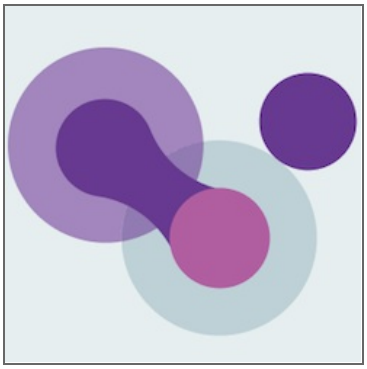

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C-SOP-501: Normalisation and Pooling of DNA Libraries for Illumina Whole Genome Sequencing

Forked from C-SOP-501: Normalisation and Pooling of DNA Libraries for Illumina Whole Genome Sequencing

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ABSTRACT

Normalisation in next-generation sequencing (NGS) is the process of equalising the concentration of multiple DNA libraries for the purpose of multiplexing. Multiplexing helps maximize the use of expensive NGS technology, enabling parallel sequencing of hundreds, to often thousands of libraries on a single flowcell, thereby driving down per sample costs.

Uneven library concentrations from different types and qualities of samples can lead to inconsistencies in data quality. Libraries with a high concentration are likely to be overrepresented on the flowcell while those with low concentration are underrepresented. Overrepresentation isn't necessarily a problem, likely increasing read depth, though it does waste the run's finite data capacity. Underrepresentation might result in poor read depth and unreliable data, wasting capacity and potentially precious library material. From a cost standpoint, wasted capacity means means additional work time re-preparing libraries, time that could be better spent on downstream analysis or preparing the next batch of libraries. From an application and outcome standpoint, analyses and decisions based on potentially inaccurate or incomplete data will at best confuse research results or lead to repetition of experiments and at worst lead to clinicians missing key information that could assist in a more appropriate treatment avenue.
Normalisation addresses these challenges ensuring every library is represented equally and sequenced to sufficient depth.

Prior to normalisation, there are several options for quantitating library preps, varying in ease and accuracy.

- The quickest and most convenient methods (i.e. spectrophotometry-based) tend not to be that accurate.

- The most accurate methods, like quantitative PCR (qPCR), take time and precision, and rely on knowing the average fragment size in each library for dilution calculations.

One crucial factor influencing the accuracy of quantitation and subsequent

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normalisation is whether the quantitation method can specifically count adaptor-ligated (i.e. amplifiable) double-stranded DNA (dsDNA) molecules. These are the only molecules that will cluster on the flowcell and contribute to sequencing output. Illumina's best practice suggests using fluorometric or qPCR-based quantitation with most types of genomic DNA libraries.

It's interesting that no single method provides all the data you need with enough accuracy for normalisation. Though fluorometry and qPCR enable the most accurate quantitation, neither can estimate average fragment size. So, it's often still necessary to check this by electrophoretic analysis (i.e. fragment analysers). However, between fluorometry and qPCR, only the latter can specifically target useful adaptor-ligated molecules by using primers complementary to the adaptor sequences. Quantifying only these viable sequencing templates gives you the best chance at normalising your libraries accurately. Adaptor ligation efficiency can vary between individual samples and batches. It's reliant on enzymatic reactions that could be affected by impurities and differences in the quality of starting material. So, quantitating with no specificity for adaptor-ligated molecules (fluorometry) means you're more likely to overestimate the sequencing-competent library concentration and over-dilute. Fluorometry (being cheaper and less accurate) can only substitute qPCR when the starting material and the end repair/adaptor ligation step of your library prep workflow are of high and consistent quality.

MATERIALS

1. Quantified and size-estimated double-stranded DNA libraries

2. Nuclease-free water or 10 mM Tris-HCl (pH 8.5)

3. Single-channel pipettes (P10, P200) with compatible tips (filter-free, sterile)

4. 96-well PCR-plate, low-profile, full skirted (ThermoFisher Scientific, Cat no. AB0800)

5. 2.0 mL microcentrifuge tubes

6. **For New England BioLabs NEBNext Library Quant Assay:**
   6a. NEBNext® Library Quant Kit for Illumina® (NEB, Cat no. E7630)

   6b. Nuclease-free water

   6c. qPCR machine

   6d. Compatible qPCR plates and seals

   6e. PCR strip tubes or microcentrifuge tubes

   6f. Conical centrifuge tubes

SAFETY WARNINGS

NEBNext Library Quant Kit for Illumina:

Dilution Buffer (10X) Safety Sheet
DNA Standard 1 Safety Sheet
DNA Standard 2 Safety Sheet
DNA Standard 3 Safety Sheet
DNA Standard 4 Safety Sheet
ROX (High) Safety Sheet
ROX (Low) Safety Sheet

Before Starting

1 Prior to initiating this protocol, ensure that all active workbenches are cleaned with 80% ethanol, all relevant personal protective clothing is worn and the work area is prepared according to local GLP guidelines for molecular methods.

2 Ensure that you have all of the following library QC metrics obtained using the recommended quantification methods described in '**C-SOP-401: Quality Control (QC) of DNA Libraries for Whole Genome Sequencing**'.

1. Mean total library fragment size (in bp) - output from the Bioanalyzer 2100 or Tapestation 4150/4200.
2. Mean library concentration (in nM) - output from a qPCR quantification assay.

> **Note**
>
> If your final mean library concentration is represented in ng/µl, convert this value to nM using the following link:
> Converting ng/µl to nM When Calculating dsDNA Library Concentration

Library Normalisation

3 Concentration normalisation is achieved by diluting an aliquot of the stock library in a diluent (nuclease-free water or 10 mM Tris-HCl pH 8.5).

> **Note**
>
> For most Illumina sequencing platforms, **2–4 nM** for each library is the preferred final concentration prior to denaturation and final loading steps; consult the respective instrument user guides for more information.

4 Calculate the dilution volumes for each library using the following equation:
$(C_1)*(V_1) = (C_2)*(V_2)$

where,
C_1 = concentration of stock library (nM)
V_1 = volume of stock library that is to be diluted (µl)
C_2 = desired concentration of the final solution (nM)
V_2 = desired total volume

Alternatively, refer **step 8** for a Normalisation and pooling calculator to compute library dilutions and pool volumes in a single step.

Note

For the most even representation of libraries in the subsequent pooling step and the most reliable cluster density on the Illumina instrument, ensure that all pipetted volumes are **preferably not less than 4µl (or at the very least 2 µl)**.

Pipetting less than 2 µl can introduce significant errors in final library pool concentration. Therefore, adjust calculations to make sure that appropriate volumes are used. For highly concentrated libraries, use one or more intermediate dilutions as shown below.

For eg. the following calculations demonstrate how to dilute 3 libraries (with different starting concentrations of 15 nM, 20 nM, and 50 nM) to a final concentration of 4 nM in a final volume of 15 µl for each library.

Starting Concentration	Volume of stock DNA (for 4 nM concentration)	Volume of diluent (for 15 ul final volume)
15 nM	4 ul	11 ul
20 nM	3 ul	12 ul
50 nM*	1.2 ul	13.8 ul

Table 1.
*The 50 nM library example only uses 1.2 µl of stock DNA to dilute down to 4 nM library. For the most accurate results, an intermediate dilution of 20 nM is necessary to achieve at least a 2 µl pipet volume.

Intermediate Dilution			Final Dilution	
Starting concentration	Volume of stock DNA (for intermediate 20 nM concentration)	Volume of diluent (for 15 ul volume)	Volume of intermediate 20 nM DNA (for 4 nM final concentration	Volume of diluent (for 15 ul volume)
50 nM	6 ul	9 ul	3 ul	12 ul

Table 2. Intermediate dilution for 50nM library.

5 Mix the calculated volumes of library and diluent together to obtain a **normalised library solution** for each sample.

Library Pool Creation (for Multiplexed Libraries)

6 **Volumetric pooling**:
Combine equal volumes of each normalised library in a 2.0 mL microcentrifuge tube.

7 Gently pipette the contents up and down 15 times to mix thoroughly. Alternatively, vortexing is also allowed.

8

> **Note**
>
> Steps 4-8 can be combined and performed using the convenient **normalisation and pooling calculator** for rapid Norm/Pool calculations.

Library Pool Confirmatory QC

9 Quantify the library pool using a robust qPCR assay, like the one described in '**C-SOP-401: Quality Control (QC) of DNA Libraries for Whole Genome Sequencing**' (NEBNext® Library Quant Kit for Illumina®) and compute the pool concentration in nM.

> **Note**
>
> i. Provided the dilution and pooling was done accurately, the final pool concentration should match the expected value chosen between 2-4 nM (step 3-4).
>
> ii. Under extreme conditions of budget limitations or reagent shortages, step 9 could be skipped. However, be advised that downstream results like clustering densities and sequencing run performance may vary greatly.

10 The normalised pool is now ready to be denatured and sequenced.

For detailed instructions on denaturation procedures, use the appropriate instrument denaturation and dilution guide provided under *Additional Information & Troubleshooting.*

Additional Information & Troubleshooting

11 Normalisation and Pooling Calculator

Library quantification and quality control quick reference guide

Converting ng/µl to nM when calculating dsDNA library concentration

For library denaturation and final loading dilution instructions:

- iSeq™ 100 System Guide
- MiniSeq™ System Denature and Dilute Libraries Guide
- MiSeq™ System Denature and Dilute Libraries Guide
- NextSeq™ 500/550 System Denature and Dilute Libraries Guide
- NextSeq 1000/2000 Sequencing System Guide
- HiSeq™ Systems Denature and Dilute Libraries Guide
    - cBot™ System Guide for denaturing and diluting libraries for the HiSeq 3000/4000
- NovaSeq™ System Guide