Methpipe Manual

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The methpipe software package is a comprehensive tool chain for analyzing whole genome bisulfite sequencing data (BS-Seq). This documentation will guide you step by step to learn how to perform the data analysis in a BS-Seq project with methpipe. To facilitate understanding of the work flow, we divide the analysis procedure into four steps: (1) Pre-mapping processing: This step include assessing read quality and pre-processing reads, such as trimming adapters; (2) Mapping: This step maps sequence reads to a reference genome; (3) Analyzing methylation status at single site: In this step we will estimate methylation frequency at single site, either CpG sites or non-CpG sites. We will also obtain information about sequencing depth and bisulfite conversion rate; (4) Higher level analysis: This step includes higher level more biologically interesting analysis, such as identifying hypo-methylated regions and/or differentially methylated regions. Later we will describe in detail the usage of tools in each step.

In the guide we will use a small project as example. Suppose that in this project you would like to study the methylation pattern in two cell types, B cells and neutrophiles. The B cell methylome is profiled with sing-end sequencing and your university's sequencing center sends back to you these sequence files:

```
bcell/s-1.fq bcell/s-2.fq bcell/s-3.fq bcell/s-4.fq.
```

The neutrophile methylome is profiled with pair-end sequencing and you have the following sequences files:

```
neut/s-1-1.fq neut/s-1-2.fq neut/s-2-1.fq neut/s-2-2.fq.
```

Now we are ready to uncover the interesting biology about DNA methylation from this dataset.

1 Pre-mapping processing

Before mapping sequenced reads to a reference genome, we need to pre-process the raw read sequences, in particular we need to trim possible adapter sequences retained in the 3' end of raw reads. Further we may be interested in examining the quality of reads in our library and visualizing raw reads in UCSC Genome Browser.

1.1 Trim adapters

As the length of reads that sequencing technology is able to produce has been increasing all the time, it is possible there are adapter sequences in the 3' end of some reads. These retained adapter sequences affects the mappability of such reads. Even if they are somehow mapped to the reference genome, the adapter sequences may introduce bias to our estimate of methylation frequency. Therefore it is necessary to trim these retained adapter sequences.

The program **trim-adapter** is used to trim adapter sequences, if any, from the 3' end of raw reads. Suppose the adapter sequences is *GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCG*, we trim the adapter sequences from raw reads in the file bcell/s-1.fq with the following command:

```
$ ./trim-adapter  s-1.fq -o s-1-clipped.fq
-a GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCG
```

If you would like to check the effective read length after trimming adapter sequences and Ns from 3' end, you can add **-l** options to specify the output file for effective read length, for example,

```
$ ./trim-adapter  s-1.fq -o s-1-clipped.fq  -l s-1-length.txt
-a GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCG
```

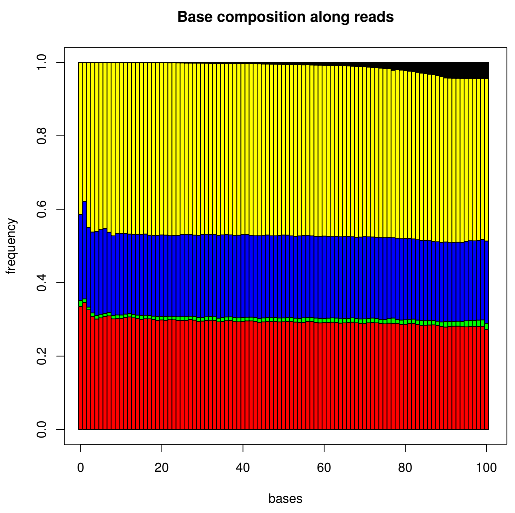
The file s-1-length.txt lists the effective read length of each read after trimming adapter sequences and Ns, which can be used to compute the statistics of effective reads lengths with any general purpose statistical package, such as R. Perform this trimming adapter processing on all sequences files and for convenience we assume the processed sequences files have their original file names thereafter.

1.2 Assessing read quality

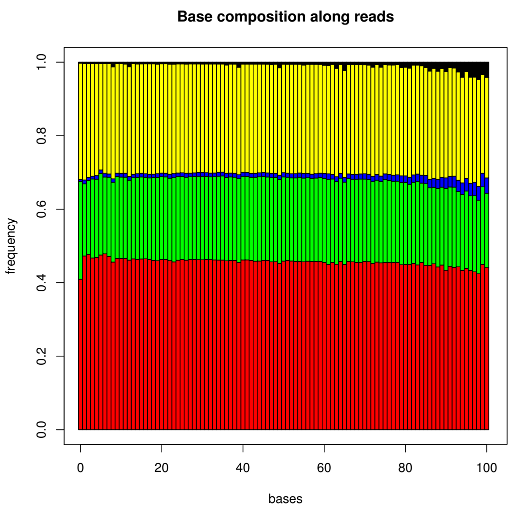
It is optional to assess read quality before mapping, however such assessment may help us to spot potential problems during library preparation and/or bisulfite sequencing. The program **read-quality-prof** is used to generate an average summary of base composition and quality scores from 5' to 3' along all reads. We run **read-quality-prof** as following

```
$ ./read-quality-prof  < s-1.fq > s-1-qual.txt
```

The output file s-1-qual.txt can be visualized with the R software. Fig. 1 shows the base composition profile in the pair-end sequencing sample, where the left panel shows T-rich reads and the right panel A-rich reads. Note the small proportion of C and G reflects the effect of bisulfite conversion.



(a) T-rich reads



(b) A-rich reads

Figure 1: Base composition

2 Mapping

In the mapping step, you will map sequence reads to a reference genome. During bisulfite treatment, unmethylated cytosines in the original DNA sequences are converted to uracils, which are in turn incorporated as thymines during PCR amplification. We call such strand T-rich and its complementary strand A-rich (adenine-rich). When mapping T-rich reads to the reference genome, either a cytosine (C) or a thymine (T) in reads is considered valid match to a cytosine in the reference genome. But for A-rich reads, a adenine or a guanine is consider valid match to a guanine in the reference genome.

If using single-end sequencing, you will get T-rich reads only. If using pair-end sequencing, you will get both T-rich reads and A-rich reads. Next we will learn how to map bisulfite treated reads with **rmapbs** from either single-end sequencing or pair-end sequencing.

2.1 Mapping single-end sequencing reads

Mapping read sequences to the reference genome is done by **rmapbs**. Before mapping, you need to get he genome sequences of appropriate organism and assembly, which is usually downloadable from UCSC Genome Browser download portal or other specific repositories of genome sequences, such as TAIR for Arabidopsis *thaliana*. Suppose that you have already downloaded the appropriate genome sequences, in our example human genome hg18, in the directory *human-genome*, to map the reads in bcell/s-1.fq to the reference genome we run

```
$ ./rmapbs -c human-genome -s fa -m 4 -o bcell/s-1.bed bcell/s-1.fq -v.
```

The option **-c** specifies the direcotory holding the reference genome and the **-s** specifies the suffix of sequence file names. The option **-m** gives the maximum number of mismatches allowed. The option **-o** specifies the output file. There are other options to fine tune the behavior of **rmapbs** which are explained on methpipe website.

2.2 Mapping pair-end sequencing reads

At present our pipeline map R-rich reads and A-rich reasd separately when mapping reads from the pair-end sequencing procedure. We map T-rich reads in the same way as for single end-reads. However we use A/G wildcards when mapping the A-rich reads, i.e. a adenine or a guanine is considered valid match to a guanine in the reference genome. The **rmapbs**, given the option **-A**, run in A/G wildcard mode. Therefore to map T-rich reads, for example, neut/s-1-1.fq, we run

```
$ ./rmapbs -c human-genome -s fa -m 4 -o neut/s-1-1.bed neut/s-1-1.fq -v.
```

To map A-rich reads, for example neut/s-1-2.fq, we run

```
$ ./rmapbs -c human-genome -s fa -m 4 -o neut/s-1-2.bed neut/s-1-2.fq -v -A.
```

The other options have the same meaning in both C/T wildcard mode and A/G wildcard mode.

2.3 Parallel mapping

Mapping is the most time-consuming and resource-demanding step in the methpipe pipeline. Fortunately we may adopt a "divide and conquer" strategy to speed up this step. In brief, we first divide large sequence files into smaller ones, map them separately in parallel and finally combining the mapped files.

A large sequence file is divided into smaller ones with the **split** utility which is a standard utility in any UNIX-like platforms. By running

```
$ split -l 16000000 bcell/s-1.fq s-1
```

you will get a series of smaller files: s-1aa, s-1ab, s-1ac, etc, which can be mapped with **rmapbs** separately as described above. To learn about the detailed usage of **split**, you may read its man page. In brief, the **-l** option specifies the number of lines in each smaller file. Since FASTQ format requires that each read consist of four lines, i.e. name line, sequence line, name line and score line, the number of lines should be multipliers of 4. The optimal number depends on the time constraints and memory contraints of your computing platform. For your information, mapping 28 millions reads to the Arabidopsis *thaliana* genome with **rmapbs** uses about 40 minutes and 5Gb memory.

3 Post-mapping processing

If everything goes well, you should already have pre-processed raw sequence reads and mapped them to the reference genome. Suppose the mapped reads files of B cell sample are

```
bcell/s-1.bed bcell/s-2.bed bcell/s-3.bed bcell/s-4.bed,
```

and the mapped reads files of the neutrophile sample methylome are

```
neut/s-1-1.bed neut/s-1-2.bed neut/s-2-1.bed neut/s-2-2.bed.
```

We will discuss some necessary processing steps, including converting A-rich reads to T-rich reads in pair-end sequencing, masking overlapping regions of paired reads, handling duplicate reads and utilities to sort and/or merge mapped reads.

3.1 Converting A-rich reads to T-rich reads

As has been noted before, if using pair-end sequencing, you will get both T-rich reads and A-rich reads. When estimating methylation frequency of a cytosine residue, we count the number of cytosines, which originate from methylated cytosines, and the number of thymines, which originate from unmethylated cytosines, and the use the ratio $\frac{\#C}{\#C+\#T}$ as the estimate of methylation frequency of that base. Therefore, A-rich reads need to be converted to T-rich reads before we are able to leverage the information from these A-rich reads. This conversion is easily done with the program **revcomp**, for example

```
$ ./revcomp < neut/s-2-2.bed > neut/s-2-2-revcomp.bed
```

Note this conversion applies to and only applies to A-rich reads in pair-end sequencing. For notation convenience, we again assume you rename the converted reads file name to its original file name thereafter.

3.2 Masking overlapping regions of paired reads

With pair-end sequencing, we obtain two reads: a T-rich read from 3' end of the orignal segments and an A-rich read from 5' end. After converting A-rich read to T-rich read, we have two T-rich reads from the orignal DNA segment, which possibly overlap for certain proportion. This overlapped region, if left as-is, will result in bias that cytosines (or thymines if unmethylated) from the same DNA molecule are counted twice.

The program **mask-overlap** identifies and masks overlapped regions. In our example project, neut/s-1-1.bed and neut/s-1-2.bed contain T-rich reads and A-rich reads (converted) respectively. We first sort the reads in these two files by name by running

```
$ ./sort -N neut/s-1-1.bed -o tmpfile && mv tmpfile neut/s-1-1.bed
$ ./sort -N neut/s-1-2.bed -o tmpfile && mv tmpfile neut/s-1-2.bed,
```

And then we run **mask-overlap** to mask overlapping regions of reads from these two files

```
$ ./mask-overlap neut/s-1-1.bed neut/s-1-2.bed
neut/s-1-1-masked.bed neut/s-1-2-masked.bed.
```

Note the commandline argument to **mask-overlap** are input-file-1, input-file-2, output-file-1 and output-file-2.

The program **mask-overlap** can also be used to obtain the following information about the pair-end sequencing data, such as the DNA fragment length distribution and the number of unpaired reads after mapping. This is done by specifying the fragment length file with the **-f** option. For example,

```
$ ./mask-overlap -f neut/s-1-length.txt  neut/s-1-1.bed neut/s-1-2.bed
neut/s-1-1-masked.bed neut/s-1-2-masked.bed.
```

In the file neut/s-1-length.txt **mask-overlap** write the fragment length based on each pair of reads. This file can then be analyzed with statistical software and standard *NIX text-processing utilities.

3.3 Sorting reads

The **sort** utility is used to sort mapped reads according to different criterion. The usage of **sort** is quite straightforward. To sort reads according to their genomic locations, we run

```
$ ./sort bcell/s-1.bed -o output-file.
```

To sort reads according to their names we run

```
$ ./sort -N bcell/s-1.bed -o output-file.
```

3.4 Merging read files

The final step before estimating methylation frequency and performing other higher level analysis is to merge read files from different sequencing lanes and/or flowcells.

If all read files are generated from the same biological library, this step is quite straightforward. We sort all read files by genomic locations with **sort** (Section 3.3), merge those files with **merge** and then remove duplicate reads with **unique** (Section 3.5). We show how to merge read files in the B cell data below.

```
$ # sort read files
$ ./sort bcell/s-1.bed -o tmpfile && mv tmpfile bcell/s-1.bed
$ ./sort bcell/s-2.bed -o tmpfile && mv tmpfile bcell/s-2.bed
$ ./sort bcell/s-3.bed -o tmpfile && mv tmpfile bcell/s-3.bed
$ ./sort bcell/s-4.bed -o tmpfile && mv tmpfile bcell/s-4.bed
$ ./merge bcell/s-*bed |./unique > bcell/bcell.bed
```

If the read files are from different libraries, it takes some extra step. The reason is that: even if two reads from different biological libraries are mapped to the same location, they are not originated from the same DNA molecule, therefore both should be kept for downstream analysis. For each library, we pool reads from this library and remove duplicate reads. Then we merge reads from different libraries.

3.5 Removing duplicate reads

Because of PCR bias and other unknown causes, there are tens of thousands of reads mapped to exactly the same location in some regions of the genome while the average sequencing depth is usually below 20. These large amounts of reads are likely orginated from the same DNA segments and will possibly bias the estimation of methylation frequency. One approach to alleviate this bias, though not completely justifiable, is to keep only one distint read at each starting position. This task is done by the program unique **unique**. For example if we would like to remove duplicate reads from the file bcell/s-1.bed, we can run

```
$ ./unique bcell/s-1.bed -o bcell/s-1-unique.bed
```

4 Methylation frequency and bisulfite conversion rate

At this step, we have precessed mapped reads and combines reads from different lanes and/or flowcells into a single file, which means that we have a bcell/bcell.bed containing all reads from the B cell sample and a neut/neut.bed containing all reads from the neutrophile sample. From now on, we will be working with these two aggregated files. In this section and the following section, we will proceed from analyzing methylation status at single loci to identifying higher methylation patterns such as hypo-methylated regions.

4.1 Estimating methylation frequency

In this section we will learn how to estimate the methylation probability at a single cytosine loci. In mammals, DNA methylation exists mostly at cytosines in the context of CpG dinucleotides. This kind of methylation is symmetric because the cytosine on the complementary strand, which is base-paired with the guanine, is also methylated due to the interesting property of maintenance DNA methyltransferases. In mammalian stem cells and particularly in plant cells, cytosines in sequence contexts other than CpG dinucleotides, such as CpHpG (H denotes adenines, thymines or cytosines), may also be methylated. This type of methylation is called asymmetric as the cytosines on the complementary strand are unnecessarily methylated. Symmetric methylation and asymmetric methylation requires different methods to estimate the methylation frequency. Simply put, in the case of asymmetric methylation we can use only reads mapped to one strand while we can use reads mapped to both strands in the case of symmetric methylation. Later we will describe the estimation methods for these two cases separately, first the case of asymmetric methylation and then the case of symmetric methylation.

After bisulfite sodium treatment, unmethylated cytosines in the original DNA sequences are converted to thymines while methylated cytosines remain the same. For convenience, we assume the bisulfite conversion is perfect for now. For a cytosine in the non-CpG context, we count the number of cytosines, which originate from methylated cytosines, and the number of thymines, which originate from unmethylated cytosines, from reads in the same strand as the cytosine. The unbiased estimator of the methylation frequency at this cytosine is simply the ratio of the number of cytosines to the number of cytosines and thymines combined. This task is carried out with the program **methcount** as following:

```
$ ./methcounts -c human_genome -non -o bcell/bcell.bed bcell/bcell-methcounts.bed,
```

where the **-c** options specifies the direcotory containing the human genome sequences and the **-non** options indicates the program should run in non-CpG mode. For each cytosine, the program reports the number of cytosines, the number of thymines and the estimation of methylation frequency in the output file bcell/bcell-methcounts.bed.

For cytosines in the context of CpG dinucleotides, we are able to use reads mapped to both strands, which means better coverage and therefore reduces variance of our estimation. We count the number of cytosines and thymines in all reads that overlap cytosines either in the positive strand or in the negative strand. And the estimator of methylation frequency is still the ratio of the number of cytosines to the number of cytosines and thymines combined. This task is done simiarly to the case of non-CpG methylation except that we ignore the **-non** option of the **methcount** program, for example,

```
$ ./methcounts -c human_genome -o bcell/bcell.bed bcell/bcell-methcounts.bed.
```

The methcount program report the methylation status of only those cytosines in the CpG context.

4.2 Estimating busilfite conversion rate

5 Higher level analysis

5.1 Identifying hypo-methylated regions

5.2 Identifying differentially methylated regions

6 Visualization

7 A sample work flow

This part shows how these tools are connected to get the methylation profile. Suppose we have the following BS-Seq library

```
reads/s_1_1.txt reads/s_1_2.txt reads/s_2_1.txt reads/s_2_2.txt
```

The final result can be obtained as following. For clarity, we show all the intermediate result. In real application, some intermediate files can be avoided by using pipes.

```
# Pre-mapping processing #

## trim adapter ##
$ ./trim-adapter reads/s_1_1.txt preprocessed/s_1_1.txt
$ ./trim-adapter reads/s_1_2.txt preprocessed/s_1_2.txt
$ ./trim-adapter reads/s_2_1.txt preprocessed/s_2_1.txt
$ ./trim-adapter reads/s_2_2.txt preprocessed/s_2_2.txt

# Mapping #
```

```
$ ./rmapbs -c genome_seq_dir -o mapped/s_1_1.mr preprocessed/s_1_1.txt
$ ./rmapbs -A -c genome_seq_dir -o mapped/s_1_2.mr preprocessed/s_1_2.txt
$ ./rmapbs -c genome_seq_dir -o mapped/s_2_1.mr preprocessed/s_2_1.txt
$ ./rmapbs -A -c genome_seq_dir -o mapped/s_2_2.mr preprocessed/s_2_2.txt

# post-mapping processing

## reverse complement A-rich strand ##
$ ./revcomp mapped/s_1_2.mr > tmpfile && mv tmpfile mapped/s_1_2.mr
$ ./revcomp mapped/s_2_2.mr > tmpfile && mv tmpfile mapped/s_2_2.mr

## mask overlapping ##
#### first sort by name ####
$ ./sort -N mapped/s_1_1.mr -o tmpfile && mv tmpfile  mapped/s_1_1.mr
$ ./sort -N mapped/s_1_2.mr -o tmpfile && mv tmpfile  mapped/s_1_2.mr
$ ./sort -N mapped/s_2_1.mr -o tmpfile && mv tmpfile  mapped/s_2_1.mr
$ ./sort -N mapped/s_2_2.mr -o tmpfile && mv tmpfile  mapped/s_2_2.mr

#### masking ####
$ ./mask-overlap mapped/s_1_1.mr mapped/s_1_2.mr masked/s_1_1.mr masked/s_1_2.mr
$ ./mask-overlap mapped/s_2_1.mr mapped/s_2_2.mr masked/s_2_1.mr masked/s_2_2.mr

#### sort by genomic location ####
$ ./sort masked/s_1_1.mr -o tmpfile && mv tmpfile  masked/s_1_1.mr
$ ./sort masked/s_1_2.mr -o tmpfile && mv tmpfile  masked/s_1_2.mr
$ ./sort masked/s_2_1.mr -o tmpfile && mv tmpfile  masked/s_2_1.mr
$ ./sort masked/s_2_2.mr -o tmpfile && mv tmpfile  masked/s_2_2.mr

## combine all result ##
#### merge ####
$ ./merge -o all.mr masked/s_1_1.mr masked/s_1_2.mr masked/s_2_1.mr masked/s_2_2.mr

#### jackpot removal #####
$ ./unique all.mr -o tmpfile && mv tmpfile all.mr

# analysis #
## methcounts ##
$ ./methcounts -c genome_sequence_file -o all-methcounts.bed all.mr
```