# 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)
   * 2.5 uL combined forward and reverse barcoded primers (10 uM)
     + Primer plates can be created with the robot method: "make\_primer\_plate"
   * 1.25 uL BSA (20 mg/mL, NEB B9000S)
   * 0.625 uL Picogreen reagent
     + 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
4. Use robot method "qPCR\_wWorklist\_altdispense" for setting up PCRs, running triplicate reactions for each sample to be sequenced.
5. 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
6. Combine triplicate PCRs for each samples, transferring samples to a new 96-well plate.
   * This can be done with the robot method: "plate\_pooling"
7. 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"
8. 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"
9. Vacuum evaporate samples to concentrate.
   * [speed-vac](../speed-vac/speed-vac.html)
   * You need >=25 uL with a concentration of 5 ng/uL
10. Quantify concentrated collection of samples using PicoGreen.
    * Make sure to have >=25 uL with a concentration of 5 ng/uL.
11. 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.
12. 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