***Library Preparation of Genomic DNA from E. coli, Sequencing and Analysis***

***Protocol by David T. Fraebel***

***Version of 11th/May/2017***

1. Read the **Nextera XT DNA Library Prep Reference Guide** (<https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-02.pdf>). This document has lots of important information and useful tips to bear in mind throughout the process.

Also read **Best Practices** (<http://support.illumina.com/sequencing/sequencing_kits/nextera_xt_dna_kit/best_practices.html>).

1. Determine and obtain whichever **MiSeq reagent kit** is needed to achieve desired coverage.

*I prefer the v3 kits- Use a 150-cycle kit if 3.8 gigabase is sufficient, otherwise use a 600-cycle kit (if 15 gigabase is insufficient you’ll have to use a HiSeq).*

1. Make sure you have enough of all the kits and reagents you’ll need to prepare and quantify (if doing Qubit yourself) all your samples.

*I use these kits:*

*DNA extraction-* ***MoBio UltraClean Microbial DNA Isolation Kit***

*Library prep-* ***Nextera XT DNA Library Preparation Kit + Nextera XT Index Kit***

*Qubit quantification-* ***Qubit dsDNA BR Kit***

*PCR cleanup-* ***Agencourt AMPure XP - PCR Purification + EpiMag HT Magnetic Separator***

1. Extract genomic DNA from overnight cultures using the MoBio kit.

*Protocol-* [*https://mobio.com/media/wysiwyg/pdfs/protocols/12224.pdf*](https://mobio.com/media/wysiwyg/pdfs/protocols/12224.pdf)

1. Quantify input DNA densities with Qubit.

*This is done at the Functional Genomics Unit, 356 Edward R. Madigan Lab (*<http://biotech.illinois.edu/functionalgenomics>*). You can either pay $3.60/sample to have it done for you (submit 3uL of each sample) or provide your own reagents and pay $20/year for unlimited self-service for your whole lab. If doing self-service use* **2uL sample + 198uL working solution**.

*Protocol-* [*https://tools.thermofisher.com/content/sfs/manuals/Qubit\_dsDNA\_BR\_Assay\_UG.pdf*](https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_BR_Assay_UG.pdf%20)

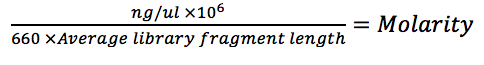
1. Dilute input DNA to 0.2ng/uL in 10mM Tris, pH 8.5.
2. Perform the first three stages of library prep as described in the **Reference Guide**: **Tagment Genomic DNA, Amplify Libraries and Clean Up Libraries.**

*IMPORTANT: Keep track of which pair of indices corresponds to each sample!*

1. Quantify cleaned-up libraries’ densities with Qubit.
2. The first time you prepare libraries it’s useful to quantify their length with Bioanalyzer.

*This is also done at the Functional Genomics Unit, up to 11 samples can fit on one $116 chip. They do it for you, you just need to submit 3uL of the cleaned-up libraries diluted to 1.0ng/uL (Do not dilute your entire sample). If you’re having them do the Qubit as well they can prepare the dilutions, submit 4uL of each in this case. The Bionanalyzer traces should look like one of the examples on page 15 of the* ***Reference Guide****.*

1. Calculate the **nanomolar** concentration of each library using the density from Qubit and the average length from Bioanalyzer:

**[nM]**

*After you’ve prepared libraries a couple times you’ll get a sense of what length distributions are typical for a given combination of kits and type of input DNA. In such cases I will skip Bioanalyzer and calculate a lower bound on concentration using a slightly longer length than observed in the past. Running the cleaned-up libraries on a gel can tell you if your assumptions are reasonable.*

1. Choose a normalization method and normalize libraries- Read this note to help you choose: <https://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/bead-based-normalization-tech-note-470-2016-007.pdf>

* If all libraries >10-15nM, do the **bead based normalization** described in the **Reference Guide.**
* If some are <10-15nM you’ll have to normalize manually:
  + For a v3 **reagent kit**, dilute each library to 4nM in 10mM Tris, pH 8.5.
  + If some are <4nM you can use one of the **Standard Normalization** recommendations in the **NextSeq Denature and Dilute Libraries Guide** (<http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/nextseq/nextseq-denature-dilute-libraries-guide-15048776-02.pdf>). If you do this, keep using this guide (instead of the MiSeq one) for step 14.

1. Pool equal volumes of each normalized library in a single tube and invert several times to mix.
2. Start thawing the **reagent cartridge** as described in the **MiSeq System Guide** (<http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-system-guide-15027617-01.pdf>) or coordinate with Betty and have her do it for you.
3. Denature and dilute the pooled library according to the normalization method you used as described in the **MiSeq Denature and Dilute Libraries Guide** (<http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-01.pdf>). If you did manual normalization you will have to choose a loading concentration based on Table 1 in this note (<http://www.illumina.com/documents/products/technotes/technote_nextera_library_validation.pdf>).
4. Prepare a PhiX control and combine with the denatured and diluted pooled library as described in the **Denature and Dilute Guide.**
5. Create a **sample sheet** using the **Illumina Experiment Manager** (<http://support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.html>).

*Set it to* ***Other- FASTQ******Only*** *and check the box for* ***Adaptor Trimming.***

1. Load the thawed **reagent cartridge** as described in **MiSeq System Guide** and start the run, or have Betty do it for you.
2. When the run completes, download the FASTQ files from **BaseSpace** (<https://basespace.illumina.com/>). This site will also have useful statistics about your run.
3. Install **Breseq** (<http://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing>) according to the documentation (<http://barricklab.org/twiki/pub/Lab/ToolsBacterialGenomeResequencing/documentation/>) and make sure it works by doing the **Test Drive** described therein.
4. Download your strain’s **Reference Genome** in .gbk form from NCBI.
5. Run **Breseq** for each of your stains using both of the FASTQ output files and the reference genome.

*I use the* ***‘-j 4’*** *option to use all 4 of my processors. If sequencing non-clonal samples I use the* ***‘-p’*** *option to predict polymorphic mutations and sometimes use a frequency cutoff to exclude low-frequency polymorphisms. Beyond that there are many more options that you can use as desired. Run* ***‘breseq –h’*** *to view a list of the advanced options.*