Fellow: Jonathan Bobek

Host: Hongmei Li-Byarlay

Post-Visit Report

I (Jonathan Bobek) arrived at Hongmei Li-Byarlay's lab at North Carolina State University, Raleigh on 1/26/2015 with 14 honeybee brains under dry ice. We first finalized project aims, deciding on a protocol which would allow me to build libraries of DNA but also attempt to extract RNA out of the same individual brain. As a result, we would be able to compare differently methylated regions with levels of gene expression and alternative splicing.

We decided to initiate with 6 of the 14 brains to act as a test for effectiveness. We first lysed 6 test brains with Proteinase K as per the Epicentre Masterpure™ Complete DNA and RNA Purification Kit protocol, using mechanical breakdown with a plastic pestle. We split our lysate in half to isolate DNA and RNA separately. RNA lysate was incubated at 65C for 15 minutes, while DNA was incubated overnight in order to maximize the yields.

For RNA isolation, total nucleic acids were precipitated as per the Epicentre protocol (Part F, page 12). As our lysate was halved, all reagents used to precipitate and remove protein and DNA in this step were halved as well. Contaminating DNA was removed with 200uL DNase I solution as recommended by protocol. We incubated samples in DNase I solution for 20 minutes for further removal of contaminating DNA. After using the recommended 2X T and C Lysis solution as well as MPC Protein Precipitation Reagent, we followed the protocol to vortex and pellet debris via cold centrifugation. RNA precipitate was pelleted by 250uL isopropanol and centrifugation, then double rinsed with 70% ethanol and resuspended in TE Buffer. We added 1uL RiboGuard™ RNase inhibitor to preserve the RNA sample. We quantified RNA via Nanodrop. Of these six samples, total yield ranged from 450 to 1400ng, with 260/280 values ranging from 2.00 to 2.10.

Total DNA precipitation was conducted the next day, following the Epicentre protocol (Part D, page 9.) Again, we used half reagents for MPC Protein Precipitation Reagent to maintain reagent ratios, precipitating with isopropanol and rinsing twice with 70% ethanol, resuspending our sample in TE buffer. We followed Qubit's dsDNA broad-range assay kit to quantify DNA. Of these six 10uL samples, two DNA samples had low yield (30-43 ng) while other four samples had a higher yield (113-275 ng).

We used the four samples with greater quantities of total DNA for the bisulfite conversion step, as the Zymo EZ DNA Methylation-Lightning™ kit protocol requires at least 100ng of DNA material. We diluted our samples to 20 uL and followed Zymo's protocol for the remaining samples, using Qubit to quantify remaining converted single-stranded DNA sample. Our results ranged from 21.1 to 29.3 ng/uL sample of 9uL eluted product. This allows us to use 50ng sample for library preparation, while the rest can be used for PCR.

For library prep, we used the Epicentre EpiGnome™ Methyl-Seq Kit and EpiGnome™ Index PCR Primers and protocol. We used the AMPure XP system for DNA purification (beads with magnetic stand)

for DNA fragment selection. The quality of the libraries were measured in a 2100 Bioanalyzer (Agilent). We found that most of our samples had low yield. In spite of using 12 cycles of the Failsafe PCR system. We plan to run 2 more cycles of PCR and test for any increased yield. In the future I will utilize the Bioanalyzer facilities at Arizona State University to test the quality. We replicated this protocol on the remaining 8 samples, yielding similar results. From conversations with the Staff Technical Applications Scientist at Illumina, we have been instructed to use 100 ng input converted DNA in future work, using 10 ng of native/unconverted DNA for comparison. We can also increase the amount of total DNA used in the Zymo EZ DNA Methylation-Lightning™ kit to 200ng.

In order to confirm successful bisulfite conversion without running Illumina sequencing analysis, we decided to use the remaining single-strand DNA from the bisulfite conversion for PCR analysis candidate genes. We chose primers using a combination of the methprimer program (http://www.urogene.org/methprimer/) as well as Zymo's bisulfite-primer-seeker (http://www.zymoresearch.com/tools/bisulfite-primer-seeker). Primers designed had at least 24bp to achieve melting temperatures above 50C, with product sizes designed to be no longer than 300bp, as BS treatment shears the DNA. As recommended through peers at ASU, we used 15-25ng product for each 25uL reaction with at least 40 runs, using an annealing gradient of four reactions between 52-60C. 1.5% agarose electrophoresis followed by ethidium bromide staining and UV imaging revealed that the highest annealing temperature (60C) resulted in the greatest intensity for most primers. This crude PCR reaction resulted in products in the 400ng/µL range. We also plan to test nested primers/touchdown PCR for greater yield than the regular PCR with better resolution. We also plan to try higher annealing temperatures and assure the quality. For identifying differently methylated regions, we used the same forward/reverse primers used for amplification for capillary sequencing, resulting in reads typically around 100bp per primer. While there are few gaps between primers, we have covered a majority of the loci of interest using our first four primer pairs.

While I have discussed many alternatives to the protocol, I was successful in acquiring the desired skills required to answer my current scientific questions. I am very thankful for Hongmei Li-Byarlay's continued help in troubleshooting my specific protocols, and I am grateful for NCSU's hospitality during my stay. I would like to extend my gratitude towards IGTRCN for giving me the opportunity to advance in my molecular skillset, as well as build relationships with other labs which can lead to possible collaborations in the future.