

AWARD NUMBER: W81XWH-14-1-0550

TITLE: Epigenetic Mediation of Endocrine and Immune Response in an Animal Model of Gulf War Illness

PRINCIPAL INVESTIGATOR: Patrick McGowan, PhD

CONTRACTING ORGANIZATION: University of Toronto,  
Toronto, Ontario, Canada, M5S 1A1

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |              |                          |                            | Form Approved<br>OMB No. 0704-0188            |                                           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|--------------------------|----------------------------|-----------------------------------------------|-------------------------------------------|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>                                                                                                                                                                                                      |              |                          |                            |                                               |                                           |
| 1. REPORT DATE<br>October 2015                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |              | 2. REPORT TYPE<br>Annual |                            | 3. DATES COVERED<br>30 Sep 2014 - 29 Sep 2015 |                                           |
| 4. TITLE AND SUBTITLE<br><br>Epigenetic Mediation of Endocrine and Immune Response in an Animal Model of Gulf War Illness                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |              |                          |                            | 5a. CONTRACT NUMBER                           |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                          |                            | 5b. GRANT NUMBER<br>W81XWH-14-1-0550          |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                          |                            | 5c. PROGRAM ELEMENT NUMBER                    |                                           |
| 6. AUTHOR(S)<br><br>Patrick O. McGowan, PhD, Gordon Broderick, PhD, James O'Callaghan, PhD, Mariana Morris, PhD, Mary Ann Fletcher, PhD<br><br>E-Mail: patrick.mcgowan@utoronto.ca, gbroderick@nova.edu, jdo5@CDC.GOV                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              |                          |                            | 5d. PROJECT NUMBER                            |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                          |                            | 5e. TASK NUMBER                               |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                          |                            | 5f. WORK UNIT NUMBER                          |                                           |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>University of Toronto, 27 King's College Cir, Toronto M5S 1A1, Canada<br><br>Centers for Disease Control, NIOSH, 1095 Willowdale Road, Morgantown, WV 26505<br><br>Nova Southeastern University, 3100 SW 9 <sup>th</sup> Avenue, Fort Lauderdale, FL 33315-3025                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |              |                          |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER      |                                           |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              |                          |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)              |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                          |                            | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)        |                                           |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |              |                          |                            |                                               |                                           |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |              |                          |                            |                                               |                                           |
| 14. ABSTRACT<br>There are now compelling human epidemiological and animal experimental data that indicate the risk of developing complex diseases is influenced by persistent epigenetic adaptations in response to environmental exposures such as toxins and stress. We propose to examine the epigenomic response to diisopropyl fluorophosphates (DFP), a sarin surrogate, and associated changes to the immune and endocrine response to lipopolysaccharide (LPS) challenge in a mouse model of Gulf War Illness (GWI), with stress hormone exposure as an experimental mediator. We will study the relationship between changes in DNA methylation and chromatin modifications in peripheral blood and the brain (specifically hippocampus and prefrontal cortex) in order to pursue a mechanistic understanding of the underlying pathology of GWI. During this reporting period, we have begun data collection on DNA methylation modifications and gene expression profiles in the brains of mice exposed to saline control, corticosterone and DFP, and have developed and refined protocols for this project in line with the GWIRC sister project. |              |                          |                            |                                               |                                           |
| 15. SUBJECT TERMS<br>Hypothalamic-Pituitary-Adrenal (HPA) axis, epigenetic, hippocampus, prefrontal cortex, lipopolysaccharide (LPS), diisopropyl fluorophosphate (DFP), corticosterone (CORT), peripheral blood mononuclear cell (PBMC), DNA methylation, Histone acetylation, immune system, genomics, mouse model                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |              |                          |                            |                                               |                                           |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                          | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                           | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | b. ABSTRACT  | c. THIS PAGE             |                            |                                               | USAMRMC                                   |
| Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Unclassified | Unclassified             | Unclassified               | 30                                            | 19b. TELEPHONE NUMBER (include area code) |

## Table of Contents

|                                                                     | <u>Page</u> |
|---------------------------------------------------------------------|-------------|
| <b>1. Introduction.....</b>                                         | <b>4</b>    |
| <b>2. Keywords.....</b>                                             | <b>4</b>    |
| <b>3. Accomplishments.....</b>                                      | <b>4</b>    |
| <b>4. Impact.....</b>                                               | <b>11</b>   |
| <b>5. Changes/Problems.....</b>                                     | <b>11</b>   |
| <b>6. Products.....</b>                                             | <b>12</b>   |
| <b>7. Participants &amp; Other Collaborating Organizations.....</b> | <b>12</b>   |
| <b>8. Special Reporting Requirements.....</b>                       | <b>14</b>   |
| <b>9. Appendices.....</b>                                           | <b>15</b>   |

## **Introduction**

Epigenetic changes, including modifications to chromatin structure and DNA methylation, alter gene expression and cellular phenotype in the absence of variation in DNA sequence. There are now compelling human epidemiological and animal experimental data that indicate the risk of developing complex diseases is influenced by persistent epigenetic adaptations in response to environmental exposures such as toxins and stress. We propose to examine the epigenomic response to diisopropyl fluorophosphates (DFP), a sarin surrogate, and associated changes to the immune and endocrine response to lipopolysaccharide (LPS) challenge in a mouse model of Gulf War Illness (GWI), with stress hormone exposure as an experimental mediator. We will study the relationship between changes in DNA methylation and chromatin modifications in peripheral blood and the brain (specifically hippocampus and prefrontal cortex) in order to pursue a mechanistic understanding of the underlying pathology of GWI. Epigenetic profiles indicative of transcriptional enhancement or repression may help identify stable biomarkers of GWI related to exposure across a variety of environmental conditions. Because epigenetic marks are potentially reversible, elucidating the manner in which environmental interventions including immune-endocrine stressors alter epigenetic marks across the blood-brain barrier in the context of toxin exposure will offer insight into mechanisms leading to stable disease states and novel routes to therapeutic intervention. The overall objective of this project is to identify epigenetic mechanisms of altered Hypothalamic-Pituitary-Adrenal (HPA) axis and immune signaling in a mouse model of environmental exposures linked to GWI. DNA methylation and histone modifications will be examined in peripheral blood and the brain using a high-throughput genome-wide approach. This proposal adds substantial value to a funded GWIRP Consortium project designed to examine gene regulatory dynamics in a model of toxicant exposure (DFP, a sarin surrogate) and stress/immune challenge (CORT, LPS).

## **Keywords**

Gulf War Illness, Hypothalamic-Pituitary-Adrenal (HPA) axis, epigenetic, hippocampus, prefrontal cortex, lipopolysaccharide (LPS), diisopropyl fluorophosphate (DFP), corticosterone (CORT), peripheral blood mononuclear cell (PBMC), DNA methylation, Histone acetylation, immune system, genomics, mouse model

## **Accomplishments**

### **What were the major goals of the project?**

The major goals of the project and associated milestones (month identified for completion/actual completion dates/percent completed) as stated in the approved Statement of Work (SOW) were:

**Task 1:** Validate mouse model and perform manipulations of animal subjects.

- Milestone: IACUC/ACURO Approval. (**month 3/December 2014, 2015/100%**)

**Task 2:** Extraction of tissue samples and blood sample processing.

- Milestone: Validation of manipulation and collection of tissue samples (**Month 3/June 2015/40%**).
- Milestone: DNA from blood prepared for Sequencing (month 5/not yet completed)
- Milestone: DNA library preparation for sequencing of blood (month 9/not yet completed)
- Milestone: Blood epigenetic paper (month 18/not yet completed)

**Task 3:** Extraction of tissue samples and brain sample processing.

- Milestone: DNA from brain prepared for Sequencing (**month 8/Sept 2015/25%**)
- Milestone: DNA library preparation for sequencing of brain (**month 10/Sept 2015/25%**)
- Milestone: Brain epigenetic paper: (month 20/not yet completed)

**Task 4:** Gene expression analysis on blood and brain

- Milestone: Gene expression profiles for blood, hippocampus and prefrontal cortex (**month 9/Sept 2015/5%**)

**Task 5:** Analysis of epigenetics and gene expression across tissue types

- Milestone: Identify epigenetic correspondence across tissue types (month 28/not yet completed)
- Milestone: Functional epigenetics paper (month 36/not yet completed).

**What was accomplished under these goals?**

- 1) The specific objectives described in the approved SOW were:

**Specific Aim 1:** Assess epigenetic changes in peripheral blood in response to DFP exposure concomitant with sustained corticosterone stress hormone administration and subsequent antigen (LPS) challenge

**Specific Aim 2:** Assess epigenetic changes in brain as a function of DFP exposure, corticosterone administration and in response to LPS challenge

**Specific Aim 3:** Assess the relationship between epigenetic changes and gene regulatory dynamics in brain and in blood, and their relationship to transcriptomic and physiological outcomes

- 2) Major Activities and results during this reporting period:

Site visits: We have made site visits to review protocols and preliminary data to ensure alignment with the overarching Consortium GW120045 (project W81XWH-12-2-0085; Morris, PI).

Feb. 24-27 2015. Dr. Broderick met with Drs. O'Callaghan and Miller at CDC/NIOSH in Morgantown, WV:

- Delivered talk to NIOSH leadership and staff
- Discussed numerical analysis protocol with laboratory research staff, specifically the alignment of time course experiments between the current award and sisters projects conducted concurrently under the broader programmatic envelope of the GWI Consortium.
- Reviewed existing preliminary data, including initial whole genome profiling short-term (6-hour post-exposure) response to DFP, LPS and corticosterone (CORT) in cortex and hippocampus conducted with the Illumina mouse microarray.
- Coordinated sharing of existing RNA sequencing data with McGowan group for first review of most promising target genes

Mar. 30-31 2015. Dr. Broderick met with Dr. McGowan at University of Toronto. The visit consisted of an onsite review of animal experimental protocols being used under overarching project W81XWH-12-2-0085 with laboratory staff, specifically the timeline for dosing and exposure protocols proposed for GWIRC studies 1 and 2.

- Delivered a Department seminar describing aspect of the proposed numerical protocol

August 17-21 2015. Dr. Broderick met again with Drs. O'Callaghan and Miller at CDC/NIOSH in Morgantown, WV. Focus of this meeting was a through review of pathway projection methods and analysis of available data, in particular Illumina array profiling gene expression in astrocytes using a novel quenching and cell separation technique (BAC-TRAP)

Animal dosing: As stated in the goal for **Task 1**, we performed baseline and challenge studies on the animal

models, followed by animal sacrifice. We have revised our ‘n’ in order to determine variability in methylation status across conditions (see Bisulfite pyrosequencing, below and **Table 1** below for dosing schedule). Tissue samples, consisting of blood and brain from 44 animals were shipped from the CDC site (Dr. O’Callaghan) to UofT (Dr. McGowan) on June 24<sup>th</sup> 2015. This group was composed of 8 saline treated animals (T0 control), 8 CORT treated animals (T1 condition), 15 DFP treated animals (T2.1 condition) and 13 DFP+CORT treated animals (T2.2 condition). Mice were treated with CORT (200 mg/L 0.6% EtOH) for 4 days in the drinking water. At day 5, mice were treated with DFP (3.0 mg/kg s.c.) and sacrifice 6 hours later.

**Table 1. Animal dosing schedule:**

| Groups | 4 Day Treatment | Time Points                         |                                       |
|--------|-----------------|-------------------------------------|---------------------------------------|
|        |                 | Day 5<br>(sac -6 hours post dosing) | 21 Day<br>(sac – 6 hours post dosing) |
| 1      | Water           | Saline (N=10) [8 received]          | -                                     |
| 2      | CORT            | Saline (N=10) [8 received]          | -                                     |
| 3      | Water           | DFP (N=10) [15 received]            | Saline (N=10)                         |
| 4      | CORT            | DFP (N=10) [13 received]            | Saline (N=10)                         |
| 5      | Water           | DFP -                               | LPS (N=10)                            |
| 6      | CORT            | Saline -                            | LPS (N=10)                            |
| 7      | CORT            | DFP -                               | LPS (N=10)                            |
| 8      | Water           | Saline -                            | LPS (N=10)                            |
|        |                 | Total                               | 100                                   |

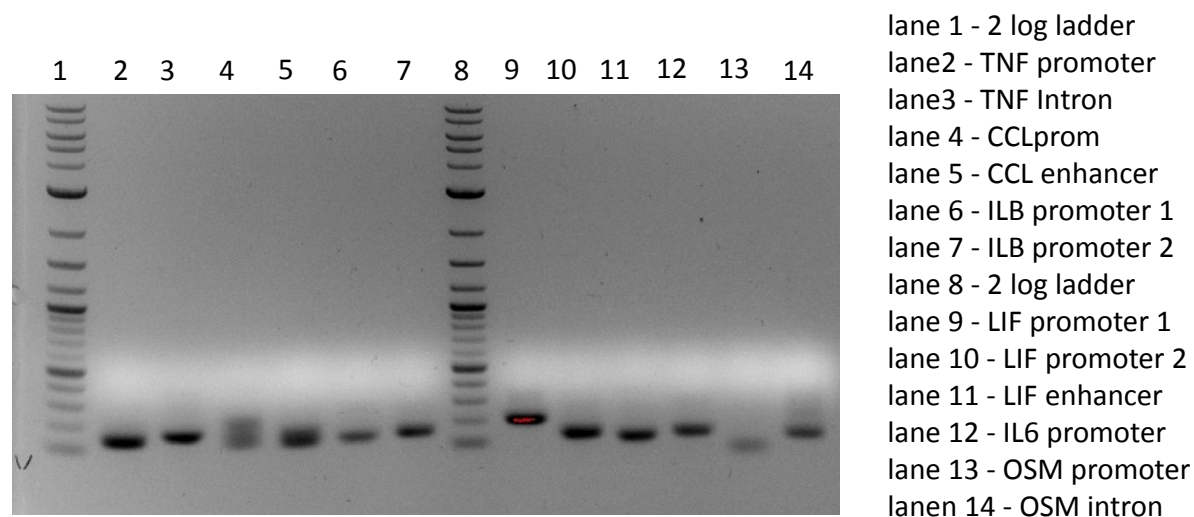
Trainee recruitment for molecular assays: We have recruited Dr. Benjamin Hing, postdoctoral fellow, Ms. Christine Lum, graduate student, and Mr. Shathveekan Sivanathan, technician, to U of T.

Blood and brain extractions: For **Task 2**, blood and brain samples were extracted and shipped from CDC to U of T. PBMC extraction was problematic from these samples, as cell separation was not ideal and thus few/no PBMCs were obtained from some samples, likely to due to shipping delays we encountered. As a result, RNA and DNA yield from PBMCs were inconsistent between samples.

Nucleotide extractions, validation and high throughput sequencing: For **Tasks 3 an 4**, we succeeded in harvesting the brain tissues (hippocampus and prefrontal cortex) described above (see Animal dosing) and have extracted good quality RNA and DNA from each of the samples above. We have made progress in DNA methylation and histone modification procedures in the following areas:

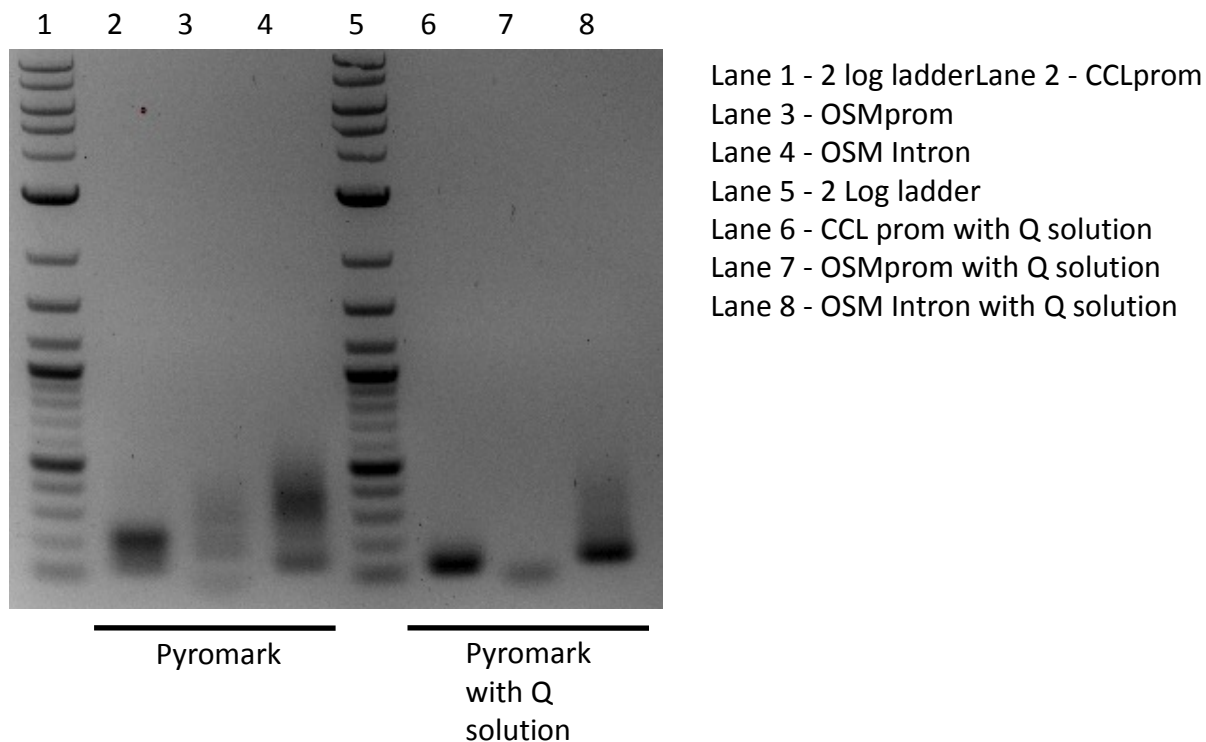
*Bisulfite-pyrosequencing for validation of DNA methylation modifications at specific loci:* It is presently unknown how variable methylation changes are between treatment groups for specific genes. This would have an impact on the number of samples required to observe robust DNA methylation changes between treatment groups by Reduced Representation Bisulfite-Sequencing (RRBS). To address this issue, we have designed bisulfite-pyrosequencing primer to validate DNA methylation changes in genes that are known to have altered gene expression levels in the mouse model of gulf war syndrome (O’Callaghan et al., J Neurochem, 2015). The genes are TNF- $\alpha$ , CCL, IL1- $\beta$ , LIF, IL6 and OSM. Primers were designed against promoter and proximal regulatory regions defined by acetylation of histone 3 lysine 27 (H3K27ac) which contained MspI restriction site (CCGG) and putative GR binding sites so that data obtained could be compared to RRBS data, which also uses MspI digested DNA for library preparation. PCR conditions have been optimised for these primer sets (see **figures 1-2** below and **Appendix 1** for preliminary data). This will be followed by pyrosequencing validation of the primer sets and profiling of the samples for each brain region. The validation is thus being done in parallel with high-throughput sequencing to maximize the efficiency of our experiments.

**Figure 1. Optimization of primer assays for Bisulfite Pyrosequencing: Part 1.**



Most genes show single bands indicating specific products. We have redone CCLprom (lane 4) with pyromark PCR and determine if pyromark PCR might improve OSM promoter (lane 13) and OSM intron (lane 14).

**Figure 2. Optimization of primer assays for Bisulfite Pyrosequencing: Part 2.**

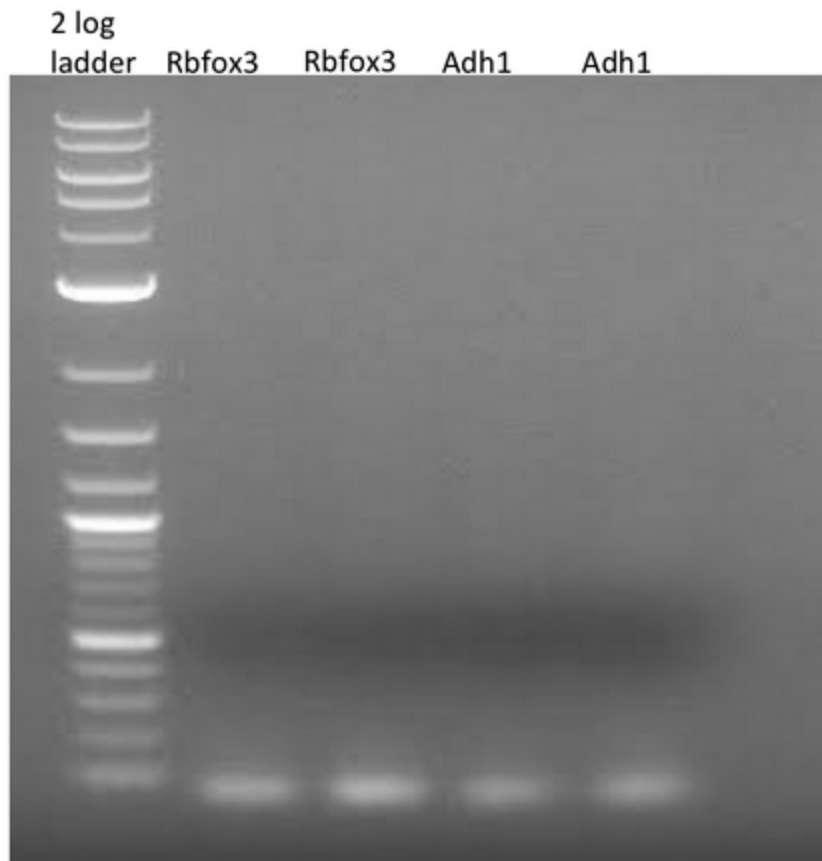


These results suggest that the addition of Q solution improves the specificity of the PCR for CCL promoter (Lane 6), OSM promoter (Lane 7), and OSM intron (Lane 8).

*Reduced Representation Bisulfite Sequencing (RRBS) for genome-wide DNA methylation modification analysis:* We have established logistic capabilities to carry out a novel one-day RRBS protocol at no additional cost to the grant, which would provide high-throughput library preparation of the samples and dramatically accelerate our objectives of preparing libraries (from an initially planned 1 week procedure). We have completed library prep of 8 samples consisting of 4 saline controls (T1) and 4 DFP+CORT (T2.2) samples in order to test our protocol. One of the saline controls will be performed in triplicate to determine technical variation of the assay. We are pooling 10 samples in a lane which should provide sufficient depth of coverage (10x) to detect at least a 10 % change in DNA methylation level. We have sent samples for DNA sequencing and are presently awaiting results from the core facility.

*Chromatin Immunoprecipitation (ChIP) sequencing for genome-wide histone modifications:* The ChIP protocol is currently in the optimization stage. We have first targeted H3K27ac, which is a histone modification mark of active regulatory regions. To validate the assay, primers were designed against alcohol dehydrogenase promoter (Adh1) and Rbfox3 which are genes expressed in the liver and in the brain, respectively. During validation of the ChIP-seq protocol, the signal produced from the pulldown by the target antibody was higher than that of the iso-type antibody showing target specificity of the target antibody. However, signal strength between Adh1 and Rbfox3 control genes were the same in the brain, which deviates from the data observed in the UCSC genomic database. Although melt curve of SYBR green qPCR showed single melt curve suggesting single amplified product. Product size was however bigger than expected suggesting off-target amplification. Using a different SYBR green mastermix (see **Figure 3** below and **Appendix 3** for preliminary data), size of the amplicon is now as expected. Another pulldown is required to determine if the change in SYBR green mastermix has resolved the issue.

**Figure 3. Validation of PCR for ChIP protocol with Adh1 and Rbfox3 control genes**



These data show single bands in each experimental well, indicating a specific product has been obtained of the correct size for each gene and that the PCR conditions have been optimized.

Nanostring quantitative transcript abundance analysis: We have initiated the studies described in **Task 4**. After

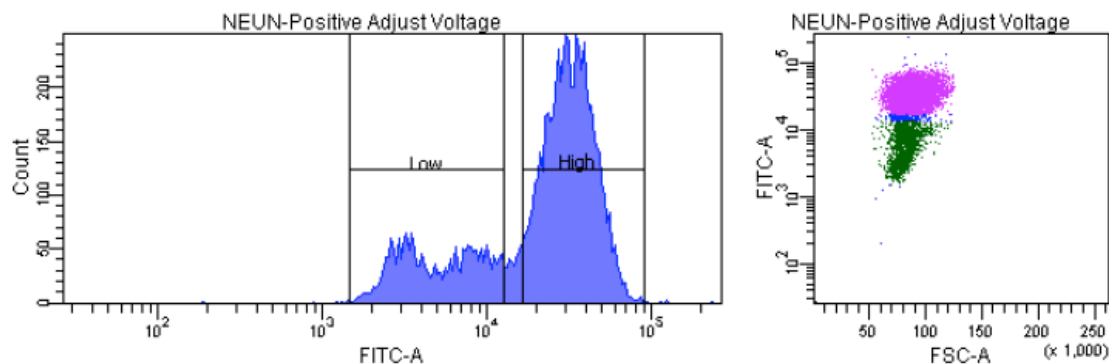


thorough review of human data available through the sister project and available nanostring kits for mouse, we have decided not to use a custom kit. Instead, we have selected the nCounter Mouse Immunology kit which provides a good coverage of pathways identified through work with our human samples at a much lower price. We have started QC runs using this chip (Dr. Nathanson, NSU).

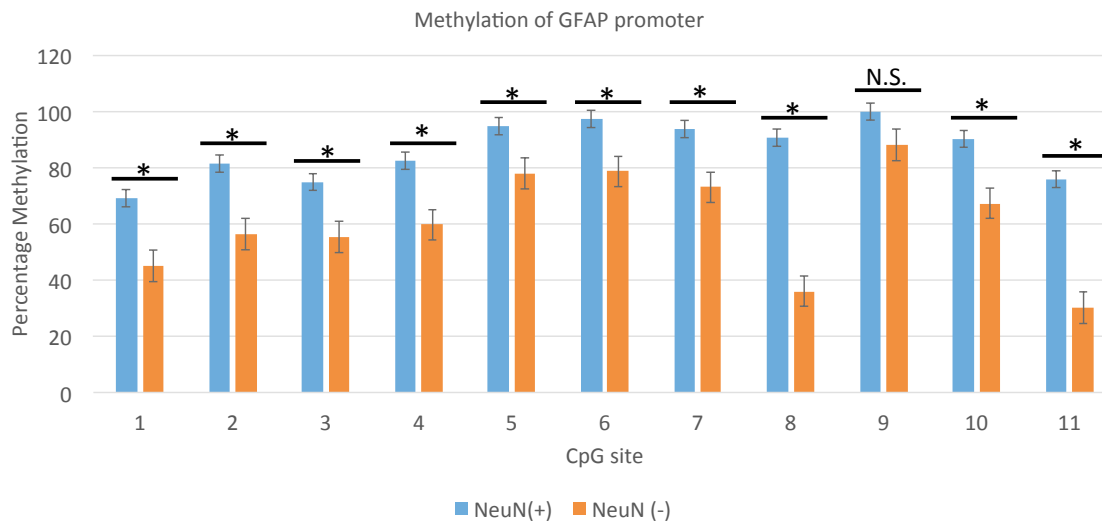
RNA sequencing: We have obtained Illumina RNA sequencing data from first exposure conditions (saline, CORT, CORT+DFP) available from the CDC database as a result of the sister project (see GWIRC annual report). We have begun to review these data to and have made adjustments to our basic software platform to broaden array of analyses possible with our epigenetic data.

Fluorescence-assisted Cell Sorting (FACS) of neurons and glia from brain tissue: DNA methylation is known to differ between neurons and glial cells (Iwamoto et al., 2011; Kozlenkov, NAR, 2014), the primary cell types in the brain. Work by our Dr. O'Callaghan (Co-PI on this project) has indicated that glia cells may drive changes in expression associated with the exposures used in this project. We conducted a pilot feasibility assessment using cell separation by Fluorescence-assisted cell sorting (FACS) of neural tissue into neurons and glia. The figures below show the results of FACS analysis of samples consisting of an n = 3 of a mix group 1 hippocampus and 2 cortices. FACS showed two peaks: A group with low level of NeuN staining and a group with a high level of NeuN staining. The NeuN group with low staining constituted ~25% of stained nuclei whereas NeuN group with high staining constituted ~70% of stained nuclei (**Figure 4** below). This is consistent with known proportion of glial cells to neurons ratio in brain regions such as the cortex. Studies have demonstrated that certain glial cells also express NeuN (Darlington et al., 2008), suggesting that the lowly stained nuclei are glial cells. Furthermore, as shown below, bisulfite pyrosequencing showed significantly higher methylation level in GFAP promoter for the highly stained nucleus compared to low stained nucleus further showing that highly stained NeuN nuclei are neurons and low stained nuclei are glial cells (**Figure 5** below). As such, the current data show that the FACS method is optimized for subsequent use to interrogate the epigenetics of neurons and glia.

**Figure 4. Cytometry with NeuN staining showing two groups of cells, assumed to be neurons and glia.**



**Figure 5. Bisulfite Pyrosequencing of DNA methylation in the GFAP promoter of NeuN+ and NeuN- cells separated by FACS, confirming neuron and glia status respectively.**



Statistical analyses: We recruited our research associate, Patrick Gourdet, into Dr. Broderick's group at NSU in late December 2014. Mr Gourdet has a background in computer science and we have been training him specifically on the use of the MatLab suite of bioinformatics tools and in the principles of high dimensional statistics as they apply to gene expression. We have also been training him on the algorithmic details of the pathway analysis technique proposed by Efroni et al. (2007).

### **What opportunities for training and professional development has the project provided?**

This project has provided technical training for postdoctoral fellow Dr. Benjamin Hing, graduate student Christine Lum and technician Shathveekan Sivanathan at UofT in laboratory activities related to the project during this reporting period. In addition, we recruited research associate Patrick Gourdet to Nova Southeastern University (NSU) and have been training him in high-dimensional data analysis including outlier detection and regression using projection methods such as Principal Component Regression (PCR) and Partial Least Squares (PLS).

### **How were the results disseminated to communities of interest?**

Some of our preliminary results were shared in reporting for the GWIRC. We have been refining our analytical protocol in collaboration with DoD with human data obtained through the sister project, and plan to apply this to the present award. Specifically, we have started pathway projection analysis on mouse data, and have completed a preliminary analysis of mouse DFP+CORT, DFP, CORT, and saline in cortex and hippocampus using Illumina RNA-seq data. We have begun to refine the resolution of the data related to quality control and pathway techniques to adjust from the highly variable human condition to the mouse condition consisting of smaller groups. Our techniques are being refined in collaboration Dr. Jacques Reifman of DoD. This will enable us to use this refinement for network analysis with epigenetic data in our mouse model.

### **What do you plan to do during the next reporting period to accomplish the goals?**

During the next reporting period, we plan to obtain LPS-treated brain and blood samples (T3 condition) and perform DNA sequencing for DNA methylation modifications for brain-PFC and blood samples. We will perform pyrosequencing validation of the primer sets for the genes TNF- $\alpha$ , CCL, IL1- $\beta$ , LIF, IL6 and OSM within each brain region. The validation is thus being done in parallel with high-throughput sequencing to maximize the efficiency of our experiments. We will be able to take advantage of existing RNA-sequencing

data to directly compare them with the epigenetic profiles we will obtain from the current work. We also plan to have optimized our ChIP-seq protocol and to perform ChIP-sequencing for histone modifications on brain and blood. We plan to perform QC analysis of sequencing data and to analyze RRBS and ChIP-seq comparisons between groups. Finally, we are continuing our feasibility analysis of FACS sorted neurons and glia to determine whether enough material for epigenetic assessments in glia can be made from each tissue source (cortex, hippocampus).

## **Impact**

### **What was the impact on the development of the principal discipline(s) of the project?**

The techniques we developed will serve our project as well as other DoD projects. Please see section on ‘results dissemination’ above for a description of the technical refinement of our analytical approach.

### **What was the impact on other disciplines?**

This work feeds back into our thinking of how certain gene regulatory circuits might fail in sister projects under the broader umbrella of the consortium.

### **What was the impact on technology transfer?**

Nothing to report.

### **What was the impact on society beyond science and technology?**

This reporting period (September 2, 2015), McGowan was invited to speak about his research program at the US Environmental Protection Agency (EPA) workshop on Epigenetics and Cumulative Risk. The goal of this workshop was “to explore and lay groundwork for practical application of epigenetic data in assessment of cumulative risk from multiple environmental factors.” The results of this project will thus likely impact public knowledge, funding and policy decisions in environmental health more broadly.

## **Changes/Problems**

### **Changes in approach and reasons for change**

As mentioned above, we have continued to refine our animal dosing schedule (number of subjects), methodological decisions (NanoString platform) data analysis platform in light of data generated from the GWIRC project in progress, at no additional cost to the current project. In addition, we have piloted a protocol to enable cell separation by Fluorescence-assisted cell sorting (FACS) of neural tissue into neurons and glia (see Section 2 of ‘Major Activities’ above for results).

### **Actual or anticipated problems or delays and actions or plans to resolve them**

We encountered an unexpected delay in the trans-border shipment of samples from CDC to UofT. This delay affected the quality and quantity of PBMCs we were able to harvest from the blood samples, with too little DNA for many samples for downstream analyses. We plan to extract PBMCs at the CDC site prior to shipping them to UofT during the next reporting period. The samples will be frozen on dry ice for shipment.

During validation of the ChIP-seq protocol (**Appendix 2**), the signal produced from the pulldown by the target antibody was higher than that of the iso-type antibody showing target specificity of the target antibody.

However, signal strength between Adh1 and Rbfox3 control genes were the same in the brain, which deviates from the data observed in the UCSC genomic database. Although melt curve of SYBR green qPCR showed single melt curve suggesting single amplified product. Product size was however bigger than expected suggesting off-target amplification. Using a different SYBR green mastermix (see **Appendix 3** for preliminary data), size of the amplicon is now as expected. Another pulldown is required to determine if the change is SYBR green mastermix has resolved the issue.

### **Changes that had a significant impact on expenditures**

Due to visa delays, Dr. Benjamin Hing, the postdoctoral fellow at UofT was not able to start until January 2015.

### **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

### **Significant changes in use or care of human subjects**

Nothing to report.

### **Significant changes in use or care of vertebrate animals**

Nothing to report.

### **Significant changes in use of biohazards and/or select agents**

Nothing to report.

### **Products**

Nothing to report.

### **Participants & Other Collaborating Organizations**

#### **What individuals have worked on the project?**

|                              |                                                                                                              |
|------------------------------|--------------------------------------------------------------------------------------------------------------|
| Name:                        | Patrick McGowan, PhD                                                                                         |
| Project Role:                | Principal-Investigator (NSU site PI)                                                                         |
| Research Identifier:         | none                                                                                                         |
| Nearest person month worked: | 3                                                                                                            |
| Contribution to Project:     | Lead PI overseeing the project. Direct supervision of molecular biology studies related to epigenetics data. |
| Funding Support:             |                                                                                                              |

|               |                               |
|---------------|-------------------------------|
| Name:         | Gordon Broderick, PhD         |
| Project Role: | Co-Investigator (NSU site PI) |

|                              |                                                                                                                                          |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Research Identifier:         | eCommons: gbroderick                                                                                                                     |
| Nearest person month worked: | 0.36                                                                                                                                     |
| Contribution to Project:     | Head of computational biology. Has worked on the computational models for animal and human research to assist in protocols and findings. |
| Funding Support:             | NIH, VA                                                                                                                                  |

|                              |                                                                                                                                                      |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------|
| Name:                        | Mariana Morris, PhD                                                                                                                                  |
| Project Role:                | Co-Investigator                                                                                                                                      |
| Research Identifier:         | eCommons: mariana                                                                                                                                    |
| Nearest person month worked: | 0.36                                                                                                                                                 |
| Contribution to Project:     | Overseeing the animal protocols and in charge of the animal research. Contributes technical expertise in animal experimentation. PI of GWIRC project |
| Funding Support:             | NIH                                                                                                                                                  |

|                              |                             |
|------------------------------|-----------------------------|
| Name:                        | Benjamin Hing, PhD          |
| Project Role:                | Postdoctoral fellow         |
| Research Identifier:         | None.                       |
| Nearest person month worked: | 9                           |
| Contribution to Project:     | Active in epigenetic assays |
| Funding Support:             |                             |

|                              |                             |
|------------------------------|-----------------------------|
| Name:                        | Ms. Christine Lum           |
| Project Role:                | Graduate student            |
| Research Identifier:         | None                        |
| Nearest person month worked: | 9                           |
| Contribution to Project:     | Active in epigenetic assays |
| Funding Support:             |                             |

|                              |                                                                         |
|------------------------------|-------------------------------------------------------------------------|
| Name:                        | Mr. Shathveekan Sivanathan                                              |
| Project Role:                | Technician                                                              |
| Research Identifier:         | None                                                                    |
| Nearest person month worked: | 1                                                                       |
| Contribution to Project:     | Assists postdoctoral fellow and graduate student with epigenetic assays |
| Funding Support:             |                                                                         |

|                              |                                                      |
|------------------------------|------------------------------------------------------|
| Name:                        | Mr. Patrick Gourdet                                  |
| Project Role:                | Research Programmer                                  |
| Research Identifier:         | None                                                 |
| Nearest person month worked: | 10                                                   |
| Contribution to Project:     | Active in data analysis and software platform design |
| Funding Support:             |                                                      |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

**What other organizations were involved as partners?**

|                      |                                                                                                     |
|----------------------|-----------------------------------------------------------------------------------------------------|
| Name:                | Centers for Disease Control and Prevention<br>National Institute for Occupational Safety and Health |
| Location:            | 1095 Willowdale Road<br>Morgantown, WV 26505                                                        |
| Contribution:        | Chemical toxicology project collaboration                                                           |
| Financial:           | None                                                                                                |
| In-kind Support:     | None                                                                                                |
| Facilities:          | None                                                                                                |
| Collaboration:       | Partner's staff works with project staff in the project.                                            |
| Personnel Exchanges: | None                                                                                                |
| Other:               | None                                                                                                |

|                      |                                                                                                                          |
|----------------------|--------------------------------------------------------------------------------------------------------------------------|
| Name:                | Nova Southeastern University                                                                                             |
| Location:            | Institute for Neuro-Immune Medicine<br>University Park Plaza<br>3440 South University Drive<br>Fort Lauderdale, FL 33328 |
| Contribution:        | Genomic profiling and computational analyses                                                                             |
| Financial:           | None                                                                                                                     |
| In-kind Support:     | None                                                                                                                     |
| Facilities:          | None                                                                                                                     |
| Collaboration:       | Partner's staff works with project staff in the project.                                                                 |
| Personnel Exchanges: | None                                                                                                                     |
| Other:               | None                                                                                                                     |

**Other.**

Nothing to report.

**Special Reporting Requirements**

Nothing to report.

## **Appendices**

### **Appendix 1. Bifulfite Pyrosequencing Optimization for validation of specific genes.**

Objective: Optimizing PCR step of bisulfite-pyrosequencing for primers designed around TNF, CCL, IL1B, LIF, IL6 and OSM (O'Callaghan et al., J. Neurochem 2015)

PCR was performed using Thermopol PCR

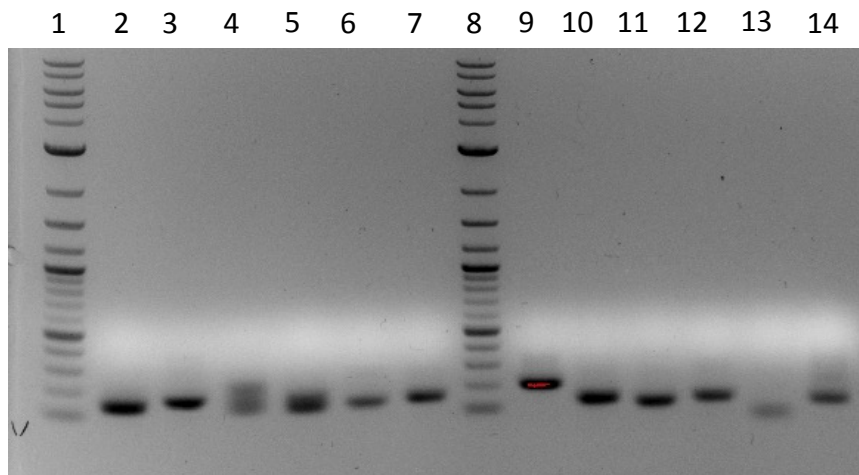
|              | Final conc. | μl     | x7 (μl)  |
|--------------|-------------|--------|----------|
| Buffer (10X) | 1X          | 2.5    | 30       |
| 10mM dNTPs   | 200μM       | 0.5    | 6        |
| Primer (5μM) | 200nM       | 1      | 12       |
| DNA          |             | 1      | 12       |
| Taq          |             | 0.125  | 1.5      |
| H2O          |             | 19.875 | 238.5    |
| Total        |             | 25     | 300      |
|              |             |        |          |
| PCR2         |             |        |          |
|              | Final conc. | μl     | x14 (μl) |
| Buffer (10X) | 1X          | 2.5    | 35       |
| 10mM dNTPs   | 200μM       | 0.5    | 7        |
| Primer (5μM) | 200nM       | 1      | 14       |
| DNA          |             | 2      | 28       |
| Taq          |             | 0.125  | 1.75     |
| H2O          |             | 18.875 | 264.25   |
| Total        |             | 25     | 350      |

First PCR thermal cycling condition:

| STEP                 | TEMP | TIME      |
|----------------------|------|-----------|
| Initial Denaturation | 95°C | 30 sec    |
| 30 Cycles            | 95°C | 30 sec    |
|                      | 57°C | 30 sec    |
|                      | 68°C | 30 sec    |
| Final Extension      | 68°C | 5 minutes |
| Hold                 | 4°C  |           |

Second PCR thermal cycling condition:

| STEP                 | TEMP | TIME      |
|----------------------|------|-----------|
| Initial Denaturation | 95°C | 30 sec    |
| 35 Cycles            | 95°C | 30 sec    |
|                      | 53°C | 30 sec    |
|                      | 68°C | 30 sec    |
| Final Extension      | 68°C | 5 minutes |
| Hold                 | 4°C  |           |



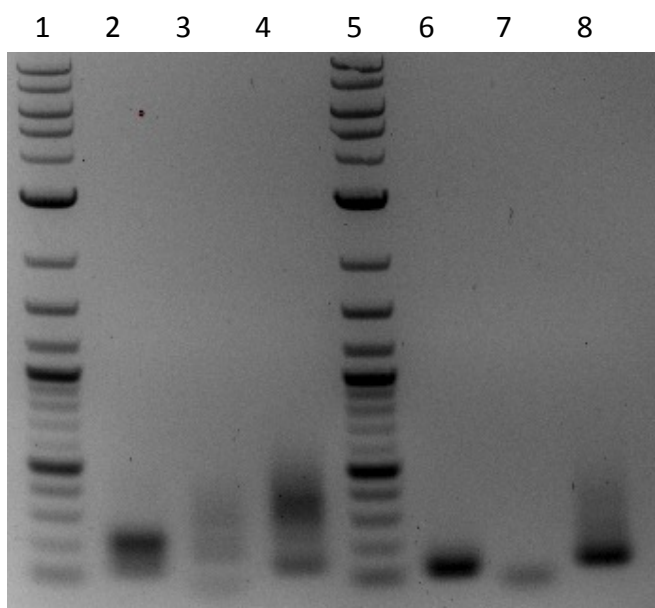
lane 1 - 2 log ladder  
lane2 - TNF promoter  
lane3 - TNF Intron  
lane 4 - CCLprom  
lane 5 - CCL enhancer  
lane 6 - ILB promoter 1  
lane 7 - ILB promoter 2  
lane 8 - 2 log ladder  
lane 9 - LIF promoter 1  
lane 10 - LIF promoter 2  
lane 11 - LIF enhancer  
lane 12 - IL6 promoter  
lane 13 - OSM promoter  
lanen 14 - OSM intron

Redo CCLprom (lane 4) with pyromark PCR and determine if pyromark PCR might improve OSM promoter (lane 13) and OSM intron (lane 14)

| PCR 1              |             | with Q solution | without Q solution |
|--------------------|-------------|-----------------|--------------------|
|                    | Final conc. | $\mu$ l         | $\mu$ l            |
| Master mix (2x)    | 1X          | 12.5            | 12.5               |
| Coral load (10x)   | 1X          | 2.5             | 2.5                |
| Q solution (5x)    | 1X          | 5               | 0                  |
| Primer (5 $\mu$ M) | 200 nM      | 1               | 1                  |
| DNA                |             | 1               | 1                  |
| H2O                |             | 3               | 8                  |
| Total              |             | 25              | 25                 |
|                    |             |                 |                    |
| PCR 2              |             | with Q solution | without Q solution |
|                    | Final conc. | $\mu$ l         | $\mu$ l            |
| Master mix (2x)    | 1X          | 12.5            | 12.5               |
| Coral load (10x)   | 1X          | 2.5             | 2.5                |
| Q solution (5x)    | 1X          | 5               | 0                  |
| Primer             | 200 nM      | 1               | 1                  |
| DNA                |             | 2               | 2                  |
| H2O                |             | 2               | 7                  |
| Total              |             | 25              | 25                 |



| PCR 1                       |      |        |
|-----------------------------|------|--------|
| Initial PCR activation step | 95°C | 15 min |
| 30 Cycles                   | 94°C | 30 sec |
|                             | 56°C | 30 sec |
|                             | 72°C | 30 sec |
| Final Extension             | 72°C | 10 min |
| Hold                        | 4°C  |        |
| PCR 2                       |      |        |
| Initial PCR activation step | 95°C | 15 min |
| 45 Cycles                   | 94°C | 30 sec |
|                             | 56°C | 30 sec |
|                             | 72°C | 30 sec |
| Final Extension             | 72°C | 10 min |



Lane 1 - 2 log ladder  
 Lane 2 - CCLprom  
 Lane 3 - OSMprom  
 Lane 4 - OSM Intron  
 Lane 5 - 2 Log ladder  
 Lane 6 - CCL prom with Q solution  
 Lane 7 - OSMprom with Q solution  
 Lane 8 - OSM Intron with Q solution

Pyromark

Pyromark  
with Q  
solution

## **Appendix 2. Native ChIP optimisation**

### **MNase Titration**

Objective: To determine length of time required for optimal MNase digestion.

Parameter: 50U of MNase used for reaction. Reaction was performed at 37°C for the length of time specified below.

|                                     |    |
|-------------------------------------|----|
|                                     | μl |
| Sample nuclei (PBS)                 | 40 |
| 10X Mnase buffer                    | 6  |
| Sodium butyrate (5mM) (stock 100mM) | 0  |
| BSA (100ng/μl) (stock 6000ng/μl)    | 1  |
| Mnase enzyme (25 U/μl)              | 2  |
| H2O                                 | 11 |
| Total                               | 60 |

Make up of other reagents:

### **BSA**

|                     |            |          |
|---------------------|------------|----------|
| stock BSA           | 10mg/ml    | 10μg/μl  |
|                     | 10000ng/μl |          |
| required 6000 ng/μl | 1.6666667  |          |
| For 20μl            | 12         | BSA (μl) |
|                     | 8          | H2O (μl) |

### **MNase**

|                   |           |
|-------------------|-----------|
|                   | μl        |
| Enzyme (2000U/μl) | 1         |
| BSA (6000ng/μl)   | 1.3333333 |
| Buffer            | 8         |
| H2O               | 69.666667 |
| Total             | 80        |

### **1% deoxycholate/1% Triton X**

|                                 |     |
|---------------------------------|-----|
| 4% deoxycholate                 |     |
| 2g in 50 ml                     |     |
|                                 | ml  |
| 1% Triton X and 1% deoxycholate |     |
| Triton X                        | 0.1 |
| 4% dexoycholate                 | 2.5 |
| H2O                             | 7.4 |
| total                           | 10  |

### **EDTA**

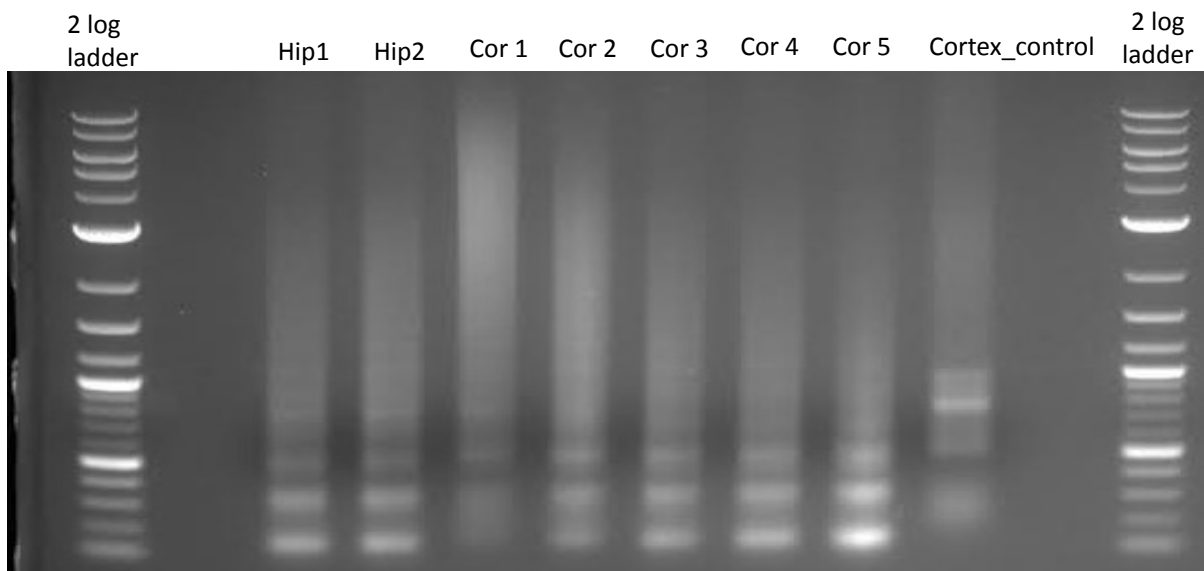
|           |     |                 |
|-----------|-----|-----------------|
| 0.5M EDTA |     |                 |
| for 100mM | 10  |                 |
| For 1ml   | 200 | μl of 0.5M EDTA |
|           | 800 | μl H2O          |

### **Results**

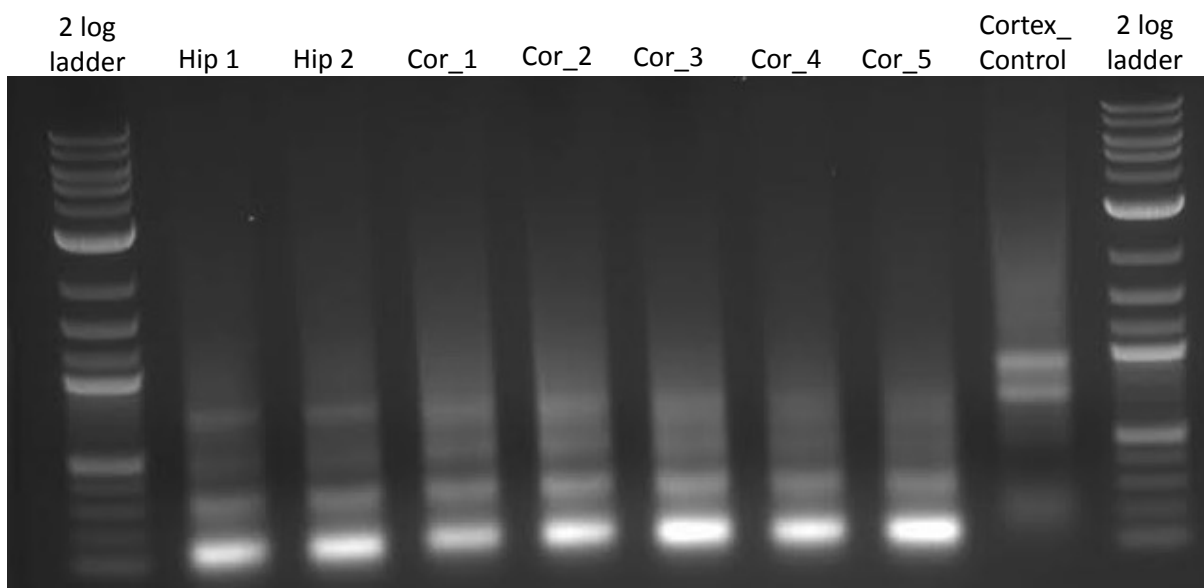
| Experiment 1   |       |         |         |                      |
|----------------|-------|---------|---------|----------------------|
|                | ng/μl | 260/280 | 260/230 | digestion time (min) |
| Hippocampus 1  | 631.9 | 1.87    | 1.95    | 10                   |
| Hippocampus 2  | 969.3 | 1.79    | 1.98    | 10                   |
| Cortex_1       | 828.4 | 1.79    | 1.98    | 10                   |
| Cortex_2       | 888.3 | 1.74    | 1.96    | 10                   |
| Cortex_3       | 877.3 | 1.75    | 1.95    | 10                   |
| Cortex_4       | 959.5 | 1.73    | 1.91    | 10                   |
| Cortex control | 631.9 | 1.93    | 1.89    | 0                    |

- Experiment not run on gel

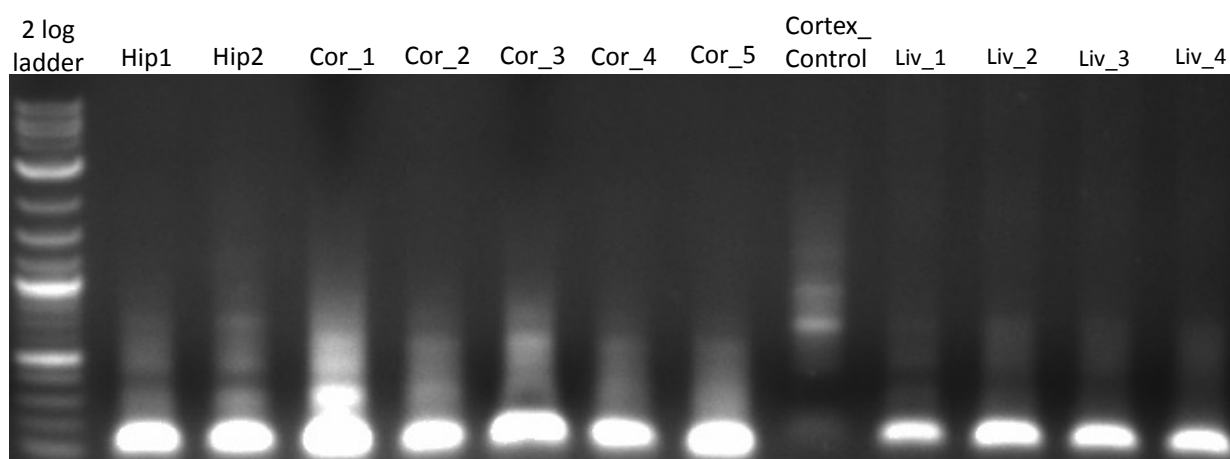
| Experiment 2   |       |         |         |                      |
|----------------|-------|---------|---------|----------------------|
|                | ng/μl | 260/280 | 260/230 | digestion time (min) |
| Hippocampus 1  | 555.7 | 1.88    | 2.01    | 5                    |
| Hippocampus 2  | 596.7 | 1.87    | 2.03    | 5                    |
| Cortex_1       | 627.2 | 1.88    | 2.01    | 2                    |
| Cortex_2       | 728.6 | 1.84    | 2.06    | 4                    |
| Cortex_3       | 556.7 | 1.88    | 1.98    | 6                    |
| Cortex_4       | 588.7 | 1.88    | 1.98    | 8                    |
| Cortex_5       | 656.5 | 1.87    | 1.97    | 10                   |
| Cortex control | 653.6 | 1.94    | 1.92    | 0                    |

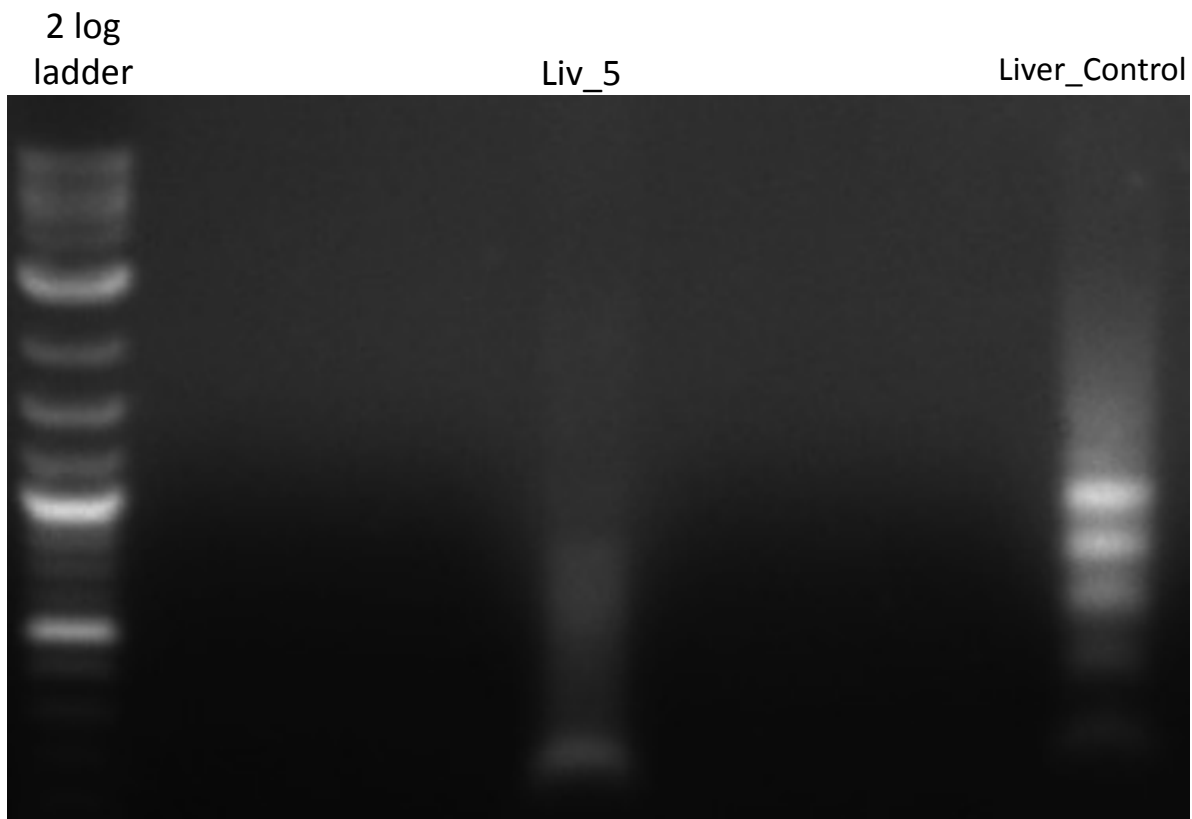


| Experiment 3   |        |         |         |                      |
|----------------|--------|---------|---------|----------------------|
|                | ng/μl  | 260/280 | 260/230 | digestion time (min) |
| Hippocampus 1  | 761.3  | 1.96    | 2.29    | 15                   |
| Hippocampus 2  | 800.2  | 1.94    | 2.25    | 15                   |
| Cortex_1       | 856.3  | 1.95    | 2.29    | 10                   |
| Cortex_2       | 983.8  | 1.98    | 2.29    | 12                   |
| Cortex_3       | 1038.2 | 1.97    | 2.17    | 15                   |
| Cortex_4       | 612.6  | 1.94    | 2.19    | 17                   |
| Cortex_5       | 850.4  | 1.96    | 2.23    | 20                   |
| Cortex control | 851.7  | 2.08    | 2.19    | 0                    |

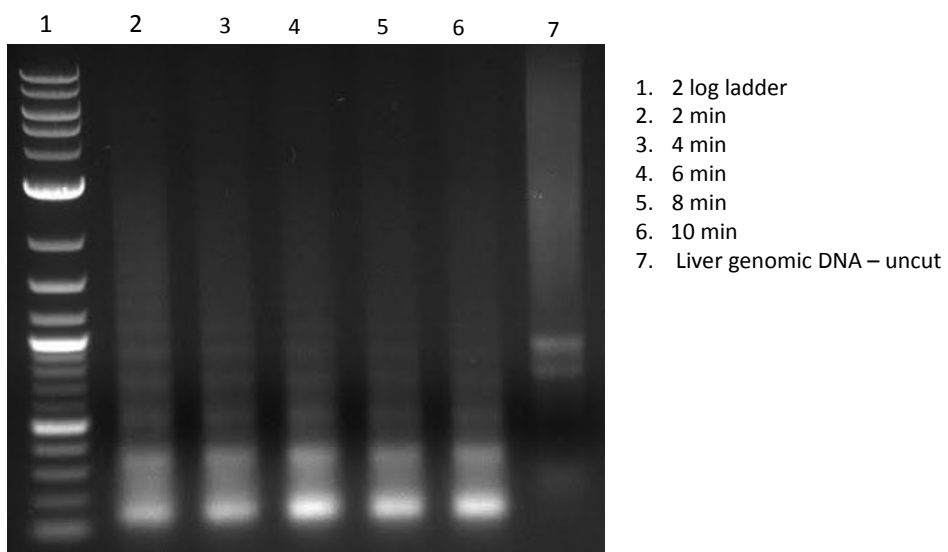


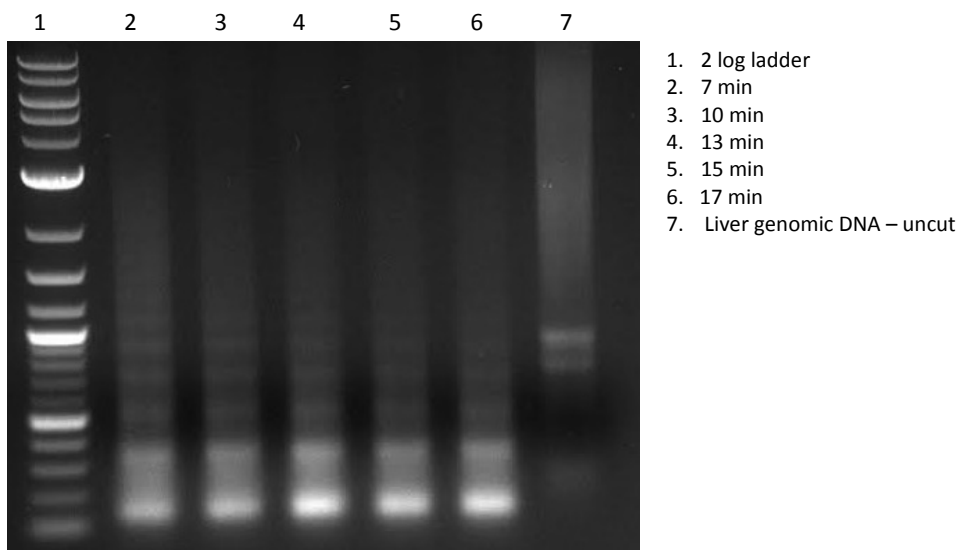
| Experiment 4   |             |         |         |                      |
|----------------|-------------|---------|---------|----------------------|
|                | ng/ $\mu$ l | 260/280 | 260/230 | digestion time (min) |
| Hippocampus 1  | 761.3       | 1.96    | 2.29    | 30                   |
| Hippocampus 2  | 800.2       | 1.94    | 2.25    | 30                   |
| Cortex_1       | 856.3       | 1.95    | 2.29    | 20                   |
| Cortex_2       | 983.8       | 1.98    | 2.29    | 25                   |
| Cortex_3       | 1038.2      | 1.97    | 2.17    | 30                   |
| Cortex_4       | 612.6       | 1.94    | 2.19    | 35                   |
| Cortex_5       | 850.4       | 1.96    | 2.23    | 40                   |
| Cortex control | 851.7       | 2.08    | 2.19    | 0                    |
| Liver_1        | 1241.3      | >1.8    | >1.8    | 10                   |
| Liver_2        | 1749.8      | >1.8    | >1.8    | 20                   |
| Liver_3        | 1586.7      | >1.8    | >1.8    | 25                   |
| Liver_4        | 1615.5      | >1.8    | >1.8    | 30                   |
| Liver_5        | 1579.4      | >1.8    | >1.8    | 35                   |
| Liver_control  | 378.3       | >1.8    | >1.8    | 0                    |





Conclusion:  
20 min is optimal digestion time for Cortex and Hippocampus.





Objective: Determining optimal annealing temperature

10ng and 50ng of DNA used for amplification for SYBR green PCR. 200g of DNA used for Thermopol PCR amplification. Please note that the annealing temperature tested were 53°C, 55°C, 57°C, 60°C, 62°C, 64°C.

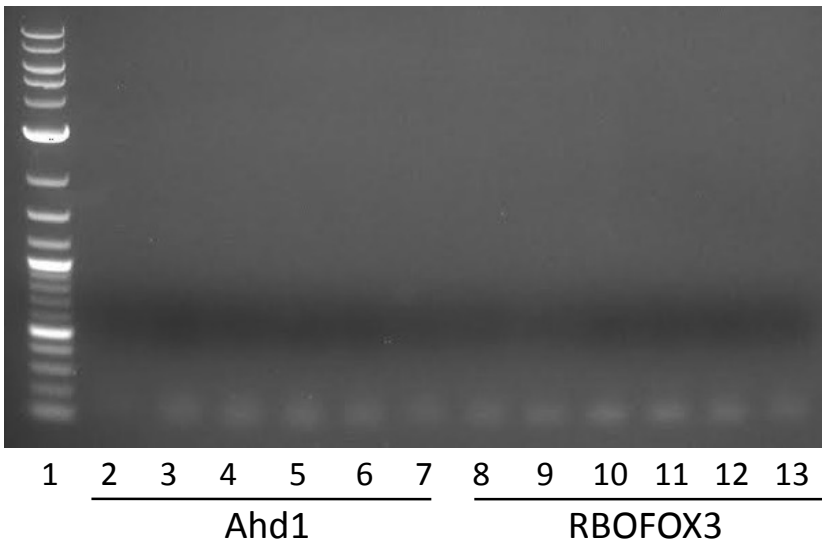
Fast SYBR green thermal cycling condition:

| Select Fast Mode                                                                                    |                                              |                  |          |        |
|-----------------------------------------------------------------------------------------------------|----------------------------------------------|------------------|----------|--------|
| Instrument                                                                                          | Step                                         | Temperature (°C) | Duration | Cycles |
| <ul style="list-style-type: none"> <li>Step One</li> <li>StepOne Plus</li> <li>7500 Fast</li> </ul> | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 3 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 30 sec   |        |
| 7900HT Fast                                                                                         | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 1 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 20 sec   |        |

Thermopol cycling condition:

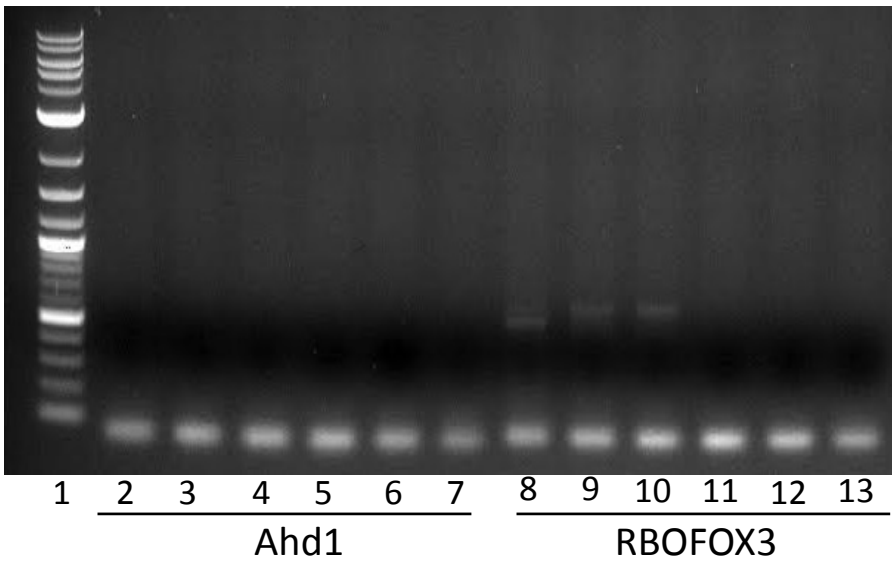
| Step            | Temp | Time       |
|-----------------|------|------------|
| Denaturation    | 95°C | 30 seconds |
| 35 Cycles       | 95°C | 30 seconds |
|                 | 60°C | 30 seconds |
|                 | 68°C | 30 seconds |
| Final extension | 68°C | 5 minutes  |
| Hold            | 4°C  |            |

### SYBR Green



1 - 2 log ladder  
2,8 - 53°C  
3,9 - 55°C  
4,10 - 57°C  
5,11 - 60°C  
6,12 - 62°C  
7,13 - 64°C

### Thermopol



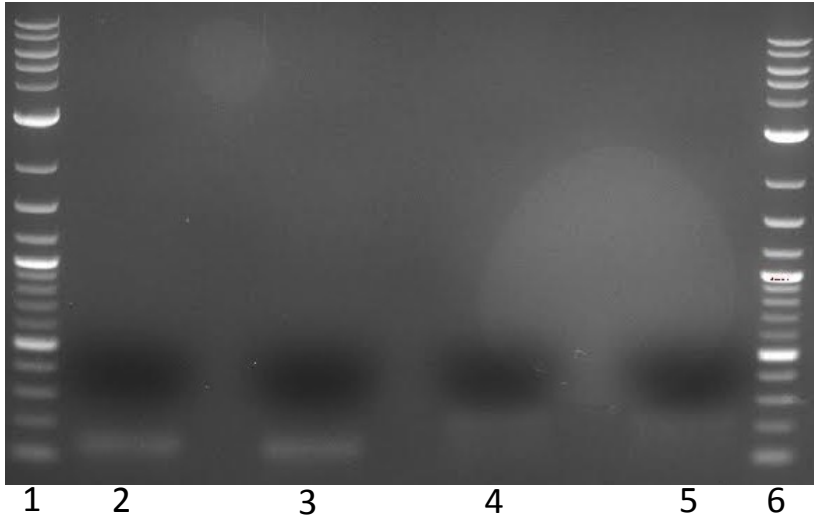
1 - 2 log ladder  
2,8 - 53°C  
3,9 - 55°C  
4,10 - 57°C  
5,11 - 60°C  
6,12 - 62°C  
7,13 - 64°C

10ng of ChIP DNA used. 30 µl reaction volume.

|            |        | Final conc. | µl |
|------------|--------|-------------|----|
| SYBR Green | 2X     | 1x          | 15 |
| Primer     | 2000nM | 200nM       | 10 |
| DNA        |        |             | 10 |
| Total      |        |             | 30 |

Fast SYBR green thermal cycling condition:

| Select Fast Mode                                                                                    |                                              |                  |          |        |
|-----------------------------------------------------------------------------------------------------|----------------------------------------------|------------------|----------|--------|
| Instrument                                                                                          | Step                                         | Temperature (°C) | Duration | Cycles |
| <ul style="list-style-type: none"> <li>Step One</li> <li>StepOne Plus</li> <li>7500 Fast</li> </ul> | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 3 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 30 sec   |        |
| 7900HT Fast                                                                                         | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 1 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 20 sec   |        |



1,6 - 2 log ladder  
 2,3 – RBFOX3  
 4,5 – ADH1

Objective: Determining optimal annealing temperature  
 400ng of DNA used for amplification. Please note that the annealing temperature tested were 55°C, 57°C, 60°C, 63°C.

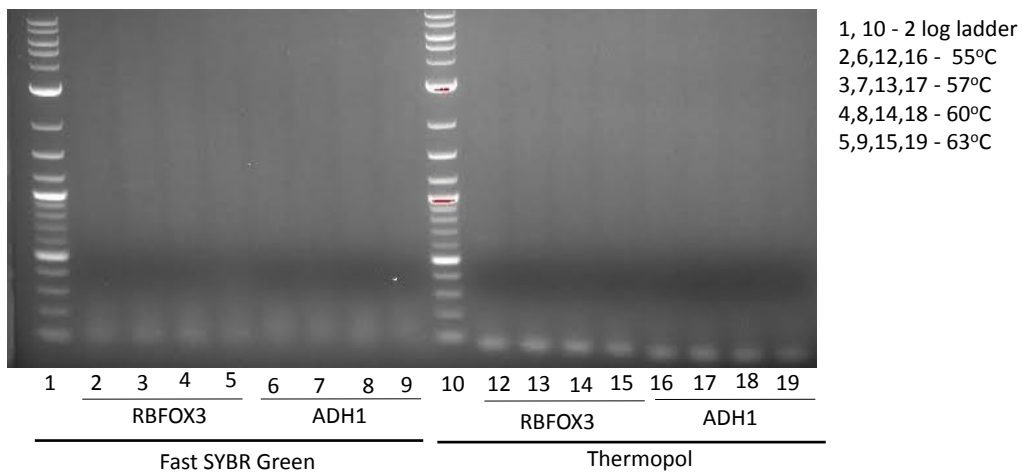
Fast SYBR green thermal cycling condition:

| Select Fast Mode                                                                                    |                                              |                  |          |        |
|-----------------------------------------------------------------------------------------------------|----------------------------------------------|------------------|----------|--------|
| Instrument                                                                                          | Step                                         | Temperature (°C) | Duration | Cycles |
| <ul style="list-style-type: none"> <li>Step One</li> <li>StepOne Plus</li> <li>7500 Fast</li> </ul> | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 3 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 30 sec   |        |
| 7900HT Fast                                                                                         | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 1 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 20 sec   |        |

Thermopol cycling condition:



| Step            | Temp | Time       |
|-----------------|------|------------|
| Denaturation    | 95°C | 30 seconds |
| 35 Cycles       | 95°C | 30 seconds |
|                 | 60°C | 30 seconds |
|                 | 68°C | 30 seconds |
| Final extension | 68°C | 5 minutes  |
| Hold            | 4°C  |            |



Objective: Titration of RBFOX3 and ADH1 primers for qPCR.  
200 ng of DNA used for amplification.

| Primer conc. | nM  | 400     | 200     | 100     | 50     |
|--------------|-----|---------|---------|---------|--------|
| nM           | 400 | 400/400 | 200/400 | 100/400 | 50/400 |
| nM           | 200 | 400/200 | 200/200 | 100/200 | 50/200 |
| nM           | 100 | 400/100 | 200/100 | 100/100 | 50/100 |
| nM           | 50  | 400/50  | 200/50  | 100/50  | 50/50  |

|                  |         |         |         |  |                  |         |         |         |
|------------------|---------|---------|---------|--|------------------|---------|---------|---------|
| Primer stock     | 2μM     |         |         |  |                  |         |         |         |
| primer conc.(nM) | 400/400 | 200/400 | 100/400 |  | primer conc.(nM) | 400/400 | 200/400 | 100/400 |
|                  | μl      | μl      | μl      |  |                  | μl      | μl      | μl      |
| SYBR green       | 10      | 10      | 10      |  | SYBR green       | 30      | 30      | 30      |
| Forward primer   | 4       | 2       | 1       |  | Forward primer   | 12      | 6       | 3       |
| Reverse primer   | 4       | 4       | 4       |  | Reverse primer   | 12      | 12      | 12      |
| DNA              | 2       | 2       | 2       |  | DNA              | 6       | 6       | 6       |
| H2O              | 0       | 2       | 3       |  | H2O              | 0       | 6       | 9       |
| Total            | 20      | 20      | 20      |  | Total            | 60      | 60      | 60      |
|                  |         |         |         |  |                  |         |         |         |
| primer conc.(nM) | 400/200 | 200/200 | 100/200 |  | primer conc.(nM) | 400/200 | 200/200 | 100/200 |
|                  | μl      | μl      | μl      |  |                  | μl      | μl      | μl      |
| SYBR green       | 10      | 10      | 10      |  | SYBR green       | 30      | 30      | 30      |
| Forward primer   | 4       | 2       | 1       |  | Forward primer   | 12      | 6       | 3       |
| Reverse primer   | 2       | 2       | 2       |  | Reverse primer   | 6       | 6       | 6       |
| DNA              | 2       | 2       | 2       |  | DNA              | 6       | 6       | 6       |
| H2O              | 2       | 4       | 5       |  | H2O              | 6       | 12      | 15      |
| Total            | 20      | 20      | 20      |  | Total            | 60      | 60      | 60      |
|                  |         |         |         |  |                  |         |         |         |
| primer conc.(nM) | 400/100 | 200/100 | 100/100 |  | primer conc.(nM) | 400/100 | 200/100 | 100/100 |
|                  | μl      | μl      | μl      |  |                  | μl      | μl      | μl      |
| SYBR green       | 10      | 10      | 10      |  | SYBR green       | 30      | 30      | 30      |
| Forward primer   | 4       | 2       | 1       |  | Forward primer   | 12      | 6       | 3       |
| Reverse primer   | 1       | 1       | 1       |  | Reverse primer   | 3       | 3       | 3       |
| DNA              | 2       | 2       | 2       |  | DNA              | 6       | 6       | 6       |
| H2O              | 3       | 5       | 6       |  | H2O              | 9       | 15      | 18      |
| Total            | 20      | 20      | 20      |  | Total            | 60      | 60      | 60      |
|                  |         |         |         |  |                  |         |         |         |
| primer conc.(nM) | 400/50  | 200/50  | 100/50  |  | primer conc.(nM) | 400/50  | 200/50  | 100/50  |
|                  | μl      | μl      | μl      |  |                  | μl      | μl      | μl      |
| SYBR green       | 10      | 10      | 10      |  | SYBR green       | 30      | 30      | 30      |
| Forward primer   | 4       | 2       | 1       |  | Forward primer   | 12      | 6       | 3       |
| Reverse primer   | 0.5     | 0.5     | 0.5     |  | Reverse primer   | 1.5     | 1.5     | 1.5     |
| DNA              | 2       | 2       | 2       |  | DNA              | 6       | 6       | 6       |
| H2O              | 3.5     | 5.5     | 6.5     |  | H2O              | 10.5    | 16.5    | 19.5    |
| Total            | 20      | 20      | 20      |  | Total            | 60      | 60      | 60      |

|                      |         |         |         |  |                      |         |         |         |
|----------------------|---------|---------|---------|--|----------------------|---------|---------|---------|
| primer conc.(nM)     | 400/400 | 200/400 | 100/400 |  | primer conc.(nM)     | 400/400 | 200/400 | 100/400 |
|                      | μl      | μl      | μl      |  |                      | μl      | μl      | μl      |
| Thermopol master mix | 2       | 2       | 2       |  | Thermopol master mix | 8       | 8       | 8       |
| Forward primer       | 4       | 2       | 1       |  | Forward primer       | 16      | 8       | 4       |
| Reverse primer       | 4       | 4       | 4       |  | Reverse primer       | 16      | 16      | 16      |
| dNTP                 | 0.5     | 0.5     | 0.5     |  | dNTP                 | 2       | 2       | 2       |
| Template             | 2       | 2       | 2       |  | Template             | 8       | 8       | 8       |
| Polymerase           | 0.125   | 0.125   | 0.125   |  | Polymerase           | 0.5     | 0.5     | 0.5     |
| H2O                  | 7.38    | 9.38    | 10.38   |  | H2O                  | 29.5    | 37.5    | 41.5    |
| Total                | 20      | 20      | 20      |  | Total                | 80      | 80      | 80      |
|                      |         |         |         |  |                      |         |         |         |
| primer conc.(nM)     | 400/200 | 200/200 | 100/200 |  | primer conc.(nM)     | 400/200 | 200/200 | 100/200 |
|                      | μl      | μl      | μl      |  |                      | μl      | μl      | μl      |
| Thermopol master mix | 2       | 2       | 2       |  | Thermopol master mix | 8       | 8       | 8       |
| Forward primer       | 4       | 2       | 1       |  | Forward primer       | 16      | 8       | 4       |
| Reverse primer       | 2       | 2       | 2       |  | Reverse primer       | 8       | 8       | 8       |
| dNTP                 | 0.5     | 0.5     | 0.5     |  | dNTP                 | 2       | 2       | 2       |
| Template             | 2       | 2       | 2       |  | Template             | 8       | 8       | 8       |
| Polymerase           | 0.125   | 0.125   | 0.125   |  | Polymerase           | 0.5     | 0.5     | 0.5     |
| H2O                  | 9.38    | 11.38   | 12.38   |  | H2O                  | 37.5    | 45.5    | 49.5    |
| Total                | 20      | 20      | 20      |  | Total                | 80      | 80      | 80      |
|                      |         |         |         |  |                      |         |         |         |
| primer conc.(nM)     | 400/100 | 200/100 | 100/100 |  | primer conc.(nM)     | 400/100 | 200/100 | 100/100 |
|                      | μl      | μl      | μl      |  |                      | μl      | μl      | μl      |
| Thermopol master mix | 2       | 2       | 2       |  | Thermopol master mix | 8       | 8       | 8       |
| Forward primer       | 4       | 2       | 1       |  | Forward primer       | 16      | 8       | 4       |
| Reverse primer       | 1       | 1       | 1       |  | Reverse primer       | 4       | 4       | 4       |
| dNTP                 | 0.5     | 0.5     | 0.5     |  | dNTP                 | 2       | 2       | 2       |
| Template             | 2       | 2       | 2       |  | Template             | 8       | 8       | 8       |
| Polymerase           | 0.125   | 0.125   | 0.125   |  | Polymerase           | 0.5     | 0.5     | 0.5     |
| H2O                  | 10.38   | 12.38   | 13.38   |  | H2O                  | 41.5    | 49.5    | 53.5    |
| Total                | 20      | 20      | 20      |  | Total                | 80      | 80      | 80      |
|                      |         |         |         |  |                      |         |         |         |
| primer conc.(nM)     | 400/50  | 200/50  | 100/50  |  | primer conc.(nM)     | 400/50  | 200/50  | 100/50  |
|                      | μl      | μl      | μl      |  |                      | μl      | μl      | μl      |
| Thermopol master mix | 2       | 2       | 2       |  | Thermopol master mix | 8       | 8       | 8       |
| Forward primer       | 4       | 2       | 1       |  | Forward primer       | 16      | 8       | 4       |
| Reverse primer       | 0.5     | 0.5     | 0.5     |  | Reverse primer       | 2       | 2       | 2       |
| dNTP                 | 0.5     | 0.5     | 0.5     |  | dNTP                 | 2       | 2       | 2       |
| Template             | 2       | 2       | 2       |  | Template             | 8       | 8       | 8       |
| Polymerase           | 0.125   | 0.125   | 0.125   |  | Polymerase           | 0.5     | 0.5     | 0.5     |
| H2O                  | 10.88   | 12.88   | 13.88   |  | H2O                  | 43.5    | 51.5    | 55.5    |
| Total                | 20      | 20      | 20      |  | Total                | 80      | 80      | 80      |

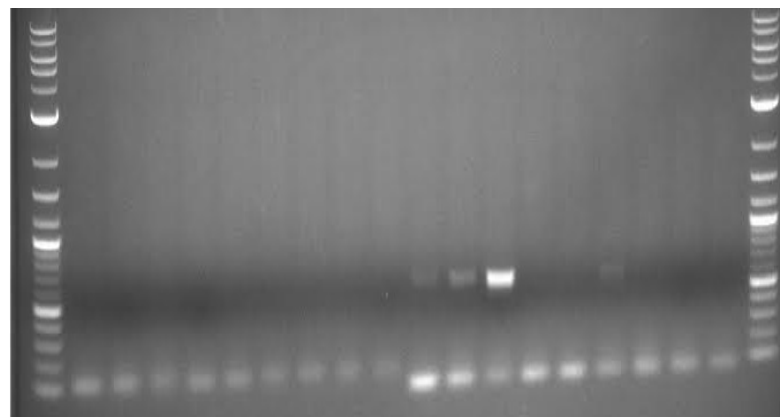
Fast SYBR green thermal cycling condition:

| Select Fast Mode                                                                                    |                                              |                  |          |        |
|-----------------------------------------------------------------------------------------------------|----------------------------------------------|------------------|----------|--------|
| Instrument                                                                                          | Step                                         | Temperature (°C) | Duration | Cycles |
| <ul style="list-style-type: none"> <li>Step One</li> <li>StepOne Plus</li> <li>7500 Fast</li> </ul> | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 3 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 30 sec   |        |
| 7900HT Fast                                                                                         | AmpliTaq® Fast DNA Polymerase, UP Activation | 95               | 20 sec   | HOLD   |
|                                                                                                     | Denature                                     | 95               | 1 sec    | 40     |
|                                                                                                     | Anneal/Extend                                | 60               | 20 sec   |        |

Thermopol cycling condition:

| Step            | Temp | Time       |
|-----------------|------|------------|
| Denaturation    | 95°C | 30 seconds |
| 35 Cycles       | 95°C | 30 seconds |
|                 | 60°C | 30 seconds |
|                 | 68°C | 30 seconds |
| Final extension | 68°C | 5 minutes  |
| Hold            | 4°C  |            |

### RBFOX3



1, 20 - 2 log ladder  
 2,11 - 400nM For/ 400nM Rev  
 3, 12 - 200nM For/ 400nM Rev  
 4, 13 - 100nM For/ 400nM Rev  
 5, 14 - 400nM For/ 200nM Rev  
 6, 15 - 200nM For/ 200nM Rev  
 7, 16 - 100nM For/ 200nM Rev  
 8, 17 - 400nM For/ 100nM Rev  
 9, 18 - 200nM For/ 100nM Rev  
 10,19 - 100nM For/ 100nM Rev

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
 Fast SYBR Green Thermopol

### Adh1



1, 20 - 2 log ladder  
 2,11 - 400nM For/ 400nM Rev  
 3, 12 - 200nM For/ 400nM Rev  
 4, 13 - 100nM For/ 400nM Rev  
 5, 14 - 400nM For/ 200nM Rev  
 6, 15 - 200nM For/ 200nM Rev  
 7, 16 - 100nM For/ 200nM Rev  
 8, 17 - 400nM For/ 100nM Rev  
 9, 18 - 200nM For/ 100nM Rev  
 10,19 - 100nM For/ 100nM Rev

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
 Fast SYBR Green Thermopol

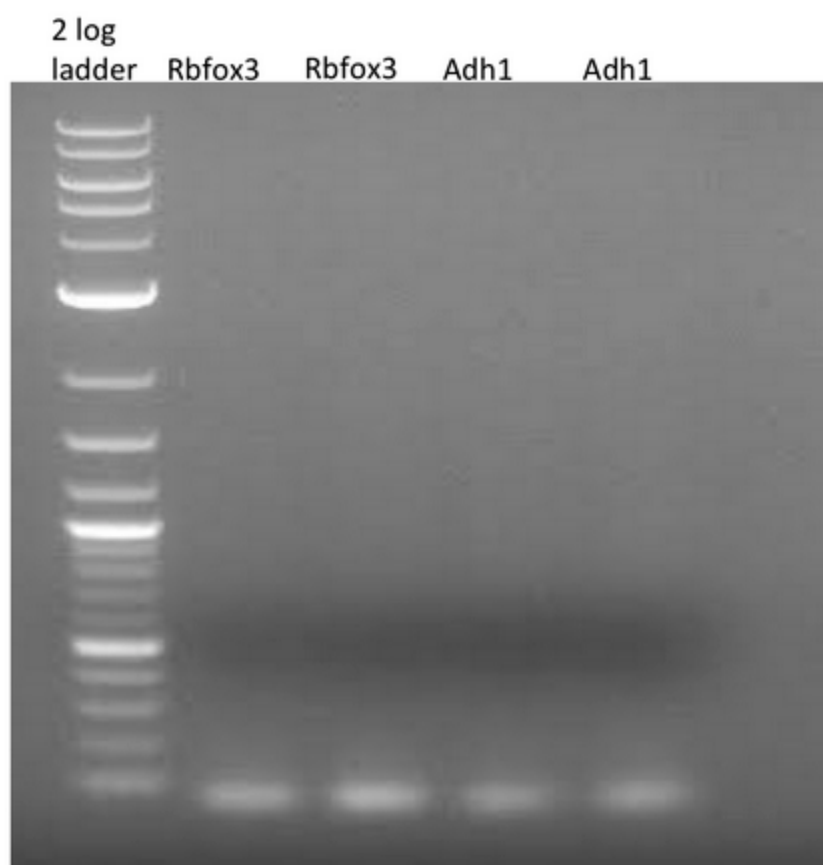
Using SYBR Green Universal Master Mix

|                              | Final conc. | μl   |
|------------------------------|-------------|------|
| 2X SYBR green PCR master mix | 1x          | 12.5 |
| Primer (5μM)                 | 200 nM      | 1    |
| Template                     |             | 2    |
| Water                        |             | 9.5  |

|       |  |    |
|-------|--|----|
| Total |  | 25 |
|-------|--|----|

| Step   | AmpliTaq Gold® Polymerase Activation† | PCR               |               |
|--------|---------------------------------------|-------------------|---------------|
|        |                                       | CYCLE (40 cycles) |               |
|        |                                       | Denature          | Anneal/Extend |
| Temp.  | 95.0 °C                               | 95.0 °C           | 60.0 °C       |
| Time   | 10 min                                | 15 sec            | 1 min         |
| Volume | 50 µL                                 |                   |               |

† The 10 min, 95°C step is required to activate the AmpliTaq Gold® DNA Polymerase.



Conclusions: These data show single bands in each experimental well, indicating a specific product has been obtained of the correct size for each gene and that the PCR conditions have been optimized.

## **References**

- Darlington, P. J., Goldman, J. S., Cui, Q. L., Antel, J. P., & Kennedy, T. E. (2008). Widespread immunoreactivity for neuronal nuclei in cultured human and rodent astrocytes. *Journal of neurochemistry*, 104(5), 1201-1209.
- Efroni S, Schaefer CF, Buetow KH. Identification of key processes underlying cancer phenotypes using biologic pathway analysis PLoS One. 2007 May 9;2(5):e425.
- Iwamoto, K., Bundo, M., Ueda, J., Oldham, M. C., Ukai, W., Hashimoto, E., ... & Kato, T. (2011). Neurons show distinctive DNA methylation profile and higher interindividual variations compared with non-neurons. *Genome research*, 21(5), 688-696.
- Kozlenkov, A., Roussos, P., Timashpolsky, A., Barbu, M., Rudchenko, S., Bibikova, M., ... & Dracheva, S. (2014). Differences in DNA methylation between human neuronal and glial cells are concentrated in enhancers and non-CpG sites. *Nucleic acids research*, 42(1), 109-127.
- O'Callaghan, J. P., Kelly, K. A., Locker, A. R., Miller, D. B., & Lasley, S. M. (2015). Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War Illness. *Journal of neurochemistry*, 133(5), 708-721.