Ig-Seq Library Quantification

Materials: Library Quantification Kit – Illumina/ABI Prism (#KK4835), library dilution buffer (10 mM Tris-HCL, pH 8.0, 0.05% Tween 20)

1. Prepare 1:10,000 dilutions of each Ig-Seq library with library dilution buffer (or other appropriate dilutions).
2. The first time you use the kit, add 1 mL of Illumina Primer Premix to the 5 mL bottle of KAPA SYBR FAST qPCR Master Mix (2x) and mix by vortexing 10sec.
3. Thaw Standards 1-6 on ice.
4. Prepare qPCR reaction plate. Each sample and standard should be measured in triplicate. For high-throughput sample processing, duplicate measurements are acceptable. For each reaction, combine the following reagents:

Reagent	Volume per sample (µL)
KAPA PCR Master Mix (2x)	6.0
Ig-Seq library or Standard	4.0

5. Seal plate. Vortex for 5 seconds. Centrifuge for 1 minute at 500 RCF.
6. Using a real-time PCR machine, begin PCR:
    a. Melt curve analysis is recommended. It may provide a useful indication of possible primer and/or adapter dimer contamination of libraries.

Step	Cycles	Temperature (ºC)	Time (minutes)
1	1	95	5:00
2	35	98	0:30
		60	1:10

    b. Annotate DNA Standards for standard curve as follows before data analysis: Standard concentrations should be input to generate standard curve.

Standard	Standard Concentration (pM)
1	20
2	2
3	0.2
4	0.02
5	0.002
6	0.0002

Data Analysis

1. Obtain the average calculated concentration of each 1:10,000 dilution for each Ig-Seq library, as determined by qPCR in relation to the concentrations of DNA Standards 1-6.
    a. Outliers distant by >0.25 C_T from the average may be omitted.
2. Perform a size adjustment calculation to account for the difference in size between average fragment length of the library (as determined by the TapeStation/Bioanalyzer) and the DNA Standard (452 bp).
3. Calculate the concentration of the undiluted library by taking account of the dilution factor
4. Sample Calculation:

Library Name	Conc. in pM calculated by qPCR instrument (triplicate data points)			Avg. conc. (pM)	Size adjusted Concentration (pM)	Conc. of undiluted library stock (pM)
Library A	A1	A2	A3	A	$A \times \frac{452}{Avg.Fragment\ Length} = W$	W x 10,000

Library Pooling
Materials: library dilution buffer (10 mM Tris-HCL, pH 8.0, 0.05% Tween 20)

Notes and Guidelines

- A scheme to pool Ig-Seq libraries for sample multiplexing should have been designed prior to addition of unique pairs of barcodes during the 2nd PCR.
- Optimal pooling achieves both even sequencing coverage for each sample and the recommended 10x coverage (10 reads/cell).
- Even sequencing coverage requires that each individual Ig-Seq library is proportionally represented in the pool, based on its input cell count, relative to input cell counts of all other libraries in the pool.

Methods

1. Pool samples, so that the final concentration of the pool is 5 nM or higher. Use 10 mM Tris-HCL, pH 8.0, 0.05% Tween 20 to dilute the pool, if necessary.

Ig-Seq Library Pool Re-quantification: Follow the same protocol from "Ig-Seq Library Quantification" to accurately assess the concentration of the pooled BCR-Seq Library.

Sequencing Ig-Seq Libraries on Illumina Platforms

Notes and Guidelines

- Illumina provides the most comprehensive documentation on library denaturation and instrumentation. This following sections are overviews of the process MiSeq uses to begin a run and highlights the custom alterations necessary to sequence Ig-Seq libraries.

MiSeq Guide

Materials

- Thawed MiSeq Reagents Kit v3 (600 cycles) (catalog# MS-102-3003)
    - Thaw for 1 hour in room temperature water bath.
- Ig-Seq Library (4 nM)
- PhiX Control v3 (catalog# FC-110-3001)

MiSeq Notes

- MiSeq Reagents Kit v3 yield up to ~15 million reads.
- To ensure optimal run quality, ~50% of the reads should be a balanced genome (Ex.: PhiX).
- Optimal cluster density: 1200-1400 K/mm^2
- Recommended loading concentration for MiSeq: 15-20 pM

Preparing Libraries for Sequencing on MiSeq

1. Thaw supplied HT1 (Hybridization Buffer) at room temperature.
2. Prepare fresh dilution of 0.2 N NaOH.
3. Start with a 4 nM Ig-Seq library.
4. Combine the following volumes of sample DNA and freshly diluted 0.2 NaOH in a microcentrifuge tube:
    - 4 nM Ig-Seq Library (5 µL)
    - 0.2 N NaOH (5 µL)
5. Vortex briefly to mix the sample solution, and then centrifuge the sample solution at 280 RCF for 1 minute.
6. Incubate for 5 minutes at room temperature to denature the DNA into single strands.
7. Add the following volume of pre-chilled HT1 to the tube containing 10 µL denatured DNA:
    - Pre-chilled HT1 (990 µL)
8. This results in a 20 pM denatured library in 1 mM NaOH. Store denatured 20 pM library on ice, until MiSeq reagent cartridge is prepared.

Preparing PhiX Control

1. Combine the following volumes to dilute the PhiX library to 4 nM.
    - 10 nM PhiX library (2 µL)
    - 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 (3 µl)
2. Combine the following volumes of 4 nM PhiX library and freshly diluted 0.2 NaOH in a microcentrifuge tube:
    - 4 nM PhiX library (5 µL)
    - 0.2 N NaOH (5 µL)
3. Vortex briefly to mix the solution, and then centrifuge the solution at 280 RCF for 1 minute.
4. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.
5. Add the following volume of pre-chilled HT1 to the tube containing 10 µL denatured DNA:
    - Pre-chilled HT1 (990 µL)
6. This results in a 20 pM denatured PhiX library in 1 mM NaOH.
7. This results in a final 20 pM denatured PhiX library, to be combined with the 20 pM denatured Ig-Seq library.

Creating Sample Sheet

1. Open *Illumina Experiment Manager* and select *Create Sample Sheet.*
2. Select *MiSeq*. Select Next.
3. Select *Other* → *FastQ Only.* Select *Next.*
4. Enter *Reagent Cartridge Barcode.*
5. Select Sample Prep Kit: *Nextera*.
6. Check Index Reads: *1*.
7. Enter *Project Name, Experiment Name, Investigator Name, Description, Date.*
8. Choose Read Type: *Paired End.*
9. Enter number of cycles for Read 1: *309*.
10. Enter number of cycles for Read 2: *309.*
11. Do not check *Custom Primer for Read 1, Custom Primer for Index, Custom Primer for Read 2,* or *Use Adapter Trimming.*
12. Select *Next.*
13. Select *Add Blank Row.* Enter "sample1" under *Sample ID.* Select *N701* under *Index 1 (i7)*. Disregard any warning and select *Finish.*
14. Save file and review in Notepad.

Starting MiSeq Sequencing Run

1. Thaw and dry MiSeq reagent cartridge, following Illumina guidelines.
2. Load final denatured 20 pM Ig-Seq with 20 pM PhiX spike-in into reagent cartridge position #17.
3. Load Index Read Primer *Index Ig (Constant) mix* (this will be a mix of the index primers for each constant region in your samples) into position #13 at a final concentration of **0.5 µM**. The total volume in position #13 is 680 µL. Mix with 1 mL serological pipette.
4. Load Read 2 Primer Mix (a mix of the constant region sequencing primers that are present in the run. I.e. IgG seq, IgM seq, IgK seq, IgL seq) into position #14 at a final concentration of **0.5 µM**. The total volume in position #14 is 680 µL. Mix with 1 mL serological pipette.
5. Carefully tap the reagent cartridge on the bench and ensure no bubbles are present in any positions.
6. Follow instructions to load flow cell and reagents through MiSeq Control Software interface.
7. Start Sequencing.
8. FastQ files with Ig-Seq reads will be output to the "Undetermined" FastQ file in the designated runs folder.