**RNA Seq Workflow for bacterial RNA using Rockhopper**

*\*Prior to first project, you will need to download the following freeware:*

*-Zipeg for Mac or 7Zip for PC; IGV and Rockhopper*

*-For EPEC you will also need to transfer a folder of replicon files from Louie For different genomes,*

*try one from the drop down menu first. If doesn’t work, will need the same*

* Download your sequence files from BaseSpace, and unzip them using Zipeg (7Zip if on a PC). Make sure they are in a well named folder on your desktop (or in other well named folder)
* Open up Rockhopper (may need admin username and password).
* For replicon name click on the green DNA strand, hit ok, and browse to folder that you got from Louie and choose the whole folder.
* For Experiment Name – write you condition 1 (eg. EPEC 23)
* Browse to your first sample in this group and choose it.
* For further samples in this group, just click on the blue plus sign and navigate to each sample in the group.
* Click on the red plus sign to add a new condition and its replicates just as in the first one.
* Finally, go under Options, Parameters, and make sure the defaults are appropriate for your experiment. You may want to click on “verbose output” if you are interested in seeing the raw data in the spreadsheet files as well as the analyzed. May help you understand your data and you can always delete unnecessary columns later.
* Once all data is loaded and parameters defined, hit submit. The program will run (time is dependent on genome size and computer power). EPEC with 2 experiments and 2 replicates each on my computer took 5 minutes.
* Once done, try hitting view in IGV. If it fails, you can load things manually as follows:
  + - All results go into a folder on your desktop called Rockhopper\_Results which was created by the program. Don’t change name or location, this will remain your results folder from here on out. Just make folder within this folder to put all pertinent results in and keep your results organized.
    - There are two text files – these are your gene and operon list files that can be open in excel and sorted how you like. I sort by Q value. Anything with a q value less than 0.01 is significant. The numbers under the corresponding expression columns are the actual sequence counts for each condition. These can be used to calculate fold change (one expression value over the other), and then take the log2 of that fold change number to get the actual fold change values we are used to. Genes of interest in the list can be further analyzed in Kegg or other database (trying to learn CummeRbund for further visuals.
    - For visualizing the data, open up IGV
    - Go to “Genomes” -> “Load from File” and navigate to your .genome file (for this first EPEC round it is in the Replicon Info folder.
    - Go to “File” -> “Load from File” and navigate to and load all files in your Rockhopper\_Results Folder, in the “GenomeBrowserFiles” (may want to only load plus or minus strands to start).
    - Right click on the data range numbers in the upper left hand corner of each track for you plus or minus reads, and from the drop down menu change the “Set Data Range” from 0-800.
    - You can zoom in on areas of interest just by boxing out a portion of the line that conveys the number of basepairs you are looking at in the window (has a little black arrow on each end)
    - You can change track height and color, and data range by right clicking on the track name, on the far left) and choosing operation from the drop down menu.
    - Enjoy the exploration☺

Addendum: Creating your own replicons

* If you need to create your own replicons either because the replicon listed is not working or your organism is not present, you will need to do the following:
  + You will need a .fna, a .ptt, and a .rnt file placed in a single folder to submit as your replicon.
  + The .fna and .ptt files are the bare essentials. .rnt is optional but is what contains the rRNA and tRNA info.
  + Most of these files can be found at: <ftp://ftp.ncbi.nih.gov/genomes/Bacteria/>
  + If you can’t find there try googling your bacteria name and .fna, etc.
  + Once you navigate to the file you need, do “Save page as” and place in your designated replicon folder.