# Illumina Miseq Barcoding Protocol

1. Quantify samples to be sequenced using Picogreen protocol.
2. Calculate sample volume necessary for addition of **5 ng** of template DNA to PCR reactions.
3. PCR reaction composition for 1 rxn:
   * 12.5 uL Mastermix (NEB Q5 High Fidelity, Hot Start PCR Mastermix - M0494)
     + 50% of total volume
   * 2.5 uL combined forward and reverse barcoded primers (10 uM)
     + 10% of total volume
     + Primer plates can be created with the robot method: "make\_primer\_plate"
   * 1.25 uL BSA (20 mg/mL, NEB B9000S)
     + 5% of total volume
   * 0.625 uL Picogreen reagent
     + 2% of total volume
     + 4x concentration, dilute the 200x stock that comes in the Picogreen kit with 1X TE
   * X uL template (5 ng/reaction)
   * PCR water up to 25 uL

* **If adding pico & BSA to master mix prior to aliquoting master mix:**
  + total\_volume = master\_mix + BSA + pico
  + volume\_master\_mix = total\_volume \* 0.870
  + volume\_BSA = total\_volume \* 0.087
  + volume\_pico = total\_volume \* 0.043

1. Use robot method "qPCR\_wWorklist\_altdispense" for setting up PCRs, running triplicate reactions for each sample to be sequenced.
2. Run the PCR plate on the qPCR thermocycler, using the following cycle:
   * 98oC for 30 seconds
   * 30 cycles of:
     + 98oC for 5 seconds
     + 50oC for 30 seconds
     + 72oC for 10 seconds
   * Final extension of 72oC for 2 minutes
   * Hold at 4oC
3. Combine triplicate PCRs for each samples, transferring samples to a new 96-well plate.
   * This can be done with the robot method: "plate\_pooling"
4. Perform Sequal PCR purification and normalization
   * SequalPrep Normalization Plates, Life Technologies, A10510-01
   * Follow the [manufacturer's instructions](https://www.lifetechnologies.com/order/catalog/product/A1051001), using 25 uL of PCR product for each sample.
   * This can be done with the robot method: "SequalPrep\_Assay"
5. Combine all Sequal'd samples (20 ul/sample) into one tube (or two, if the volume too large).
   * Pre-weigh the tube to help with the next step (speed-vac).
   * This can be done with the robot method: "plate\_pooling"
6. Vacuum evaporate samples to concentrate.
   * [speed-vac](../speed-vac/speed-vac.html)
   * You need >=25 uL with a concentration of 5 ng/uL
7. Quantify concentrated collection of samples using PicoGreen.
   * Make sure to have >=25 uL with a concentration of 5 ng/uL.
8. Run concentrated sample on a gel to ensure expected, cleaned product for sequencing.
   * If additional, unexpected bands are seen, consider [Gel extraction](../gel_extraction/gel_extraction.html) of the concentrated sample.
     + This may result in higher sequence quality.
9. Submission to Cornell Sequencing Facility:
   * Place 20 uL of sample with a concentration of 5 ng/uL into sequencing tube.
   * Additionally, submit 10 uL of 100 uM sequencing primers:
     + forward sequencing primer
     + reverse sequencing primer
     + reverse index read primer

## Usual comments provided with the sequencing order:

### Comments for 515f-806r primers

We have generated our dual-indexed custom barcoded library as described in Kozich et al., (2013). Along with the submitted library, we have provided 10 ul of 100 uM custom sequencing primers: Primer 1, Index Primer, Primer 2. We request the following MiSeq run specifications: a **cluster density of 650-750k/mm^2** (under-shooting the cluster density is better than over-shooting), a **PhiX spike-in of 5%**. These MiSeq run specifications are described in Kozich et al., (2013); see 'Run Monitoring' in the Supplemental Materials. Please let us know if you have any questions about the primers or requested run parameters. Thank you! [Citation: Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. (2013). Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol 79:5112-5120.]

### Comments for 515f-927r primers

We would like to run the 2x300 paired end read platform. Our submitted sample is a pooled set of already barcoded samples. We have supplied custom primers (labeled Primer 1, Index Primer 1, and Primer 2) for the sequencing as well, as follows: Primer 1: combined pad, linker, and gene-specific primer sequence at the 5' end of the region (pad+linker+515F primer) that will produce a long sequence read from the 5' end of the sequence. (Read 1) Index Primer 1: reverse complement of the pad, linker, and gene-specific primer from the 3'end (pad+linker+927R) that will provide an index read of the 3' end of the sequence. (Read 2) Primer 2: Combined pad linker, and gene-specific primer sequence at the 3' end of the region (pad+linker+927R primer) that will produce the second sequence read (300 nt) from the 3' end of the sequence (Read 4). The primers are described in more detail in [Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. (2013). Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol 79:5112-5120]. 10ul of 100uM primer has been supplied. Please let us know if you have any questions about the primers.