge. For each sample prepare an Amicon® filter provided with the kit as shown in Figure 3 on page 19 of the manual and transfer the sample to the Amicon® filter (approx. 500 µL). Avoid touching the filter as not to damage the filter membrane and affect the diafiltration step. Centrifuge filter for 5 min at 14000 x g and discard the flow through.

5. Add 450 µL of Desulfonation Buffer to the filter, centrifuge for 5 min at 14000 x g and discard the flow through.

6. Add 450 µL of Desulfonation Buffer to the filter and incubate for 5 min at room temperature before spinning for 5 minutes at 14000 x g. Discard the flow through.

7. Add 450 µL of Wash Buffer to the filter, centrifuge for 5 minutes at 14000 x g and discard the flow through. Repeat wash step twice more.

8. Elute the sample by inverting the Amicon® filter into a new 1.5 mL Amicon® collection tube as shown in Figure 4 page 20 of the manual provided with the kit and centrifuge at 2000 x g for 2 min. The bisulfite-converted sample should be around 10 µl total volume. Purified DNA can be stored up for 24 hours 2-8°C. For longer term storage, freeze at -20°C.

3.4 PCR amplification

An amplification step must be added to enrich for NGS adapted and multiplexed and (oxidised) bisulfite converted DNA prior to sequencing. The number of PCR cycles should be adapted to the input material used for NGS library making. The polymerase provided in the kit has been engineered to resist uracil stalling, therefore allowing accurate quantitation of bisulfite- converted libraries (*see* **Note 7**). The primers used are Illumina® TruSeq Primers for indexed adapters. For the PCR amplification, we only use half of the bisulfite-converted samples, keeping half of the sample as a back-up. Alternative strategies could be used to estimate optimal cycle numbers.

1. Thaw all reagents on ice prior to use, mix and centrifuge briefly. Prepare the PCR mix (outlined in Table 6) in 0.2 mL PCR tube or strip adding all reagents in order. If multiple samples are processed, prepare a master mix without the TrueMethyl™ DNA polymerase. Add the TrueMethyl™ DNA polymerase last and mix by pipetting. Amplify the DNA using the thermal cycling settings as outlined in Table 7.

Table 6: PCR reaction master mix

Solution	Volume per reaction (µL)
Nuclease Free Water	31.5
5x TrueMethyl^{TM} polymerase Buffer	10
10mM dNTP	1
PCR Illumina TruSeq primers	2
Bisulfite converted DNA	5
TrueMethyl^{TM} DNA polymerase (2U/µL)	0.5
Total volume	50

Table 7: Thermal cylcer conditions for PCR amplification

Number of Cycles	Time (seconds)	Temperature (°C)
1	120	95
	30	95
15x repeat	20	60
	45	72
1	Hold	4

2. Clean up the PCR products with Agencourt® AMPure® XP Beads. Use 1x beads to volume of PCR reaction. Mix beads and PCR reaction and incubate for 5 min at room temperature. Put tube on magnetic stand to separate beads from supernatant until the solution is clear (~5 min). Remove the supernatant without disturbing the beads.

3. Wash the beads with 1 mL freshly prepared 80% ethanol per tube whilst on the magnetic stand. Discard supernatant. Perform wash step with 80% ethanol once more and discard the supernatant again. Let the beads air dry for 5 min until the beads are completely dry and/or look cracked.

4. Keep tube on the magnetic stand and add 20 µL Ultra Pure water supplied with TrueMethyl^{TM}6 Kit to elute the DNA from the beads. Remove the tubes from the magnet and re-suspend the beads completely in water by pipetting. Place the tubes onto the magnetic stand until solution has cleared. Remove and keep the supernatant as input for quantification and quality control steps.

5. Run Agilent High Sensitivity DNA Bioanalsyer ChIP and Quant- it ™Pico Green® dsDNA assay before submission for sequencing. Note: If sample has adapter dimer contamination clean with 0.65x to 0.8x Agencourt® AMPure® XP Beads as above.

6. We recommend using 100 bp paired end sequencing on a Illumina® HiSeq platform.

3.8   Data analysis

Sequencing costs can be a deterrent when performing whole genome bisulfite sequencing. The protocol described here resorts to the subtraction of the oxidative bisulfite sequencing (oxBS-seq) from bisulfite sequencing (BS-seq) dataset to obtain the levels of hydroxymethylation (5hmC). Plotting the values for BS-seq versus oxBS-seq in scatter plots allows a preliminary analysis of how many regions display higher methylation in BS-seq (as expected) or in oxBS (which might be a measure for false-positives and noise in the system). Higher overall sequencing depth can reduce this noise; however this would imply increased sequencing costs. Combining the protocol described here with a downstream analysis that pools regions of biological interest, can be used to gain depth over groups of features without the need for further sequencing. Depending on the biological question/ system being studied it is possible to analyse relevant categories of genes that are known to be modulated with the treatment/ biological process under study and investigate if 5hmC plays a role in the known variation in the expression of these genes. Alternatively, it is possible to do an unfiltered analysis to understand if modulation of 5hmC is enriched in any specific biological category (*see* **Note 8**). This subheading is meant as a brief overview on how to do an initial exploration of the oxBS-seq data sets. For data exploration we use SeqMonk after the data has been mapped using Bismark [3]. A comprehensive protocol on how to map oxBS/BS data using Bismark is available online [4] and beyond the scope of this chapter.

1. Import methylation calls into SeqMonk using the generic text import; to keep the file size small import only CpG calls. SeqMonk will import methylated and unmethylated Cs individually as one bp reads. The methylation state is encoded in the strandedness of the read (forward = methylated, reverse = unmethylated). Bisulphite datasets can be extensive. If you want to look at non-CG methylation import CpG and non-CG separately.

2. To exclude mapping artefacts or repeats that result in regions having an unnaturally high coverage, generate 1kb tiled probes over the genome, check the probe-read-histogram and exclude all regions where the read coverage appears to be an outlier; we tend to look at the probe value distribution histogram and exclude anything that lies beyond the majority of reads, which is typically 2^{10} (1024) reads per 1kb. If you wanted to make this a bit more scientific you could maybe say the top 1% of values, e.g. using the value distribution filter. These regions can be excluded from the data set re-importing active data stores and selecting Exclude > regions to exclude.

3. Adding all methylated and unmethylated base calls within a region of interest can give biased results due to either too few base calls or uneven coverage, where most calls would come from a subset of loci. Choosing the Quantitation pipelines > bisulfite methylation over features option enables you to

apply a minimum coverage filter over every base call. All base calls meeting the coverage requirements are counted individually and weighed equally to calculate the average methylation level. In addition one can filter for a minimum number of cytosine positions (which also need to meet the coverage criteria) per feature before it is considered measured well enough. For 5mC levels compare the oxBS data sets.Here we run through an example to compare CGIs for a reasonably deep sequencing experiment (*e.g.* 10 x).

4. Run the Quantitation pipelines > bisulfite methylation over features option with settings being: Features to quantitate > CGI, Minimum count to include position >4 (coverage over individual CpG), Minimum observations to include filter > 5 (minimum number of CpGs above threshold for features to be counted), this has been replaced in the latest version of SeqMonk to produce NaN values (not a number), which means that they do no longer have to be filtered out as they do not count as numbers at all (similar to the R concept of NA values)> Run pipeline. This will yield a single value (percent methylation) for each CGI in the genome.

5. Create probes first. This can be done as unbiased tiled probes (e.g. 1kb or 5kb), choosing the tile length so that the tile is covered which will depend on the overall methylation level. Alternatively, probes can be chosen covering certain number of CpG positions or covering certain features – such as, promoters, CGI, gene bodies or any other group of functional relevance, e.g. gene ontology (GO) terms. Lastly, by using windows (of variable length) spanning a fixed number of consecutive CpG positions, e.g. 50 CpG, probes can be generated across the genome.

6. To get a value for hydroxymethylation use the methylation over feature pipeline to determine average values of the feature of interest and then subtract oxBS-seq from the BS-seq data set using the Relative Quantitation method.

7. Doing trend plots of hydroxymethylation around features of interest (+/- a few kb up and downstream) shows if 5hmC is important in the modulation known to occur in the treatment/ biological process of interest.

8. By generating probes over features, then generating running window probes within the features and quantitating the features as described above one can get CGIs of interest. Filtering using the windowed replicate test filter makes it possible to identify regions where there is a certain trend within the features. These can then be analysed to identify categories of interest using GO analysis.

4. Notes

1. At the moment Illumina® TruSeq adapters (which come methylated) allow for multiplexing of up to 24 samples.

2. Accurate library quantitation is essential, indeed is more important than in standard workflows. Any existing bias in the library pool going into the chemical conversion reactions will be carried through to the final libraries for sequencing, which will result in uneven coverage.

3. Spike-in controls can be added here as there is no clean up step before adapter ligation as the spike in controls are too small to be recovered by SPRI beads if they are not adapter ligated. We found very consistent conversion rates between multiplexed samples. It could therefore be considered whether it is sufficient to add the spike-in control to only one or two of the pooled samples.

4. Before pooling we recommend to run a Kapa Library Quantification kit for Illumina® platforms to accurately quantify each library to achieve optimal pooling conditions.

5. When using 80% ethanol instead of acetonitrile make sure that beads are completely dry before elution step (cracks starting to show).

6. The Bisulfite Reagent comes in aliquots for four samples each. Once the Bisulfite Reagent is dissolved it cannot be stored. Considering the coverage two aliquots can be used for nine samples. When performing more than four reactions, mix the different aliquots of dissolved Bisulfite solution prior to use.

7. Kappa HiFi Uracil+ can be used as an alternative and works well with low amounts of DNA. See Table 8 for reaction mix and Table 9 for PCR conditions.

Table 8: PCR reaction master mix for Kapa HiFi Uracil +

Solution	Volume per reaction (µL)
Nuclease Free Water	18
PCR Illumina TruSeq primers	2
Bisulfite converted DNA	5
Kapa HiFi Uracil 2x Master Mix	25
Total volume	

Table 8: Thermal cylcer conditions for Kapa HiFi Uracil + PCR amplification

Number of Cycles	Time (s)	Temperature (°C)
1	120	95
15x repeat	20	95
	30	65
	30	72
1	300	72
1	Hold	4

8. It should be mentioned that while this chapter focuses on the combination of oxBS-seq and BS-seq for the identification of 5hmC levels, oxBS-seq by itself is superior to BS-seq to accurately map 5mC levels because BS-seq 5mC calls include 5hmC calls.

5. References

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Figure 1: Workflow for multiplexing of samples prior to bisulfite and oxidative bisulfite conversion. Protocol steps are summarised as text with blue arrows indicating the order of the workflow.

Figure 2: Bioanalyser electropherogram of a oxBS library. Representative profile of a library derived from a multiplexed sample.
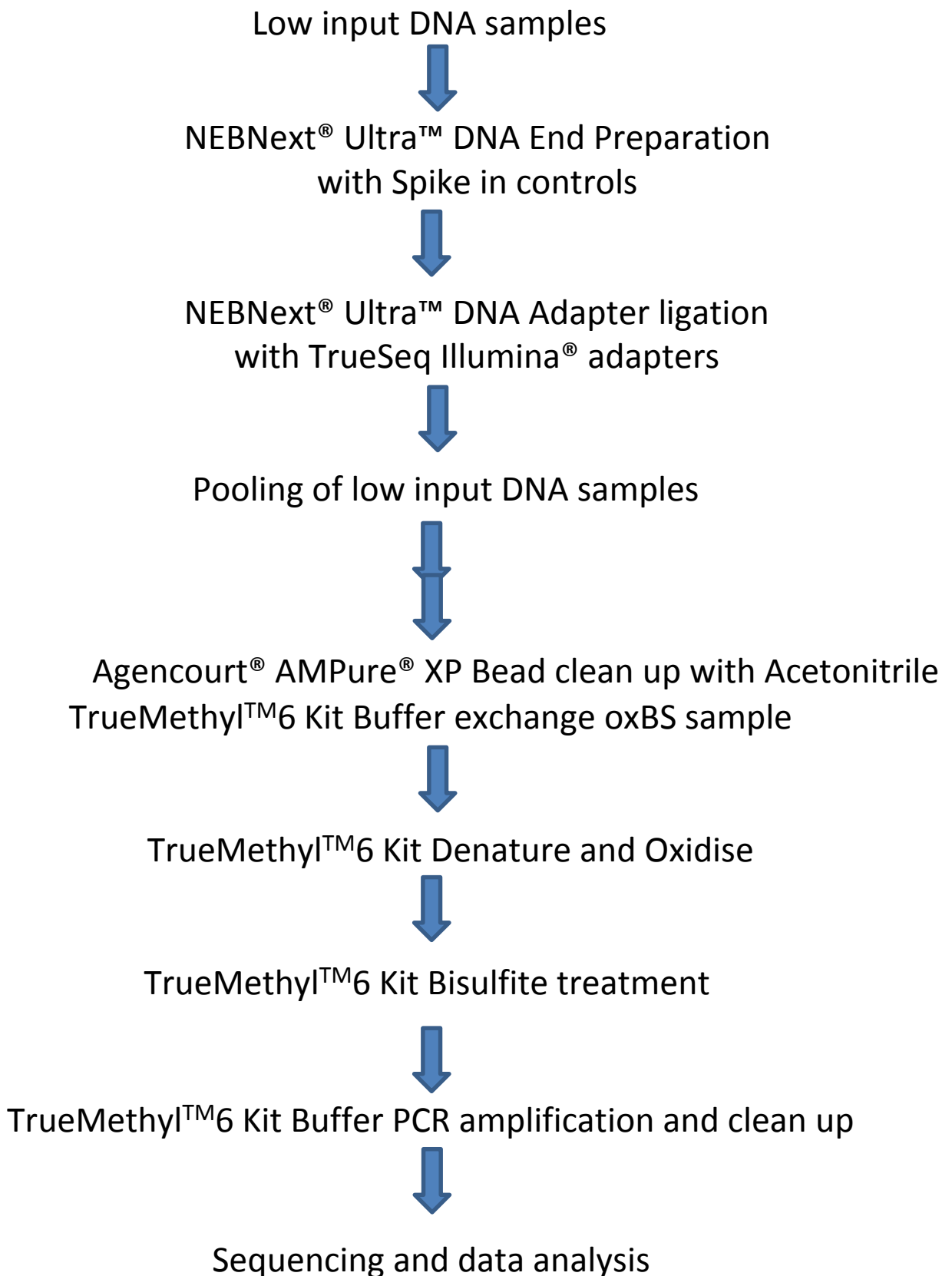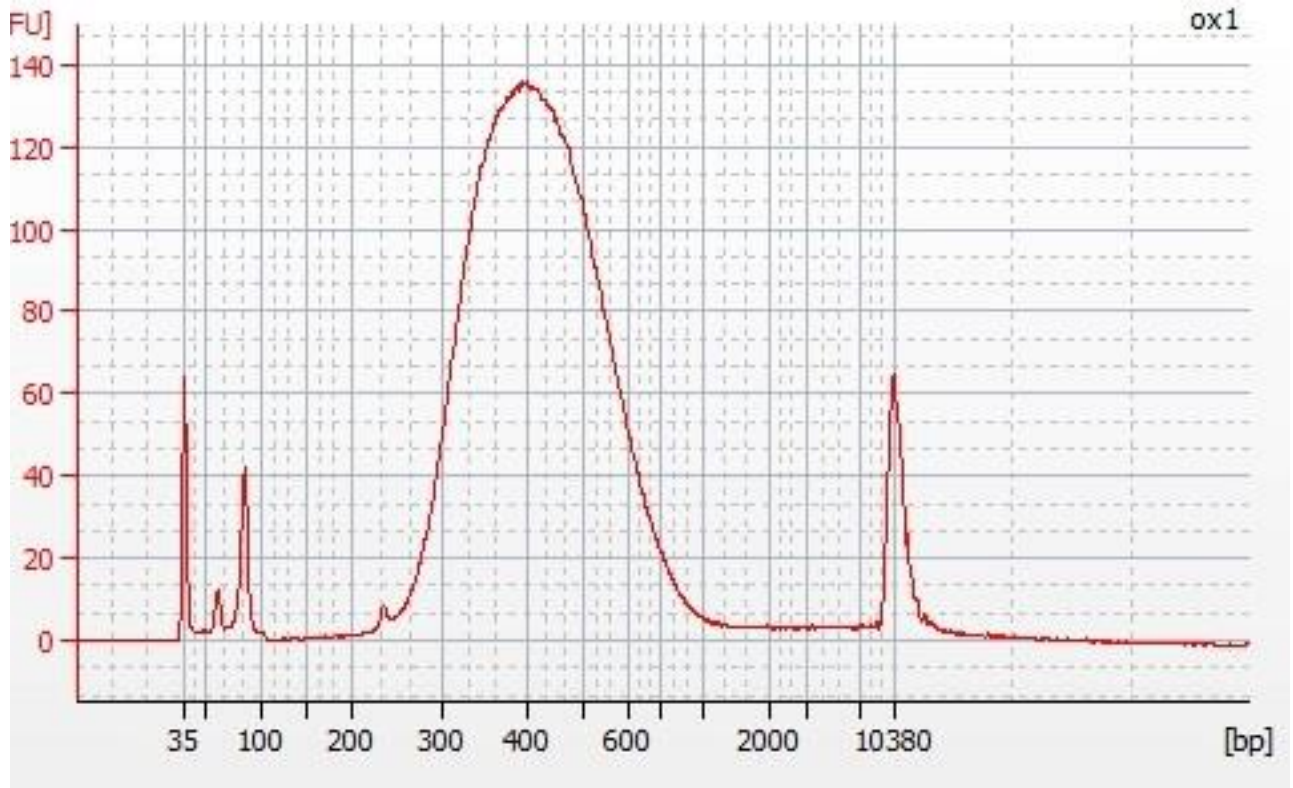
Low input DNA samples

⬇

NEBNext® Ultra™ DNA End Preparation
with Spike in controls

⬇

NEBNext® Ultra™ DNA Adapter ligation
with TrueSeq Illumina® adapters

⬇

Pooling of low input DNA samples

⬇

Agencourt® AMPure® XP Bead clean up with Acetonitrile
TrueMethyl^{TM}6 Kit Buffer exchange oxBS sample

⬇

TrueMethyl^{TM}6 Kit Denature and Oxidise

⬇

TrueMethyl^{TM}6 Kit Bisulfite treatment

⬇

TrueMethyl^{TM}6 Kit Buffer PCR amplification and clean up

⬇

Sequencing and data analysis

Figure 1

Figure 2