

# **Exercises: QC and Mapping of BS-Seq data**

## Licence

This manual is © 2014-21, Felix Krueger & Simon Andrews.

This manual is distributed under the creative commons Attribution-Non-Commercial-Share Alike 2.0 licence. This means that you are free:

- to copy, distribute, display, and perform the work
- to make derivative works

Under the following conditions:

- Attribution. You must give the original author credit.
- Non-Commercial. You may not use this work for commercial purposes.
- Share Alike. If you alter, transform, or build upon this work, you may distribute the resulting work only under a licence identical to this one.

Please note that:

- For any reuse or distribution, you must make clear to others the licence terms of this work.
- Any of these conditions can be waived if you get permission from the copyright holder.
- Nothing in this license impairs or restricts the author's moral rights.

Full details of this licence can be found at

<http://creativecommons.org/licenses/by-nc-sa/2.0/uk/legalcode>

## Introduction

In this session we will use small subsets of publicly available Bisulfite-Seq data in FastQ format, perform initial QC and trimming steps and align the data to the mouse genome. The basic steps we're going to perform are:

- Initial quality control with FastQC
- Adapter and quality trimming using Trim Galore (a wrapper around Cutadapt)
- Alignment of reads to the mouse genome using Bismark
- Deduplicate aligned data and extract methylation calls (used for analysis later on)
- Generate graphical HTML analysis report

## Software

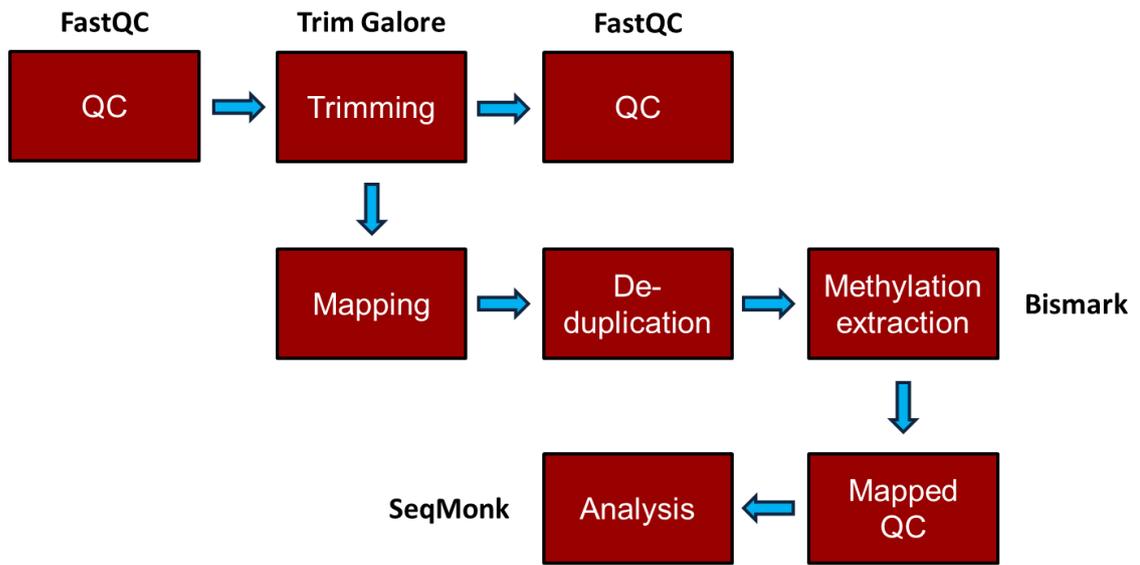
The data processing described in this practical is intended to be run as a set of command line tools and should work on Linux or Mac platforms.

- FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)
- Trim Galore ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/))
- Cutadapt (<https://code.google.com/p/cutadapt/>)
- Bismark (<http://www.bioinformatics.babraham.ac.uk/projects/bismark/>)
- Bowtie2 (<http://bowtie-bio.sourceforge.net/bowtie2/>)

## Data

The data in this practical comprises 200,000 reads from a paired-end dataset (GSM1027571). The data used in this course may be downloaded from the Babraham Bioinformatics web site (<http://www.bioinformatics.babraham.ac.uk/training.html>).

**Exercise outline**



We will talk you through a way to connect to a server on the AWS (Amazon Web Services) before this practical.

## Exercise 1 – Initial QC

The data for this section is in the QC\_and\_Mapping subfolder of your main Meth\_Course\_Data data folder. You should be able to see two files in there:

```
$ cd Meth_Course_Data
$ cd QC_and_Mapping
$ ls
Sample1_PE_R1.fastq.gz
Sample1_PE_R2.fastq.gz
```

In this exercise, we are going to run a full primary analysis of bisulfite treated reads, starting from raw FastQ files as they might come off an Illumina sequencer from any sequencing facility. Since modern sequencers generate huge data volumes (~30M sequences for a MiSeq, ~200M for a lane on the HiSeq and ~450M for a NextSeq) the analysis of a full run may take up to a couple of days to complete (depending on several parameters such as read length, genome size, repeat content, alignment parameters).

The two files for Sample1 are Read 1 (R1) and Read 2 (R2) of a paired-end (PE) experiment.

First, we would like to get an initial idea about the sequencing quality of the files we are going to use.

You can run FastQC on all samples in one go by using its non-interactive mode with the command:

```
$ fastqc *fastq.gz
```

Once the analysis has completed you can look at the HTML reports using Firefox or your preferred local browser e.g.:

```
firefox *.html
```

What can you tell about the files without knowing the origin of or having aligned the data? Try to find answers to the following questions:

- What does the sequence quality look like?
- Does the data appear to contain read-throughs into the Illumina adapter sequence?
- Are there overrepresented sequences/contaminants?
- Is there a noticeable difference between Read 1 and Read 2 for the paired-end experiment?

## Exercise 2: Adapter and quality trimming

In Exercise 1 we have seen that the sequencing quality in our test samples looks fairly good. Especially with longer reads or RRBS data one should still get into the habit of trimming the data to improve mapping efficiency and reduce the chance of misalignments.

Sample 1 is a paired-end experiment, so it is important to make this known to the trimming program with the option --paired:

```
$ trim_galore --paired --fastqc Sample1_PE_R1.fastq.gz Sample1_PE_R2.fastq.gz
```

This command automatically removes:

- base calls with a Phred score of 20 or lower (assuming Sanger encoding)
- any traces of standard Illumina adapter sequence from the 3' end
- Sequence pairs where either read became too short as a result of trimming (<20 bp)
- Keeps Reads 1 and Read 2 of sequence pairs in sync (required by all aligners)

### Exercise 3: Read alignments using Bismark

Alignments and methylation calling are carried out with Bismark (using the underlying short read aligner Bowtie 2 (default)). The subfolder 'Genomes' already contains the reference sequence file for chromosome 1 of the mouse genome (GRCm38 build) as well as its bisulfite genome indexes for Bowtie 2, so we can use them straight away (the indexing of a human or mouse sized genome takes ~2 hours, so we have already prepared this in advance).

To run Bismark for Sample 1 (paired-end), type:

```
$ bismark --genome ../Genomes/ -1 Sample1_PE_R1_val_1.fq.gz -2  
Sample1_PE_R2_val_2.fq.gz
```

The alignments should finish within a couple of minutes. If you wanted to find out how well the alignment step went you can look at the Bismark mapping reports in the terminal window, like so:

```
$ cat Sample1_PE_R1_val_1_bismark_bt2_PE_report.txt
```

## Exercise 4: Deduplication

The deduplication is carried out using a script which is part of the Bismark package, called `deduplicate_bismark`. In its default mode it will use the first alignment for a given genomic region, which is basically equivalent to using a random alignment per position. The script works out whether the file to be deduplicated is a single-end or paired-end file.

```
$ deduplicate_bismark Sample1_PE_R1_val_1_bismark_bt2_pe.bam
```

## Exercise 5: Methylation extraction

Extracting methylation calls from the Bismark BAM files automatically generates a small report detailing the methylation calls that were found during the process (which might or might not look somewhat different to the stats in the Bismark mapping report, depending on whether or not the file was heavily duplicated). The methylation extractor also detects if the input file is a single-end or a paired-end file, and will set the option `--no_overlap` for paired-end files automatically. The option `--bedGraph` will produce the coverage files, which are very intuitive to read and may serve as input file for SeqMonk or R-packages later on. The coverage files could also be submitted to GEO as processed data when it comes to publishing your data.

The methylation extractor command for Sample 1 is:

```
$ bismark_methylation_extractor --bedGraph --gzip  
Sample1_PE_R1_val_1_bismark_bt2_pe.deduplicated.bam
```

This all needs to go on one line in your shell.

This command

- generates strand and context specific cytosine output files
- counts overlapping parts of read pairs only once
- generates an M-bias report
- produces a bedGraph and coverage file
- generates an overall count report for (splitting report)

## Exercise 6: Bismark HTML report

The module `bismark2report` generates a visual HTML report from the Bismark alignment, deduplication, methylation extraction and M-bias reports. It attempts to automatically detect all relevant files in the current working directory folder for you (which should work just fine here), but files may also be specified if desired. Just run:

```
$ bismark2report
```

The report is meant to get you an impression very quickly of how well the experiment has worked. To view the reports, please open these two reports locally using your preferred browser:

```
Sample1_PE_R1_val_1_bismark_bt2_PE_report.html
```

Take a look at the report and try to answer the following questions:

- Was the mapping efficiency good or were there any major problems?
- Does the average methylation level look as expected?
- Can you comment on the level of bisulfite conversion efficiency in the sample? (You might want to look at the levels of non-CG methylation for this)
- Are there any notable technical methylation biases in the results? If so what might be the causes and what could you do to remove them?

The Bismark report should look something like this:



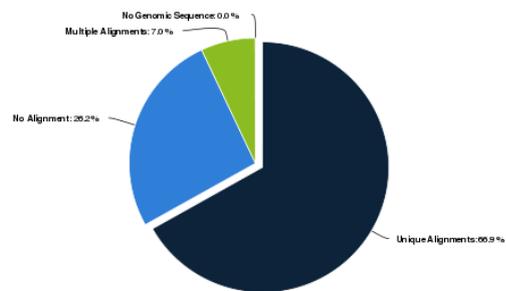
## Bismark Processing Report

Sample1\_PE\_R1.fastq.gz and Sample1\_PE\_R2.fastq.gz

Data processed at 17:15 on 2014-12-10

### Alignment

Sequence pairs analysed in total	200,000
Paired-end alignments with a unique best hit	133,718
Pairs without alignments under any condition	52,309
Pairs that did not map uniquely	13,973
Genomic sequence context not extractable (edges of chromosomes)	0



### Cytosine Methylation

Total C's analysed	4,946,951
Methylated C's in CpG context	222,397
Methylated C's in CHG context	9,398
Methylated C's in CHH context	20,320
Methylated C's in Unknown context	0
Unmethylated C's in CpG context	92,677
Unmethylated C's in CHG context	1,282,894
Unmethylated C's in CHH context	3,319,265
Unmethylated C's in Unknown context	2
Percentage methylation (CpG context)	70.6%
Percentage methylation (CHG context)	0.7%
Percentage methylation (CHH context)	0.6%
Methylated C's in Unknown context	N/A%

