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WORKS FOR ME

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## Library pooling and quality control for Illumina sequencing

COMMENTS 0

DOI

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### ABSTRACT

The ability to multiplex many samples on the same run makes Illumina sequencing a powerful and affordable tool for many researchers. When pooling samples for sequencing it is important that they are pooled at equal molarity to prevent over or under representation of any individual sample in the same run. Care also has to be taken in deciding how many samples can be combined to achieve the required sequencing depth, a factor that is further complicated in viral sequencing where the majority of the reads may originate from the host and only a small proportion corresponding to viral reads.

Also of major importance is the final pool quality control (QC) to ensure that all contaminating adapter dimers are removed, generate accurate size distribution and quantification of the final pool. Accurate QC ensures accurate loading on the sequencer, overestimation of the pool molarity can cause under clustering resulting in fewer reads and can even cause run failure. Underestimation of pool molarity causes over clustering, lowering the read quality and risking run failure.

Adapter dimers are small fragments containing full length adapter sequences which can bind to the flow cell and cluster. Due to their small size, they cluster more efficiently than the longer library fragments and so reduce the library-specific read depth as well as causing over clustering which reduces the data quality possible run failure. In addition free adapter can be incorporated into the library clusters resulting in index hopping and incorrect assignment of library barcodes.

Here we describe our standard workflow for determining the appropriate sequencing depth and pooling by equal molarity of Illumina sequencing libraries, along with our procedure for pool QC.

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### PROTOCOL CITATION

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MATERIALS TEXT

**Reagents:**

⊗ Agencourt AmPure XP beads <b>Contributed by users Catalog #A63880</b>	Step 4
⊗ Qubit® dsDNA HS Assay Kit <b>Thermo Fisher Scientific Catalog #Q32854</b>	Step 5.2
⊗ High Sensitivity D5000 ScreenTape <b>Agilent Technologies Catalog #5067-5592</b>	Step 6.1
⊗ High Sensitivity D5000 Reagents <b>Agilent Technologies Catalog #5067-5593</b>	Step 6.1

**Additional reagents:**

10 mM Tris pH8

Nuclease-free water

Absolute Ethanol

**QC Equipment:**

Equipment	
Qubit	NAME
Flurometer	TYPE
Invitrogen	BRAND
Q33228	SKU
<a href="https://www.thermofisher.com/order/catalog/product/Q33228">https://www.thermofisher.com/order/catalog/product/Q33228</a>	LINK
	

## Equipment

### 4200 TapeStation System

Electrophoresis tool for DNA and RNA sample quality control.

TapeStation Instruments

G2991AA

<https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263>

NAME

TYPE

BRAND

SKU

LINK

## SAFETY WARNINGS

Qubit reagent is known to bind nucleic acid and is provided as a solution in DMSO. Treat the Qubit dsDNA HS Reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

## BEFORE STARTING

It is recommended that individual libraries are cleaned up and QC'd following the protocol [Library clean up and quality control for Illumina sequencing](#). Each Illumina library should have an accurate size and quantification and be free from contaminants.

## Pooling

- 1 Before pooling the samples determine the number of samples per pool. The estimated reads per sample will depend on the required depth, genome length and expected percentage of on-target reads. The number of samples per pool will also depend on the type of Illumina sequencer/cartridge used and the read length.

You can use the attached calculator to determine the samples/cartridge and how many pools are required for your experiment.

 SequencingDepth.xlsx

- 2 For each pool calculate the volume required so that each library is present in equal quantities.

### Note

It is important to pool by molarity rather than mass as this will ensure equal numbers of molecules of each library. Otherwise longer or shorter fragment sizes will be under or over represented respectively.

Library molar concentration can be calculated from the library fragment size and mass concentration as follows:

$$\text{Conc (nM)} = \frac{\text{sample (ng/}\mu\text{l)}}{\text{size (bp)} * 660 \text{ (g/mol)}} * 1000000$$

You can use the attached calculator to determine the pooling volumes.

 EquimolarPooling.xlsx

You may be required to pool sub-pools instead of individual libraries. If so an additional weighting by the number of samples per sub-pool is required. See the attached calculator.

 EquimolarPooling\_subPools.xlsx

- 3 Using a fresh 1.5 mL DNA LoBind tube carefully add the required volume of each library.

#### Note



If possible use low-retention tips to improve pipetting accuracy.

- 4 Optional: Concentrate pool using 1.4X Ampure XP.


Equilibrate Ampure XP beads to  Room temperature and vortex well to mix.

 Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

- 4.1 Measure the total volume of the pool and add 1.4X volume of Ampure XP beads and mix well.

- 4.2 Incubate at  Room temperature room temperature for  00:05:00

5m



- 4.3 Place on a magnetic rack for  00:05:00 until beads and solution have fully separated.

5m

#### Note

It is important to wait until the beads have fully separated, the time it takes will vary depending on the ratio of Ampure beads:sample and the level of DNA.

4.4 Carefully add  200 µL 80% Ethanol (freshly prepared) without disturbing the beads.

4.5 Incubate at  Room temperature for  00:00:30 .

30s

4.6 Place on magnetic rack for  00:01:00 until the beads and solution have fully separated.

1m


4.7 Keeping on the magnet and carefully remove supernatant without disturbing the beads.

4.8 Repeat wash with  200 µL 80% Ethanol (freshly prepared) .

4.9 Remove all traces of ethanol.

#### Note

Sample can be briefly centrifuged and placed back on the magnet to remove residual ethanol.



4.10 Keeping on the magnet air dry for up to  00:03:00 .

3m

#### Note

Take care not to over dry the beads.

4.11 Add the required volume of 10 mM Tris pH 8.0, remove from the magnet and mix well to fully suspend the beads.

4.12 Incubate at  Room temperature for at least  00:02:00 to elute the DNA.

2m

4.13 Place back on magnet until beads and solution have fully separated.

4.14 Transfer the supernatant containing the pool DNA to a fresh 1.5 mL DNA LoBind tube.

## Pool QC

2m

5 It is recommended that library quantification is performed using a fluorometric method (e.g. Qubit) rather than an absorbance based method (e.g. Nanodrop) as it is more specific, sensitive and accurate.

### Equipment

Qubit	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33228	SKU
<a href="https://www.thermofisher.com/order/catalog/product/Q33228">https://www.thermofisher.com/order/catalog/product/Q33228</a>	LINK



5.1 Prepare 0.5 mL thin-walled PCR tubes, including 2 tubes for standard solutions and 3 for each pool. Label the tube lids and not the sides.

#### Note

For increased accuracy it is recommended that multiple readings are taken per pool.

- 5.2 Prepare the Qubit dsDNA High Sensitivity master mix for the total number of samples and standards with an excess.

A	B
Component	Volume (μl)
Qubit dsDNA HS Buffer	199
Qubit dsDNA HS Reagent	1
Total	200

 Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

#### Safety information

Qubit reagent is known to bind nucleic acid and is provided as a solution in DMSO. Treat the Qubit dsDNA HS Reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

#### Note

Ensure that the Qubit standards are equilibrated to room temperature for 30 min before use.

- 5.3 Aliquot the Qubit dsDNA High Sensitivity master mix into the assay tubes as follows:

Standards  190 μL Qubit master mix

Samples  199 μL Qubit master mix

- 5.4 Add  10 μL Qubit standard to each standard assay tube.

- 5.5 Add  1  $\mu$ L pool to each sample assay tube.
- 5.6 Vortex assay tubes and briefly centrifuge.
- 5.7 Incubate at  Room temperature for  00:02:00 .
- 5.8 Select dsDNA high Sensitivity assay on the Qubit Fluorimeter and press "Read Standards".
- 5.9 Insert Standard 1 and 2 into the sample chamber when prompted, close the lid and and press "Read Standard".
- 5.10 Select "Run samples" and select the sample volume as  1  $\mu$ L .
- 5.11 Insert a sample tube into the sample chamber, close the lid and press "Read tube".
- 5.12 Record the concentration of the Qubit sample in ng/ $\mu$ L.
- 5.13 For each pool ensure all readings are within the same range.

**Note**

If required additional readings can be taken.



5.14 Calculate the average pool concentration from the replicates ensuring any outliers are removed.

6 It is recommended that the libraries are visualised using capillary electrophoresis, we describe visualisation with a TapeStation and High Sensitivity D5000 ScreenTape. Alternatives such as the BioAnalyzer or Fragment Analyzer can also be used.

The purpose is to provide a size for the library fragments to give accurate molar quantification and to determine the quality of the library.

#### Equipment

##### 4200 TapeStation System

NAME

Electrophoresis tool for DNA and RNA sample quality control.

TYPE

TapeStation Instruments



BRAND

G2991AA


SKU

<https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263>

LINK

6.1 Ensure that the D5000 ScreenTape and Reagents are equilibrated to  Room temperature at least  00:30:00 before use, vortex and briefly centrifuge.

 High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

 High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

30m

#### Note

D5000 ScreenTape has a sizing range of 100-5000 bp. The D1000 ScreenTape and Reagents could be used as an alternative which has a sizing range of 35-1000 bp.

6.2 Prepare a dilution of each pool to approximately 1 ng/μL.

6.3 In fresh PCR strip tubes prepare the ladder assay tube as follows:

A	B
Component	Volume (µl)
D5000 sample buffer	2
D5000 ladder	2
Total	4

6.4 Prepare the sample assay tubes as follows:

A	B
Component	Volume (µl)
D5000 sample buffer	2
Diluted Pool	2
Total	4

6.5 Spin down, using IKA vortexer mix at  2000 rpm, 00:01:00 then spin down again.

1m

6.6 Load the assay tubes and ScreenTape into the TapeStation instrument.

6.7 Select the required sample/ladder positions in the TapeStation software and click "start".

**Note**

The TapeStation analysis software will open automatically at the end of the run to display the results.

6.8 Analyse the results, the pool should generate a similar trace to the individual libraries.

If needed follow the same troubleshooting guidelines as in the protocol [Library clean up and quality control for Illumina sequencing](#).

## 6.9 Determine the pool fragment peak size in bp.

### Note

If your pool doesn't give a single sharp peak then a region size can be used instead.

## 6.10 Calculate the pool molar concentration using the fragment size (Step 6.9) and mass concentration (Step 5.14).

$$\text{Conc (nM)} = \frac{\text{sample (ng/}\mu\text{l)}}{\text{size (bp)} * 660 \text{ (g/mol)}} * 1000000$$

### Note

The final pool should have a concentration at least 10 nM.