

# Correcting Methylation Calls in Low-Mappability Regions

Caiden M. Kumar, Ariel Erijman, Bradley W. Langhorst

July 27, 2021

## Abstract

DNA methylation is an important component in vital biological functions such as embryonic development, carcinogenesis, and heritable regulation. Accurate methods to assess genomic methylation status are crucial to its effective use in many scenarios, especially in the detection and diagnosis of disease. Methylation aligners, such as Bismark and bwa-meth, frequently assign MapQ values to reads which are significantly higher than can be supported by the uniqueness of the region they are mapped to. These incorrectly high MapQs result in inappropriate methylation calling in repetitive regions. We observe reads that should map to separate locations (possibly having different methylation states) actually end up mapping to the same locus, causing apparent mixed methylation at such loci. Methylation calling can be improved by using Bismap mappability data to filter out insufficiently unique reads. Simply filtering out Cs in insufficiently unique regions is not adequate as it is prone to overfiltering Cs in small mappability dips. These Cs can in fact often be called using reads anchored in a nearby mappable region. We have created a patch for the MethylDackel methylation caller to perform read-based filtering. Read-based filtering resolves some of the apparent mixed methylation to either 0% or 100% methylation. We examined methylation calls with and without read-based filtering in or near the 7830 <check this number> genes containing ClinVar variants in a methylation sequencing data set from the NA12878 cell line and in tumor samples. Examining low mappability Cs in the NA12878 data set revealed 1405 mixed methylation Cs were corrected to 0% methylation, and 2577 mixed methylation Cs were corrected to 100% methylation.

## Introduction

As DNA methylation status can have a significant biological function [26], it is important that there be an accurate way of calling methylation on a genome. Although there are multiple varieties of DNA methylation, a significant type is methylation of cytosine to 5-methylcytosine [5]. Data on DNA cytosine methylation state can be gathered using a methylation sequencing technique

(see Figure 1), for example bisulfite sequencing [6]. In bisulfite sequencing, unmethylated cytosines are deaminated to uracil by the addition of sodium bisulfite. 5-methylcytosines are not affected. Since uracil sequences as thymine and 5-methylcytosine sequences as cytosine, positions of unmethylated Cs in a reference sequence can be identified by C->T transitions[6].

It is also possible to use an enzymatic method, EM-seq, employing TET2 to oxidize 5-methylcytosine and an APOBEC enzyme to deaminate unmodified cytosines to uracil. While sodium bisulfite treatment produces other DNA damage, this enzymatic method deaminates with more precision [28].

Whichever method is used to deaminate cytosines, sequence data is typically aligned to a reference genome using a methylation-aware aligner [9], which is specifically designed to handle the C->T transitions in methylation sequencing data when aligning the reads to a reference. Once aligned, the data can be passed through a methylation caller such as MethylDackel [25] or bismark\_methylation\_extractor [17], which will use the resulting read alignments to determine the methylation status of particular cytosines (see Figure 2). The resulting data shows the methylation status of each cytosine in the genome and can therefore be used to find and study biologically significant DNA methylation sites.

A read must be unambiguously placed if it is to provide information about a specific locus. Reads that equally match more than one area of the reference genome should not be used to assess methylation of any given C. To avoid calling Cs using reads derived from multiple genomic loci, methylation aligners (and read aligners in general) assign a MapQ value to each read alignment (see Figure 2). According to the SAM specification [10], MapQ is defined as: “ $-10 \log_{10} \Pr\{\text{mapping position is wrong}\}$ , rounded to the nearest integer”. MapQ indicates how uniquely placed a read alignment is, that is, in how many other places could the read align to the reference. A low MapQ means that the read may align in many places throughout the genome (for instance, a read of centromeric satellite DNA would likely have a very low MapQ). A high MapQ indicates that the read likely aligns where it is placed and nowhere else in the genome.

A methylation caller can use accurate MapQ values to filter out reads with multiple placements in the genome, allowing the resulting methylation calls to accurately reflect their specific loci.

## Results

While evaluating the methylation aligner bwa-meth [23], we observed a significant number of reads with unexpectedly high MapQ values in repetitive regions (e.g. centromeres). After observing these high MapQ reads in the centromere and larger repetitive regions, we investigated to see if smaller regions might also be too repetitive to support the high aligner MapQ estimates observed. We identified repetitive regions using data from Bismap [14], a tool that counts the number of occurrences of every single K-mer of a particular length (in this

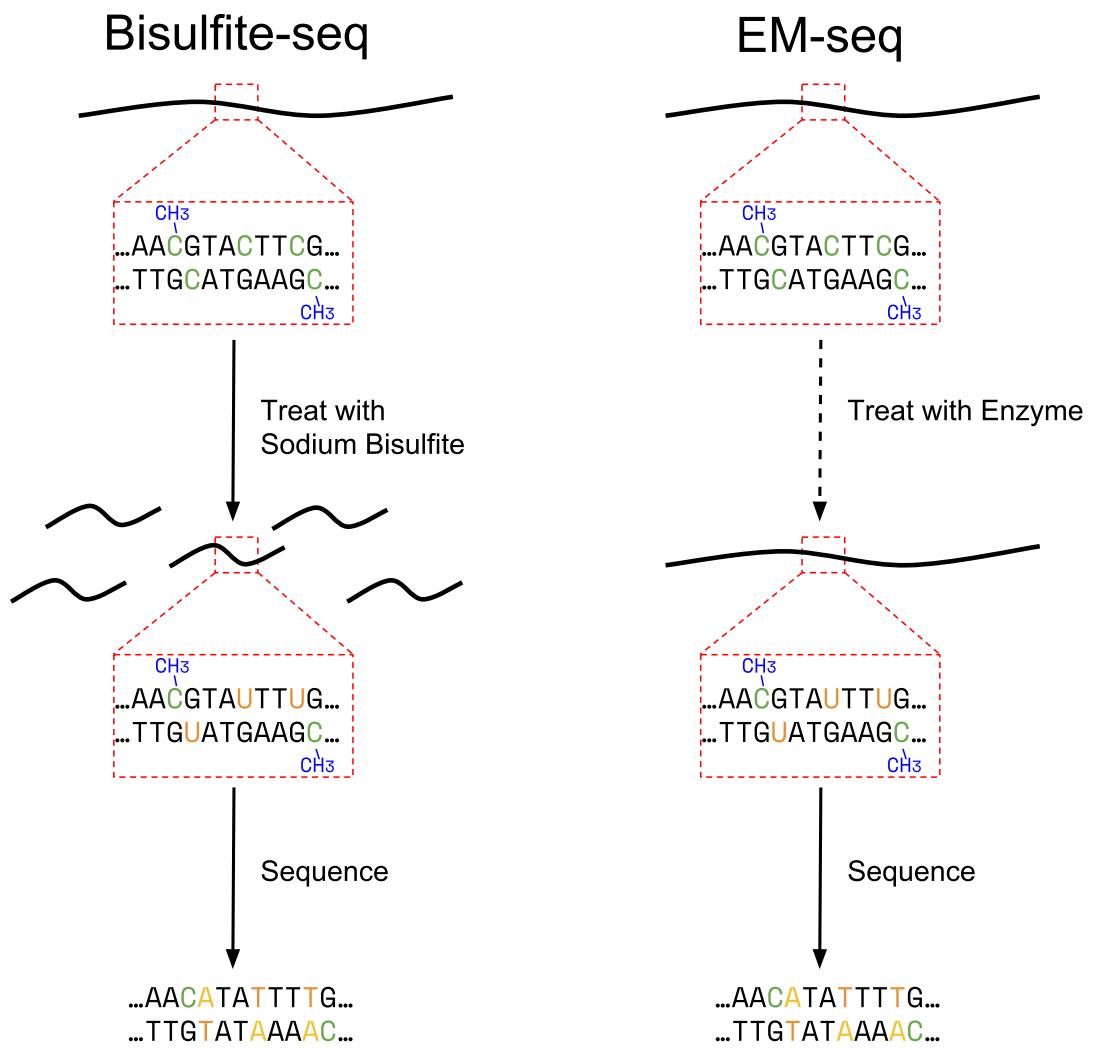


Figure 1: Overview of Methylation Sequencing Methods. <todo: Treat with Enzyme -> Tet2-> APOBEC

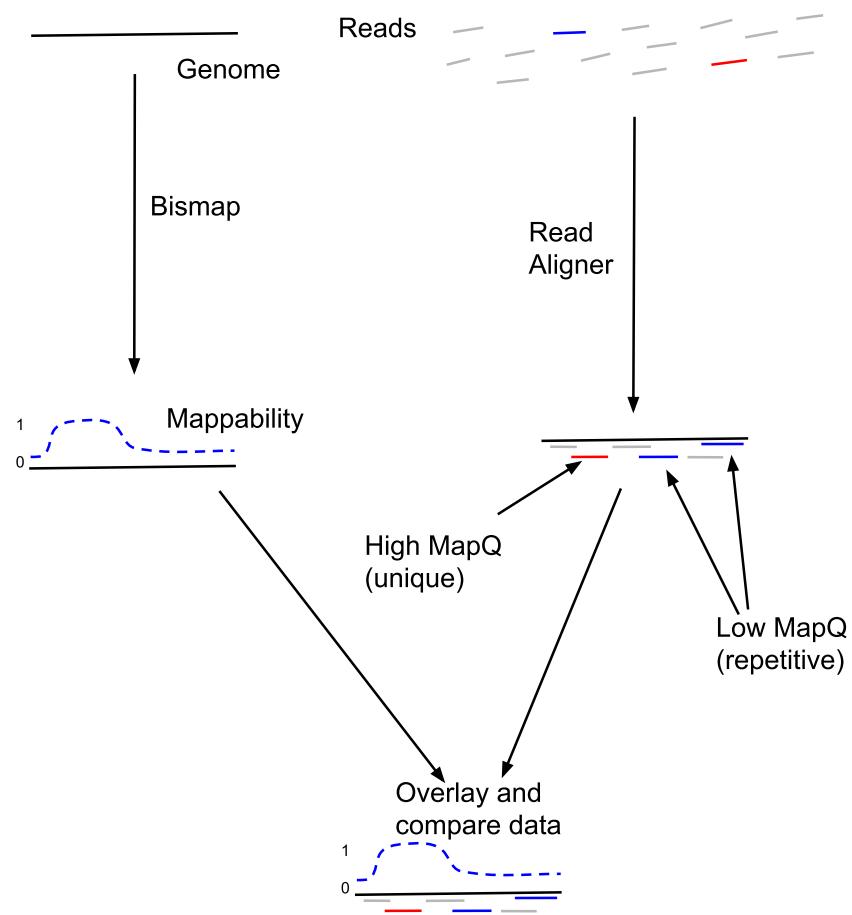


Figure 2: Experimental Overview

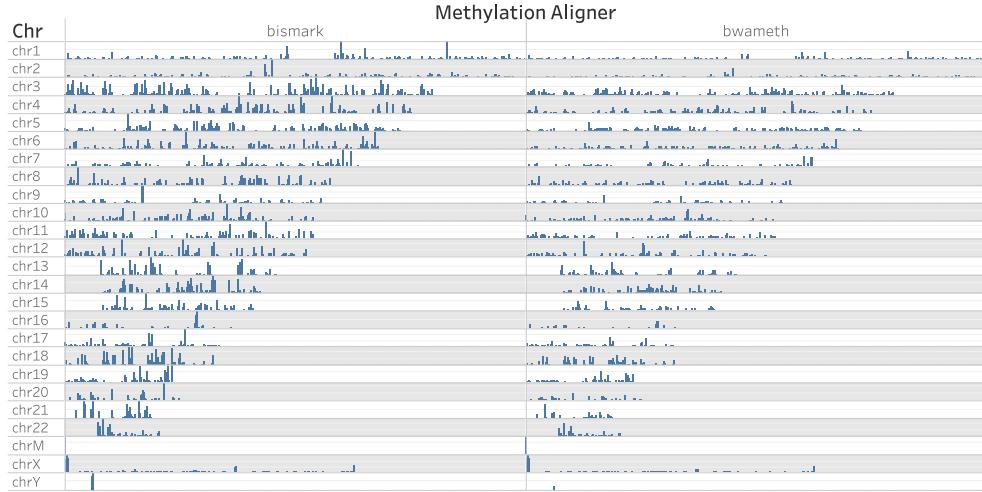


Figure 3: Cs called in low mappability regions after MapQ filtering are represented across all chromosomes...

case,  $k=100$ ) in the genome to create a mappability score (ranging from 0 to 1 in increments of 0.01) for every base in the GRCh38 reference. <bases this on assumption that each locus is either 0 or 100% <what does this note mean exactly? I forgot.>> Bismap takes the effect of C->T conversion into account and therefore produces data which is applicable in the context of methylation sequencing. Reads entirely contained within a region of low mappability should not have high MapQ values due to their repetitiveness, however we observed many high-MapQ reads in regions with very low or zero Bismap mappability (see Figure 4).

While low MapQ can indicate repetitiveness, even stringent MapQ thresholds cannot reliably select reads for safe methylation calling in regions containing repetitive DNA. We considered excluding methylation calls on Cs found in low-mappability regions (e.g. using bedtools), but rejected this approach because it is prone to both over- and underfiltering. Cs in short, unique regions would be kept (underfiltered) even if the surrounding DNA is repetitive (see Figure 6). However, this situation was not commonly observed. Overfiltering of Cs in short repetitive regions is a larger problem though. In this scenario, a C located in a small dip in mappability would be eliminated (overfiltered) despite coverage from read pairs anchored in nearby unique regions (see Figure 7). In practice, underfiltering is rare ( $x\%$  of Cs in GRCh38) but overfiltering is much more common ( $y\%$  of Cs in GRCh38) <need to fill in data here> (see Figure 5). In cases where reads from multiple low-mappability regions are placed onto one region (creating a coverage spike), filtering by coverage could possibly also remove the problematic region, but in cases where reads are simply mixed among multiple low-mappability regions (not creating a coverage spike), coverage filtering would

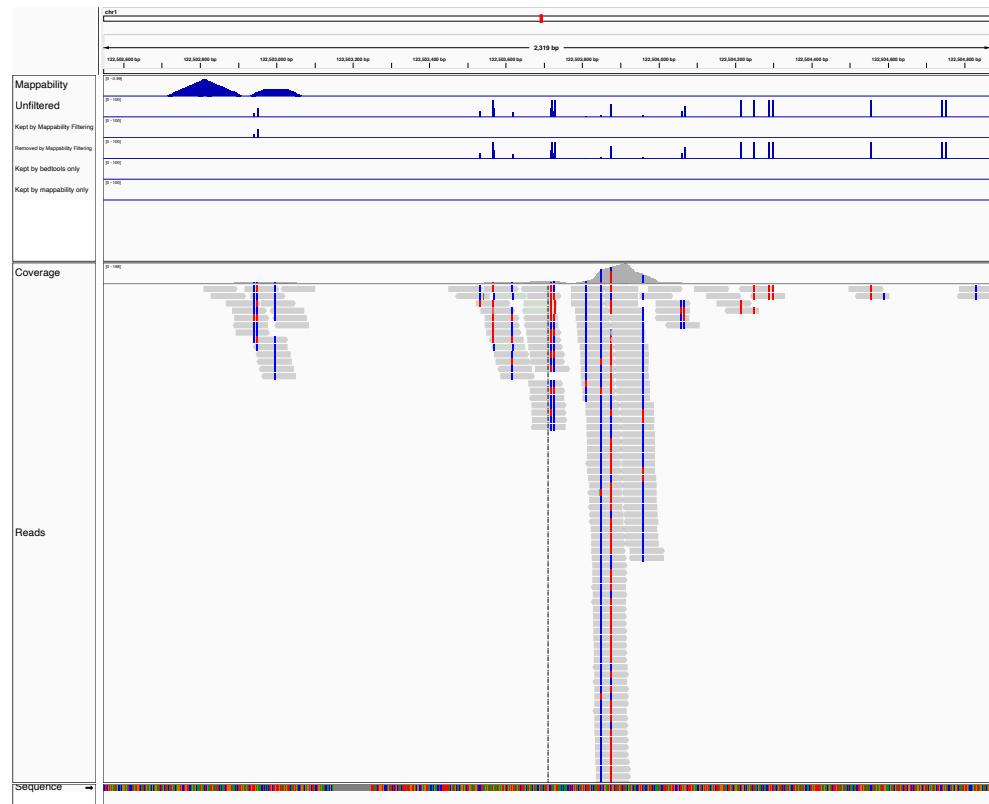


Figure 4: MapQ filtering does not remove all poorly mapped reads.

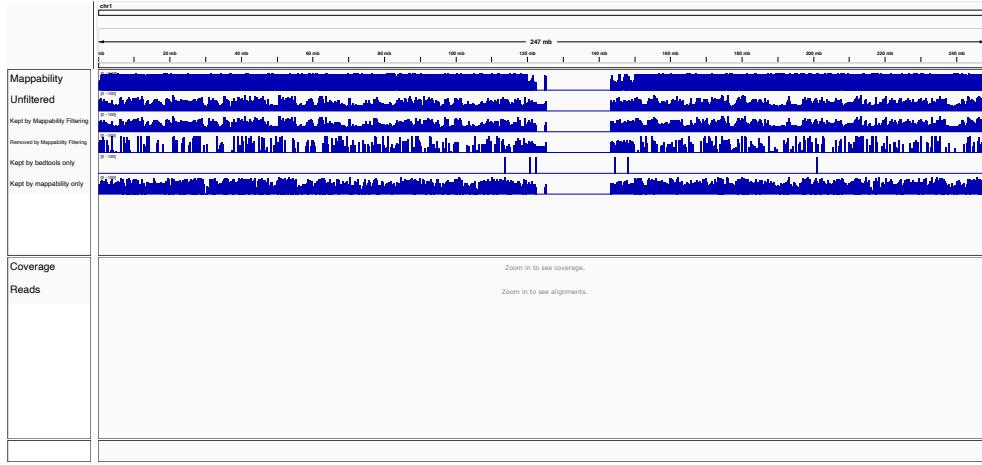


Figure 5: <I think we need to be more explicit about what is being plotted here>An overview of the occurrences of these scenarios in GRCh38 chromosome 1.

not be effective at removing the affected regions.

To reliably filter out only problematic read pairs (those where both mates are placed in low mappability regions) we modified the MethylDackel methylation caller to accept a bigWig file of low-mappability regions to exclude from analysis. This per-read filtering approach precisely eliminates only those reads in repetitive regions and does not incur a significant cost in terms of execution speed (10:14 min with the patch 9:55 min without, when run with 20 threads on the GRCh38 reference genome <update these numbers?>). (see computational methods for details)

To focus on the incorrect alignments and methylation calls that have biological and medical significance, we examined methylation calls in GENCODE genes [11] that contain variants listed in the ClinVar [18] database of disease-associated variants. Using our alignment filtering approach we successfully avoided calling 15,539 <update this number after re-run> Cs in these important regions in a 50ng EM-seq sample which have insufficient mappability to support methylation calling. Briefly, to determine that we avoided methylation calling in a region that has insufficient mappability, we intersected read alignments and MapQ data with Bismap low mappability regions, counting Cs in regions where the MapQ is higher than should be possible given the mappability (see Figure 2). <is this how we did it? or did we compare filtered/unfiltered?>

## Filtering Effect

We compared the effect of per-read mappability filtering on number of C's called, as well as number of C's called in low-mappability regions (as defined by per-C filtering), on EM-seq samples of 3 sample masses aligned with the bwa-meth

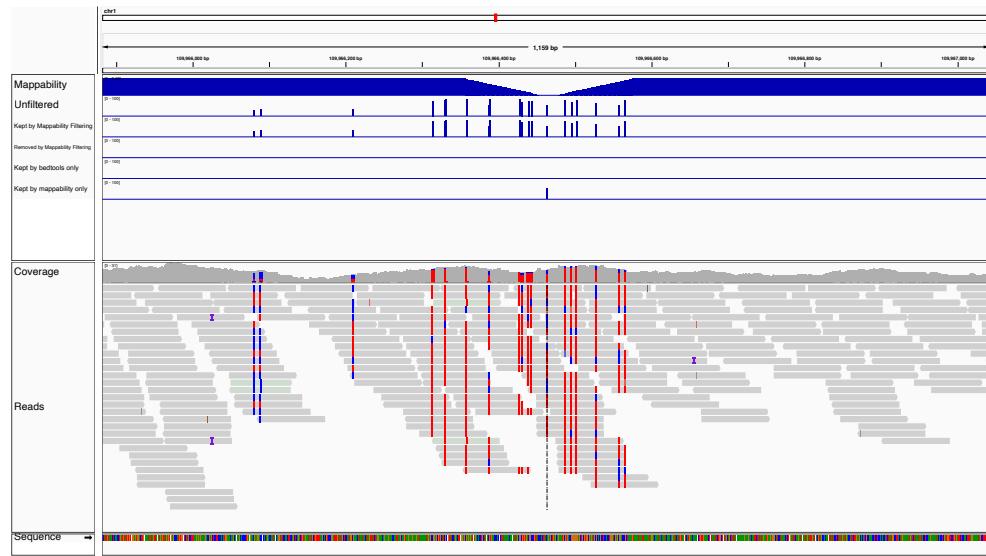


Figure 6: An example of a C incorrectly discarded by coordinate filtering.

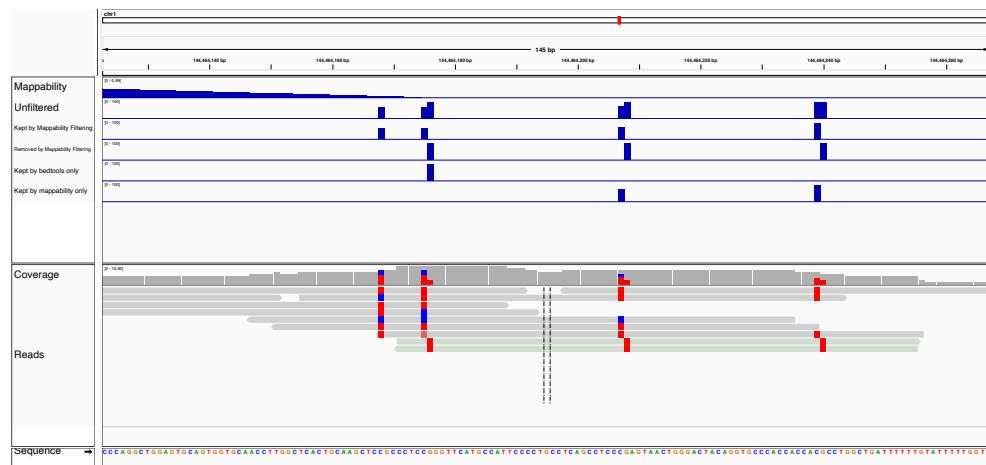


Figure 7: <needs an expanded explanation>An example of a C incorrectly kept by coordinate filtering.

We also examined how many mixed methylation ( $0\% < \% \text{Methyl} < 100\%$ ) Cs were resolved to either 0% or 100% methylation, and found <numbers> (see Figure <NUM>), showing how unlike per-C filtering, per-read filtering can clean up methylation calls without always having to entirely delete them.

## EM-seq vs. WGBS

Due to the reduced DNA damage and resulting longer read lengths that occur in EM-seq as opposed to WGBS (whole genome bisulfite sequencing), we compared two sets of sequencing runs, each with the same 3 sample masses, to see if there is a difference between the two aligners with regard to the number of miscalls, is it is possible longer reads could allow for more low-mappability Cs to be called using reads extending into higher-mappability regions. We found that, as we had predicted, <ADD DATA> (see Figure <NUM>).

## Bwa-meth vs. Bismark

For comparison, we also examined the Bismark methylation aligner [17] in the same manner. We observed that compared to bwa-meth, Bismark produced more high MapQ reads in repetitive regions (see Figure 3). In addition, Bismark and bwa-meth use different systems to define values for MapQ. Bismark (as a result of using Bowtie2 for alignment) reports MapQ based on number of reference mismatches, producing values between 0 and 42 [1, 13]. Bwa-meth (using BWA-MEM for alignments) follows the SAM specification in estimating probabilities in the MapQ field. Overall, we observed somewhat more miscalls with Bismark than we did with bwa-meth <ADD DATA>, as shown in Figure <NUM>.

## GRCh38 vs. T2T

We also examined the effect of using the T2T reference [22] in place of GRCh38, since the T2T reference is much more complete than GRCh38, including repetitive regions such as centromeres, telomeres, and rDNA, which could play a role in where methylation is called and what areas of the genome have unique enough sequence to support calling. We found that, as we predicted, there were many fewer miscalls in the T2T data as compared to the GRCh38 data <ADD AND VERIFY DATA>.

todo: filtering effect em-seq vs wgbs (fig) for bwa-meth (in pipeline)

todo: filtering effect em-seq-vs wgbs for bismark (this is the data I'm working on) (in pipeline)

todo: # cs affected (worst) bismark vs filtered vs bwa-meth vs filtered (best) (fig?) (I can get this from the emseq/wgbs data, it's a simple bedtools operation) (in pipeline, somewhat)

todo: group Cs by magnitude of change in methylation post filtering (fig, heatmap?) (best done by comparing both %methyl and read count, maybe we should discuss how to layout this figure though)

todo: effect on ClinVar (in pipeline)

todo: examples of specific effects in ClinVar (I have 2 of these from my talk)

todo: find/examine relevant human (GEO?) dataset (maybe, depends on time/progress) (sequencing, not chip) (ideally, clinically relevant EM-seq)

todo: T2T data (repeat analysis on this and compare effect) (in pipeline)

## Materials and Methods

### DNA Methylation Sequencing

#### Materials

Libraries were prepared from 10, 50, and 200 ng of genomic DNA from the NA12878 cell line (Coriel). This input was supplemented with a small amount of fully-methylated Xp-12 DNA (isolated from Xp12 phage following the procedure in Y. J. Lee, P. R. Weigle, Detection of modified bases in bacteriophage genomic DNA. Methods Mol. Biol. 2198, 53-66 (2021).[19]) <is there a specific source for this, e.g. a company and/or product name <https://www.pnas.org/content/118/26/e2026742118.full#ref-35?>>, lambda phage DNA (NEB #N3011), and a pUC19 plasmid (NEB #N3041) treated with M.SssI CpG Methyltransferase (NEB #M0226).

#### Whole Genome Bisulfite Libraries

Libraries were prepared using the Ultra II DNA library prep kit (NEB E7645) before being Bisulfite converted according to <protocol>.

#### EM-seq Libraries

<anything about the EM-seq samples?>

#### Sequencing

Libraries were pooled and sequenced with diverse libraries (~10%) on 2 flow cells of an Illumina Novaseq 6000 [12] using the S2 chemistry. We acquired 1.55 billion 99 bp paired-end reads for the EM-seq method and 1.60 billion paired-end reads for the Bisulfite converted libraries <get this from samtools stats on the bams what mass is this for? need counts for all masses unless they're identical>.

#### Computational Methods

The following data and tools were used for the analysis:

The GRCh38.p11 analysis set (hereafter referred to as “GRCh38”) supplemented with phage T4, phage lambda, phage Xp12, and pUC19 contigs was

used throughout [7]. A VCF file of disease-associated variant sites was downloaded from ClinVar (see supplemental data for file date) and a GFF file of the GENCODE v31 gene annotations were used. The mappability data was the 100bp multi-read bigWig file downloaded from Bismap (see supplemental materials for link). A detailed diagram of the analysis pipeline is found in Figures 9 and 10. Tools used include bedtools [24], samtools [20], GNU awk, MethylDackel, bigWigToBedGraph [15], BEDOPS [21], GNU sort, GNU head, bwa-meth, and Bismark. GNU sort and head are required since some of the functionality needed (specifically, the ability to parallelize sorting and the ability to use a negative value with the -n option for head to count lines from the end of the file) is not present in BSD sort and head.

The sequencing reads were aligned using the bwa-meth aligner using default options. Methylation calling was performed on the resulting BAMs using MethylDackel v0.6.0. MethylDackel uses a default value of 10 as a minimum MapQ which should include mostly single locus reads, however inaccurate MapQs can lead to reads being incorrectly included in methylation calling. In order to eliminate reads in low-mappability regions, a patch was created for MethylDackel which allows it to take as an input a bigWig file which is then used to filter out read pairs (this patch currently only supports paired-end reads) where neither mate intersects a high-mappability region. The patch allows for user configuration of the low mappability threshold and the number of bases which must be equal to or above that threshold in order for the read pair to be kept (the defaults are a low mappability threshold of 0.01 and to require 15 bases that are greater than or equal to that threshold in a single read). The filtering algorithm has been optimized both by loading the mappability data into memory before calling, and through the use of a custom run-length compressed binary file format that we here term BBM (Binary BisMap). This format can store the mappability data for the hg38/GRCh38 human genome in 143 MB, compared to 1.11 GB when stored as a bigWig, giving a compression ratio for this dataset of 7.78:1. <mention the trimming settings?>

The ClinVar VCF and GENCODE GFF were combined using bedtools and BEDOPS into a single BED file listing all GENCODE annotation regions that overlap one or more ClinVar variants (these regions will be referred to as “ClinVar regions”), which will be called the “ClinVar regions BED”. The Bismap bedGraph (which, as is standard for bedGraph files, did not contain zeroes) and an FAI index of the GRCh38 reference genome FASTA file were processed with bedtools, awk, and GNU sort to obtain a bedGraph containing all mappability data, including zeroes. This file will be referred to as the “Bismap complete bedGraph”. It was then combined with the ClinVar regions BED using bedtools map to create a file containing the minimum and mean mappability for each gene with a ClinVar variant.

The Bismap complete bedGraph was then filtered using awk to produce a file containing only low mappability regions (mappability  $< 0.01$ ). The file with minimum and mean mappability for every ClinVar region was filtered likewise on minimum mappability. The two resulting files (one of low-mappability regions, one of ClinVar regions with low minimum mappability) were combined to

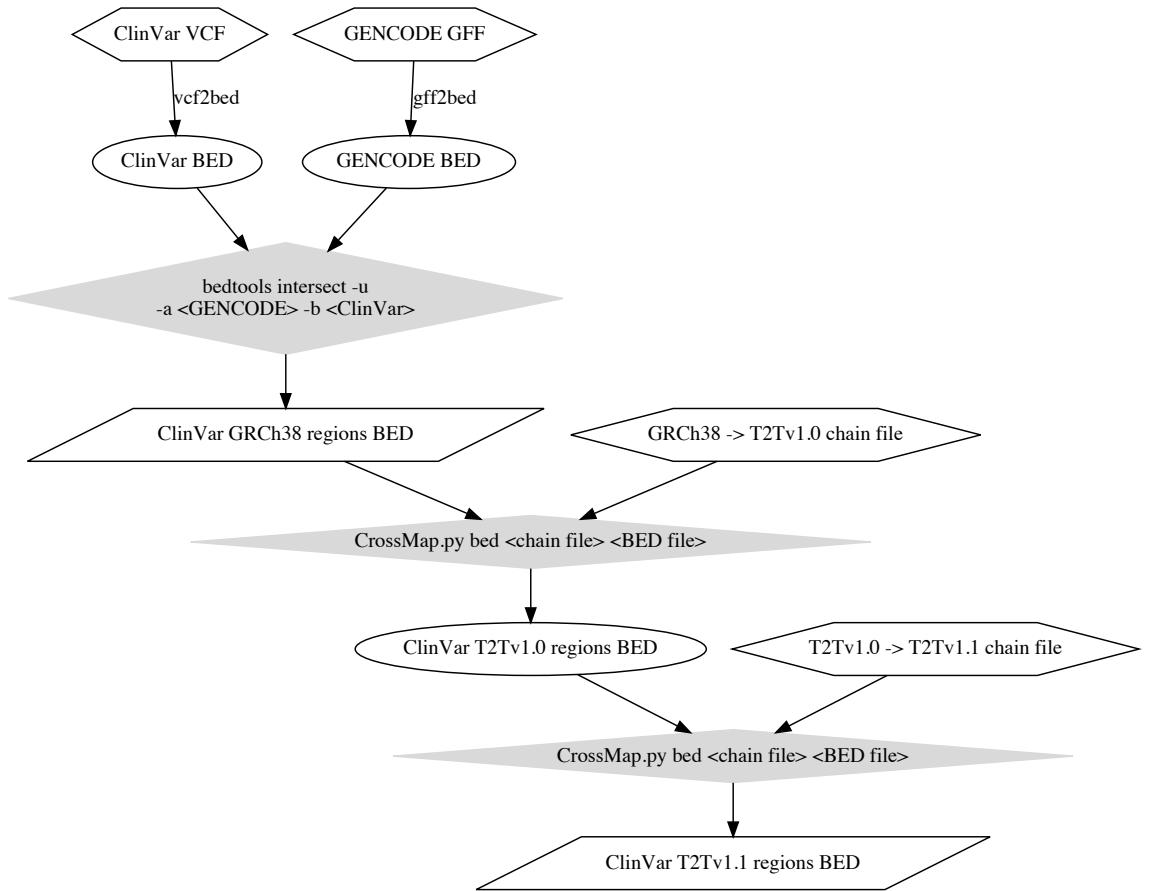


Figure 8: Detailed description of the procedure used to annotate the T2Tv1.1 reference using the GRCh38 ClinVar and GENCODE data. Hexagons are input files. Ovals are intermediate files. Gray rhombuses are processing steps with multiple arguments. Parallelograms are output files. Labels on edges are processing steps with one input and one output.

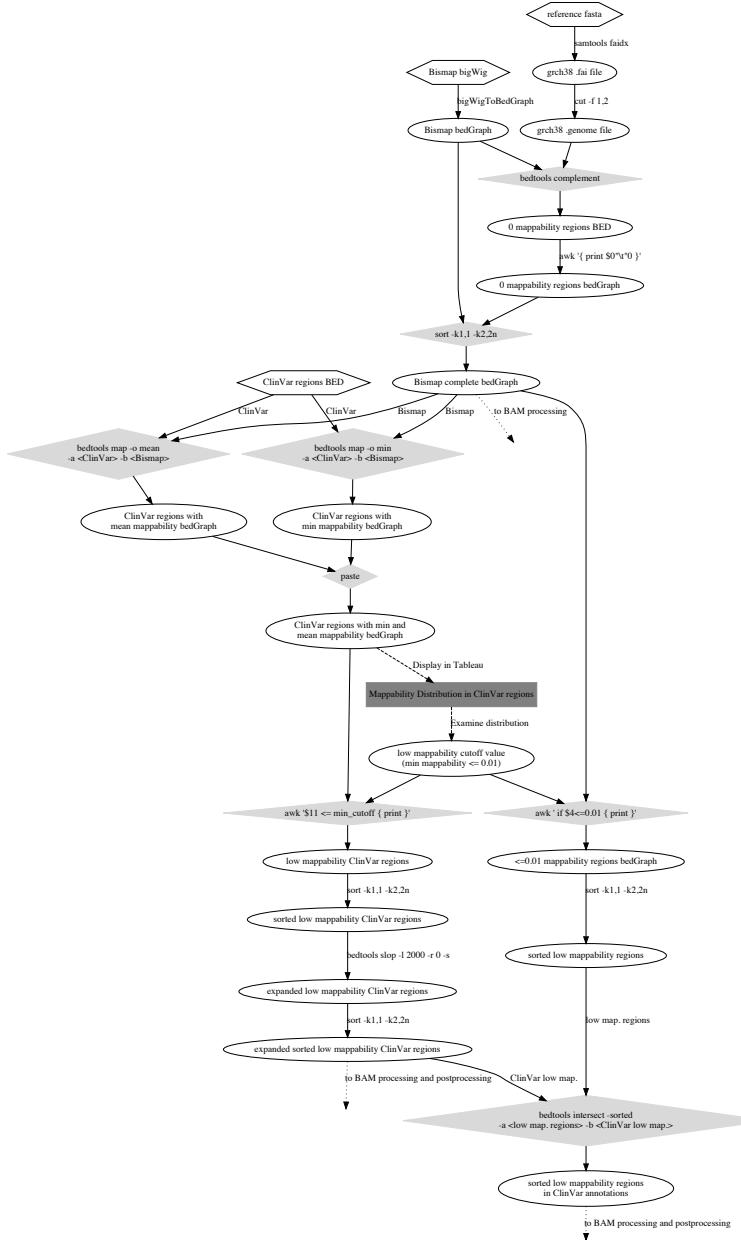


Figure 9: Detailed description of preprocesing steps used to identify biologically relevant low-mappability regions.. Ovals are intermediate files. Gray rhombuses are processing steps with multiple arguments. Parallelograms are output files. Labels on edges are processing steps with one input and one output (except edges directly entering rhombuses, which simply indicate which lines come from which steps and go to which arguments). Dotted lines indicate inputs that go to the BAM processing steps in Figure 10. Dark gray rectangles indicate steps involving manually examining the data.<sup>13</sup>Dashed lines indicate manual steps.

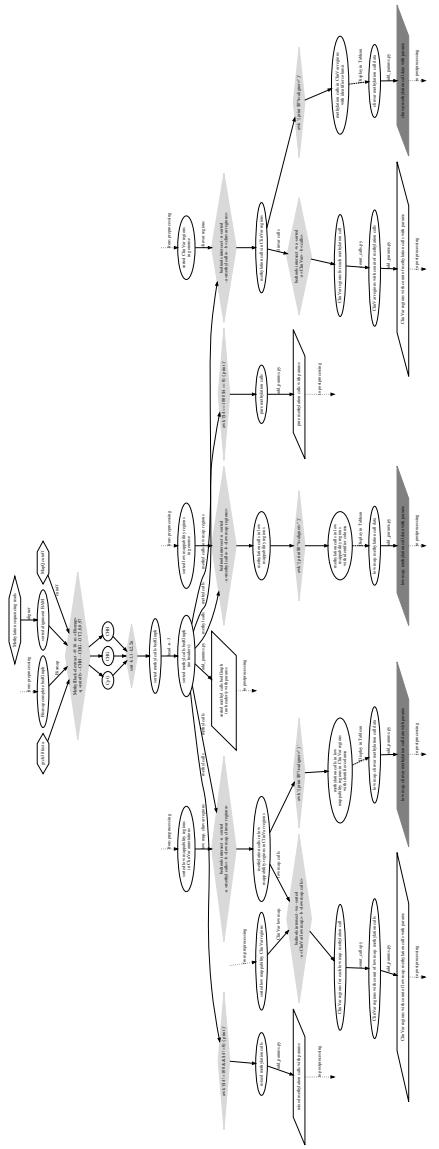


Figure 10: Detailed description of steps for calling methylation and intersecting the resulting calls with the low mappability regions determined in the preprocessing steps. Ovals are intermediate files. Gray rhombuses are processing steps with multiple arguments. Parallelograms are output files. Labels on edges are processing steps with one input and one output (except edges directly entering rhombuses, which simply indicate which lines come from which steps and go to which arguments). Dotted lines indicate inputs that come from the preprocessing steps in Figure 9. Dark gray parallelograms indicate final outputs that are examined manually. Dashed lines indicate manual steps.

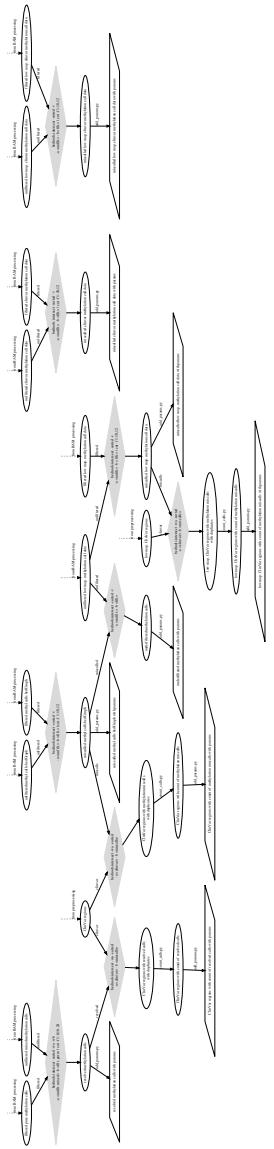


Figure 11: Detailed description of steps for calculating methylation miscalls from the data output from the preprocessing and methylation calling steps. Ovals are intermediate files. Gray rhombuses are processing steps with multiple arguments. Parallelograms are output files. Labels on edges are processing steps with one input and one output (except edges directly entering rhombuses, which simply indicate which lines come from which steps and go to which arguments). Dotted lines indicate inputs that come from the preprocessing steps in Figure 9 and methylation calling steps in Figure 10. Dark gray parallelograms indicate final outputs that are examined manually. Dashed lines indicate manual steps.

produce a file of all low mappability regions that are in ClinVar regions. <what did I do for partial overlaps here??>

Alignments of 2x99-bp paired-end EM-seq and whole genome bisulfite reads were processed using MethylDackel (with and without the custom patch) using a minimum MapQ cutoff of 10 and the default settings mentioned above and combined with the file of all low mappability regions in ClinVar regions to produce a list of all methylation calls in low mappability regions (this will be referred to as the “low mappability calls file”). A low-mappability methylation call, as used here, is defined as a methylation call that is in a region with Bismap mappability less than 0.01, as defined by the position of the C alone (positions of anchoring reads are not considered when determining this).

The low mappability calls file was intersected with the file of ClinVar regions with low minimum mappability to produce a file of ClinVar regions with low mappability calls. The -wa option for bedtools intersect was used here, which writes a copy of the ClinVar region to the output file for each low mappability call in the region, in order that this file would contain multiple copies of each region, one per low mappability call in the region. These duplicates were then used to count low mappability calls by feeding the data to a custom Python script (found in the Nextflow script in the supplemental materials ) which counted and combined the duplicates, producing a list of all ClinVar regions with low mappability calls and how many low mappability calls are in each region.

Since this analysis was run for three different input masses and two sequencing protocols, the low mappability calls files were also processed through a custom Python script (see supplemental materials) to add a field specifying which input mass, sequencing protocol, and MethylDackel filtering setting were used. As the input mass would have been difficult to parse out of the BAM name due to inconsistent formatting, a CSV mapping BAM file names to input masses was created and given as input to this step. The same field was added to the lists of all ClinVar regions with low mappability calls and counts described previously. We also produced similarly-annotated files for all methylation calls (regardless of mappability), all methylation calls in GENCODE genes which contained ClinVar variants, all methylation calls in low-mappability regions that were also in GENCODE genes which contained ClinVar variants, counts of calls in in GENCODE genes which contained ClinVar variants regardless of mappability, methylation calls that are either 0% or 100% (regardless of mappability), and methylation calls which are neither 0% nor 100% (also regardless of mappability)..

To assess miscalls (i.e. which Cs are filtered out by per-read filtering), as well as underfiltering/overfiltering with respect to per-C filtering (discussed above), and resolution of mixed methylation calls to 0% or 100%, pairs of these files were compared using bedtools to produce files showing the difference between filtered and unfiltered data. All files were examined and compared in Tableau®.

To compare the behavior of Bismark with bwa-meth, this analysis was re-run with the Bismark aligner using default settings and deduplicated using deduplicate\_bismark according to the documented protocol [16]. The name of the aligner was added to the field specifying the input mass, protocol, and

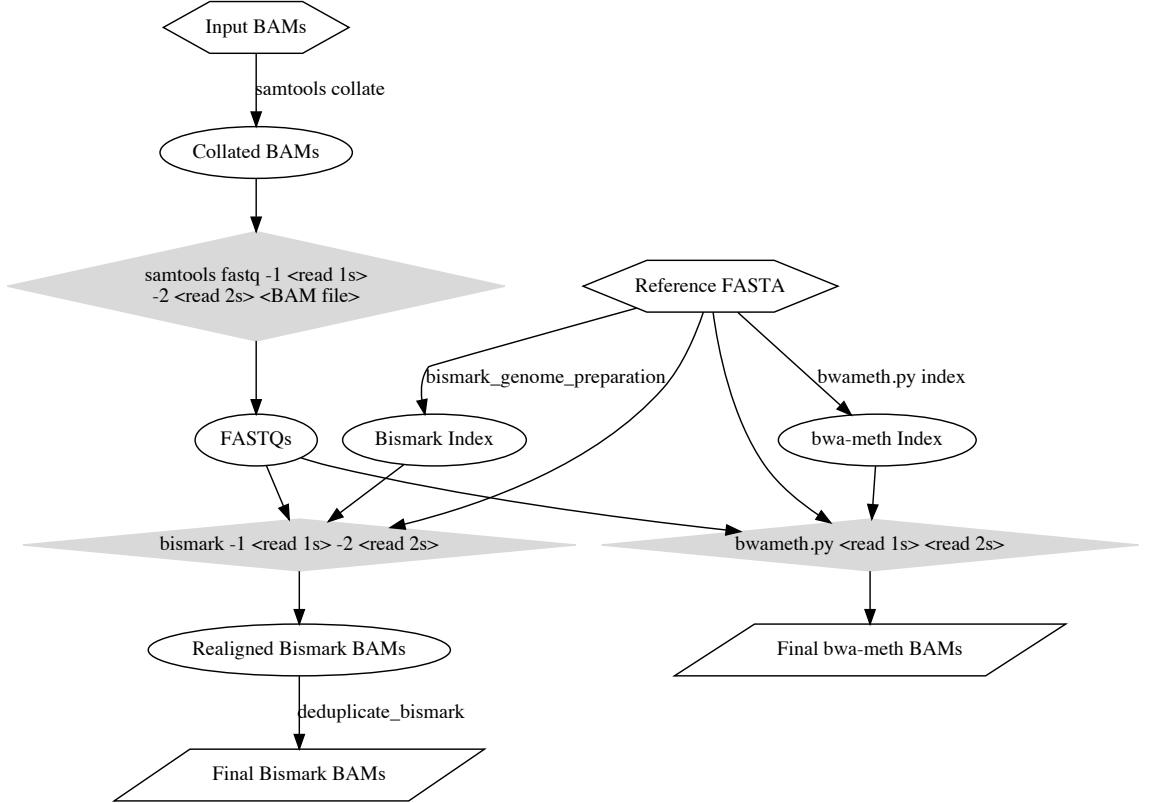


Figure 12: Detailed description of BAM realignment steps. Hexagons are input files. Ovals are intermediate files. Gray rhombuses are processing steps with multiple arguments. Parallelograms are output files. Labels on edges are processing steps with one input and one output.

MethylDackel filtering settings present in all output files containing methylation calls or counts of such calls in ClinVar regions (using the same custom Python script).

To compare the need for and the effect of mappability filtering on the more complete T2T v1.1 reference genome [22] (hereafter referred to as “T2T”), the analysis was re-run using the T2T reference in place of GRCh38 (supplemented with phage T4, phage lambda, phage Xp12, and pUC19 just as with GRCh38). To use the ClinVar and GENCODE datasets, which were respectively originally obtained as VCF and GFF files containing genomic coordinates in GRCh38, with the T2T reference, the positions were transferred over to the T2T reference using CrossMap [29]. The lift-over was done in two steps with separate chain files. First, the GRCh38 regions were lifted over using CrossMap to T2Tv1.0 using a chain file published on GitHub by Nico Alavi [4]. Then, the lifted-

over T2Tv1.0 annotations were lifted over again to T2Tv1.1 using a chain file from the T2T Consortium [2]. Specifically, the ClinVar regions BED, with the ClinVar and GENCODE data already combined, was lifted over. This converted file was used in place of the ClinVar regions BED for analysis with the T2T reference. As there was no Bismap mappability file for the T2T reference, we downloaded the Bismap tool and generated a k=100 multi-read Bismap mappability dataset for this reference genome. In total, this analysis was run on all combinations of sequencing method (WGBS or EM-seq), aligner (Bismark or bwa-meth), input mass (10, 50, or 200 ng), filtering (read-based filtering or no read-based filtering), and reference genome (GRCh38 or T2T), using a Nextflow[8] pipeline (see supplemental materials ) which managed the execution of all the analysis tools needed.

## Discussion

Reads placed with falsely high confidence have cascading detrimental effects on methylation calling, differential methylation assessment, and assement of phenotypes associated with methylation status. Because of the more accurate MapQ values, decreased run time, and more flexibility to separate methylation calling from alignment, we recommend the use of bwa-meth for alignment and MethylDackel with MapQ > 10 for methylation calling. To further improve accuracy of methylation assessment, reads with both mates in low mappability regions should be excluded.

<waiting on results>

## Supplemental Materials

The Nextflow scripts used to analyze this data can be found at [https://github.com/nebiolabs/low\\_bismap\\_methyl\\_calls](https://github.com/nebiolabs/low_bismap_methyl_calls) . Individual tools used in the analysis include sambamba[27], vcf2bed, gff2bed, bigWigToBedGraph, MethylDackel, awk, python, GNU sort, GNU head, bedtools, and BEDOPS. Versions of all tools used are specified in the nextflow 21.04.0 scripts using conda[3] dependency resolution.

The custom Python script that adds the input mass, sequencing protocol, MethylDackel filtering setting, and aligner name can be found at [https://github.com/nebiolabs/low\\_bismap\\_methyl\\_calls](https://github.com/nebiolabs/low_bismap_methyl_calls).

The pull request for the patch adding mappability support to MethylDackel can be found at <https://github.com/dpryan79/MethylDackel/pull/80>. It was merged into MethylDackel in version 0.5.0.

The Bismap file used for this analysis was downloaded from <https://www.pmggenomics.ca/hoffmanlab/proj/bismap/trackhub/hg38/k100.Bismap.MultiTrackMappability.bw>

The ClinVar VCF was the July 22, 2019 version of ClinVar's variants VCF, with a file name of clinvar\_20190722.vcf.gz

## References

- [1] QC Fail Sequencing » MAPQ values are really useful but their implementation is a mess.
- [2] marbl/CHM13, July 2021. original-date: 2019-02-28T16:00:16Z.
- [3] Miniconda, April 2021.
- [4] Nico Alavi. burgshrimps/liftover\_t2t, May 2021. original-date: 2020-11-03T21:50:35Z.
- [5] Achim Breiling and Frank Lyko. Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. *Epigenetics & Chromatin*, 8:24, 2015.
- [6] Gary G. Chen, Jeffrey A. Gross, Pierre-Eric Lutz, Kathryn Vaillancourt, Gilles Maussion, Alexandre Bramouille, Jean-François Théroux, Elena S. Gardini, Ulrike Ehlert, Geneviève Bourret, Aurélie Masurel, Pierre Lepage, Naguib Mechawar, Gustavo Turecki, and Carl Ernst. Medium throughput bisulfite sequencing for accurate detection of 5-methylcytosine and 5-hydroxymethylcytosine. *BMC Genomics*, 18:96, Jan 2017. 28100169[pmid].
- [7] Deanna M. Church, Valerie A. Schneider, Tina Graves, Katherine Auger, Fiona Cunningham, Nathan Bouk, Hsiu-Chuan Chen, Richa Agarwala, William M. McLaren, Graham R. S. Ritchie, Derek Albracht, Milinn Kremitzki, Susan Rock, Holland Kotkiewicz, Colin Kremitzki, Aye Wollam, Lee Trani, Lucinda Fulton, Robert Fulton, Lucy Matthews, Siobhan Whitehead, Will Chow, James Torrance, Matthew Dunn, Glenn Harden, Glen Threadgold, Jonathan Wood, Joanna Collins, Paul Heath, Guy Griffiths, Sarah Pelan, Darren Grahams, Evan E. Eichler, George Weinstock, Elaine R. Mardis, Richard K. Wilson, Kerstin Howe, Paul Flicek, and Tim Hubbard. Modernizing reference genome assemblies. *PLoS biology*, 9(7):e1001091, July 2011.
- [8] Paolo Di Tommaso, Maria Chatzou, Evan W. Floden, Pablo Prieto Barja, Emilio Palumbo, and Cedric Notredame. Nextflow enables reproducible computational workflows. *Nature Biotechnology*, 35(4):316–319, April 2017. Bandiera\_abtest: a Cg\_type: Nature Research Journals Number: 4 Primary\_atype: Correspondence Publisher: Nature Publishing Group Subject\_term: Computational biology and bioinformatics;Data publication and archiving Subject\_term\_id: computational-biology-and-bioinformatics;data-publication-and-archiving.
- [9] Francine E. Garrett-Bakelman, Caroline K. Sheridan, Thadeous J. Kacmarczyk, Jennifer Ishii, Doron Betel, Alicia Alonso, Christopher E. Mason, Maria E. Figueroa, and Ari M. Melnick. Enhanced reduced representation bisulfite sequencing for assessment of dna methylation at base pair resolution. *J Vis Exp*, (96):52246, Feb 2015. 25742437[pmid].

- [10] The SAM/BAM Format Specification Working Group. Sequence Alignment/Map Format Specification, May 2018. <https://samtools.github.io/hts-specs/SAMv1.pdf>.
- [11] Jennifer Harrow, Adam Frankish, Jose M. Gonzalez, Electra Tapanari, Mark Diekhans, Felix Kokocinski, Bronwen L. Aken, Daniel Barrell, Amonida Zadissa, Stephen Searle, If Barnes, Alexandra Bignell, Veronika Boychenko, Toby Hunt, Mike Kay, Gaurab Mukherjee, Jeena Rajan, Gloria Despacio-Reyes, Gary Saunders, Charles Steward, Rachel Harte, Michael Lin, Cédric Howald, Andrea Tanzer, Thomas Derrien, Jacqueline Chrast, Nathalie Walters, Suganthi Balasubramanian, Baikang Pei, Michael Tress, Jose Manuel Rodriguez, Iakes Ezkurdia, Jeltje van Baren, Michael Brent, David Haussler, Manolis Kellis, Alfonso Valencia, Alexandre Reymond, Mark Gerstein, Roderic Guigó, and Tim J. Hubbard. Gencode: The reference human genome annotation for the encode project. *Genome Res*, 22(9):1760–1774, Sep 2012. 22955987[pmid].
- [12] Illumina. Novaseq 6000. <https://www.illumina.com/systems/sequencing-platforms/novaseq/specifications.html>.
- [13] Johnnrbanggenome. Biofinysics: How does bowtie2 assign MAPQ scores?, May 2014.
- [14] Mehran Karimzadeh, Carl Ernst, Anshul Kundaje, and Michael M. Hoffman. Umap and bismap: quantifying genome and methylome mappability. *Nucleic Acids Research*, 2018.
- [15] W. J. Kent, A. S. Zweig, G. Barber, A. S. Hinrichs, and D. Karolchik. Bigwig and bigbed: enabling browsing of large distributed datasets. *Bioinformatics*, 26(17):2204–2207, 2010. <http://genome.ucsc.edu/>.
- [16] Felix Krueger. FelixKrueger/Bismark, July 2021. original-date: 2015-11-07T18:14:13Z.
- [17] Felix Krueger and Simon R. Andrews. Bismark: a flexible aligner and methylation caller for bisulfite-seq applications. *Bioinformatics*, 27(11):1571–1572, 2011.
- [18] Melissa J Landrum, Jennifer M Lee, Mark Benson, Garth R Brown, Chen Chao, Shanmuga Chitipiralla, Baoshan Gu, Jennifer Hart, Douglas Hoffman, Wonhee Jang, Karen Karapetyan, Kenneth Katz, Chunlei Liu, Zenith Maddipatla, Adriana Malheiro, Kurt McDaniel, Michael Ovetsky, George Riley, George Zhou, J Bradley Holmes, Brandi L Kattman, and Donna R Maglott. Clinvar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Research*, 46(D1):D1062–D1067, 2018.
- [19] Yan-Jiun Lee and Peter R. Weigle. Detection of Modified Bases in Bacteriophage Genomic DNA. In Alexey Ruzov and Martin Gering, editors, *DNA Modifications: Methods and Protocols*, Methods in Molecular Biology, pages 53–66. Springer US, New York, NY, 2021.

- [20] Heng Li, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, and 1000 Genome Project Data Processing Subgroup. The sequence alignment/map format and samtools. *Bioinformatics*, 25(16):2078–2079, 2009.
- [21] Shane Neph, M. Scott Kuehn, Alex P. Reynolds, Eric Haugen, Robert E. Thurman, Audra K. Johnson, Eric Rynes, Matthew T. Maurano, Jeff Vierstra, Sean Thomas, Richard Sandstrom, Richard Humbert, and John A. Stamatoyannopoulos. Bedops: high-performance genomic feature operations. *Bioinformatics*, 28(14):1919–1920, 2012.
- [22] Sergey Nurk, Sergey Koren, Arang Rhie, Mikko Rautiainen, Andrey V. Bzikadze, Alla Mikheenko, Mitchell R. Vollger, Nicolas Altemose, Lev Uralsky, Ariel Gershman, Sergey Aganezov, Savannah J. Hoyt, Mark Diekhans, Glennis A. Logsdon, Michael Alonge, Stylianos E. Antonarakis, Matthew Borchers, Gerard G. Bouffard, Shelise Y. Brooks, Gina V. Caldas, Haoyu Cheng, Chen-Shan Chin, William Chow, Leonardo G. de Lima, Philip C. Dishuck, Richard Durbin, Tatiana Dvorkina, Ian T. Fiddes, Giulio Formenti, Robert S. Fulton, Arkarachai Fungtammasan, Erik Garrison, Patrick G. S. Grady, Tina A. Graves-Lindsay, Ira M. Hall, Nancy F. Hansen, Gabrielle A. Hartley, Marina Haukness, Kerstin Howe, Michael W. Hunkapiller, Chirag Jain, Miten Jain, Erich D. Jarvis, Peter Kerpeljiev, Melanie Kirsche, Mikhail Kolmogorov, Jonas Korlach, Milinn Kremitzki, Heng Li, Valerie V. Maduro, Tobias Marschall, Ann M. McCartney, Jennifer McDaniel, Danny E. Miller, James C. Mullikin, Eugene W. Myers, Nathan D. Olson, Benedict Paten, Paul Peluso, Pavel A. Pevzner, David Porubsky, Tamara Potapova, Evgeny I. Rogaev, Jeffrey A. Rosenfeld, Steven L. Salzberg, Valerie A. Schneider, Fritz J. Sedlazeck, Kishwar Shafin, Colin J. Shew, Alaina Shumate, Yumi Sims, Arian F. A. Smit, Daniela C. Soto, Ivan SoviÄ, Jessica M. Storer, Aaron Streets, Beth A. Sullivan, FranÃ§oise Thibaud-Nissen, James Torrance, Justin Wagner, Brian P. Walenz, Aaron Wenger, Jonathan M. D. Wood, Chunlin Xiao, Stephanie M. Yan, Alice C. Young, Samantha Zarate, Urvashi Surti, Rajiv C. McCoy, Megan Y. Dennis, Ivan A. Alexandrov, Jennifer L. Gertron, Rachel J. O'Neill, Winston Timp, Justin M. Zook, Michael C. Schatz, Evan E. Eichler, Karen H. Miga, and Adam M. Phillippy. The complete sequence of a human genome. *bioRxiv*, page 2021.05.26.445798, May 2021. Publisher: Cold Spring Harbor Laboratory Section: New Results.
- [23] B. S. Pedersen, K. Eyring, S. De, I. V. Yang, and D. A. Schwartz. Fast and accurate alignment of long bisulfite-seq reads. *ArXiv e-prints*, January 2014.
- [24] Aaron R. Quinlan and Ira M. Hall. Bedtools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, 26(6):841–842, 2010.
- [25] Devon Ryan. MethylDackel. <https://github.com/dpryan79/MethylDackel>.

- [26] Dirk Schübeler. Function and information content of dna methylation. *Nature*, 517:321 EP –, Jan 2015.
- [27] Artem Tarasov, Albert J. Vilella, Edwin Cuppen, Isaac J. Nijman, and Pjotr Prins. Sambamba: fast processing of ngs alignment formats. *Bioinformatics*, 31(12):2032–2034, 2015.
- [28] Romualdas Vaisvila, V. K. Chaithanya Ponnaluri, Zhiyi Sun, Bradley W. Langhorst, Lana Saleh, Shengxi Guan, Nan Dai, Matthew A. Campbell, Brittany S. Sexton, Katherine Marks, Mala Samaranayake, James C. Samuelson, Heidi E. Church, Esta Tamanaha, Ivan R. CorrÃ³a, Sriharsa Pradhan, Eileen T. Dimalanta, Thomas C. Evans, Louise Williams, and Theodore B. Davis. Enzymatic methyl sequencing detects DNA methylation at single-base resolution from picograms of DNA. *Genome Research*, June 2021. Company: Cold Spring Harbor Laboratory Press Distributor: Cold Spring Harbor Laboratory Press Institution: Cold Spring Harbor Laboratory Press Label: Cold Spring Harbor Laboratory Press Publisher: Cold Spring Harbor Lab.
- [29] Hao Zhao, Zhifu Sun, Jing Wang, Haojie Huang, Jean-Pierre Kocher, and Liguo Wang. CrossMap: a versatile tool for coordinate conversion between genome assemblies. *Bioinformatics (Oxford, England)*, 30(7):1006–1007, April 2014. Edition: 2013/12/18 Publisher: Oxford University Press.