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Methyl-MiniSeq Service Report:
Genome-Wide Bisulfite Sequencing

Workflow Checklist

Sample Received	✓
Sample Quality Check	✓
Sample Prepared for Sequencing	✓
Next-Gen Sequencing	✓
Sequence Quality Check	✓
Bioinformatics Analysis	✓
Data/Results	✓

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Materials & Methods

The samples were processed and analyzed using the Classic RRBS Service: Genome-wide bisulfite sequencing (Zymo Research, Irvine, CA).

DNA Extraction: If DNA extraction was performed, one of two different DNA extraction kits was used depending on the sample amount. The manufacturer's instructions were followed, unless otherwise stated. The kit used in this project is marked below.

☐ Quick-DNA Plus Kit Miniprep Kit (Zymo Research, Irvine, CA)

☐ Quick-DNA Plus Kit Microprep Kit (Zymo Research, Irvine, CA)

☒ N/A (DNA Extraction Not Performed)

Additional Notes: NA

Methyl-MiniSeq® Library preparation: 200 ng of starting input genomic DNA was sequentially digested with 60 units of TaqαI followed by 30 units of MspI (NEB) and then purified with Zymo Research DNA Clean & Concentrator™-5 (Cat#: D4003). Fragments were ligated to pre-annealed adapters containing 5'-methyl-cytosine instead of cytosine according to Illumina's specified guidelines. Adaptor-ligated fragments of 150-250 bp and 250-350 bp in size were recovered from a 2.5% NuSieve 1:1 agarose gel using Zymoclean™ Gel DNA Recovery Kit (Cat#: D4001). The fragments were then bisulfite-treated using the EZ DNA Methylation-Lightning™ Kit (Cat#: D5030). Preparative-scale PCR was performed and the resulting products were purified with DNA Clean & Concentrator™-5 (Cat#: D4003) for sequencing on an Illumina HiSeq.

Sequence alignments and data analysis: Sequence reads from bisulfite-treated Classic RRBS libraries were identified using standard Illumina base calling software and then raw FASTQ files were adapter, filled-in nucleotides, and quality trimmed using TrimGalore 0.6.4. FastQC 0.11.8 was used to assess the effect of trimming and overall quality distributions of the data. Alignment to the hg19 reference genome was performed using Bismark 0.19.0. Methylated and unmethylated read totals for each CpG site were called using MethylDackel 0.5.0. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Fisher's exact test was performed for groups without replicates(Tumor_2 vs NAT_2, Tumor_3a vs NAT_3a, Tumor_4 vs NAT_4) and T-test was performed for groups having replicates(Tumor vs NAT). These tests were performed on each CpG site that has at least five reads coverage. In order to identify significantly differential methylated cytosines, methylation value between groups has to be minimum 10% with a p-value (unadjusted) of < 0.05. Promoter, gene body, and CpG island annotations were added for each CpG included in the comparison where available.

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Sample Information

Sample ID	Original Sample Label	Total Read Number (M)	Mapping Efficiency (%)
in013_1	Tumor_4	17.763210	46.98%
in013_2	NAT_4	22.375362	41.51%
in013_3	Tumor_2	19.062438	44.74%
in013_4	NAT_2	18.124028	39.87%
in013_5	Tumor_3a	21.587385	53.46%
in013_6	NAT_3a	22.246479	47.33%

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Tables and Graphical Data Information

Terminology

Chr/pos/Site	Chromosomal coordinates for the site
Sample Name	DNA methylation ratio that's the measured number of methylated cytosine divided by total number of cytosines covered at that site
methdiff	short for methylation difference – the difference in methylation ratios between two samples at the specified site determined by subtracting the value for the second sample (to the right) from the value for the first sample (to the left)
pvalue	Quantification of the statistical significance of the methylation difference by either the Student's t-test, the Fisher's Exact test, or ANOVA depending on the analysis type
Padj_fdr	FDR-adjusted p-value of the test
Padj_slim	Slim-adjusted p-value of the test
Promoter/exon/intron	Gene annotation of a particular region
Cgi	A 'Y' indicates the region falls within a CpG island
Meth_cov	The total number of methylated cytosine covered at that site
Total_cov	The total number of cytosines covered at that site

Graphical Data Definition

Heatmaps with Hierarchical Clustering	Samples are grouped based on similarity for the top 3000 or all differentially methylated CpG sites covered in the assay. Red represents high levels of DNA methylation, and blue represents low levels of DNA methylation.
Coverage Plots	The charts show the sequencing coverage of CpG Sites in the samples
Violin Plots (Labelled "MethByAnno_...")	Display the overall methylation level of individual sample for each annotated genomic feature (CpG islands aka "cgi", gene bodies, promoters). A small circle inside the violin represents the median methylation ratio, the black bars extending from the circle represent the interquartile range (25^{th} and 75^{th} percentiles), and the whiskers extend out to 1.5x of the interquartile range. The width of the "violin" represents a smoothed probability density of CpG sites with a specific methylation ratio.

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Project Data

Comparison Results

Top 3000 Differential CpG Sites (XLSX format)

Tumor vs NAT
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_xlsx/Tumor_vs_NAT_top3000.xlsx

Tumor_2 vs NAT_2
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_xlsx/Tumor_2_vs_NAT_2_top3000.xlsx

Tumor_3a vs NAT_3a
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_xlsx/Tumor_3a_vs_NAT_3a_top3000.xlsx

Tumor_4 vs NAT_4
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_xlsx/Tumor_4_vs_NAT_4_top3000.xlsx

All Differential CpG Sites (CSV format)

Tumor vs NAT
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_csv/Tumor_vs_NAT.csv

Tumor_2 vs NAT_2
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_csv/Tumor_2_vs_NAT_2.csv

Tumor_3a vs NAT_3a
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_csv/Tumor_3a_vs_NAT_3a.csv

Tumor_4 vs NAT_4
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/diff_csv/Tumor_4_vs_NAT_4.csv

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Project Data (Cont.)

Heatmaps (show top 3000 differential sites)

Tumor vs NAT
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/Tumor_vs_NAT.png

Tumor_2 vs NAT_2
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/Tumor_2_vs_NAT_2.png

Tumor_3a vs NAT_3a
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/Tumor_3a_vs_NAT_3a.png

Tumor_4 vs NAT_4
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/Tumor_4_vs_NAT_4.png

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Project Data (Cont.)

Global Plots

Read Coverage Distributions

https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/Coverage.pdf

Global Methylation Distribution in Annotation Regions (Violin Plots)

CpG Island
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/MethByAnno_cgi.pdf

Gene Body
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/MethByAnno_genes.pdf

Promoter
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/output/MethByAnno_promoter.pdf

Raw Data

Trimmed FASTQ Files

List of Downloadable URLs:
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/download_fastq.txt

BAM Alignments

https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/download_bam.txt

Methylation Calling Tables

https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/download_cytosine_report.txt

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Genome Browser Tracks

Please see on the next pages detailed instructions for loading custom tracks in UCSC Genome Browser.

BigWig Group Averages

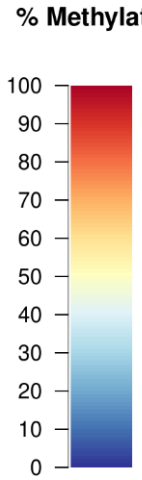
Track files were produced in BigWig format by taking the average of samples within each group which had a coverage ≥ 5 at each individual cytosine. A custom tracks file contains the URLs for each track file, which can be cut and pasted into the UCSC Genome Browser for viewing.

Download Custom Tracks File:
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/tracks/tracks_avg.txt

Genome Tracks for Individual Samples with Methylation Ratio and Coverage
Track files were generated in BigBed format. The tracks show each covered CpG site with coverage of ≥ 5 reads in the genome for each sample. Each CpG site is labeled with a fraction, which represents the number of methylated cytosines over total number of cytosines covered by sequencing reads at that site. A greater number of total reads at a cytosine increases confidence in the resulting methylation ratio. CpG sites are colored using a color scale (see below) matching the methylation ratio (the division of methylated reads over total reads for each CpG). The tracks can be viewed on UCSC Genome Browser using the same procedure as for the group average tracks above.

Download Custom Tracks File:
https://epiquest.s3.amazonaws.com/epiquest_in013/NM7wkUqufpLLDK45GKHJJK/CG/tracks/tracks_meth.txt

Color Scale representing CpG methylation level in genome tracks for individual samples:



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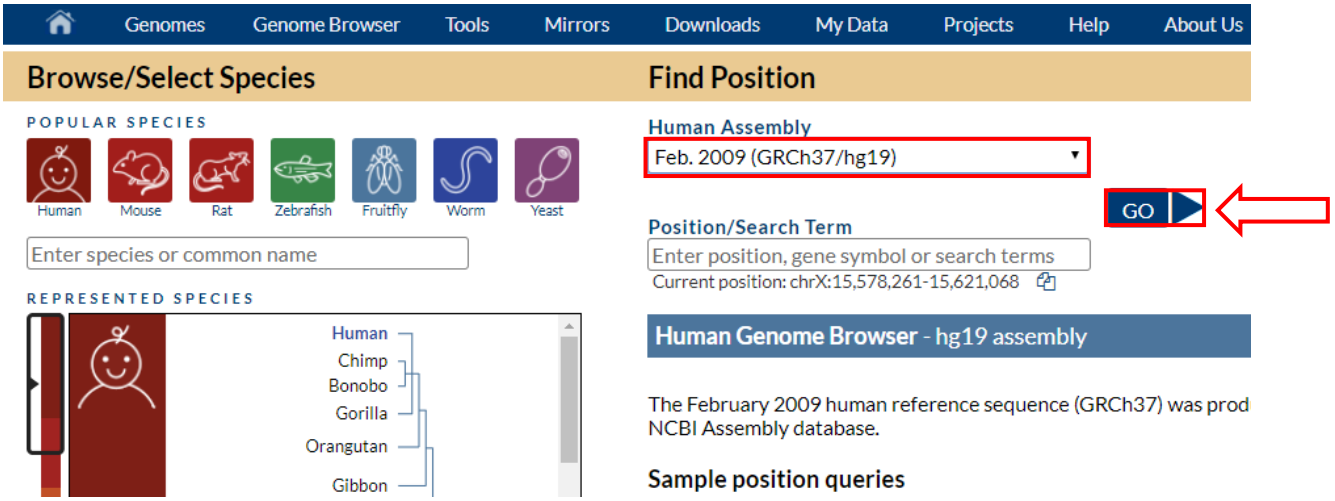
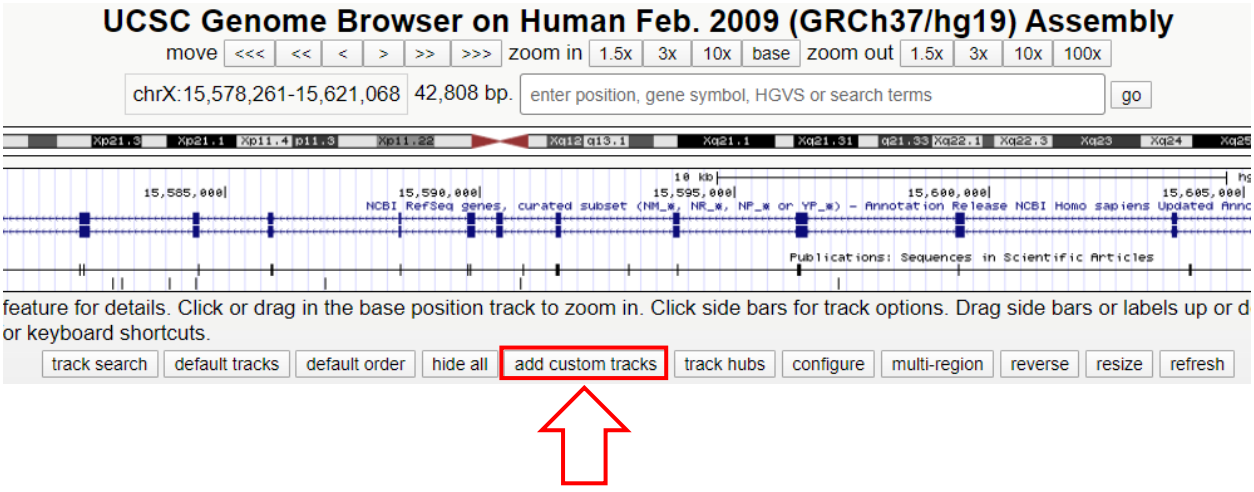
Genome Browser Tracks Visualization

To Visualize genome browser tracks on UCSC, please follow steps below:

1. Go to UCSC Genome Browser, select the **genome** and **assembly** specific to your samples and then click **add custom tracks**. Note: Current assembly used for human is "Feb. 2009 (GRCh37/hg19)"





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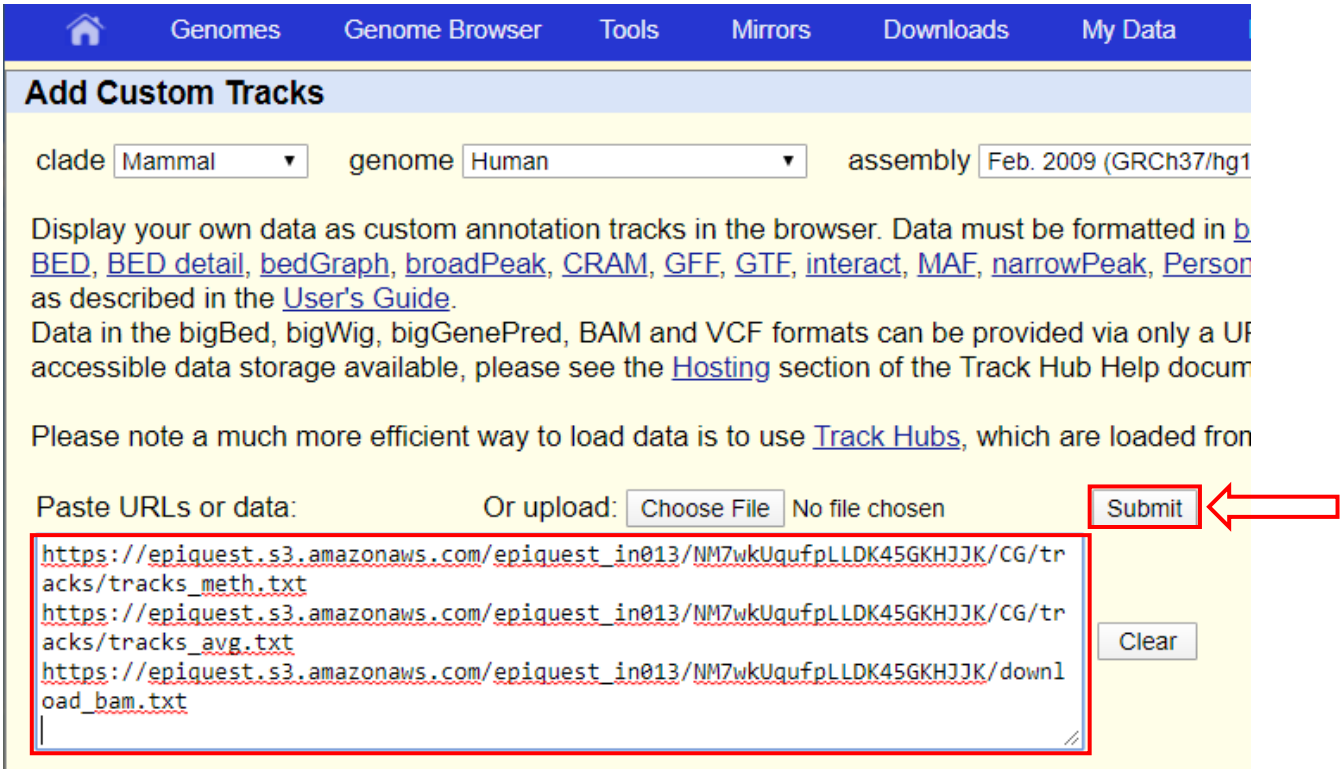
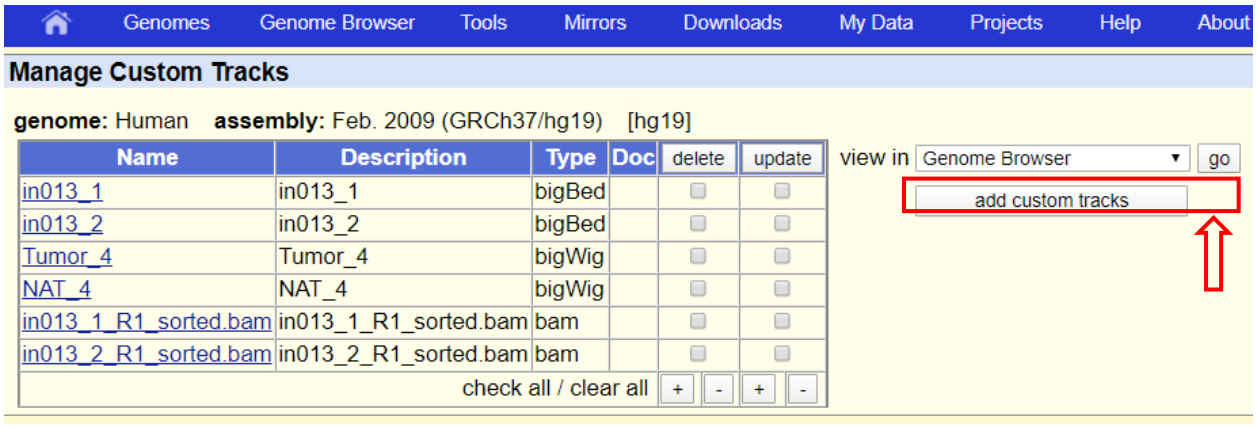
Genome Browser Tracks Visualization (Cont.)

2. Paste the links provided and then **Submit** to upload your browser track (this may take some time)



3. Click **go to genome browser** once all desired tracks are loaded and navigate the genome to look for areas of interest



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Genome Browser Tracks Visualization (Cont.)

Example of methylation tracks and sequence read tracks in the genome browser:

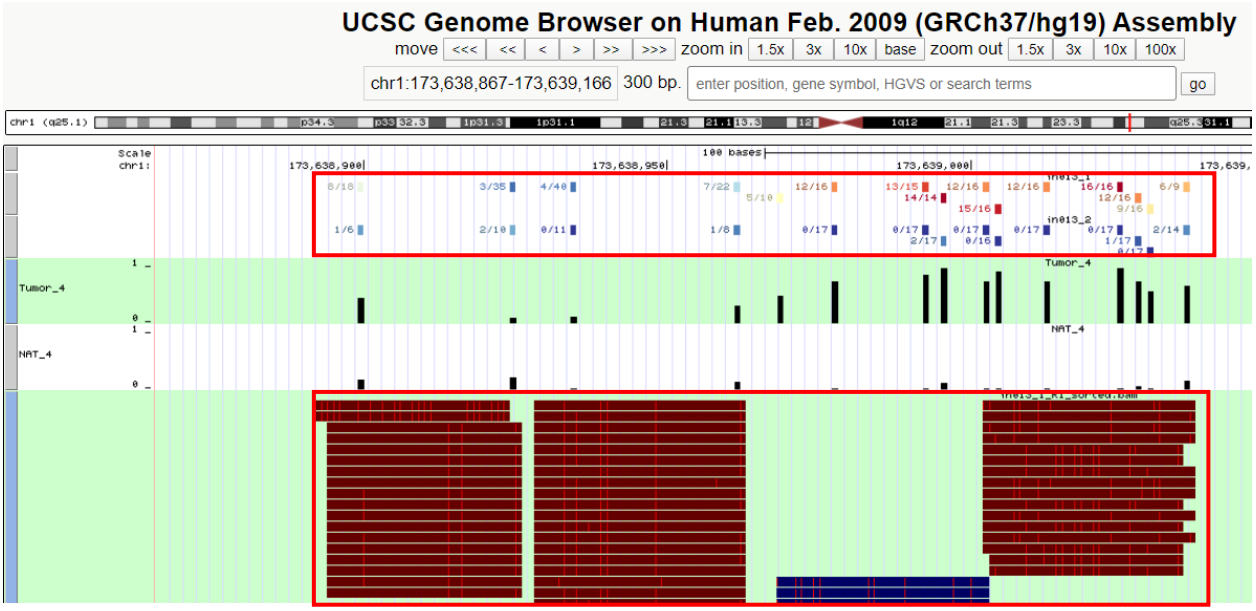
The **CpG Methylation** track:

1. The fraction denotes methylation ratio which is the number of methylated cytosines over total number of cytosines covered by sequencing reads at that site.
2. Red corresponds to higher methylation levels, while blue corresponds to lower methylation levels.
3. Methylation ratio is displayed next to each CpG site when display density is set to "pack" (as in the example above) or all the way to the left when display density is set to "full".

The **Read** track:

1. Shows the aligned reads with mismatches (unmethylated cytosines converted to thymines are shown as mismatches).
2. Blue reads align to the "+" strand and red reads align to the "-"strand.

The genome browser may have trouble loading or not be able to load when there are many custom tracks expanded and you are trying to navigate to different areas of the genome. If problems occur, try altering the display density of the tracks to "dense" or "squish" and then try again.



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