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TITLE:

A Rat Methyl-Seq Platform to Identify Epigenetic Changes Associated with Stress Exposure

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SUMMARY:

Here, we describe the protocol and implementation of Methyl-Seq, an epigenomic platform, using a rat model to identify epigenetic changes associated with chronic stress exposure. Results demonstrate that the rat Methyl-Seq platform is capable of detecting methylation differences that arise from stress exposure in rats.

ABSTRACT:

As genomes of a wider variety of animals become available, there is an increasing need for tools that can capture dynamic epigenetic changes in these animal models. The rat is one particular model animal where an epigenetic tool can complement many pharmacological and behavioral studies to provide insightful mechanistic information. To this end, we adapted the SureSelect Target Capture System (referred to as Methyl-Seq) for the rat, which can assess DNA methylation levels across the rat genome. The rat design targeted promoters, CpG islands, island shores, and GC-rich regions from all RefSeq genes.

To implement the platform on a rat experiment, male Sprague Dawley rats were exposed to chronic variable stress for 3 weeks, after which blood samples were collected for genomic DNA extraction. Methyl-Seq libraries were constructed from the rat DNA samples by shearing, adapter

ligation, target enrichment, bisulfite conversion, and multiplexing. Libraries were sequenced on a next-generation sequencing platform and the sequenced reads were analyzed to identify DMRs between DNA of stressed and unstressed rats. Top candidate DMRs were independently validated by bisulfite pyrosequencing to confirm the robustness of the platform.

Results demonstrate that the rat Methyl-Seq platform is a useful epigenetic tool that can capture methylation changes induced by exposure to stress.

INTRODUCTION:

Advances in high-throughput sequencing have led to a wealth of genomic sequences for both model and non-model organisms. The availability of such sequences has facilitated research in genetics, comparative genomics, and transcriptomics. For instance, available genomic sequences are highly useful for aligning sequencing data from ChIP-Seq experiments that enrich DNA based on its association with histone modifications[1], or bisulfite sequencing, which measures DNA methylation by detecting uracil formed from bisulfite conversion of unmethylated cytosines[2]. However, there have been delays in the implementation of epigenomic platforms that incorporate available genomic sequencing data in their design due to a lack of annotated data of species-specific regulatory sequences that can influence gene function.

In particular, DNA methylation is one of the most widely studied epigenetic modifications on DNA that can leverage available genomic data for building a methylomic platform. One such example is an array-based platform for the human methylome[3], which has been widely used in various disciplines from oncology to psychiatry[4,5]. Unfortunately, similar platforms for non-human animal models are scarce, as there are virtually no widely-used platforms that have taken advantage of the genomic sequence in their initial design.

A common method to assess the methylomic landscape of non-human animal models is reduced representation bisulfite sequencing (RRBS)[6]. This approach overcomes the cost of whole-genome bisulfite sequencing that, while providing a comprehensive methylomic landscape, provides lower read-depth coverage due to cost and limited functional information in large gene-poor areas of the genome[2]. RRBS involves restriction digest and size-selection of genomic DNA to enrich for highly GC-rich sequences such as CpG islands that are commonly found near gene promoters and thought to play a role in gene regulation[7]. While the RRBS method has been used in a number of important studies, its reliance on restriction enzymes is not without notable challenges and limitations. For instance, enrichment of GC-rich sequences in RRBS is entirely dependent on the presence of specific sequences recognized by the restriction enzyme and subsequent size selection by electrophoresis. This means that any genomic areas that do not contain these restriction sites are excluded during size selection. Also, cross-species comparisons are challenging unless the same restriction sites are present in the same loci among the different species.

One approach to overcoming the limitations of RRBS is to use an enrichment method that takes advantage of the published genomic sequence in the design of the platform. The array-based human platform uses primer probes designed against specific CpGs for allele-specific (CG vs. TG

after bisulfite conversion) target annealing and primer extension. Its design reflects not only the available human genomic sequence, but experimentally-verified regulatory regions acquired from multiple lines of inquiry, such as ENCODE and ENSEMBL[8]. Despite its wide use in human methylomic investigations, a similar platform does not exist for model animals. In addition, the array-based format places significant constraint on the surface area available for probe placement. In the past several years, efforts have been made to combine the target-specificity afforded by capture probe design and the high-throughput feature of next-generation sequencing. Such an endeavor has resulted in the sequencing-based target enrichment system for the mouse genome (mouse Methyl-Seq), which was used to identify brain-specific or glucocorticoid-induced differences in methylation[9,10]. Similar platforms for other model and non-model animals are needed to facilitate epigenomic research in these animals.

Here, we demonstrate the implementation of this novel platform to conduct methylomic analysis on the rat. The rat has served as an important animal model in pharmacology, metabolism, neuroendocrinology, and behavior. For example, there is an increasing need to understand the underlying mechanisms that give rise to drug toxicity, obesity, stress response, or drug addiction. A high-throughput platform capable of capturing methylomic changes associated with these conditions would increase our understanding of the mechanisms. Since the rat genome still lacks annotation for regulatory regions, we incorporated non-redundant promoters, CpG islands, island shores[11], and previously identified GC-rich sequences into the rat Methyl-Seq platform[12].

To assess successful design and implementation of the SureSelect Target Enrichment (generically referred to as Methyl-Seq) platform for the rat genome, we employed a rat model of chronic variable stress (CVS)[13] to identify differentially methylated regions between unstressed and stressed animals. Our platform design, protocol, and implementation may be useful for investigators who may want to conduct a comprehensive and unbiased epigenetic investigation on an organism whose genomic sequence is already available but remains poorly annotated.

PROTOCOL:

All experiments were completed in accordance and compliance with all relevant regulatory and institutional guidelines, including the Institutional Animal Care and Use Committee at the Johns Hopkins School of Medicine.

1. Animals

1.1. Obtain male adolescent Sprague-Dawley rats at 4 weeks of age. House the animals in polycarbonate rat cages in a temperature-and humidity-controlled room on a 12 h light, 12 h dark cycle with light onset at 0600 h. Provide the animals with *ad libitum* access to water.

1.2. Allow rats to acclimate for 1 week to reduce stress associated with transportation. Pair-house the animals (N=16) to preclude isolation stress, and at 5 weeks of age, begin the chronic variable stress (CVS) regimen for 3 weeks.

2. Chronic Variable Stress

2.1. Administer the CVS regimen once in the morning (9-11 AM) and once in the afternoon (1-3 PM) at irregular times to keep the routine unpredictable. Incorporate overnight mild stressors. The CVS regimen includes: 1) 3 h in a restraint cylinder; 2) 10 min swim; 3) 3 h cage tilt 4) 1 h slow shaking platform; and 5) 1 h in the 4 °C cold room.

Note: Overnight stressors include social crowding (5 per cage), social isolation, wet bedding, food restriction, and lights-on. A typical weekly schedule of the stress regimen is provided in **Table 1**.

3. Endocrine Assays

3.1. Determine levels of corticosterone (CORT) using tail blood (~50 μL) samplings collected at the same time (9 AM) twice per week throughout the experiment, prior to CVS regimen to establish baseline hormone levels (Day 0), once during the middle of the weekly CVS (Days 4,11, and 18), after every 7 days of CVS (Days 7 and 14), and at the conclusion of CVS (Day 21). Collect blood samples prior to the daily stress regimen.

3.1.1. Collect one final trunk blood sample during euthanasia (Day 25) for RIA and genomic DNA extraction.

3.2. Centrifuge all blood samples (600 x g, 4 °C, 10 min) to separate the plasma from the blood cells. Pipet out the plasma (supernatant) and store the samples at -80 °C.

3.3. Thaw and use the plasma to determine CORT levels by radioimmunoassay (RIA). Ensure that the 3-week plasma CORT levels are elevated in the stressed animals to verify the robustness of the stress regimen.

4. Behavior

4.1. After the CVS regimen (Days 23-24), assess each animal for anxiety-like behavior on the elevated plus maze (EPM)[14].

4.2. Using a video camera, record the animals on the EPM apparatus for 300 s and score the time spent in the center, closed arms, and open arms.

5. Design of the Rat Methyl-Seq

5.1. Using the UCSC Genome Browser, obtain non-redundant genomic coordinates (rat Nov 2004 rn4 assembly) for CpG islands and island shores (± 1 kb flanking CpG islands), promoters (± 1 kb of each TSS) of each RefSeq gene, and other sequences that may be available from relevant literature.

Note: For the rat Methyl-Seq, additional GC-rich sequences from a previous array-based

methylation platform was added[12]. For regions greater than 5 kbps, alternating regions of 500 bps were sampled followed by 1 kbps that were skipped. The final rat Methyl-Seq design consists of 111 Mbps, 2.3 million CpGs; and an average region size of 594 bps. It targets 228,800 unique loci.

5.2. Enter a compiled list of genomic coordinates into a commercially-available target capture design software for appropriate probe design.

6. Construction of the Rat Methyl-Seq Library from Genomic DNA

Note**:** To eliminate batch effects, process multiple samples at the same time, and scale up the master mixes accordingly. Extract DNA using a commercially available DNA extraction kit. Column- or precipitation-based methods both yield high-quality genomic DNA (260/280 ratio ~1.8). Use of phenol-based methods are not recommended. Elute or resuspend DNA in Low TE buffer (10 mM TE, 0.1 mM EDTA, pH 8.0).

6.1. Sample Preparation

Note: For every step using DNA-binding magnetic beads, make sure the beads are acclimated to room temperature for at least 30 min and well mixed before use.

6.1.1. Shear DNA

6.1.1.1. Use a fluorometer to determine initial double-stranded DNA concentration of each sample. Dilute >1 µg of gDNA to 50 µL with Low TE buffer (10 mM TE, 0.1 mM EDTA, pH 8.0) in low DNA-binding microcentrifuge tubes.

6.1.1.2. Shear samples using an isothermal sonicator (10% Duty Cycle, 5 Intensity, 200 Cycles per Burst, 6 cycles of 60 s, Frequency sweeping, 4 °C).

6.1.1.3. Assess quality of DNA using an electrophoresis-based system that measures DNA size and quantity.

Note: The DNA amount recommended is 1 µg, or 3 µg. If there is limited starting material, the lowest input amount should be >500 ng, as lower amounts will adversely affect the quantity and quality of the libraries generated.

6.1.2. Repair DNA ends.

6.1.2.1. Use the rat Methyl-Seq kit to prepare the End-repair Master Mix on ice. Add 52 µL of mix to each sample and incubate in a thermal cycler without a heated lid (20 °C for 30 min, 4 °C hold).

End-repair Master Mix (per sample):
35.2 µL of Water

10 µL of End Repair Buffer (10x)
1.6 µL of dNTP Mix
1 µL of T4 DNA Polymerase
2 µL of Klenow DNA Polymerase
2.2 µL of T4 Polynucleotide Kinase

6.1.2.2. Purify samples using 180 µL of DNA-binding magnetic beads and 400 µL of freshly prepared 70% ethanol per sample. Add 180 µL of beads to each sample and incubate for 5 min at room temperature. Pellet beads, remove supernatant and resuspend pellet in 200 µL of 70% ethanol. Remove ethanol and repeat wash once.

6.1.2.3. Use a magnetic plate to pellet beads and remove as much ethanol as possible. Dry in a 37 °C heatblock for 3 - 5 min until the bead pellet is completely dry. Resuspend in 44 µL of nuclease-free water and collect approximately 42 µL of supernatant.

Stopping Point: After repairing DNA ends, samples may be sealed and stored at -20 °C.

6.1.3. Adenylate the 3’ ends.

6.1.3.1. Prepare Adenylation Master Mix on ice. Add 9 µL mix to each sample and incubate in a thermal cycler without a heated lid (37 °C for 30 min, 4 °C hold).

Adenylation Master Mix (per sample):
5 µL of Klenow buffer
1 µL of dATP
3 µL of Klenow DNA Polymerase

6.1.3.2. Purify samples using 90 µL of DNA-binding magnetic beads and 400 µL of freshly prepared 70% ethanol per sample. Add 90 µL of beads to each sample and incubate for 5 min at room temperature. Pellet beads, remove supernatant and resuspend pellet in 200 µL of 70% ethanol. Remove ethanol and repeat wash once.

6.1.3.3. Use a magnetic plate to pellet beads and remove as much ethanol as possible. Dry in a 37 °C heatblock for 3 - 5 min until the bead pellet is completely dry. Resuspend in 35 µL of nuclease-free water and collect approximately 33.5 µL of supernatant.

6.1.4. Ligate the methylated adapter.

6.1.4.1. Prepare Ligation Master Mix on ice and add 16.5 µL of mix to each sample. Incubate in a thermal cycler without a heated lid (20 °C for 15 min, 4 °C hold).

Ligation Master Mix (per sample):
2.5 µL of Water
2.5 µL of Methyl-Seq Methylated Adapter

10 µL of T4 DNA Ligase Buffer (5x)
1.5 µL of T4 DNA Ligase

6.1.4.2. Purify samples using 90 µL of DNA-binding magnetic beads and 400 µL of freshly prepared 70% ethanol per sample. Add 90 µL of beads to each sample and incubate for 5 min at room temperature. Pellet beads, remove supernatant, and resuspend pellet in 200 µL of 70% ethanol. Remove ethanol and repeat wash once.

6.1.4.3. Use a magnetic plate to pellet beads and remove as much ethanol as possible. Dry in a 37 °C heatblock for 3 - 5 min until the bead pellet is completely dry. Resuspend in 22 µL of nuclease-free water and collect approximately 22 µL of supernatant. Assess quality using a bioanalyzer.

Note: If the total amount of DNA is less than 500 ng, shear and process additional DNA prior to proceeding with the subsequent steps. If the average DNA size does not increase by more than 30 bps, check to ensure that the reagents are new, as T4 DNA polymerase, Klenow, and/or T4 ligase may be old.

Stopping Point: After ligating methylated adapter, samples may be sealed and stored at -20 °C.

6.2. Hybridization

6.2.1. Transfer samples to low DNA-binding microcentrifuge tubes and use a heated vacuum concentrator to reduce sample volume to less than 3.4 µL. Reconstitute samples to 3.4 µL.

Note: Concentrate the samples to approximately ~3 µL to ensure samples are removed from vacuum concentrator before all liquid evaporates.

6.2.2. Prepare hybridization buffer at room temperature and Methyl-Seq Block Mix on ice. Add 5.6 µL of Methyl-Seq Block Mix to each sample and incubate in thermal cycler (95 °C for 5 min, 65 °C for 2 min, 65 °C hold).

Hybridization Buffer (per sample):
6.63 µL of Methyl-Seq Hyb 1
0.27 µL of Methyl-Seq Hyb 2
2.65 µL of Methyl-Seq Hyb 3
3.45 µL of Methyl-Seq Hyb 4

Methyl-Seq Block Mix (per sample):
2.5 µL of Methyl-Seq Indexing Block 1
2.5 µL of Methyl-Seq Block 2
0.6 µL of Methyl-Seq Block 3

6.2.3. Prepare RNase Block Mix and the Capture Library hybridization mix. Add 20 µL of Capture

Library Hybridization Mix to each sample and incubate at 65 °C for at least 16 h.

RNase Block Mix (per sample):
0.5 µL of RNase Block
1.5 µL of Water

Capture Library Hybridization Mix (per sample):
13 µL of Hybridization Buffer
2 µL of RNase Block Mix
5 µL of Rat Methyl-Seq Capture Library

Note: **Keep reactions at 65 °C** when adding Hybridization Mix to prevent non-specific binding.

6.2.4. Aliquot 50 µL of streptavidin magnetic beads per sample into a new 8-well strip tube. Wash beads with 200 µL of Methyl-Seq Binding Buffer. Use magnetic plate to pellet beads and remove supernatant between each wash for a total of 3 washes. After the final wash, resuspend streptavidin beads in 200 µL of Methyl-Seq Binding Buffer.

6.2.5. Add samples to 200 µL of washed streptavidin magnetic beads and incubate at room temperature for 30 min using a rotating mixer. While mixing, aliquot 200 µL of Methyl-Seq Wash Buffer 2 into triplicate wells of a 96-well plate per sample and place in a thermal cycler to pre-warm to 65 °C.

6.2.6. After incubation, pellet streptavidin magnetic beads using magnetic plate and resuspend the beads in 200 µL Methyl-Seq Wash Buffer 1. Incubate for 15 min at room temperature. Use a magnetic plate to pellet and discard supernatant.

6.2.7. Wash beads 3 times with Methyl-Seq Wash Buffer 2: resuspend bead pellet in 200 µL of Wash Buffer 2 (pre-warmed in step 6.2.5.), incubate beads in thermal cycler (65 °C, 10 min), and pellet beads. Discard supernatant after each wash using a magnetic plate.

Note: Maintain hybridization reactions at 65 °C when adding Wash Buffer 2 to prevent non-specific binding.

6.2.8. Add 20 µL of Methyl-Seq Elution Buffer to the washed beads and incubate at room temperature for 20 min. Use a magnetic plate to pellet beads and transfer supernatant to a new strip tube. Discard the beads.

Note: While incubating, prepare bisulfite conversion reagent.

6.3. Bisulfite Conversion

Note: Perform bisulfite conversion of the eluted ssDNA using appropriate reagents and instructions from a commercially-available bisulfite conversion kit.

6.3.1. Add 130 µL prepared bisulfite conversion reagent to supernatant from previous step. Divide each of the 150 µL reactions equally into two wells. Incubate in a thermal cycler (64 °C for 2.5 h, 4 °C hold).

Note: The 150 µL reaction is divided equally into two separate wells to ensure homogenous temperature. After incubating for 2.5 h, immediately proceed to the next step.

6.3.2. Bind samples to spin columns by adding 600 µL of Binding Buffer and wash once with 100 µL of Wash Buffer. Centrifuge columns (15,000 x g, 1 min) between all bisulfite conversion steps and discard flow through.

6.3.3. Desulphonate samples by adding 200 µL of Desulphonation Buffer to columns. Incubate at room temperature for 15 - 20 min. Repeat centrifugation and discard flow through.

6.3.4. Wash columns twice with 200 µL of Wash Buffer. Elute each sample by adding 10 µL of Elution Buffer to the column, incubating for 3 min at room temperature, and centrifuging (15,000 x g, 1 min). Repeat elution step for a total of 20 µL.

6.3.5. Prepare PCR reaction Master Mix 1 on ice. Add 82 µL of mix to each sample. Incubate in a thermal cycler with the following program.

PCR Reaction Master Mix 1 (per sample):
30 µL of Water
50 µL of Methyl-Seq PCR Master Mix
1 µL of Methyl-Seq PCR1 Primer F
1 µL of Methyl-Seq PCR1 Primer R

Thermal Cycler Program:
Stage 1, 1 cycle: 95 °C 2 min
Stage 2, 8 cycles: 95 °C 30 s, 60 °C 30 s, 72 °C 30 s
Stage 3, 1 cycle: 72 °C 7 min
Stage 4, 1 cycle: 4 °C Hold

6.3.6. Purify samples using 180 µL of DNA-binding magnetic beads and 400 µL of freshly prepared 70% ethanol per sample. Add 180 µL of beads to each sample and incubate for 5 min at room temperature. Pellet beads, remove supernatant and resuspend pellet in 200 µL of 70% ethanol. Remove ethanol and repeat wash once.

6.3.7. Use a magnetic plate to pellet beads and remove as much ethanol as possible. Dry in a 37 °C heatblock for 3 - 5 min until the bead pellet is completely dry. Resuspend in 21 µL of nuclease-free water and collect approximately 19.5 µL of supernatant.

6.4. Indexing

6.4.1. Prepare PCR reaction Master Mix 2 on ice. Add 25.5 µL Master Mix 2 to each sample. Add 5 µL commercial indexing primers to individual samples and incubate in a thermal cycler.

PCR Reaction Master Mix 2 (per sample):
25 µL Methyl-Seq PCR Master Mix
0.5 µL Methyl-Seq Common Indexing Primer

Thermal Cycler Program:
Stage 1, 1 cycle: 95 °C 2 min
Stage 2, 6 cycles: 95 °C 30 s, 60 °C 30 s, 72 °C 30 s
Stage 3, 1 cycle: 72 °C 7 min
Stage 4, 1 cycle: 4 °C Hold

Note: Additional cycles (2 - 3) may be necessary if the starting DNA concentration is below recommended values.

6.4.2. Purify samples using 90 µL of DNA-binding magnetic beads and 400 µL of freshly prepared 70% ethanol per sample. Add 90 µL of beads to each sample and incubate for 5 min at room temperature. Pellet beads, remove supernatant and resuspend pellet in 200 µL of 70% ethanol. Remove ethanol and repeat wash once.

6.4.3. Use a magnetic plate to pellet beads and remove as much ethanol as possible. Dry in a 37 °C heatblock for 3 - 5 min until the bead pellet is completely dry. Resuspend in 24 µL of nuclease-free water and collect approximately 24 µL of supernatant.

6.4.4. Assess concentration and bp size using the high-sensitivity DNA detection reagents on a bioanalyzer.

Note: If the bioanalyzer fails to detect the presence of the library DNA, repeat the preparation steps with additional DNA.

Stopping point: After purification, indexed samples may be sealed and stored at -20 °C.

6.4.5. Pooling Samples for the appropriate next-generation sequencing platform used.

6.4.5.1. Using the concentration data from the bioanalyzer, which determines DNA molarity based on library size and quantity in a given volume, dilute with Low TE buffer (6.1.1.1) and combine all samples to a final concentration of 15 pM.

Note: A more sensitive method of quantifying the library is by quantitative real-time PCR using primers that target the ligated adapters.

6.4.5.2. Run pooled samples on the number of lanes that are sufficient for 4 samples per lane on

a next-generation sequencer.

Note: For instance, if 16 library samples have been uniquely indexed and combined, run the libraries over 4 lanes, equivalent to 4 samples per lane.

7. Sequencing on a Next-Generation Sequencer

7.1. Send the samples to the institutional sequencing core for clustering of the Methyl-Seq library, followed by sequencing on a next-generation sequencing machine.

8. Analysis to Identify DMRs

8.1. Implement Bismark[15], which invokes Bowtie 2.0 as an internal sequence aligner[16,17], to align raw input reads to bisulfite-converted, plus-strand genome. Following alignment, use the Bismark_methylation_extractor to perform quality control and assign an estimated methylation value to each CpG.

8.2. Generate a list of DMRs with the BS-Seq package[18] in Bioconductor. Filter the DMRs based on having greater than 3 consecutive CpGs and P-value < 0.05.

Note: Generate a DMR list that includes genomic coordinates, distance to the nearest RefSeq gene, number of CpGs within each DMR, average% CpG methylation value across the DMR for the two comparison groups (*e.g.,* stressed vs. unstressed), the P-value, and the FDR (false discovery rate) value. Use the DMR list, *i.e*., genomic coordinates, to design pyrosequencing primers for validation.

9. Validation by Bisulfite Pyrosequencing

9.1. Primer Design

9.1.1. Design primers for bisulfite PCR and pyrosequencing. Design two sets of PCR primers (outside and nested) so that the nested PCR will amplify 150-400 bps of a DMR.

Note: In general, designed primers are at least 24 bases long with at least 4 - 5 non-consecutive G's (C's for the reverse primer) to account for reduced annealing temperature from loss of sequence complexity. One of the nested primers will be biotin-labeled and HPLC-purified. However, standard primers should be ordered first to optimize the PCR step by resolving the reactions on an agarose gel.

9.1.1.1. Design the pyrosequencing assay primer so that it targets the complementary biotinylated strand just 1 - 2 bases upstream of the CpGs to be assayed. Design multiple pyrosequencing primers as necessary, as each pyrosequencing primer can reliably assay 30 bps downstream.

9.1.2. For the Rt1-m4, use the following:

rRT1M4 Outside – F	TGTAYGATTTTGGTTATYGTAAAT
rRT1M4 Outside – R	AACTTACAAATTTCACCAACTCA
rRT1M4 Nested – F	GTGGGTTAYGTGGATAATATATAG
rRT1M4 Nested – R	AATCACTTACCATTCTCTCTCTAACTA
rRT1M4 Pyro1	TAYGTGGATAATATATAGAT
rRT1M4 Pyro2	GATAGTTATTTGGYGAGTTAG
rRT1M4 Pyro3	GAGTATTTGGAGGAGTTGAT
rRT1M4 Pyro4	GGATTTTAATATTTGGT

9.2. Use a commercially-available kit for bisulfite conversion of rat blood gDNA.

Note: The bisulfite conversion steps have been adapted from the commercially-available kit with the following modifications: In step 1, add 50-100 ng of blood gDNA and dilute with water to 20 µL. In step 9, elute 20 µL per sample.

9.2.1. Prepare bisulfite conversion reagent according to the manufacturer's protocol and combine with diluted gDNA. Incubate in thermal cycler (64 °C for 2.5 h, 4 °C hold).

9.2.2. Add Binding Buffer to converted gDNA in spin columns and centrifuge (15,000 x g, 1 min). Wash columns once then add Desulphonation Buffer to the columns and incubate for 15 min at room temperature. Centrifuge (15,000 x g, 1 min).

9.2.3. Wash column with Wash Buffer and centrifuge (15,000 x g, 1 min). Repeat wash step with centrifugation (15,000 x g, 2 min). Add 20 µL Elution Buffer and centrifuge (15,000 x g, 1 min) to elute.

9.3. PCR amplification

9.3.1. Prepare Outside PCR Master Mix. Add 21.5 µL of Master Mix to 3.5 µL bisulfite-converted gDNA and run thermal cycler program.

Outside PCR Master Mix:
16.25 µL of Water
2.5 µL of Polymerase Buffer [10x]
0.5 µL of dNTP [10 mM]
1 µL of Forward Primer [0.1 µM]
1 µL of Reverse Primer [0.1 µM]
0.25 µL of Taq DNA Polymerase [5000 U/mL].

Thermal cycler program:
Stage 1, 1 cycle: 94 °C 4 min
Stage 2, 47 cycles: 94 °C 1 min, 53 °C 30 s, 72 °C 1 min

Stage 3, 1 cycle: 72 °C 8 min, 4 °C Hold

9.3.2. Prepare Nested PCR Master Mix. Add 23 µL of Master Mix to 2 µL of sample from outside PCR and repeat the outside PCR thermal cycler program. Assess PCR product quality through gel electrophoresis (1x TAE buffer, 1% agarose gel).

Nested PCR Master Mix:
17.75 µL of Water
2.5 µL of Polymerase Buffer [10x]
0.5 µL of dNTP [10 mM]
1 µL of Forward Primer [0.1 µM]
1 µL of Reverse Primer [0.1 µM]
0.25 µL of Taq DNA Polymerase [5000 U/mL]

Note: For nested PCR, either the forward or the reverse primer must be biotinylated.

9.4. Pyrosequencing

9.4.1. Make a master mix containing 38 µL of Binding Buffer, 35 µL of water, and 2 µL of streptavidin-coated sepharose beads per sample. In a 96-well plate, add 75 µL of master mix and 5 µL of nested PCR product. Shake on a plate shaker for 15 - 60 min.

9.4.2. While shaking, add 12 µL of primer (0.5 µM, diluted in annealing buffer) into the wells of a pyrosequencing assay plate.

9.4.3. After shaking, perform wash steps using binding reaction wash buffers. Place vacuum tool in trough filled with water then collect samples from plate. Submerge vacuum tool in half-filled troughs containing 70% ethanol, NaOH (0.2 M), and Tris acetate buffer (10 mM, pH 7.4). Disconnect from vacuum and place vacuum tool in HS assay plate to transfer beads.

9.4.4. Place plate on heat block and incubate at 80 °C for 2 min. Allow plate to cool for 5 min then begin pyro program.

REPRESENTATIVE RESULTS:

A successful implementation of the rat Methyl-Seq platform depends on several criteria. **Figure 1** shows the overall workflow of the study and highlights specific quality control (QC) steps that are needed before moving forward. One of the first factors to consider is the robustness of the animal model and the stress regimen, which determine the magnitude of epigenetic changes that occur across the methylome. Since our animal work is predicated on our previous observation that corticosterone (CORT) exposure can lead to changes in DNA methylation[19,20], our chronic variable stress (CVS) regimen needed to be of sufficient rigor to produce stressed rats with elevated plasma CORT levels. A typical weekly CVS regimen is shown in **Table 1** and consisted of daily stressors in the morning, afternoon, and overnight that are constantly changed to prevent habituation and diminished stress response. Throughout the 3-week regimen, the stressed

animals exhibited significantly elevated levels of mean plasma CORT [Days 4-21, Control: 32.7 ± 3.7 ng/mL, Stress: 103.0 ± 11.9 ng/mL (mean ± SEM), P = $2.2x10^{-4}$, **Figure 2A**] over those of unstressed, control animals. Consistently, these animals also showed greater anxiety-like behavior on the elevated plus maze (EPM), as indicated by the significantly more time spent in the closed arms of the EPM and less time in the open arms (**Figure 2B**). These results demonstrate that the CVS exposure led to significant endocrine and behavioral changes, leading us to investigate whether these changes were associated with specific DNA methylation signatures.

We emphasize several checkpoints that are crucial for the successful construction of the Methyl-Seq library. Starting with a sufficient quantity of DNA is necessary, as sonication, multiple wash/purification, target enrichment, and bisulfite conversion steps successively reduce the quantity of DNA in the finished library. Although several PCR amplification steps alleviate the loss of DNA template, excessive PCR cycle numbers can introduce higher duplicate reads. For the current rat Methyl-Seq study, 2 μg of blood gDNA per rat was used. We note that Methyl-Seq libraries can be made with starting DNA amount as low as 500 ng. Smaller starting material allows users to generate libraries from DNA isolated by FACS (fluorescence-activated cell sorting) or needle punches, although there is increased risk of producing an insufficient amount of libraries for subsequent sequencing. QC is performed by electrophoresis of 1 μL of the sample on a bioanalyzer, which provides DNA molecular weight, quantity, and molarity. Three critical steps that require the use of the bioanalyzer are: 1) following sonication step to ensure sufficient shearing of DNA (~170 bp, red, **Figure 3**); 2) following adapter ligation step indicated by a shift in the average size of the sheared DNA (~200 bp, blue, **Figure 3**) to ensure their subsequent amplification by PCR; and 3) following final library purification step to ensure the quantity and size of the library for sequencing.

The R-packages BSSeq and BSmooth in Bioconductor were used for analyzing the bisulfite sequencing data[18]. They include tools and methods for aligning the sequence reads, performing quality control, and identifying differentially methylated regions (DMRs). BSmooth software invokes Bowtie 2.0[16,17] as an internal sequence aligner to obtain CpG-level measurement summaries, by alignment of raw input reads to bisulfite-converted genomic sequences. The aligned reads are then filtered through rigorous quality control procedures that seek to identify systematic sequencing and base-calling errors that may skew downstream analyses. A series of plots are generated to visually aid in this process of filtering. Sequencing metrics are also generated to document relevant information such as number of aligned reads, % target, and per CpG coverage, among others (**Table 2**). Once the data are filtered, a smoothing/normalization algorithm is performed, where every CpG is assigned an estimated methylation value based on all QC reads from each sample and estimates from neighboring CpGs to ensure more accurate calling of methylation status even in cases where the sequence coverage is low. This value provides a smoothed estimate of the probability of methylation at each CpG site. By comparing the mean of the smoothed methylation estimates of each sample between the two treatment groups and ranking genomic regions from the most significantly different to least, a list of DMRs is generated (**Table 3**).

The top DMR between stressed and unstressed groups was located in the promoter of the rat

major histocompatibility gene *Rt1-m4*, with stressed animals exhibiting higher methylation levels across all CpGs than unstressed animals (**Figure 4A**). To confirm successful implementation of the Methyl-Seq platform and the data analysis, primers were designed against the DMR, and blood DNA methylation levels in the entire cohort of stressed and unstressed animals (8 sequenced by Methyl-Seq and 8 not sequenced) were assessed by bisulfite pyrosequencing. Results demonstrate significant increase in DNA methylation across 10 out of the 12 CpGs assayed (5.1-10.4 change in % methylation, $P < 0.037$, **Figure 4B**). KEGG pathway analysis was performed on all of the nominally significant DMRs to identify pathways associated with stress. Consistently, DMR-associated pathways implicated diseases associated with chronic stress exposure, such as diabetes, cardiovascular disease, and cancer (**Table 4**).[21-23] To demonstrate an association between the epigenetic data and the degree of exposure to stress, methylation levels at CpG-10 were compared to the mean 3-week CORT levels for each animal. Results showed a modest correlation between the endocrine and methylation data (R^2=0.54, P=0.001, **Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1. **Overall schematic workflow for the rat Methyl-Seq platform.** One μg of the genomic DNA extracted from the blood of stressed and control rats is first processed for constructing the Methyl-Seq libraries for sequencing, analysis, and target identification. Another 100 ng of DNA is used for independent validation of the identified epigenetic targets by bisulfite pyrosequencing.

Figure 2. **Exposure to chronic variable stress (CVS) leads to endocrine and behavioral changes in rats.** (A) Multiple samplings of corticosterone (CORT) demonstrate the robustness of the 3-week CVS regimen. Blood samples were collected in the morning prior to the daily stress regimen. (B) Stressed animals spent more time in the closed arms and less time in the open arms of the elevated plus maze (EPM). Boxplots with data point for each animal are shown. Student's T-test was performed for statistical significance. *P<0.05, **P<0.01, and ***P<0.001.

Figure 3. **Quantitation of sheared and adapter-ligated rat DNA on a bioanalyzer.** The red and blue curves show the quantity and size of genomic DNA (red) following shearing in an isothermal sonicator and adapter ligation, respectively. Each line represents one sample and the red and blue curves reflect both loss of DNA during the several steps (end-repair, 3'-adenylation, and sample cleanup) and increase in bp size due to the ligation of the adapters. Sharp peaks at 25 bp and 1500 bp are standard markers that have been added to the loading buffer.

Figure 4. **CVS-induced epigenetic changes are detected by rat Methyl-Seq.** (A) Analysis of the rat Methyl-Seq data implicated the promoter of the gene *Rt1m4* as a differentially methylated region (DMR) between stressed (red) and control (blue) rats. The graphical output for the *Rt1m4* DMR (pink shaded region) displays each CpG (vertical gray line), the four samples in each group (red or blue lines), and the% methylation levels for each animal (red or blue dot). (B) Twelve CpGs within the DMR were validated by bisulfite pyrosequencing. The bar graphs are represented as mean ± SEM, and a Student's T-test was performed for statistical significance. *P<0.05.

Figure 5. Linear regression analysis showed a modest correlation between % DNA methylation

at CpG-10 of *Rt1m4* and the 3-week mean plasma CORT levels of both stressed and control animals (N=16). Data from stressed animals are represented by red circles.

Table 1: A typical weekly schedule of the chronic variable stress regimen (CVS).

Table 2. Sequencing metrics obtained from the rat Methyl-Seq platform.

Table 3. Top 10 differentially methylated regions. For each DMR, the output table shows from the left to right column: chromosomal location (chr), coordinates (start/end), gene name, distance from the transcription start site, differential area statistics between stressed and control groups (areaStat), mean differential methylation (meanDiff), mean methylation levels across each DMR for stressed and control groups (stress/control), and direction of methylation change from controls.

Table 4. KEGG Pathway analysis of DMRs identified from the rat Methyl-Seq.

DISCUSSION:

In this study, we designed and implemented the Methyl-Seq platform for the rat genome. By demonstrating its utility with a rat model of stress, we demonstrated that the experimental and analytical pipeline can provide differentially methylated regions between two comparison groups.

To ensure a successful implementation of the platform, several critical steps need to be observed. First, initial DNA quality and quantity has a significant impact on the quality and quantity of the final Methyl-Seq library. We used a fluorometer, rather than a spectrophotometer, to ensure that our DNA measurement reflected the quantity of double-stranded DNA present. The bioanalyzer was used to measure the molecular size and quantity of DNA following shearing and after adapter ligation. Verifying the molecular size "shift" between these steps is crucial to confirm the presence of adapters at the ends of each DNA fragment that will undergo adapter-mediated PCR in the subsequent steps. The quantity of DNA remaining at the end of the adapter ligation step is also important, since at least 100 ng of the library product is needed at this step to ensure sufficient quantity is available after the target enrichment and bisulfite conversion steps. A final high-sensitivity measurement was performed on the constructed Methyl-Seq library so that the library can be properly diluted for subsequent clustering on the next-generation sequencer. Finally, bisulfite pyrosequencing was employed as a highly-quantitative, independent method to assess the accuracy of the analytical pipeline. The final validation using the original samples and replication using additional animals are crucial steps to ensure that the experiment can detect biologically significant changes in DNA methylation.

We also include several guidelines in the event of deviation from the protocol or if problems are encountered. First, it is possible to lose too much DNA during end-repair, adapter ligation, or magnetic bead purification steps. Alternatively, starting amounts of DNA could be small (< 200 ng) due to limited tissue/DNA availability or implementation of various enrichment methods such as fluorescence activated cell sorting. Increasing the cycle number during the two library

amplification steps may be able to compensate for the excessive loss of DNA or low starting DNA amount throughout the library construction protocol. However, no more than an additional 2 - 3 cycles are recommended, as excessive template amplification is likely to lead to an increase in the number of duplicate reads being sequenced. These duplicates are excluded during the alignment step to prevent bias in percent methylation calculations. Second, if the average DNA size does not increase by more than 30 bps, check to ensure that the reagents are new, as T4 DNA polymerase, Klenow, and/or T4 ligase may be old. Commercially available replacement reagents can be used.

Additionally, it is possible that the predicted DMRs might not validate by pyrosequencing, where DNA methylation differences do not exist or are significantly less than those predicted by analysis. Poor validation of candidate regions is a problem too common for many genome-wide analyses, such as when pyrosequencing results do not confirm differential methylation or the effect size is much smaller than that predicted by the analysis. BSmooth is one analytical package that "smoothes" the methylation levels across a window of multiple CpGs. For the current experiment, BSmooth implicated a DMR whose methylation levels were validated by bisulfite pyrosequencing. However, there will likely be discrepancies between methylation levels predicted by BSmooth and those verified by pyrosequencing. The discrepancies arise from the smoothing function that estimates the average methylation values across all of the CpGs within a DMR, including consecutive CpGs that may differ in DNA methylation by more than 50% or CpGs whose methylation values were excluded due to sub-threshold read depth. R-packages such as MethylKit[24] can be used to identify smaller windows of CpGs or even single CpGs whose methylation levels correlate strongly with those validated by pyrosequencing. Implementing different packages and testing their predicted regions or CpGs of differential methylation by pyrosequencing will ensure robustness of data. Alternatively, original Methyl-Seq libraries can be resequenced and added to the read files to increase read depth. Since determination of methylation levels are semi-quantitative and dictated by the number of reads [(# of CpGs)/(# of TpGs+CpGs)], increasing the read depth for a given CpG will increase the accuracy of its percent methylation value. In this study, we only considered CpGs whose methylation values were determined by at least ten reads and achieved an overall read coverage of 19x for each CpG.

The rat Methyl-Seq platform is not without its limitations. While it is more cost effective than whole-genome bisulfite sequencing, it is considerably more expensive than other methods. Nevertheless, most of the cost was for purchasing lanes on the sequencer and not for the capture system. Depending on the read depth necessary, with cross-tissue comparisons requiring less due to large (25-70%) differences[12] in DNA methylation, the cost can be reduced by multiplexing more samples per lane and using a higher-capacity platform. Also, the sample preparation is more time-consuming than other methods. While similar to other pulldown approaches that incorporate next-generation sequencing, the added bisulfite conversion and purification steps add to the work load. Overall, the Methyl-Seq platform is a cost-effective alternative to whole-genome sequencing and provides base-pair resolution at more than 2.3 million CpGs, which is considerably more than those assayed by microarray-based platforms. To date, the commercially-available human and mouse Methyl-Seq platforms have been used to document alcohol-dependent changes in the macaque brain[25,26], neurodevelopmental genes in the mouse

brain[9], and blood-brain targets of glucocorticoids[10]. Further, the ability to target specific regions regardless of sequence recognition by restriction enzymes makes it an ideal platform for cross-species comparisons. For this study, we designed the Methyl-Seq platform for the rat, for which many pharmacological, metabolic, and behavioral experiments are performed without the benefit of a genome-wide methylomic tool. Our data show that it can be used to detect DMRs in a rat model of stress and correlated other physiological parameters such as overall plasma CORT levels.

The Methyl-Seq platform is ideal for epigenetic experiments in animals with sequenced genomes that may not have enough experimental evidence documenting regulatory regions. When such regions are made available, additional regions may be custom-designed and attached to the current version. Further, the platform is ideal for comparative genomics, since the target enrichment is not constrained by restriction enzyme recognition. For instance, the promoter region of any gene of interest can be captured regardless of whether it harbors a specific restriction site. Similarly, any regulatory regions, such as those identified in mouse or humans, which are conserved in the genome of interest can be captured.

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Figure 1

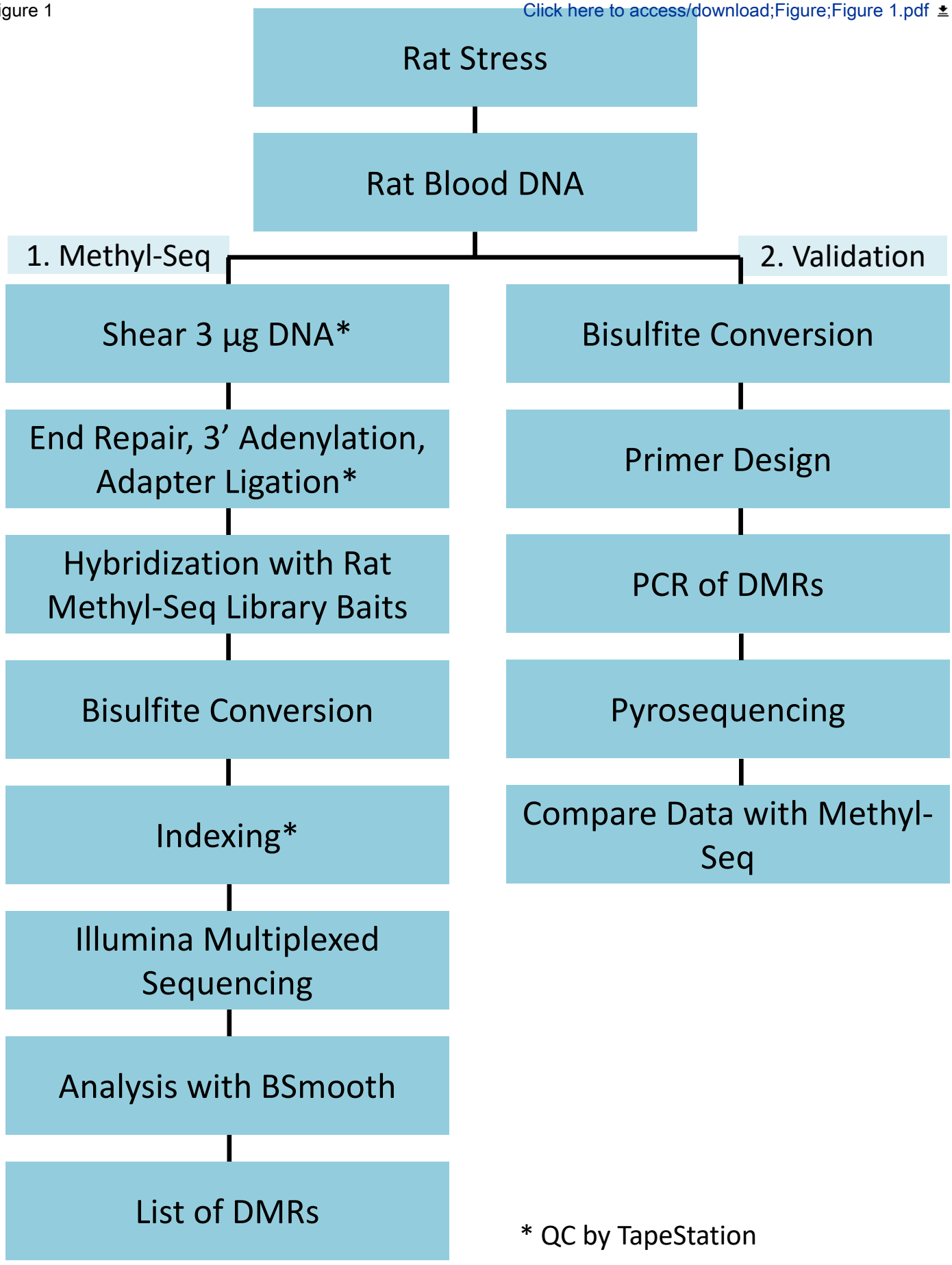
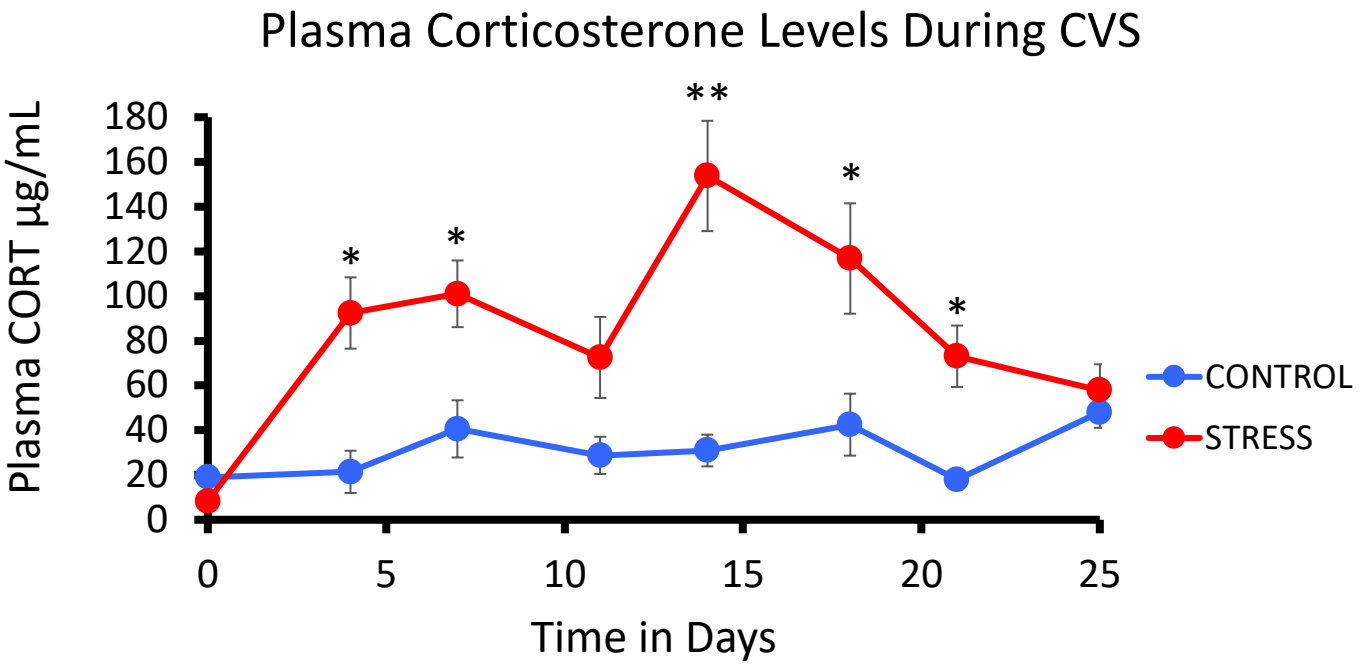


Figure 2

A)



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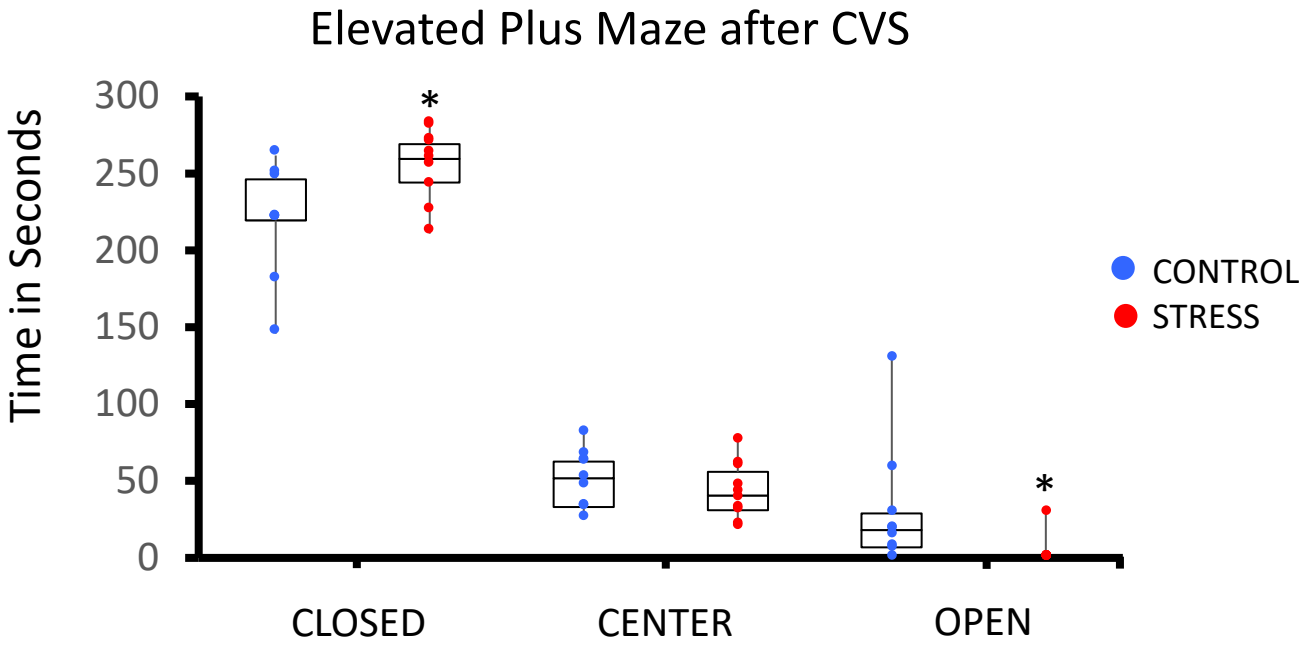

Figure 3

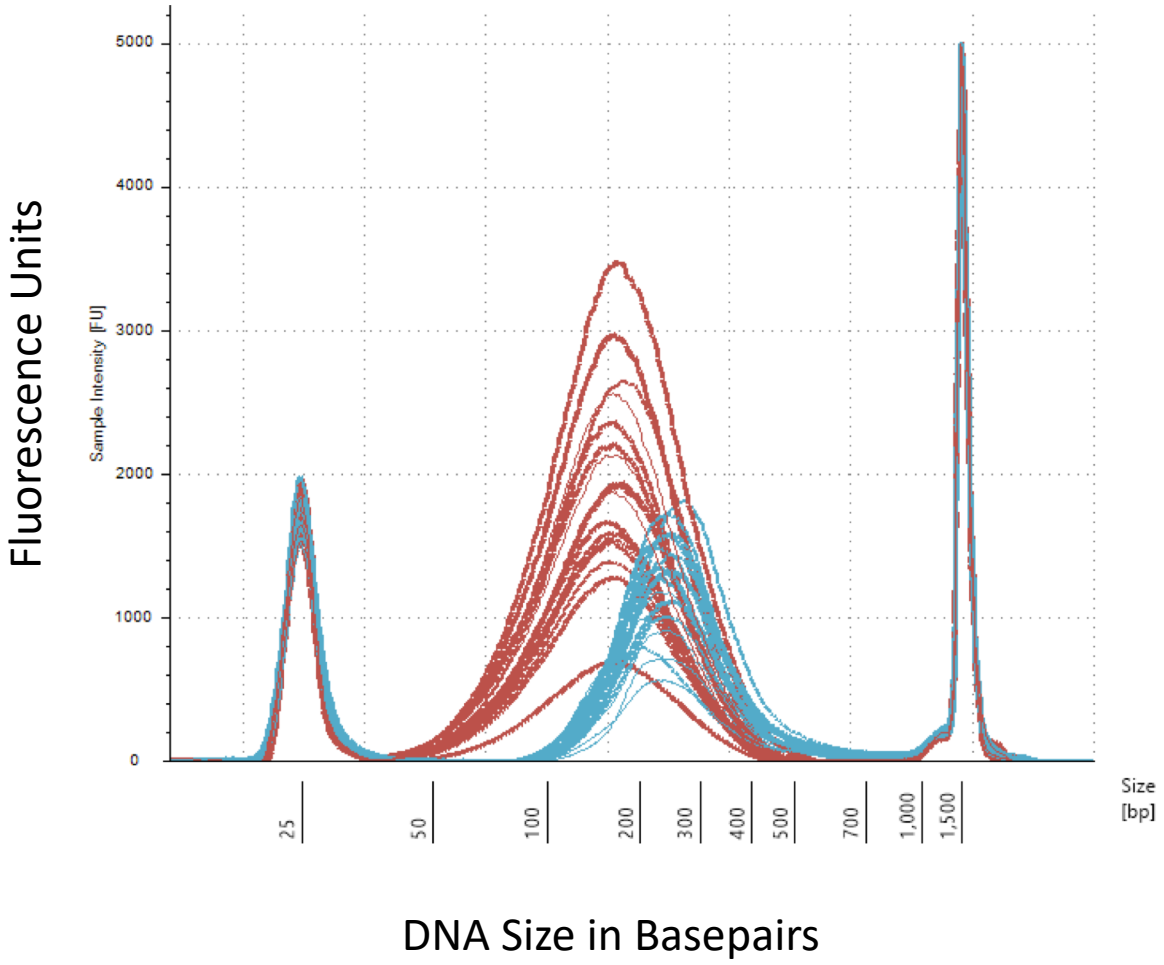

Figure 4

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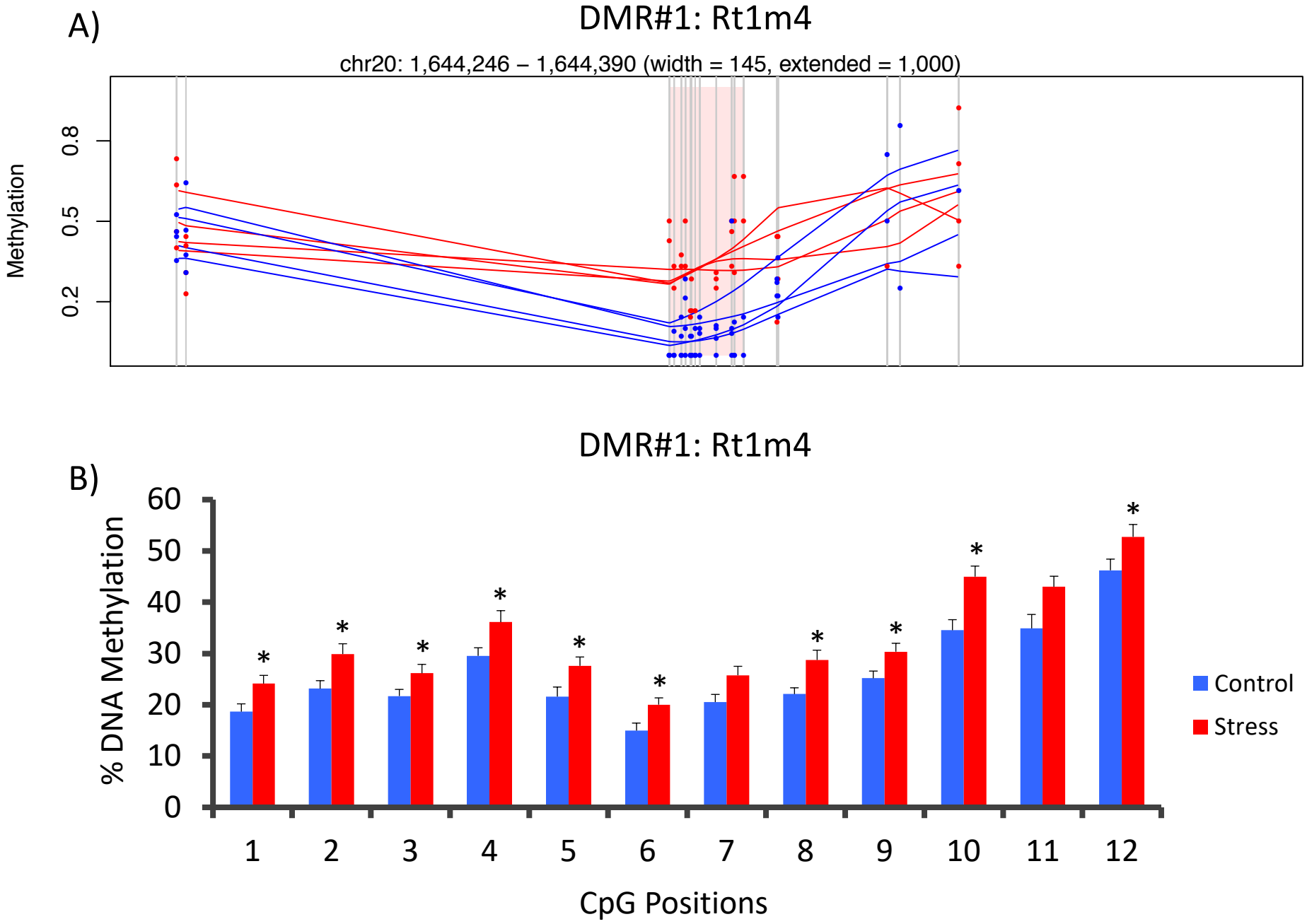

Figure 5

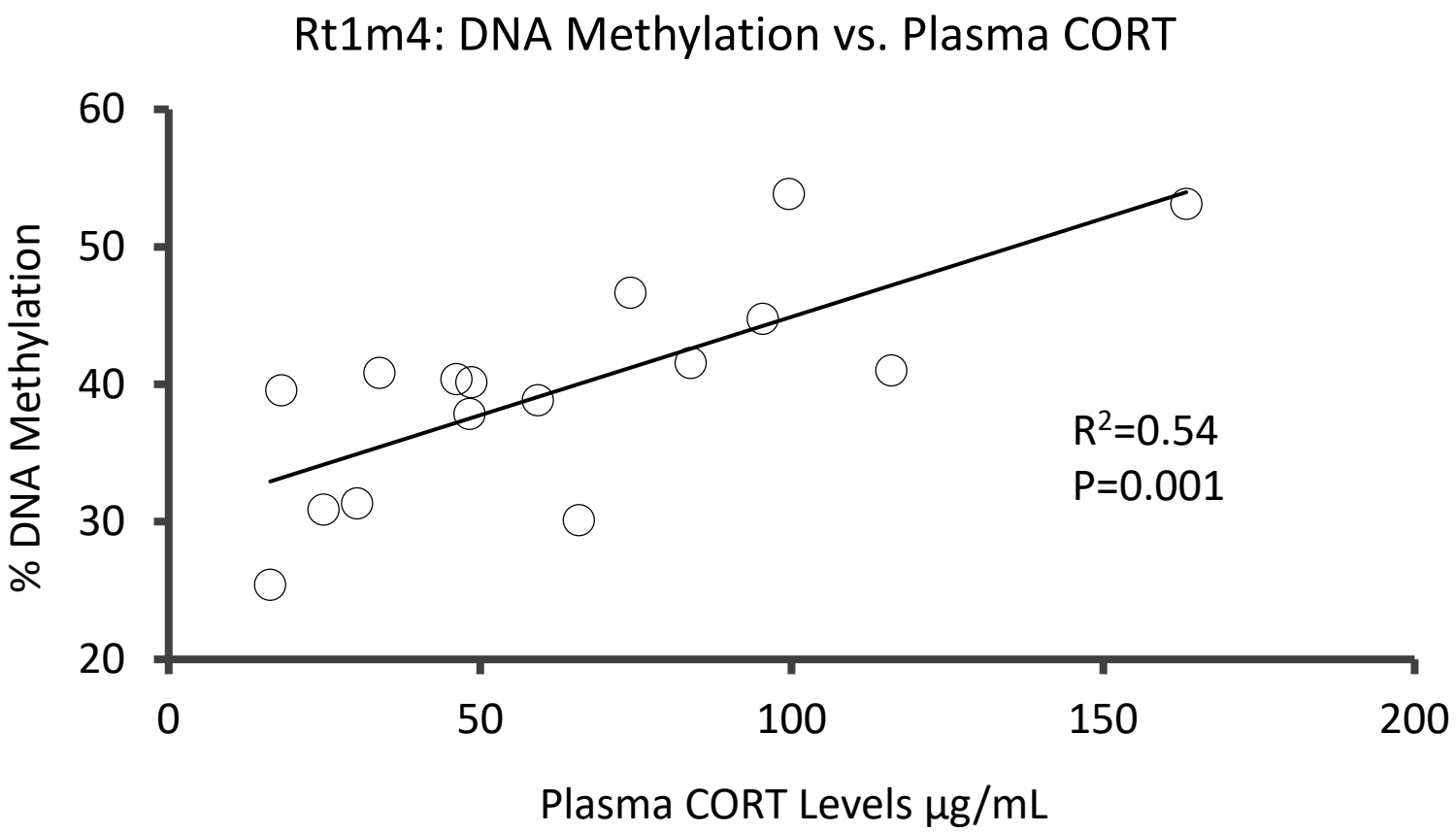

Table 1: A typical weekly schedule of the chronic variable stress regimen (CVS).

Week	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
AM	Restraint	Swim	Cold Room	Swim	Restraint	Shaker
PM	Shaker	Cage Tilt	Restraint	Shaker	Cold Room	Restraint
Overnight	Food Restrict	Wet Bedding	Isolation	Light On	Crowding	Light On

Day 7
Swim
Cold Room
Wet Bedding

Table 2. Sequencing metrics obtained from the rat Methyl-Seq platform

Sequencing Metrics	Stress[1] (n = 4)
Paired End Reads (PER)	89,290,397
Uniquely Mapped Paired End Reads (UMPER)	39,200,255
Alignment Rate/Mapping Efficiency (UMPER/PER)	44%
Duplicate Reads (% of UMPER)	73%
Deduplicated UMPER	10,481,031
Average Read Depth Coverage (x) (ARDC)	6x
CpGs (N)	12,056,878
ARDC (x) of CpGs	2x
CpGs with at least 10 reads (N)	481,383
ARDC (X) of CpGs with at least 10 reads	19
On Target CpGs (complete overlap with Probe Target Regions)	1,923,872
On Target ARDC (x) of CpGs	7x
On Target CpGs with at least 10 reads (N)	428,249
On Target ARDC (x) of CpGs with at least 10 reads	18X
On Target (PER with 1 or more Base Pair overlap with Probe Target Regions) (UMPER)	8,277,715
% On Target (of Deduplicated UMPER)	78%
On Target (Total Bases Mapped) Mb	125Mb
On Target Average Read Depth Coverage (x) (ARDC)	9x

[1]Sequencing metrics based on averages across subjects in each group

Control[1] (n = 4)
80,165,674
35,013,406
44%
65%
12,306,018
6x
12,056,878
2x
595,850
19
2,007,638
8x
531,419
18X
9,369,523
77%
128Mb
10x

Table 3. Top 10 Differentially Methylated Regions

chr	start	end	gene	dista
chr20	1,644,246	1,644,390	RT1-M4	in_g
chr5	160,361,352	160,361,564	LOC690911	in_g
chr3	61,138,281	61,138,330	RGD1564319	265
chr2	143,064,811	143,065,010	Ufm1	85
chr7	30,764,111	30,764,284	Ntn4	in_g
chr17	12,469,112	12,469,218	Idnk	419
chr7	47,101,725	47,101,930	Pawr	in_g
chr5	76,111,248	76,111,822	Txndc8	151
chr11	80,640,132	80,640,356	Dgkg	in_g
chr8	71,759,248	71,759,411	Mir190	210

ance	areaStat	meanDiff	stress	control	direction
gene	93.03	0.22	0.33	0.11	gain
gene	-70.75	-0.19	0.72	0.91	loss
569	61.79	0.21	0.94	0.72	gain
69	-59.48	-0.11	0.13	0.24	loss
gene	57.04	0.21	0.94	0.73	gain
996	-50.91	-0.13	0.74	0.88	loss
gene	-50.54	-0.12	0.64	0.76	loss
703	-50.38	-0.11	0.85	0.96	loss
gene	-50.07	-0.16	0.73	0.89	loss
226	-47.84	-0.17	0.58	0.75	loss

Table 4: KEGG Pathway analysis of DMRs identified from the rat Methyl-Seq

KEGG Pathway Terms	Gene Count	%	P-value	Benjamini
Diabetes				
Type II diabetes mellitus	12	0.1	$3.6x10^{-4}$	$9.8x10^{-3}$
Cardiovascular Disease				
Vascular smooth muscle contraction	18	0.1	$1.6x10^{-3}$	$3.6x10^{-2}$
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	13	0.1	$4.0x10^{-3}$	$7.1x10^{-2}$
Dilated cardiomyopathy	14	0.1	$7.6x10^{-3}$	$1.2x10^{-1}$
Neuron Function				
Long-term potentiation	11	0.1	$1.5x10^{-2}$	$1.4x10^{-1}$
Signaling				
MAPK signaling pathway	35	0.2	$2.4x10^{-4}$	$9.9x10^{-3}$
Calcium signaling pathway	22	0.1	$1.2x10^{-2}$	$1.4x10^{-1}$
Chemokine signaling pathway	21	0.1	$1.2x10^{-2}$	$1.3x10^{-1}$
Cancer				
Pathways in cancer	42	0.3	$4.1x10^{-5}$	$3.4x10^{-3}$
Glioma	15	0.1	$4.4x10^{-5}$	$2.4x10^{-3}$
Non-small cell lung cancer	10	0.1	$7.9x10^{-3}$	$1.1x10^{-1}$
Colorectal cancer	13	0.1	$8.4x10^{-3}$	$1.1x10^{-1}$
Chronic myeloid leukemia	12	0.1	$1.2x10^{-2}$	$1.3x10^{-1}$

Table of Materials

Click here to access/download;Table of Materials;Materials List JoVE Final.xlsx

Name of Material/ Equipment	Company	Catalog Number
Radioimmuno assay (RIA)	MP Biomedicals	7120126
Master Pure DNA Purification Kit	Epicentre/Illumina	MC85200
Thermal-LOK 2-Position Dry Heat Bath	USA Scientific	2510-1102
Vortex Genie 2	Fisher	12-812
Ethyl alcohol, Pure	Sigma-Aldrich	E7023
Centrifuge 5424 R	Eppendorf	-
Qubit 2.0	ThermoFisher Scientific	Q32866
Qubit dsDNA BR Assay Kit	ThermoFisher Scientific	Q32850
Qubit dsDNA HS Assay Kit	ThermoFisher Scientific	Q32851
Qubit Assay Tubes	ThermoFisher Scientific	Q32856
SureSelectXT Rat Methyl-Seq Reagent Kit	Agilent Technologies	G9651A
SureSelect Rat Methyl-Seq Capture Library	Agilent Technologies	931143
IDTE, pH 8.0	IDT DNA	11-05-01-09
DNA LoBind Tube 1.5 mL	Eppendorf	22431021
Covaris E-series or S-series	Covaris	-
microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm (25)	Covaris	520045
Water, Ultra Pure (Molecular Biology Grade)	Quality Biological	351-029-721
Veriti 96 Well-Thermal Cycler	Applied Biosystems	4375786
AMPure XP Beads	Beckman Coulter	A63880
96S Super Magnet	ALPAQUA	A001322
2200 TapeStation	Agilent Technologies	G2965AA
D1000 ScreenTape	Agilent Technologies	5067-5582
D1000 ScreenTape High Sensitivity	Agilent Technologies	5067-5584
D1000 Reagents	Agilent Technologies	5067-5583
D1000 Reagents High Sensitivity	Agilent Technologies	5067-5585
DNA110 SpeedVac	ThermoFisher Scientific	-
Dynabeads MyOne Streptavidin T1 magnetic beads	Invitrogen	65601
Labquake Tube Rotator	ThermoFisher Scientific	415110Q
EZ DNA Methylation-Gold Kit	Zymo Research	D5006
Illumina Hi-Seq 2500	Illumina	-

PCR and Pyrosequencing Primers	IDT DNA	Variable
Taq DNA Polymerase with ThermoPol Buffer - 2,000 units	New England BioLabs	M0267L
Deoxynucleotide (dNTP) Solution Set	New England BioLabs	N0446S
Pyromark MD96	QIAGEN	-
Ethyl Alcohol 200 Proof	Pharmco-Aaper	111000200
Sodium Hydroxide Pellets	Sigma-Aldrich	221465
Tris (Base) from J.T. Baker	Fisher Scientific	02-004-508
PyroMark Gold Q96 Reagents (50x96)	QIAGEN	972807
PyroMark Annealing Buffer	QIAGEN	979009
PyroMark Binding Buffer (200 ml)	QIAGEN	979006
Streptavidin Sepharose High Performance Beads	GE Healthcare	17-5113-01
PyroMark Q96 HS Plate	QIAGEN	979101
Eppendorf Thermomixer R	Fisher Scientific	05-400-205
SureDesign Website	Agilent Technologies	-
UCSC Genome Browser	University of California Santa Cruz	-
Agilent Methyl-Seq Protocol	Agilent Technologies	-

Comments/Description
Corticosterone, 125I labeled
Used with 1.5 mL tubes Vortex Mixer 100% Ethanol, molecular grade Must be capable of 20000 x g Fluorometer
High sensitivity DNA detection reagents
Reagents for preparing the Methyl-Seq library RNA baits for enrichment of rat targets 10 mM TE, 0.1 mM EDTA
Isothermal sonicator
DNA-Binding magnetic beads Magnetic plate for purification steps Electrophresis-based bioanalyzer
Vacuum Concentrator
Streptavidin magnetic beads
Nutator Mixer is also acceptable Bisulfite conversion kit. Contains Binding, Wash, Desulphonation, and Elution buffers Next-generation sequencing machine

Pyrosequencing machine
70% Ethanol solution
0.2 M NaOH denature buffer solution
10 mM Tris Acetate Buffer wash buffer solution
Reagents required for pyrosequencing

Streptavidin-coated sepharose beads
Pyrosequencing assay plate
Plate mixer. 96-well block sold separately (cat. No 05-400-207)
Target capture design software (https://earray.chem.agilent.com/suredesign/)

rat Nov 2004 rn4 assembly

https://www.agilent.com/cs/library/usermanuals/public/G7530-90002.pdf

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Title of Article:	**Implementation of the Rat Methyl-Seq platform to identify epigenetic changes associated with stress exposure**
Author(s):	**Jenny L. Carey, Olivia H. Cox, Fayaz Seifuddin, Leonard Marque, Kellie L.K. Tamashiro, Peter P. Zandi, Gary S. Wand, Richard S. Lee**

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CORRESPONDING AUTHOR

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Title:	**Dr.**

Signature: Richard S. Lee Date: July 27, 2018

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- Done.

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- Most of the terms bearing commercial language have been removed. We only mention "SureSelect" only at the beginning as a means to identify the platform used.

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- The term "SureSelect" has been removed from most of the document except at the beginning.

5. *Please remain neutral in tone when discussing commercial products (e.g., line 584 needs to be revised). The accompanying video cannot appear to be an advertisement.*

- Specific product names have been removed and replaced by generic terms throughout the manuscript.

6. *Figure 2: Please change ug/mL to µg/mL. Please define error bars in the figure legend. Please line up the panels better and ensure that they have the same dimension if possible. Also, what statistical test was used to produce p-values?*

- Appropriate changes to the unit symbols have been made. We used a Student's T-test for generating most of the P-values. Figure legends have been edited to include information regarding the test used.

7. *Figure 4: Please define the error bars and other symbols in the figure legend.*

- We have now added: "The bar graphs are represented as mean±SEM, and a Student's T-test was performed for statistical significance. *P<0.05."

8. *Figure 5: Please change ug/mL to µg/mL. Please note that the asterisk symbols defined in the figure legend are not shown in Figure 5; also, what statistical test was used here?*

- The figure legend has been edited: "Linear regression analysis showed a modest correlation between % DNA methylation at CpG-10 of *Rt1m4* and the 3-week mean plasma CORT levels of both stressed and control animals (N=16)."

9. *Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.*

- Done.

10. *Please provide an email address for each author.*

- Done.

11. *Please shorten the Summary to 10-50 words to concisely describe the protocol and its applications.*

- The summary has been condensed to 10-50 words.

12. *Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.*

- These changes have been made throughout the manuscript.

13. *Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.*

- Done.

14. *Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.*

- Done.

15. *The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.*

- The protocol has been simplified.

16. *Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.*

- Additional details have been added throughout the protocol.

17. *Protocol step 3: What happens after centrifugation? Is the plasma discarded or kept?*

- We have now added the following: "Centrifuge all blood samples (600 x g, 4 °C, 10 min) to separate the plasma from the blood cells. Pipet out the plasma (supernatant) and store the samples at -80 °C."

18. *Step 4: Please add more details about the EPM or provide a relevant reference.*

- The relevant reference on the EPM has been added since this part is not a focus of the manuscript.

19. *Steps 6.1.1, 6.1.2, 6.1.3, 6.3, etc.: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol and refer back and forth in order to complete this protocol. Please remove the references to the specific steps of the other manual.*

- Additional steps have been added to make the protocol more self-sufficient.

20. *After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

- Done.

21. *Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*

- Done.

22. *Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

- Done.

23. *Disclosures: Please disclose that the manuscript is part of a contest prize from Agilent Technologies.*

- The appropriate language has been added to the disclosure section.

24. *References: Please do not abbreviate journal titles, and please include dois where applicable.*

- Full journal names and DOIs have been added when applicable.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this manuscript #JoVE58617, the authors describe the methods to apply the SureSelect MethylSeq strategy using the rat genome. This is highly important, as this animal model is increasingly being used in different research fields. As the authors state, DNA methylation analysis in rat has been limited by the widely used methods that do not rely on genome sequence. In addition, this method could be improved when additional regulatory information is available. Although more expensive than other approaches, the benefits are large.

Major Concerns:
No major concerns.

Minor Concerns:

Keywords:

1. *HPA axis is never mentioned in the manuscript, while crucial for stress, I would recommend removing it.*

- The term “HPA axis” has been removed.

Introduction:

2. *Reorganization of paragraphs: there seems to be a break in the flow of the manuscript in the first 3 paragraphs. I would recommend moving paragraph 2 (lines 77-89) right after line 69. Then move and combine lines 70-75 with paragraph 3.*

- We thank the reviewer for this suggestion. However, since the second paragraph introduces DNA methylation, we thought that it would be better served to keep that paragraph in place. Rearranging the paragraphs would introduce RRBS, which is a method for assessing DNA methylation, followed by DNA methylation as one epigenetic modification.

3. *Line 69, I suggest changing "animals" by "species".*
- The sentence has been changed to clarify what we meant by functional data: "However, there have been delays in the implementation of epigenomic platforms that incorporate available genomic sequencing data in their design due to a lack of annotated data of species-specific regulatory sequences that can influence gene function."

4. *Add references on the use of 450k (line 73). Add refs for 450K uses.*
- Two references have been added.

5. *Paragraph 3 has more detailed description of the 450k and barely no description of the SureSelect approach that has been designed and used for human and for mouse (by the same authors), what regions are covered, number of CpGs, description of the CpGs, etc. In addition, the authors could include discussion of the Illumina TruSeq Methyl Capture EPIC library prep kit.*
- We have been advised to not mention specific commercial names, except at the beginning of the manuscript only for items that are essential. Therefore, we cannot include a discussion of the Illumina TruSeq Methyl Capture EPIC library prep kit. We have now added details for the description of the rat Methyl-Seq platform in the step 5.1 of the protocol: "Note: The final rat Methyl-Seq design consists of 111 Mbps, 2.3 million CpGs; and an average region size of 594 bps."

6. *Paragraph 4 (lines 106-111): the rat is also useful for neuroendocrinology studies, for instance, the authors use the rat for stress studies, and others also use them as a model to study puberty (for example), so the use of rat could be expanded to include, at least, the area of interest of the authors.*
- We have added the following: "The rat has served as an important animal model in pharmacology, metabolism, neuroendocrinology, and behavior."

7. *Line 109: it is unclear what the authors refer with "changes". Changes due to what in particular?*
- We have changed the sentence to: "For example, there is an increasing need to understand the underlying mechanisms that give rise to drug toxicity, obesity, stress response, or drug addiction. A high-throughput platform capable of capturing methylomic changes associated with these conditions would increase our understanding of the mechanisms."

Protocol:

8. *Animals: under which light/dark conditions were the animals maintained prior to the beginning of CVS? Food and water was available ad libitum? Please add these details.*
- Information on animal husbandry has been added: "House the animals in polycarbonate rat cages in a temperature-and humidity-controlled room on a 12 h light, 12 h dark cycle with light onset at 0600 h. Provide the animals with *ad libitum* access to water."

9. *Endocrine assays: this section states that blood for CORT analysis is collected at 10AM, and later it says prior to the daily stress regimen, but the daily stress regimen seems to start at 8AM. Please clarify this discrepancy.*
- Thank you for catching this discrepancy. We have added the following: "House the animals in polycarbonate rat cages in a temperature-and humidity-controlled room on a 12 h light, 12 h dark cycle with light onset at 0600 h. Provide the animals with *ad libitum* access to water."

And “Determine levels of corticosterone (CORT) using tail blood (~50 μL) samplings collected at the same time (9 AM) two times per week throughout the experiment…”

10. *Line 151: add "use the plasma to determine…".*
    - Done.

11. *The CVS treatment lasts 3 weeks, according to the description, however, there is an additional measure at day 25 but no description on why and how this measure was taken.*
    - We have added the following sentence: “Collect one final trunk blood sample during euthanasia (Day 25) for RIA and genomic DNA extraction.”

12. *Design of the rat methylseq: add the references that were used in line 164. How many CpGs are covered?*
    - We have added the reference of the study used to generate a subset of the rat Methyl-Seq probes. Users interested in identifying potential regulatory regions for their own species have to find the appropriate references, if any. Additional information on the number of CpGs have been added in section 5.1.

13. *There is no description on how DNA was isolated*
    - We have had success using several methods based on columns or protein precipitation. Given that there are several methods, we added the following in the note section: “Extract DNA using a commercially available DNA extraction kit. Column- or precipitation-based methods both yield high-quality genomic DNA (260/280 ratio ~1.8). Use of phenol-based methods are not recommended.”

14. *There is no description on which blood time point was used to create the Methy-Seq libraries.*
    - We used genomic DNA extracted from trunk blood obtained on Day 25. This information is now added.

Sample preparation:

15. *The current Agilent protocol suggests the use of 1ug or 3ug of DNA, while the authors recommend the use of >2ug. Do the authors advice the use of more DNA than the minimum recommended by the protocol for every case? Are there particular cases where one amount or the other may be advisable?*
    - We advise users to follow the Agilent protocol closely by using either 1 μg or 3 μg of DNA. However, when those amounts are unavailable, we recommend using at least 500 ng of DNA. We have now added the following: “Note: The DNA amount recommended is 3 μg, or 1 μg. If there is limited starting material, the lowest input amount should be >500 ng, as lower amounts will adversely affect the quantity and quality of the libraries generated.”

16. *Maybe the authors could suggest a kit to use to purify DNA samples of poor quality.*
    - We have been advised to exclude mentioning specific items by manufacturers unless used in the protocol. We recommend DNA cleanup kits to get rid of excess salts and contaminants.

17. *A little bit more detail on the methods would be helpful. The idea would be for the users to refer to this paper instead to the agilent protocol, so a few recommendations at the beginning of the protocol's steps may be useful. For instance, a common comment could be that AMPure beads need to be at room temperature and well mixed before using them, do not let the pellet dry as this will reduce the recovery amount, etc. More specific comments for each step could summarize what is the goal of each step.*

- Additional details about AMPure beads and drying the pellets have been added throughout the protocol.

18. *At which points is it advisable to run the samples on a bioanalyzer to check the quality of the library?*
- We recommend at three time points: immediately after shearing to assess average fragment size, following adapter ligation to confirm increased fragment size, and after the last step of the Methyl-Seq library construction to assess average fragment size and quantity. These crucial points have been added in the protocol and discussion.

19. *Step 6.2.4, add the volume of wash buffer.*
- Done.

20. *Bisulfite conversion: explain why the sample is split into two wells (I assume is to ensure the temperature is maintained homogenously through the whole volume since the thermocycler maximum volume may be 100ul?).*
- Reviewer is correct. We have now added the following: "Note: The 150 µL reaction is divided equally into two separate wells to ensure homogenous temperature. After incubating for 2.5 h, immediately proceed to the next step."

21. *6.3.2. and 6.4.1. Change mixture by Master Mix to be consistent.*
- Thank you. Done.

22. *It would also be helpful to show an example on how the final concentration of the library is calculated, accounting for the size of the library, etc…*
- The bioanalyzer software calculates the area under the curve (of library bp size and abundance in loading volume) to calculate molarity. Each library sample is then diluted to 15 pM final concentration. We have now added the following to section 6.4.3.1: "Using the concentration data from the bioanalyzer, which determines DNA molarity based on library size and quantity in a given volume, dilute and combine all samples to a final concentration of 15 pM."

23. *Please also mention why the concentration is taken from the D1000 Screen Tape, in my experience, the concentration from Qubit is more accurate than that on the chips. So, a reasoning for the users on why is important to rely on the D100 Screen Tape may be important.*
- We agree with the reviewer that the Qubit is more accurate. We use the Qubit to determine dsDNA quantity prior to shearing. We use the D1000 Screen Tape afterwards because the TapeStation (generation 2 bioanalyzer) also provides the average fragment size, which we need to confirm successful adapter ligation. This is a critical part of preparing libraries because inefficient adapter ligation has ruined many library samples.

24. *Also, the authors could offer the option of using qPCR to quantify the libraries.*
- A more sensitive method of quantifying the library is by quantitative real-time PCR using primers that target the ligated adapters. We have now added: "Note: A more sensitive method of quantifying the library is by quantitative real-time PCR using primers that target the ligated adapters."

25. *Please, also explain how the pooling could be done, providing an example.*
- After obtaining the concentration of each sample (see response #22), which is at the 100-300nM range, the samples are diluted to 2 nM concentration in 150 µL volume. For the current experiment, 8 samples have been pooled to run on two lanes of the HiSeq-2500

platform. By combining 150uL of all 8 samples together at 2 nM concentration, we obtained a 250 pM library pool. The pooled samples are submitted to the sequencing core where the they are further diluted to 15 pM concentration prior to sequencing.

26. *Analysis to identify DMRs: the pipeline will give information on differentially methylated CpGs and Regions (DMCs and DMRs). But the authors only focus on DMRs, could you give a reasoning of why?*

- As indicated, the analysis pipeline also allows the user to identify DMCs. We used the dmrFinder function of BSseq/BSmooth to reduce the number of loci that implicate the same genes and to reduce the number of false positives, since identification of at least three consecutive CpGs that change in the same direction increases the probability of finding a real difference in methylation.

27. *There is no description of the statistical analysis used to identify DMCs or DMRs.*

- Since the statistical analysis is not the focus of the manuscript and not particularly insightful for the video portion of the publication, we did not include the details. Some of the details for the statistical analysis is as follows: BSseq was used to analyze the CpG level data across the samples. Using the genomic location and matrices consisting of M (methylation) and Cov (read coverage of CpGs) values, BSseq smoothes the M-values across CpGs using the BSmooth function, computes t-statistics between groups of samples using the function BSmooth.tstat and establishes the threshold levels of the t-statistics to identify DMRs using the function dmrFinder. For smoothing, default parameters were used with the exception of the smoothing window size set to 500, and the minimum number of CpGs within the smoothing window set to 20. For computing the t-statistics between the CONTROL and STRESS groups, CpGs with at least 10x coverage across all samples were used. The t-statistics were not smoothed or corrected. To determine the threshold of the t-statistics to use in the dmrFinder, quantiles for the t-statistics for the entire genome was calculated. The threshold for including consecutive CpGs in a DMR was the 99th percentile (tail ends) of the t-statistic distribution. Regions with 3 or more CpGs and greater than or equal to 10% mean methylation difference between CONTROL vs. STRESS groups were ranked and displayed by “areaStat” and subjected to further investigation.

28. *The bioinformatics steps could be more detailed, the sequence of each step could be added. Not everyone will be familiar with the pipeline which is crucial to identify DMRs, so a more extensive description of the steps and statistical analysis is advised. For instance, the size of the DMR is probably going to depend on the parameters selected in the pipeline, and these details are important. Is there deconvolution applied?*

- A more detailed analysis is described above. Once again, we refrain from expanding the analysis section because while crucial part of the study presented, it is not the focus of the JOVE publication. Unfortunately, no deconvolution dataset is available for the rat methylome as they do for humans.

29. *There is also no description of what power is needed for this type of studies.*

- Since this is a methods paper that uses the stress model as an example, no power calculation was performed prior to the study. However, a posthoc calculation shows that a sample size of N=8 per group has greater than 90% power for effect sizes as low as approximately 1.25 (by Cohen’s D). We note that in our previous work with the Methyl-Seq platform (Seifuddin et al., 2017) and the CVS animal model, we have observed differential DNAm differences of greater than 10% (minimum effect size = 1.8) in VEHICLE vs. CORT-treated or unstressed vs. stressed animals.

30. *Describe FDR (false discovery rate)*

- Done.

31. *Do the authors use any software to design the pyrosequencing primers? If so, please add.*
    - The PyroMark software by Qiagen allows users to design nested primers only. However, do not use this software, but instead use the guidelines included, since the software has often failed to provide usable primer sets.

32. In here, the authors could give an example of the primers used to amplify the DMR in RT1-M4.
    - The primers have been included.

rRT1M4 Outside - F	TGTAYGATTTTGGTTATYGTAAAT
rRT1M4 Outside - R	AACTTACAAATTTCACCAACTCA
rRT1M4 Nested - F	GTGGGTTAYGTGGATAATATATAG
rRT1M4 Nested - R	AATCACTTACCATTCTCTCTCTAACTA
rRT1M4 Pyro1	TAYGTGGATAATATATAGAT
rRT1M4 Pyro2	GATAGTTATTTGGYGAGTTAG
rRT1M4 Pyro3	GAGTATTTGGAGGAGTTGAT
rRT1M4 Pyro4	GGATTTTAATATTTGGT

33. *A schematic on how the primers are designed would be helpful. It is a bit unclear what is the pyrosequencing primer and the nested primer.*
    - We have edited the primer design step to better distinguish the nested vs. pyro sequencing primers: “Note: Two sets of PCR primers (outside and nested) are designed so that the nested PCR will amplify 150-400 bps of a DMR. In general, designed primers are at least 24 bases long with at least 4-5 non-consecutive G’s (C’s for the reverse primer) to account for reduced annealing temperature from loss of sequence complexity. One of the nested primers will be biotin-labeled and HPLC-purified. However, standard primers should be ordered first to optimize the PCR step by resolving the reactions on an agarose gel.”

34. *9.2. in the note add that those steps are from the Zymo kit and not from your protocol here.*
    - Since we cannot provide commercial names in the methods section, the name of the manufacturer (Zymo Research) has already been added in the Materials List.

35. *In step 9.3.1.1. would the amount of DNA added (3.5ul) the same whether the original DNA amount was 50 or 100ng?*
    - Elution from 100 ng of DNA results in approximately twice the amount of bisulfite-converted DNA available compared to that from 50 ng of DNA. Using the same volume of 3.5ul does not adversely affect PCR quality, since both amounts are sufficient for bisulfite PCR.

36. *Could you also distinguish the Forward and Reverse primers used in the outside and nester PCRs, please? In both case they are labelled the same.*
    - Forward and reverse primers are designated by “F” and “R,” respectively.

37. *Is there purification of the outside PCR prior to the nested PCR?*
    - 2 uL of the outside reaction can be used directly for nested PCR.

38. *You could also add the gel image for the resulting pyrosequencing reaction.*

- The pyrosequencing reaction does not provide a PCR product that can be visualized on a gel. The reaction involves luminescence during primer extension. The final images are pyrograms of the peak heights that reflect nucleotide incorporation. Since our main goal of including pyrosequencing is to validate the Methyl-Seq, we chose not to include additional details on pyrosequencing.

39. *The authors could also add explanations of what could go wrong at the different steps (not all, but at least some of them), and provide options. For instance, if when running a bioanalyzer chip the size is different than expected, what could be the cause and what the user could do.*

- We have added the following: “Second, if the average DNA size does not increase by more than 30 bps, check to ensure that the reagents are new, as T4 DNA polymerase, Klenow, and/or T4 ligase may be old. Commercially available replacement reagents can be used.”

RESULTS:

40. *No statistical analysis for the CORT results or the pyrosequencing validation data.*

- We have added the following statistical analysis that better reflects the figures: “Throughout the three-week regimen, the stressed animals exhibited significantly elevated levels of mean plasma CORT [Days 4-21, Control: 32.7 $\pm$ 3.7 ng/mL, Stress: 103.0 $\pm$ 11.9 ng/mL (mean $\pm$ SEM), $P=2.2x10^{-4}$]” and “Results demonstrate significant increase in DNA methylation across 10 out of the 12 CpGs assayed (5.1-10.4 change in % methylation, $P < 0.037$).”

41. *During the CVS paradigm, what happened to the unstressed/control animals? Where they in the same room as the stressed animals?*

- All of the stressors are administered in a different part of the animal facility away from the unstressed rats to minimize olfactory and auditory contact between the two groups.

42. *What is the CORT measure on day 25?*

- The CORT measurement is shown on day 25. However, the measurement was made from the plasma derived from trunk blood during euthanasia.

43. *I assume the EPM was performed on day 21, and not on day 25, right?*

- EPM was performed on Days 23-24 prior to euthanasia. We gave all of the animals, especially the stressed group, one day of inactivity prior to behavior testing. We have now added: “After the CVS regimen (Days 23-24), assess each animal for anxiety-like behavior on the elevated plus maze (EPM).”

44. *Figure 2B could be represented as boxplots with each data point represented, since the sample size is small.*

- Done.

45. *After line 496 you could add: "… significant endocrine and behavioral changes, leading us to investigate if these changes were associated with specific DNA methylation signatures…"*

- Thank you for the suggestion. The text has been edited accordingly.

46. *I would remove "Further" from line 497.*

- Done.

47. *The authors explain the reasons why starting with high quality DNA is important, and they start with 2ug of DNA. This amount could be a problem for some studies, where such amount of DNA is not available. The agilent protocol recommends the use of 1ug (or 3ug) of DNA, and the Illumina EPIC works with 500ng of DNA. This could be more appealing for users that have limited availability*

of DNA. Suggesting the use of 2ug of starting DNA could be discouraging for some users. Could the authors add a bit more of discussion on what to do when the amount of DNA is below 2ug or even 1ug? What would be the options for still being able to use the Methyl-Seq with smaller amounts?

- We have now added the following: “We note that Methyl-Seq libraries can be made with starting DNA amount as low as 500 ng. Smaller starting material allows users to generate libraries from DNA isolated by FACS (fluorescence-activated cell sorting) or needle punches, although there is increased risk of having insufficient libraries available for sequencing.”

48. *It is unclear what the authors are trying to explain in lines 505-509. Please clarify.*

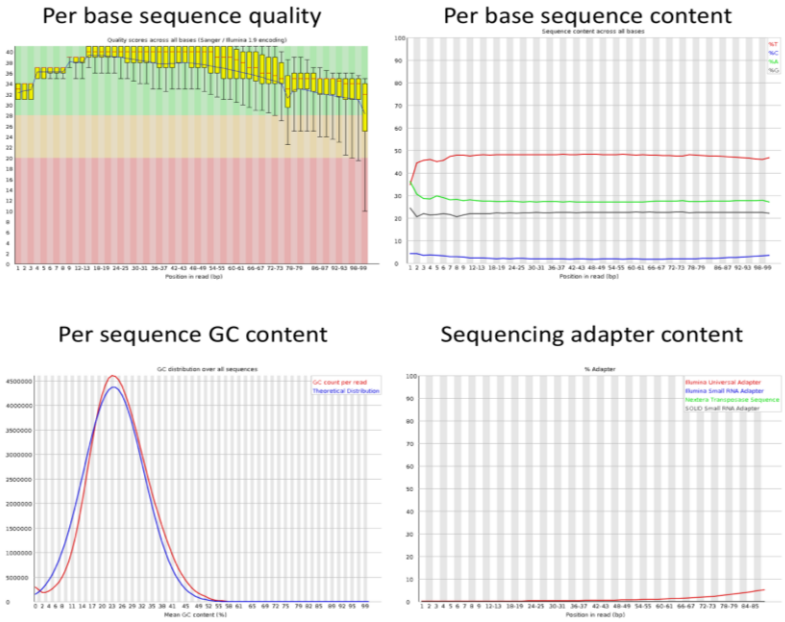
- We apologize for the incoherent language. We wanted to emphasize the specific times when bioanalyzer assessment of the samples were necessary. We have edited the sentences as follows: “Three critical steps that require the use of the bioanalyzer are: 1) following sonication step to ensure sufficient shearing of DNA (~170 bp, red, **Figure 3**); 2) following adapter ligation step indicated by a shift in the average size of the sheared DNA (~200 bp, blue, **Figure 3**) to ensure their subsequent amplification by PCR; and 3) following final library purification step to ensure the quantity and size of the library for sequencing.”

49. *BSmooth is not introduced in the methods section. Getting back to the point that a more detailed bioinformatics analysis will be needed. Some information from lines 511-528 could go to methods.*

- We agree with the reviewer that the pipeline would be best served in the methods section. However, we had difficult time incorporating the directions into commands without invoking the script lines that were used. Since our main focus was in generating the libraries that *can be* analyzed and independently validated, we decided to keep the text in the representative results section.

50. *Could you add some of the plots to guide the user in what to expect or what he/she should look forward?*

- We have added some of the many QC figures here for the reviewer. However, we do not include these figures since the sequencing aspect of Methyl-Seq is not a focus of the current study and not amenable for the video portion of JOVE. Following figures show four of the many sequencing QC plots that are generated.



51. *I assume the additional 8 animals used for validation, went through the same CVS protocol and showed the same results. No information is given.*

- All of the animals (N=16) were stressed or unstressed at the same time. We have edited the text to state the following: "To confirm successful implementation of the Methyl-Seq platform and the data analysis, primers were designed against the DMR, and blood DNA methylation levels in the entire cohort of stressed and unstressed animals (8 sequenced by Methyl-Seq and 8 not sequenced) were assessed by bisulfite pyrosequencing."

52. *How is the KEGG analysis done in all the DMRs? Do you refer to the genes mapped by the DMRs?*

- The KEGG analysis was performed using identities of the closest gene to each DMR.

53. *Why only CpG10 is chosen for correlation analysis?*

- We chose CpG-10 since it showed the biggest difference in DNA methylation between the groups. Several other CpGs showed similar correlation between DNA methylation and mean CORT levels. However, correlation coefficient was not as high. We thought that this approach was acceptable as a primary example, as our purpose was to just show a representative result.

54. *The authors could also use TRANSFAC or similar to predict transcription factors binding to the DMR.*

- We thank the reviewer for this suggestion. Initial analysis showed that many of the DMRs were putative binding sites for the glucocorticoid receptor among many other factors. However, since our main goal for this study was to demonstrate a meaningful biological association between DNA methylation stress exposure, we deemed such an analysis beyond the scope of the study to merit its inclusion.

55. *Is there any direct relationship between the genes identified and the CORT levels?*

- We have not done any background search to assess whether a statistically significant number of genes are related to glucocorticoid signaling. However, we note that our KEGG pathway analysis has implicated type 2 diabetes, cardiovascular disease, and cancer, all of which are associated with chronic exposure to stress.

Figures and tables:

56. *Figure 1 legend: instead of a fraction, use the amount of DNA (or range) that is used.*

- We have added the following: "Approximately one μg of the genomic DNA extracted from the blood of stressed and control rats is first processed for constructing the Methyl-Seq libraries for sequencing, analysis, and target identification. Another 100 ng is used for independent validation of the identified epigenetic targets by bisulfite pyrosequencing."

57. *Figure 2: change bar plots to boxplots with data points. Add statistical method used in the legend.*

- Done.

58. *Figure 4A: it looks like data from all 4 animals per group, was not available per each CpG. Could you explain? Was that data missing or discarded?*

- On specific CpGs where not all 4 data points are present, it is possible that a data point may have overlapped with another, especially near 0% methylation, or some of the CpGs did not meet the threshold of 10X coverage. The smoothing function borrows CpG methylation levels from nearby CpGs to estimate overall methylation average across the DMR.

59. *Figure 4B: change the bar plots by boxplots with data points. Also add statistical analysis in legend.*

- For this one we chose to keep the bar graph, as the methylation values were consistent within each group (unlike the Figure 2B EPM data) and all of our previous methylation data have been displayed as such. We have added the statistical analysis used in the figure legend.

60. *Figure 5: could you differentiate the controls and stressed animals using different dot type of color?*

- The stressed animals are now represented by red circles.

61. *Figure 5 legend: add statistical analysis.*

- Done.

62. *Table 2: could you describe the different measurements taken and what the differences are.*

- All of the terms such as PER, UMPER, and ARDC have been defined in the first column of the table. The purpose of this table is to show the various assessments that were made from sequencing of the Methyl-Seq libraries before and during alignment and CpG read depth coverage. We displayed the metrics for both control and stressed animals separately to show that the metrics are similar for both groups.

63. *Table 3: add description of the columns headings.*

- We have added the following to the Table 3 description: "For each DMR, the output table shows from the left to right column: chromosomal location (chr), coordinates (start/end), gene name, distance from the transcription start site, differential area statistics between stressed and control groups (areaStat), mean differential methylation (meanDiff), mean methylation levels across each DMR for stressed and control groups (stress/control), and direction of methylation change from controls)."

DISCUSSION

64. Line 599. This sentence is unclear and it does not make sense with the rest of paragraph.

- We have edited the sentence as follows: "We also include several guidelines in the event of deviation from the protocol or if problems are encountered."

65. *If additional PCR cycles are needed when working with low amounts of DNA, at which PCR do the authors recommend them to be added?*

- We recommend performing additional cycles (2-3) at 6.4.1, when the samples are getting amplified with the index primers.

66. *Also, do the authors have experience using this protocol with <200ng of DNA or using it with FACS cells? If that is the case, what where the modifications employed in these circumstances?*

- We do have experience using the Methyl-Seq protocol following FACS when the amount of DNA was between 100 to 500 ng. We have had limited success when the amount was close to 100 ng. We have also employed alternative methods where bisulfite conversion is performed prior to adapter ligation. Unfortunately, the percentage of alignable reads were less than 10%. We found that the original protocol described here works well with DNA amounts as low as 300 ng.

67. *It is unclear if BSmooth is one of the reasons why Methyl-Seq and pyrosequencing may differ, could the authors clarify this section?*

- The reviewer is correct in suggesting that BSmooth is the reason for the different results observed between pyrosequencing vs. Methyl-Seq. Since the smoothing function takes the average across all of the CpG values within a DMR in determining percent methylation differences, pyrosequencing assays that assay across a subset of those CpGs in a DMR will provide values that may be different. In addition, Methyl-Seq is semi-quantitative in nature given the limited read depth. Methylation values obtained from such a semi-quantitative assessment stands to be different from a much more quantitative methods such as pyrosequencing. We have now added the following: "For the current experiment, BSmooth implicated a DMR whose methylation levels were validated by bisulfite pyrosequencing. However, there will likely be discrepancies between methylation levels predicted by BSmooth and those verified by pyrosequencing. The discrepancies arise from the smoothing function that estimates the average methylation values across all of the CpGs within a DMR, including consecutive CpGs that may differ in DNA methylation by more than 50% or CpGs whose methylation values were excluded due to sub-threshold read depth. R-packages such as MethylKit[24] can be used to identify smaller windows of CpGs or even single CpGs whose methylation levels correlate strongly with those validated by pyrosequencing."

68. *Also the pipeline is quite different between MethylSeq and pyrosequencing, could this be contributing to differences in both methodologies?*

- Yes, the Methyl-Seq provides comprehensiveness (genome-wide) at the expense of accuracy. On the other hand, pyrosequencing is thought to be much more accurate given its focus on one candidate region. The key difference lies in the number of template DNA assessed (read depth) for determining % DNA methylation. This language has been added at the end of the fourth paragraph in the discussion.

69. *Is there any other methodology that could be used for validation, instead of pyrosequencing only? Or that is the best method the authors recommend?*

- For assaying candidate DMRs, pyrosequencing is widely considered to be the gold standard. However, for assessing DNA methylation at multiple loci simultaneously, the Mi-Seq platform can be used. The Mi-Seq platform enables multiple DMR amplicons to be sequenced at much higher read depth (>10K) to enable fairly accurate assessment of DNA methylation.

70. *The authors refer to testing different packages for DNA methylation analysis, could you refer to published papers that list them?*

- For those interested in additional packages for DNA methylation analysis, they can read, "Strategies for analyzing bisulfite sequencing data," Wreczycka et al., Journal of Biotechnology, 2017.

71. *Overall, paragraph 3 of the discussion, may need some rewriting to clarify what the point of this paragraph is.*

- We agree that paragraph 3 is confusing. We have now changed the first sentence and edited the paragraph: "We also include several guidelines in the event of deviation from the protocol or if problems are encountered."

72. *Would the cost of the Methyl-Seq be reduced by pooling more samples per lane and using HiSeq4000? There is no information on how many samples were sequenced per lane.*

- The cost per sample of Methyl-Seq can be reduced by running the pooled samples on a higher-capacity platform than the HiSeq2500 that was the platform used for rat Methyl-Seq.

We have now added additional information about the number of pooled samples run per lane: “Run pooled samples on the number of lanes that are sufficient for four samples per lane on a next-generation sequencer. Note: For instance, if 16 library samples have been uniquely indexed and combined, run the libraries over 4 lanes, equivalent to 4 samples per lane.”

73. *Paragraphs 4 and 5 could be combined, since 5 describes the pros of using this method. An additional advantage of using this method is in the number of CpGs investigated as compared for example with the 450k. The Methyl-Seq has been successfully applied to other species, like mouse and non-human primates.*

- We agree with the reviewer, and the paragraphs have been combined. We added the following: “Overall, the Methyl-Seq platform is a cost-effective alternative to whole-genome sequencing and provides base-pair resolution at more than 2.3 million CpGs, which is considerably more than those assayed by microarray-based platforms. To date, the commercially-available human and mouse Methyl-Seq platforms have been used to document alcohol-dependent changes in the macaque brain, neurodevelopmental genes in the mouse brain,[9] and blood-brain targets of glucocorticoids.”

74. *Would it be possible to use the probes designed in the human version and select the homologous regions in mouse or rats, using a conservation threshold, to perform cross-species comparisons of regions of interest?*

- We advise against using the human Methyl-Seq to enrich for homologous regions in rodents. Since the Methyl-Seq design targets non-coding regulatory regions, most of which are not strongly conserved, it is unclear how many of the homolgous loci can be enriched. To maximize the number of loci that can be analyzed from other species, it might be necessary to design a new human Methyl-Seq platform based on non-exonic sequence conservation rather than gene regulation in the event of poor overlap of loci. However, we do note that a cross-species comparison can be performed when the two species are closely related. For instance, Cervera-Juanes et al. (2017) have used the human Methyl-Seq platform to capture macaque DNA. This is possible due to the high degree of conservation in the non-coding regions of both genomes.

Reviewer #2:

Manuscript Summary:
present their protocol using a rat model of chronic stress, to demonstrate the rat-methyl-seq platform. They use the SureSelect Methyl-Seq Target Enrichment (using Agilent SureDesign) for CpG islands, promoters and shores located close to genes in the rat genome that are then sequenced using Illumina.

Major Concerns:
1. *I think to more focus on the methodology rather than inclusion of the CORT data and discussions of the model.*

- We agree with the reviewer. The central methodology revolves around the Methyl-Seq platform. We have included the information on the CORT data and short discussion of the model only to provide motivation and the justification to examine DNA methylation. We had wanted to not only demonstrate the Methyl-Seq platform per se, but the platform in the context of answering a specific question.

2. *I think the pyrosequencing could be shown as a validation of the method - but this does not need to be part of this protocol as it distracts from the main message. Same with the endocrine assay and behaviour.*

- We mulled over the inclusion of the pyrosequencing protocol. However, we decided to include it to serve as a comparison for the more lengthy Methyl-Seq protocol.

3. *More information on the SureSelect target enrichment is needed. How many regions, what coverage is needed etc. Perhaps a table? This would also help inform how many samples to pool.*
- We have added the following in section 5: “Note: The final rat Methyl-Seq design consists of 111 Mbps, 2.3 million CpGs; and an average region size of 594 bps. It targets 228,800 unique loci.” We have also added suggestions for the number of sample and lanes for sequencing: “Note: For instance, if 16 library samples have been uniquely indexed and combined, run the libraries over 4 lanes, equivalent to 4 samples per lane.”

4. *Have other used this on other tissues than brain.*
- We have added the following sentence: “To date, the commercially-available human and mouse Methyl-Seq platforms have been used to document alcohol-dependent changes in the macaque brain, neurodevelopmental genes in the mouse brain,[9] and blood-brain targets of glucocorticoids.”

5. *How was the DNA extracted? What purity and amounts are needed.*
- For the current study, we used the Epicentre MasterPure DNA Extraction Kit. However, we have also extracted good-quality DNA (260/280 ratio ~1.8) using column-based kits such as ones available from Qiagen. We have added the required purity and amount in the text: “Extract DNA using a commercially available DNA extraction kit. Column- or precipitation-based methods both yield high-quality genomic DNA (260/280 ratio ~1.8). Use of phenol-based methods are not recommended. Elute or resuspend DNA in low TE buffer (10 mM TE, 0.1 mM EDTA, pH 8.0).”

6. *Title and method would be best to state SureSelect Methyl-Seq: Implementation of the Rat "SureSelect" Methyl-Seq platform to identify epigenetic changes associated with stress exposure.*
- Unfortunately, we have been advised to omit commercial names from the manuscript as much as possible. The SureSelect name is mentioned in the introduction.

7. *The analysis to identify DMRs could provide more overview, detail and figures - in addition to the tables that are useful.*
- Please refer to our response to the first reviewer’s comment #27.