

<b>GeneLab SOP for TruSeq Total RNA library pooling, normalization and QC</b>	Document No.:	GL-SOP-6.5
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	Last revised by:	Valery Boyko

### Purpose/Scope:

This SOP describes the steps used by NASA GeneLab to pool, normalize, and check the quality of TruSeq Total RNA libraries using an Agilent TapeStation and an Illumina iSeq.

### Reagents:

1. Agilent D1000 DNA ScreenTape – (Cat#5067-5582, stored at 4°C)
2. Agilent D1000 DNA Reagents – (Cat#5067-5583, stored at 4°C) Including D1000 DNA Sample Buffer and D1000 ladder.
3. Qubit dsDNA HS Assay Kit (Thermo Fisher Cat#Q32854/51) or Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Cat#Q33231/30)
4. 8-strip PCR tubes or 96-well plate
5. cDNA libraries
6. Plate seals
7. Nuclease-Free water
8. TruSeq Resuspension Buffer (RSB)
9. Microplate centrifuge
10. Table top vortex
11. Table to micro-tube centrifuge
12. 1.5mL microtubes
13. 1-2 N NaOH
14. Tris-HCl pH 8.5
15. Tris-HCl pH 8.0

### Procedure:

[Click on the procedure step for quick navigation.]



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### **Individual library QC:**

This procedure describes how to obtain average library size [nt]. Average library size for each library in the planned multiplexed pool should be determined prior sample multiplexing. +/-60nt is tolerated, if the distribution is more spread out, expect the shorter fragments to cluster more abundantly to the flow cell.

It is best to perform this step on the day that the library construction is completed.

1. Take out all Agilent D1000 TapeStation components and let equilibrate to room temperature for 30min: Screen Tape, Sample Buffer and Sample Ladder (optional).
2. Only work with cDNA libraries when fully thawed.
  - a. To thaw, place the plate on ice for 10-20min.
  - b. Make sure the adhesive foil is tightly attached to the plate and vortex the plate.
  - c. To avoid cross contamination, spin the plate using a microplate centrifuge before removing the adhesive cover.
3. Follow SOP#004.
4. Record the average fragment size and the estimated sample concentration in the experiment master-sheet.
5. Use the estimated sample concentration [nG/uL] and average library size [nt] to convert the values to molarity [nM]. Use the following formula:

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

6. Assess the spread of the library concentrations in the pool by calculating the least concentrated library and the most concentrated library.
7. Calculate average library size [nt], it will be used to assess the pool molarity.

Note: TapeStation is not an accurate measure of quantity for dsDNA libraries. It's only acceptable for this workflow that combines iSeq sequencing and individual library clustering ability for pool normalization.

### **Creating equal volume library pool:**

1. Make sure all the libraries that need to be multiplexed are thawed:
  - a. To thaw, place the plate on ice for 10-20min.
  - b. Make sure the adhesive foil is tightly attached to the plate and vortex the plate.
  - c. To avoid cross contamination, spin the plate using a microplate centrifuge before removing the adhesive cover.
2. Combine 2uL of each library in to a 1.5mL tube.
  - a. If working with many plates, use a multi-channel pipette and 8-tube strip to combine 2uL of each plate row. Combine the content of the 8-tube strip into one 1.5mL tube.
  - b. Create a pool for each plate.
  - c. Combine all pools.
3. Label the tube clearly.
4. Gently vortex the equal volume pool and place on ice until use.
5. Safely seal the library plates with adhesive seal and store at -20°C for later use.

### **iSeq run 1 - Equal volume pool:**

1. Follow iSeq User Guide to properly thaw the sequencing cartridge:

Method	Thaw Time	Instruction
20°C to 25°C water bath	6 hours, not exceeding 18 hours	<ul style="list-style-type: none"> <li>• Use 6 L (1.5 gal) water per cartridge.</li> <li>• Set a temperature-controlled water bath to 25°C <b>or</b> mix hot and cold water to achieve 20°C to 25°C.</li> <li>• Face the bag label up, submerge the cartridge completely, and apply ~2 kg (4.5 lbs) weight to prevent floating.</li> <li>• Do not stack cartridges in the water bath unless it is temperature-controlled.</li> </ul>
2°C to 8°C refrigerator	36 hours, not exceeding 72 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.
Room-temperature air (20°C to 25°C)	9 hours, not exceeding 18 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.

2. Make sure RSB buffer is at room temperature prior use.
3. To measure the pool concentration, use Qubit dsDNA HS or Qubit 1X dsDNA HS reagent.
4. Record pool concentration; deduct 20% from the value given by Qubit.
5. Use previously calculated average fragment size and Qubit concentration -20% to calculate pool molarity following the formula:

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

6. Take out the Flow Cell from 4°C storage.
7. Use “Loading conc calculator” for help with calculating dilution and:
  - a. Dilute the equal volume library pool to 1nM with RSB buffer in a low bind 1.5mL microtube. Vortex and spin down.
  - b. Dilute the equal volume library pool to loading concentration (50pM for TruSeq Total RNA kit) with RSB buffer in a low bind 1.5mL microtube. Vortex and spin down.
  - c. Dilute PhiX control to same loading concentration (50pM) and add 1% to the library pool.
8. Follow the iSeq user guide and SOP#022 Sequencer Set Up to load the library on to the sequencer.
9. Safely store the remaining PhiX and the library pools in -20°C.

### **Equal volume iSeq analysis:**

1. Transfer BCL files from the iSeq to the processing computer.
2. Perform BCL2FASTQ conversion.
3. Perform HTStream (follow “To\_generate\_HTStream\_iSEQ-QC-report\_on\_MMOC\_20190930”) to obtain:
  - a. %\_total\_reads value – clustering percentage of the library.
  - b. %\_Mus\_rRNA – percentage of the ribosomal RNA.

**Note:** GeneLab Sample Processing Laboratory rRNA% threshold is 5%. If a library presents higher ribosomal contamination, it should be brought up to the GL Science group for discussion prior proceeding with the sequencing.

4. Open “Use\_iSeq\_HTStream\_output\_to\_calculate\_library\_pooling\_values” and read the “READ ME” tab.
5. Follow instructions on sheet titled “To\_calc\_repool\_vol” to calculate the volumes of each libraries that need to be combined to create new normalized pool.

### **Creating normalized library pool:**

1. If more than 50 libraries combined, it is strongly advised to follow this protocol with another person whereas one performing the pipetting steps and the other reading out the sample ID's and the volumes.
2. Make sure all the libraries that need to be multiplexed are thawed:
  - a. To thaw, place the plate on ice for 10-20min.
  - b. Make sure the adhesive foil is tightly attached to the plate and vortex the plate.
  - c. To avoid cross contamination, spin the plate using a microplate centrifuge before removing the adhesive cover.
3. Print out the sample ID, sample location on the plate and the volume that needs to be combined. Arrange the information in a clear way, for example in a plate layout.
4. Combine pre-calculated volumes of each library in to 1.5mL tube.
  - a. If working with many plates, create a pool for each plate.

- b. Combine all pools.

**Note:** If library clustering percentage distribution in the pool requires too high of a volume for the least concentrated library, sub-pooling is possible (Follow document “Use\_iSeq\_HTStream\_output\_to\_calculate\_library\_pooling\_values”).

5. Label the pool tube clearly.
6. Gently vortex the normalized pool and place on ice until use.
7. Safely seal the library plates with adhesive seal and store at -20°C for later use.

### **iSeq run 2 - normalized pool:**

1. Follow iSeq User Guide to properly thaw the sequencing cartridge:

Method	Thaw Time	Instruction
20°C to 25°C water bath	6 hours, not exceeding 18 hours	<ul style="list-style-type: none"> <li>• Use 6 L (1.5 gal) water per cartridge.</li> <li>• Set a temperature-controlled water bath to 25°C <b>or</b> mix hot and cold water to achieve 20°C to 25°C.</li> <li>• Face the bag label up, submerge the cartridge completely, and apply ~2 kg (4.5 lbs) weight to prevent floating.</li> <li>• Do not stack cartridges in the water bath unless it is temperature-controlled.</li> </ul>
2°C to 8°C refrigerator	36 hours, not exceeding 72 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.
Room-temperature air (20°C to 25°C)	9 hours, not exceeding 18 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.

2. Make sure RSB buffer is at room temperature prior use.
3. To measure the pool concentration, use Qubit dsDNA HS or Qubit 1X dsDNA HS reagent.
4. Record pool concentration; deduct 20% from the value given by Qubit.
5. Use previously calculated average fragment size and Qubit concentration -20% to calculate pool molarity following the formula:

$$\frac{(\text{concentration in ng/}\mu\text{l})}{(660 \text{ g/mol} \times \text{average library size in bp})} \times 10^5 = \text{concentration in nM}$$

6. Take out the iSeq 100 Flow Cell from 4°C storage.
7. Use “Loading conc calculator” for help with calculating dilution and:
  - a. Dilute the normalized library pool to 1nM with RSB buffer in a low bind 1.5mL microtube. Vortex and spin down.

- b. Dilute the normalized library pool to loading concentration (50pM for TruSeq Total RNA kit) with RSB buffer in a low bind 1.5mL microtube. Vortex and spin down.
  - c. Dilute PhiX control to same loading concentration (50pM) and add 1% to the library pool.
8. Follow the iSeq user guide and SOP#022 Sequencer Set Up to load the library on to the sequencer.
9. Safely store the remaining PhiX and the library pools in -20°C.

**Normalized iSeq analysis:**

1. Transfer BCL files from the iSeq to the processing computer.
2. Perform BCL2FASTQ conversion.
3. Perform HTStream (follow “To\_generate\_HTStream\_iSEQ-QC-report\_on\_MMOC\_20190930”) to obtain:
  - a. %\_total\_reads value – clustering percentage of the library.
  - b. %\_Mus\_rRNA – percentage of the ribosomal RNA.
4. Open “Use\_iSeq\_HTStream\_output\_to\_calculate\_library\_pooling\_values” and read the “READ ME” tab.
5. Follow instructions on sheet titled “Balanced\_pool\_read\_estimate” to estimate the reads/sample for the subsequent NovaSeq run.
6. If the pool is balanced and the desired amount of reads/sample is reached, proceed to NovaSeq run.

**NovaSeq run:**

1. Follow NovaSeq 6000 User Guide to properly thaw the sequencing cartridge in a room temperature water bath:

Cartridge	Duration of Thaw
SP, S1, and S2 SBS cartridge	4 hours
SP, S1, and S2 cluster cartridge	Up to 2 hours
S4 SBS cartridge	4 hours
S4 cluster cartridge	Up to 4 hours

- Make sure RSB buffer is at room temperature prior use.
- Use normalized pool at original concentration from “iSeq run 2 - normalized pool” step. Make sure the pool is fully thawed. Keep pool on ice until use.
- Take the NovaSeq Flow Cell out from 4°C storage and let equilibrate to room temperature.
- Make the following consumables (prepare a fresh dilution each run!):
  - 0.2 N NaOH
  - 10mM Tris-HCl pH 8.5
  - 400mM Tris-HCl pH 8.0
- For PCR amplified pools such as TruSeq Total RNA, the pooled library concentration shall be 1.5-3.0nM. GeneLab SPL uses 1.5nM as a baseline.
- Use “Loading conc calculator” for help with calculating dilution and:
  - Dilute the normalized library pool to 1.5nM with RSB buffer in a low bind 1.5mL microtube. Vortex and spin down. Volume of the dilution is dependent upon the flow cell used:

Mode	Final Volume (µl)
SP/S1	100
S2	150
S4	310

- Dilute PhiX control with 10mM Tris-HCL to same concentration of 1.5nM add 1% to the library pool.
- Add 0.2 N NaOH to the tube of non-denatured pool with 1% PhiX. Volume of NaOH is dependent upon the flow cell used:

Flow Cell	0.2 N NaOH	Nondenatured Library Pool (µl)	Resulting Volume
SP/S1	25	100	125 µl, or 125.6 µl with PhiX
S2	37	150	187 µl, or 187.9 µl with PhiX
S4	77	310	387 µl, or 388.9 µl with PhiX



9. Cap the tube, vortex briefly and centrifuge.
10. Incubate at room temperature for 8 minutes.
11. Add 400mM Tris-HCl to the denatured library tube:

Mode	400 mM Tris-HCl, pH 8.0 (µl)	Resulting Volume
SP/S1	25	150 µl, or 150.6 µl with PhiX
S2	38	225 µl, or 225.9 µl with PhiX
S4	78	465 µl, or 466.9 µl with PhiX

12. Cap the tube, vortex briefly and centrifuge.
13. Transfer the full volume of denatured library with PhiX to the library tube provided with the NovaSeq 6000 reagent kit.
14. Immediately proceed to loading the library tube into the cluster cartridge and setting up the run.  
Note: denatured libraries must be used within 1 hour.
15. Follow the NovaSeq 6000 user guide and SOP#022 Sequencer Set Up to initiate the run.
16. Make sure all tubes are properly labeled and safely store the remaining PhiX and the library pools in -20°C.