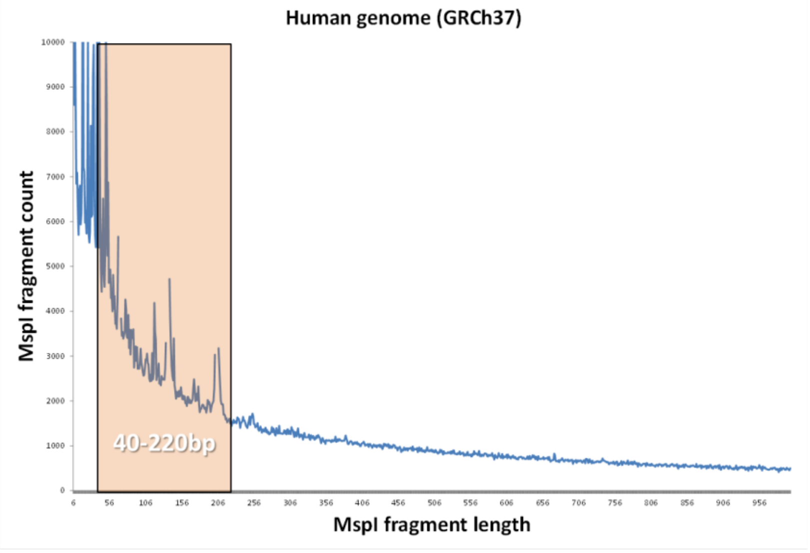
Reduced Representation Bisulfite-Seq – A Brief Guide to RRBS

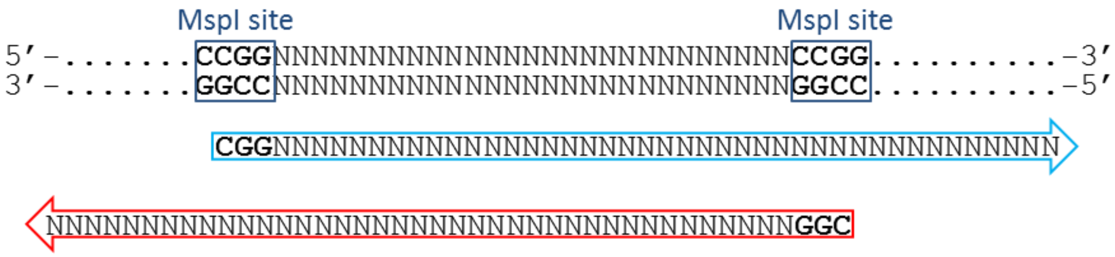
January 29, 2012

What is RRBS?

Typically, RRBS samples are generated by digesting genomic DNA with the restriction endonuclease MspI. This is followed by end-repair, A-tailing, adapter ligation and finally bisulfite conversion. Often, the library is also size-selected for fragments between 40 and 220bp in length. This fragment size has been shown to be plentiful in the sample and yield information on the vast majority of CpG islands (CGIs) in the human or mouse genome. Fig. 1 shows that quite a few MspI-MspI fragments (generated in silico for the mouse genome) are even shorter than 40bp. Since the size selection process if not as good as it is in theory, often a sizeable number of fragments below 40bp can end up in the RRBS library.



The fairly small fragment size of RRBS fragments can become a potential problem especially for sequencing reads with high read length (e.g. > 75bp or >100bp). If the read length is longer than the MspI-MspI fragment itself, the sequencing read may continue to read into the adapter sequence on the 3' end:

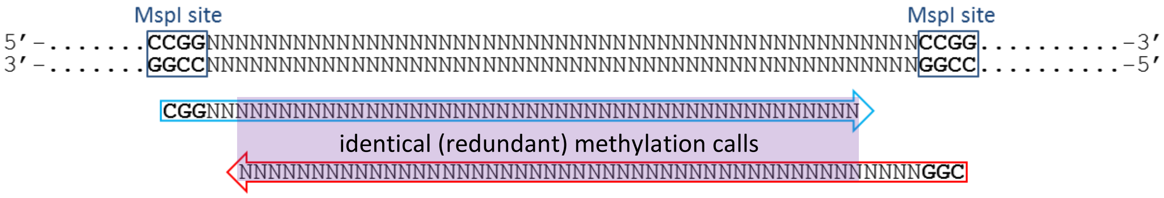

MspI site
MspI site
5'-.......CCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGG...........-3'
3'-.......GGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCC...........-5'
CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC

Such 'adapter contamination' may result in a lower mapping efficiency if the read does not align at all, or it may lead to false alignments which can result in incorrect methylation calls. As a simple rule, the longer the read length the higher the proportion of reads with adapter contamination. If such adapter contamination is not spotted and removed appropriately, a longer read length is most likely resulting in a lower mapping efficiency!

If the read length is longer than the MspI fragment one will also read (and perform a methylation call) for a cytosine that has been filled in with a predefined methylation state during the end-repair step. This is discussed further below.

Single-end or paired-end?

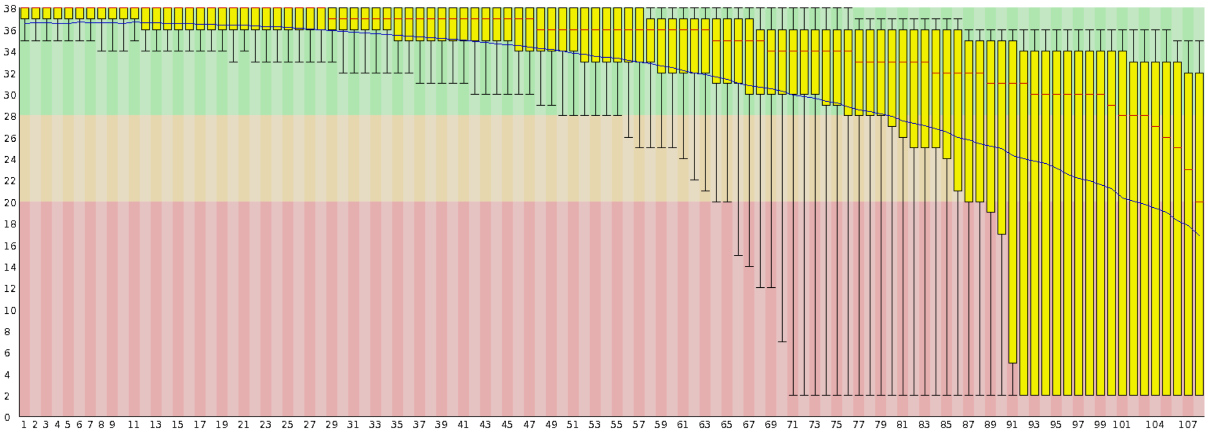
It seems to be a common misconception, that paired-end reads yield methylation results for both the forward and the reverse strand. In reality, a paired-end read results from PCR amplification of either the original top strand (OT), or the original bottom strand (OB). Thus, the other ends that are sequenced in the second round are sequences from the strand complementary to OT (CTOT), or complementary to OB (CTOB). These complementary strands are also informative for the same strand as their partner reads. As a consequence, paired-end reads that overlap in the middle yield redundant methylation information for the same strand:


MspI site
MspI site
5'-.......CCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGG...........-3'
3'-.......GGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCC...........-5'
CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
identical (redundant) methylation calls
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC

Granted, the paired-end nature might result in a somewhat increased mapping efficiency of paired-end reads over single-end reads. However, in addition to reading into the adapter on the other side, paired-end reads face the additional problem of generating potentially redundant methylation information. Redundant methylation calls need to be discarded if positions are filtered for a certain coverage by independent reads, since regions of overlap for paired-end reads would be over-represented. In short, because of the redundant overlapping parts paired-end RRBS reads are not simply 'twice as many reads therefore twice as many methylation calls'. Single-end experiments with the same number of reads as both paired-end reads added together are more likely to yield more genuine methylation information, as long as the read length is long enough to allow for a fair mapping efficiency for single-end reads (40-50bp reads are probably long enough to get mapping efficiencies in the range of 60 to 70%).

Other read length effects

Current sequencing on the Illumina platform often produces data whose quality deteriorates towards later cycles. We have received feedback from numerous sources, or downloaded data from public archives, which looks similar to this read quality profile:



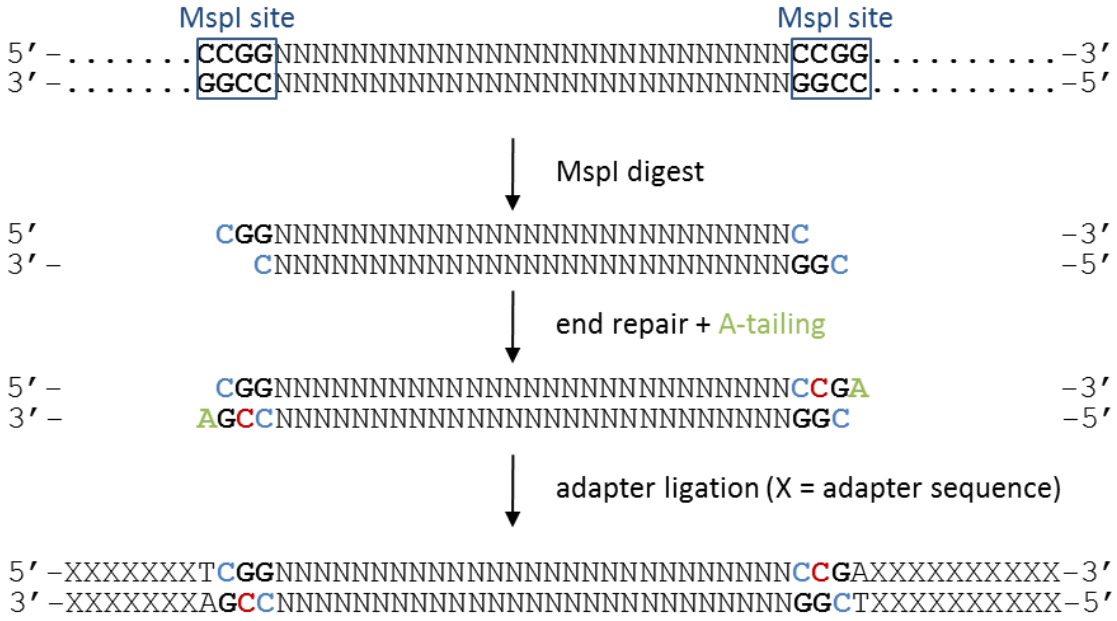
Up to a read length of around 60-70bp, the basecall quality of these reads is excellent (> Phred 30). After that, however, Phred scores tend to drop dramatically in a fairly large number of sequences, which means the rates at which bases are called erroneously increases. Base call errors in reads can result in reads not being aligned at all (reduced mapping efficiency), incorrect methylation calls or, in the worst case, mis-alignments (which will most likely also generate incorrect methylation calls). Some of these aspects have been recently discussed in the following review: Krueger F., Kreck B. et al., DNA Methylome Analysis Using Short Bisulfite Read Data, 2012).

In a nutshell:
RRBS reads suffer disproportionally from problems associated with long read lengths because they are, compared to other -Seq applications, size-selected for rather short fragment sizes. In the following sections, I will discuss some further aspects that need to be considered when analysing RRBS samples, and I will introduce our way of dealing with all sorts of read length related problems or experimentally introduced biases for RRBS libraries.

Potential biases for directional RRBS libraries (OT, OB strands only)

Directional sequencing, the most wide-spread way of (RR)BS, only ever sequences reads originating from the original top (OT) or original bottom (OB) strands. For simplicity, I have refrained from drawing out all four bisulfite DNA strands for the illustration below.

The sequential steps of RRBS are:

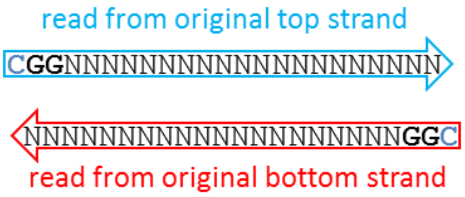

MspI site
MspI site
5′-.......CCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGG...........-3′
3′-.......GGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCC...........-5′
MspI digest
5′ CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNC -3′
3′- CNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC -5′
end repair + A-tailing
5′- CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGA -3′
3′- AGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC -5′
adapter ligation (X = adapter sequence)
5′-XXXXXXXTCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXXXXXX-3′
3′-XXXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCTXXXXXXXXXX-5′

Cytosines in blue retain the original genomic methylation state, whereas **cytosines in red** are introduced experimentally during the fragment end-repair reaction. This can be accomplished with either unmethylated or methylated cytosines, the trend seems to be that unmethylated cytosines are being used primarily now.

After the adapters are attached, the sequences are treated with sodium bisuflite, which converts unmethylated cytosines into thymines. Thus, the first three bases of (almost) all RRBS reads are either **CGG** or **TGG**, depending on their genomic methylation state. This applies to reads from both the OT and OB strand, and as nearly all reads in a directional RRBS experiment start with one of these two options, every read provides information on at least one CpG right in the start.

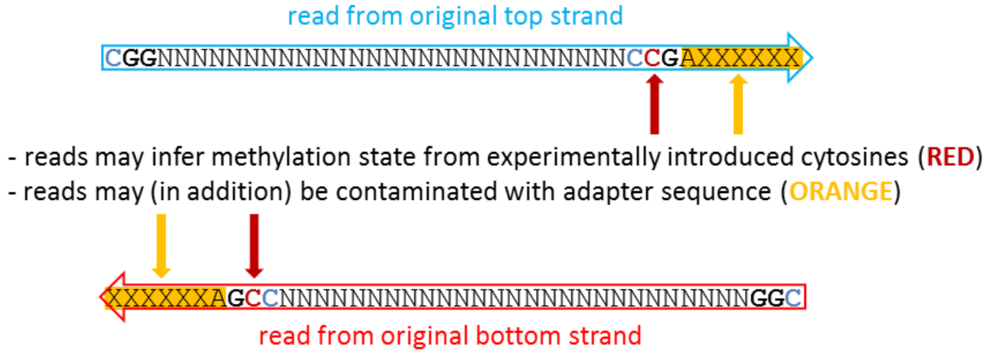
For directional libraries one can then discrimate the following two cases:

A) The read length is shorter than the MspI fragment


read from original top strand
CGGNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNGGC
read from original bottom strand

In this case, the entire read can be used for alignments and methylation calls. The first position resembles the true genomic methylation state (which can be C or T).

B) The read length is longer than the MspI fragment size


read from original top strand
CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXX
XXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC
read from original bottom strand

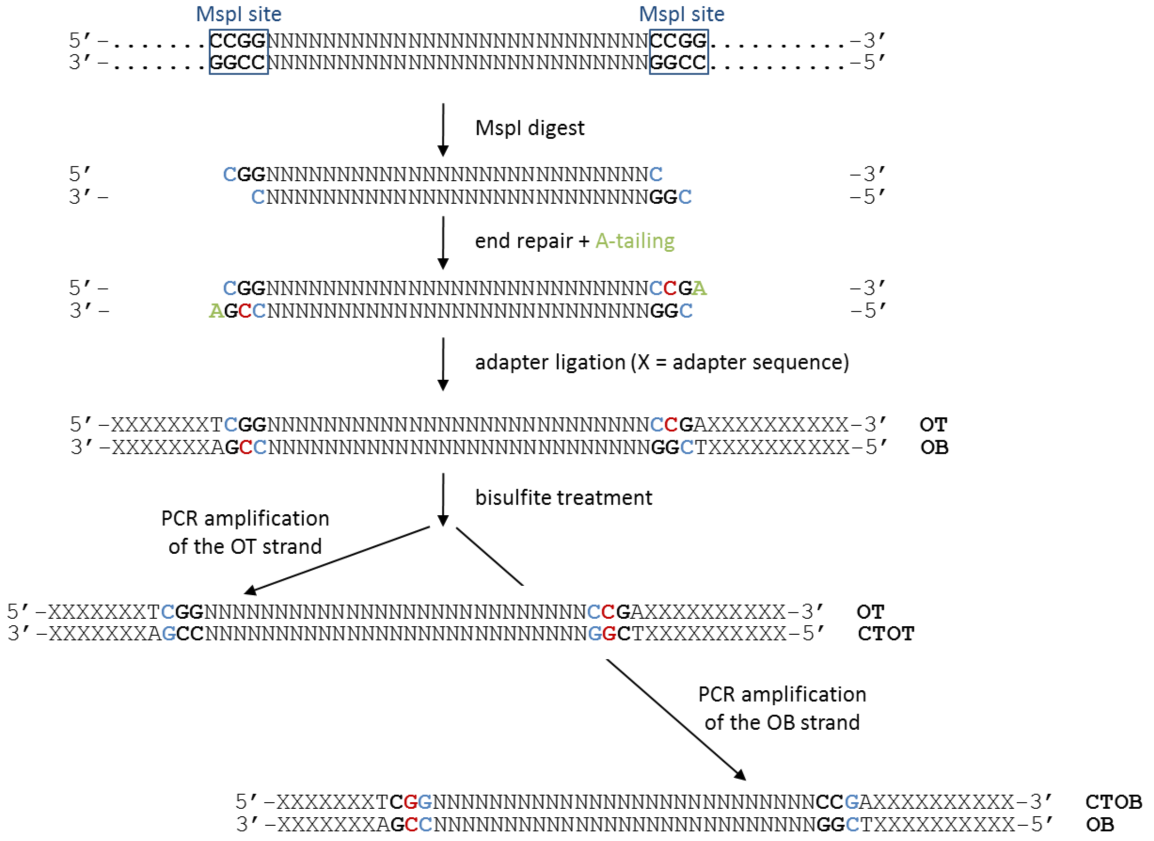
- reads may infer methylation state from experimentally introduced cytosines (**RED**)
- reads may (in addition) be contaminated with adapter sequence (**ORANGE**)

In this case, the sequencing read will contain the position which has been filled in during the end repair step (marked in **RED**), as well as read into the adapter sequence on the 3’ end of the read (marked in **ORANGE**). Retaining either the biased position or adapter contamination in the sequence read is highly undesirable.

Non-directional or paired-end RRBS libraries

Non-directional bisulfite sequencing is less common, but has been performed in a number of studies (Cokus et al. (2008), Popp et al. (2010), Smallwood et al. (2011), Hansen et al. (2011), Kobayashi al. (2012)). In this type of library, sequence reads may originate from all four possible bisulfite DNA strands (original top (OT), complementary to OT (CTOT), original bottom (OB) or complementary to OB (CTOB)) with roughly the same likelihood.

Paired-end reads do by definition contain one read from one of the original strands as well as one complementary strand. Please note that the CTOT or CTOB strands in a read pair are reverse-complements of the OT or OB strands, respectively, and thus they carry methylation information for the exact same strand as their partner read but not for the other original DNA strand. Similar to directional single-end libraries, the first read of directional paired-end libraries always comes from either the OT or OB strand. The first read of non-directional paired-end libraries may originate from any of the four possible bisulfite strands.

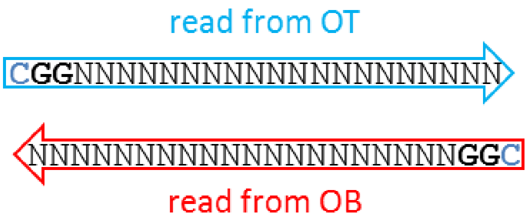

MspI site
MspI site
MspI digest
end repair + A-tailing
adapter ligation (X = adapter sequence)
OT
OB
bisulfite treatment
PCR amplification of the OT strand
OT
CTOT
PCR amplification of the OB strand
CTOB
OB

Again, **cytosines in blue** retain the original genomic methylation state, whereas **cytosines in red** are introduced experimentally during the fragment end-repair reaction. This can be accomplished with either unmethylated or methylated cytosines, the trend seems to be that unmethylated cytosines are being used primarily now.

After bisulfite conversion, the first three bases of non-directional RRBS reads that originated from the OT or OB strands will also be either **CGG** or **TGG**, depending on their genomic methylation state. In addition, however, non-directional libraries may contain reads which originated from the CTOT or CTOB strands. These reads will have **CAA** or **CGA** at the start, depending on whether unmethylated or methylated cytosines were used for the end-repair reaction, respectively. (Theoretically, the sequence could also be CAG or CGG, but this would assume that a C in CHH context was methylated on the other strand, and this is arguably very rarely the case for CpG-rich sequences. For simplicity it is therefore left out here). In either case, the second base would incur a methylation call of a base that does no longer reflect the genomic methylation state, which is illustrated below.

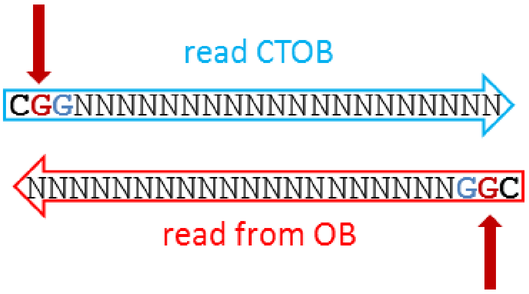
For non-directional libraries, one can discrimate the following four cases:

A) The read length is shorter than the MspI fragment, OT or OB alignments



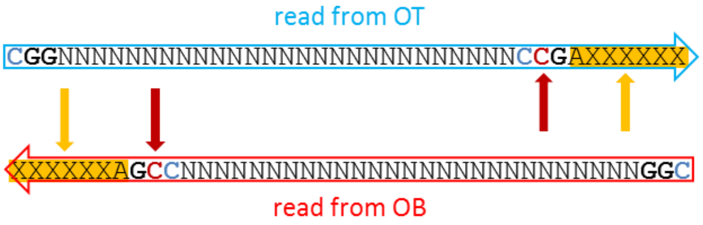
In this case, the entire read can be used for alignments and methylation calls. The first position resembles the true genomic methylation state (which can be C or T).

B) The read length is shorter than the MspI fragment, CTOT or CTOB alignments



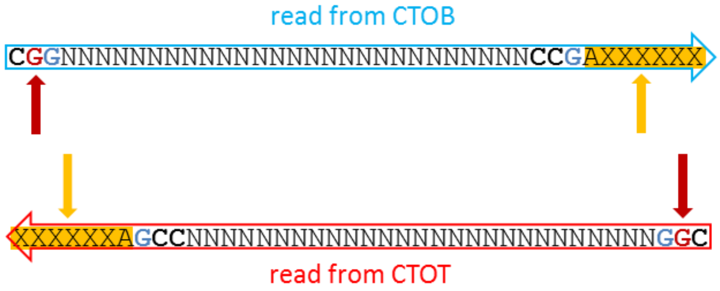
In this case, the read will start with CAA or CGA (the filled-in and the other cytosine in CHG context on the opposing strand are expected to be fully bilsufite converted), whereby the position marked in **RED** infers the methylation state from a cytosine that was experimentally introduced. The positions in **BLUE** carries genomic methylation information. As a consequence, methylation information of the second base would bias the results depending on the methylation state of the cytosine used for end-repair and needs to be excluded from methylation analysis.

C) The read length is longer than the fragment length, OT or OB alignments


read from OT
CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXX
XXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC
read from OB

Analogous to directional libraries, the sequencing read will contain the position that was filled in during the end-repair step (marked in **RED**), as well as read into the adapter sequence on the 3’ end of the read (marked in **ORANGE**). Retaining either the biased position or adapter contamination in the sequence read is highly undesirable.

D) The read length is longer than the fragment length, CTOT or CTOB alignments


read from CTOB
CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXX
XXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC
read from CTOT

- reads may infer methylation state from experimentally introduced cytosines (**RED**)
- reads may (in addition) be contaminated with adapter sequence (**ORANGE**)

Similar to case **B)** the read does now contain the filled-in base at position 2 in the read (typically CAA or CGA, **RED**), as well as adapter contamination at the 3’ end (**ORANGE**). Retaining either the biased position or adapter contamination in the sequence read is highly undesirable.

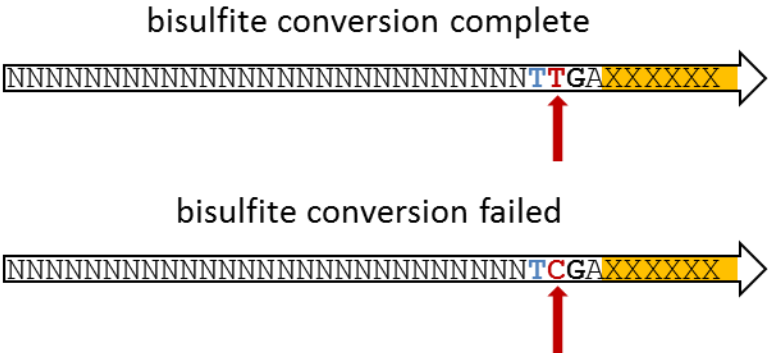
Taking appropriate QC measures for RRBS libraries

As for almost all high throughput sequencing applications we would recommend to perform some quality control on the data, as it can often straight away point you towards the next steps that need to be taken (e.g. with FastQC, http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). As outlined above, we believe that thorough QC and taking appropriate steps to remove problems is absolutely critical for proper analysis of RRBS libraries since they are susceptible to a variety of errors or biases that one could probably get away with in other sequencing applications. In summary, the examples discussed here were:

- poor qualities – affect mapping, may lead to incorrect methylation calls and/or mis-mapping
- adapter contamination – may lead to low mapping efficiencies, or, if mapped, may result in incorrect methylation calls and/or mis-mapping
- positions filled in during end-repair will infer the methylation state of the cytosine used for the fill-in reaction but not of the true genomic cytosine
- paired-end RRBS libraries (especially with long read length) yield redundant methylation information if the read pairs overlap
- RRBS libraries with long read lengths suffer more from all of the above due to the short size-selected fragment size

Exploiting the filled-in position to determine the bisulfite conversion efficiency

If the end-repair was performed using unmethylated cytosines, this position can theoretically be exploited as a built-in bisulfite conversion efficiency control. To assess the bisulfite conversion efficiency one needs to identify sequences that contain the adapter on their 3' end, and count up the number of times the filled in position (marked in **RED**) was not converted to `T`.


bisulfite conversion complete
NNNNNNNNNNNNNNNNNNNNNNNNNNNTTGAXXXXXX
bisulfite conversion failed
NNNNNNNNNNNNNNNNNNNNNNNNNNNTCGAXXXXXX

The percentage of non-bisulfite converted cytosines at the fill-in position can be calculated as:

$$\% \text{ non-conversion} = C_X / (T_Y + C_X) * 100$$

(with X and Y being the number of times these residues were observed).

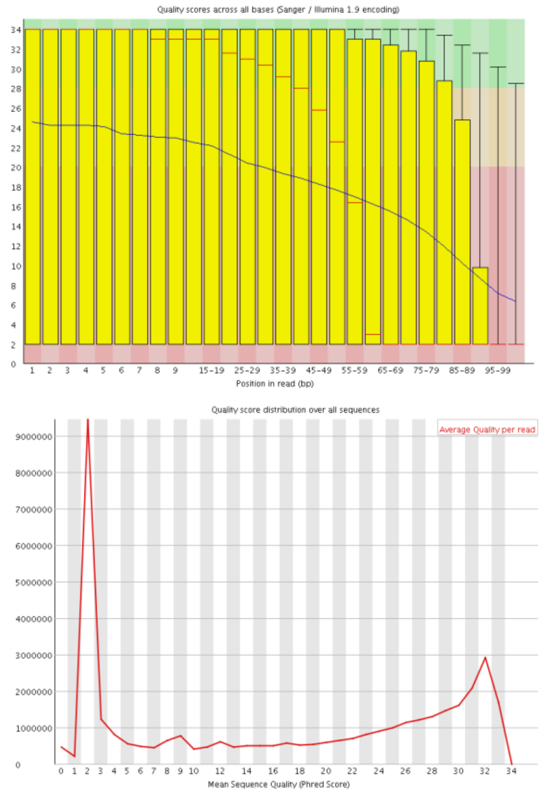
To make this calculation more reliable one can vary the required overlaps with the adapter sequence, e.g. requiring 3, 5, 7bp etc., before the non-conversion rate of the filled-in position is determined.

Adaptive quality and adapter trimming with `trim_galore`

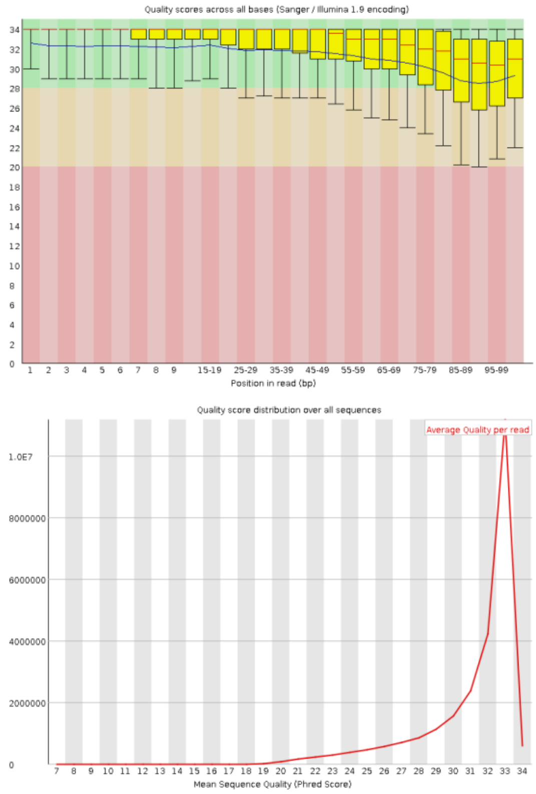
We have tried to implement a method to rid RRBS libraries (or potentially all kinds of sequencing datasets) of potential problems in one convenient process. For this we have developed a wrapper script (`trim_galore`) that makes use of the publically available adapter trimming tool `Cutadapt` and `FastQC` for optional quality control once the trimming process has completed.

Even though `trim_galore` works for any (base space) high throughput dataset (e.g. downloaded from the SRA) this section describes its use mainly with respect to RRBS libraries.

- In the first step, low-quality base calls are trimmed off from the 3' end of the reads before adapter removal. This efficiently removes poor quality portions of the reads. Here is an example of a dataset downloaded from the SRA which was trimmed with a Phred score threshold of 20 (data set DRR001650_1 from Kobayashi et al., 2012).

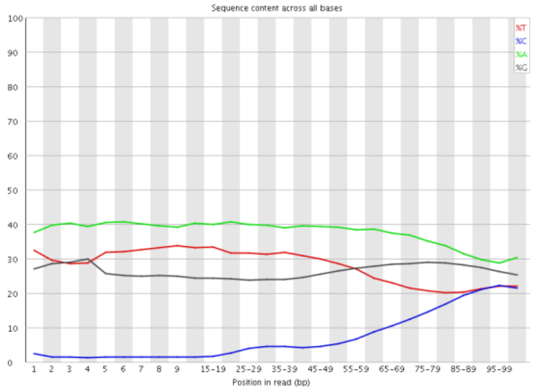


before trimming

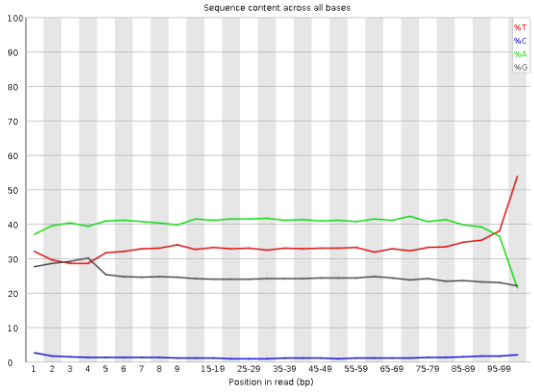


after trimming

In the next step, Cutadapt finds and removes adapter sequences from the 3’ end of reads. If no sequence was supplied it will use the first 13 bp of the standard Illumina paired-end adapters ('AGATCGGAAGAGC'), which recognises and removes adapters from most standard libraries. To control the stringency of the adapter removal process one gets to specify the minimum number of required overlap with the adapter sequence, else it will default to 1. This default setting is extremely stringent, i.e. an overlap with the adapter sequence of even a single bp is spotted and removed. This may appear unnecessarily harsh, however, as a reminder adapter contamination may in a bisulfite-Seq setting lead to mis-alignments and hence incorrect methylation calls, or result in the removal of the sequence as a whole because of too many mismatches in the alignment process. Tolerating adapter contamination is most likely detrimental to the results, but we realize that this process may in some cases also remove some genuine genomic sequence. It is unlikely that the removed bits of sequence would have been involved in methylation calling anyway (since only the 4th and 5th adapter base would possibly be involved in methylation calls (for directional libraries that is)), however, it is quite likely that true adapter contamination – irrespective of its length – would be detrimental for the alignment or methylation call process, or both.



before trimming



after trimming

This example (same dataset as above) shows the dramatic effect of adapter contamination on the base composition of the analysed library, e.g. the C content rises from ~1% at the start of reads to around 22% **(!)** towards the end of reads. Adapter trimming with Cutadapt gets rid of most signs of adapter contamination efficiently. Note that the sharp decrease of A at the last position is a result of removing the adapter sequence very stringently, i.e. even a single trailing A at the end is removed.

- trim_galore also has an ‘--rrbs’ option, which identifies sequences that were adapter-trimmed and removes another 2 bp from their 3' end. This is to avoid that the filled-in cytosine position (marked in **RED** in the above examples) close to the second MspI site in a sequence is used for methylation calls. Sequences which were merely trimmed because of poor quality will not be shortened any further.

- trim_galore also has a ‘--non_directional’ option, which will screen adapter-trimmed sequences for the presence of either CAA or CGA at the start of sequences and clip off the first 2 bases if found. If `CAA` or `CGA` are found at the start, no bases will be trimmed off from the 3’ end even if the sequence had some contaminating adapter sequence removed (in this case the sequence read likely originated from either the CTOT or CTOB strand).

- Lastly, since quality and/or adapter trimming may result in very short sequences (sometimes as short as 0bp), `trim_galore` can filter trimmed reads based on their sequence length (default: 20bp). This is to reduce the size of the output file and to avoid crashes of alignment programs which require sequences with a certain minimum length. Note that is not recommended to remove too short sequences if the analysed FastQ file is one of a pair of paired-end files since this confuses the sequence-by-sequence order of paired-end reads which is again required by many aligners. In the case of paired-end files we would disable length filtering (by setting the `-l` value to 0), and run a paired-end validation script on both _1 and _2 FastQ files which removes entire read pairs if one of the two sequences is shorter than a certain threshold. This `validate_paired_end_files` script is available upon request.

Applying these steps to both self-generated and downloaded data can ensure that you really only use the high quality portion of the data for alignments and further downstream analyses and conclusions.