**MCB 525, Fall 2016**

**1.6: PCR Amplification and Barcoding**

**Objective:** In this section, the ligation products are amplified and sample-specific barcodes are introduced, along with sites needed so the library fragments will bind to the Illumina flow-cell during sequencing. There are 4 primers in this PCR reaction: the longer ILL-HT/ILL-BC primers (containing sample-specific barcodes) and a higher concentration of the shorter ILL-Lib1 and ILL-Lib2, which take over after a few cycles. We perform a “test-scale” PCR to determine optimum cycle number, or the minimum cycle at which you can visualize the product. The full 2bRAD protocol (including the prep-scale PCR and gel purification of the target band through a nifty “freeze-and-squeeze” method) can be referenced on the Meyer lab website at <http://people.oregonstate.edu/~meyere/docs/2bRAD_11Aug2015.pdf>

**Protocol:**

1. We will perform 3 PCR reactions for each of your ligation products. Prepare a master mix based on the following recipe for a single reaction:

Nuclease-free H2O 4.3 μL

10 mM dNTP 0.4 μL

10 μM ILL-Lib1 0.4 μL

10 μM ILL-Lib2 0.4 μL

1 μM ILL-HT 1.0 μL

1 μM ILL-BC 1.0 μL

5X HF buffer 4.0 μL

Phusion polymerase 0.5 μL

1. Combine 12 μL master mix with 8 μL of the ligation product from step 1.5.
2. Amplify on the following profile:

98°C for 10 sec

98°C for 5 sec

x *N* cycles 60°C for 20 sec

72°C for 10 sec

1. Remove one tube for each template at N= 10, 15, and 20 cycles.
2. To determine optimum cycle number, run these products on a **2% agarose** gel.
3. This time, we are interested in resolving a small PCR fragment (~166 bp) rather than visualizing genomic DNA so run the gel with **the 50 bp ladder**.
4. Also use the **loading dye diluted 1:10 with 50% glycerol** instead of the 6X loading dye to avoid the shadow on the gel that often occurs with the 6X loading dye.

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1. *Can you identify the desired product that you would cut out of the gel? Do you have any secondary products to be aware of?*
2. *What cycle number would you choose for the full-scale PCR reaction*
3. *Given the following desired library fragment (total size: 166 bp) and sequences for the primers, during which stage of the protocol was each underlined portion originally introduced?*

***Desired library fragment:***

*5’*- AATGATACGG CGACCACCGA GATCTACAC**[BC2]** ACACTCTTTC CCTACACGAC GCTCTTCCGA TCT – (N)12CGA(N)6TGC(N)12 - AGATCGGAAGA GCACACGTCT GAACTCCAGT CAC**[BC1]**ATCTCG TATGCCGTCT TCTGCTTG *-3'*

1 2

3 4

5

***Primers:***

*ILL-HT: 5’-* AATGATACGG CGACCACCGA GATCTACAC**[BC2]** ACACTCTTTC CCTACACGAC GCTCTTCCGA TCT -*3’ \*\*BC1 and BC2 represent unique 5-6 bp barcode sequences\*\**

*ILL-BC: 5’-* CAAGCAGAAG ACGGCATACG AGAT**[BC1]**GTGAC TGGAGTTCAG ACGTGTGCTC TTCCGATC -*3*’

*ILL-Lib1: 5’-* AATGATACGG CGACCACCGA *-3’*

*ILL-Lib2: 5’-* CAAGCAGAAG ACGGCATACG A *-3’*

*5ILL-NN: 5’-* CTACACGACG CTCTTCCGAT CTNN *-3’*

*3ILL-NN: 5’-* CAGACGTGTG CTCTTCCGAT CTNN *-3’*

*Anti-ILL: 5’-* AGATCGGAAG AGC(InvdT)-*3’*