Pooling your Illumina Libraries for Sequencing

Updated 01-Mar-2024 by Katie Murphy

BACKGROUND:

Illumina libraries should be pooled in equimolar amounts prior to sequencing submission. Doing so should result in the most even distribution of reads across your samples for a given library pool.

HELPFUL HINTS:

- LAB has a spreadsheet available that can assist in your molarity and pooling calculations. We highly recommend referring to this spreadsheet when following the protocol below.

MATERIALS:

- "Library_pooling_calculations.xlsx" spreadsheet from LAB
  - Can be found on LAB's SharePoint site.
- Individual library quantification results from Qubit or Quant-iT
- Individual library size assessment from TapeStation or Bioanalyzer
- Libraries to be pooled

PROTOCOL:

Step 1 – Pooling

1.1 Convert each library's concentration to molarity (nM) using your ng/ul from HS Qubit or Quant-iT and your average size (bp) from Tapestation or Bioanalyzer.
   a. The conversion equation can be found in the "ADDITIONAL INFORMATION" section at the end of this protocol.

1.2 Pick a set volume (µL) to pool for *either* your most dilute library (lowest nM) or your most concentrated library (highest nM).
   a. If choosing your most dilute library, this sample will have the greatest volume being pooled.
   b. If choosing your most concentrated library, this sample will have the lowest volume being pooled.

1.3 Multiply the nM value of that library (nmol/liter, or fmol/uL) by the volume pooled (uL), to get the number of femtomols being pooled for your chosen library. *This exact femtomol value is what you will use when calculating the volume to pool for each of your remaining samples.*

1.4 Now calculate the volume (µL) to pool for your remaining samples by dividing the femtomols from above by the nM of each sample.

1.5 As in step 3, calculate the femtomols pooled for each individual sample. This step is simply to check your math, to make sure you haven't mistyped/miscalculated anything. You should have the same value for all samples.

1.6 Verify that all of your volumes to pool are feasible. If any are too high (require more library than you have) or are too low to pipette accurately, then adjust the volume you used in step 2 and recalculate your other values.

1.7 Calculate your expected nM for your pool by summing up the total femtomols and dividing by the total volume.

1.8 Also calculate the expected ng/µL for your pool by summing up the total ng pooled and dividing by the total volume.

Step 2 – Pooling QC

2.1 Use HS dsDNA Qubit or Quant-iT to measure the concentration (ng/µL) of your library pool.

2.2 Run 2 uL of your pool on a HS D1000 or HS D5000 tape to determine the average fragment size for the pool and to verify that no adapter-dimer is present.

2.3 Calculate the molarity (nM) of your pool using the equation given in the "Additional Information" section of this guide.

2.4 Optional: If required or recommended by the sequencing facility, use qPCR to most accurately quantify your library pool.

Your pool is now ready to submit for sequencing!

ADDITIONAL INFORMATION:

- When you need to convert from ng/ul to nM, please use the following equation. Average size is determined using the TapeStation or Bioanalyzer, and concentration in ng/µL measured using Qubit or Quant-iT.

$$\frac{(\text{concentration in ng}/\mu\text{L})}{(660\ \text{g/mol}) \times (\text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

- If the pool shows an adapter-dimer peak around 130 bp on the TapeStation or Bioanalyzer, you should perform an additional 0.8X bead cleanup.

- iTru y-yoke adapter and index sequences can be found in Glenn *et al* (2019).

REFERENCES:

1. Glenn TC, Nilsen RA, Kieran TJ, Sanders JG, Bayona-Vásquez NJ, Finger JW, Pierson TW, Bentley KE, Hoffberg SL, Louha S, Garcia-De Leon FJ, del Rio Portilla MA, Reed KD, Anderson JL, Meece JK, Aggrey SE, Rekaya R, Alabady M, Belanger M, Winker K, Faircloth BC. 2019. Adapterama I: universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext). PeerJ 7:e7755 http://doi.org/10.7717/peerj.7755.