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High-Throughput Library Pooling for NGS

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Protocol status: Working
We use this protocol in our group for RNA library prep on the CZ Biohub Echo. Please provide any feedback or comments so we can make it as user-friendly as possible!

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Abstract

Preparation of high-quality sequencing libraries is a costly and time-consuming component of metagenomic next generation sequencing (mNGS). While the overall cost of sequencing has dropped significantly over recent years, the reagents needed to prepare sequencing samples are likely to become the dominant expense in the process. Furthermore, libraries prepared by hand are subject to human variability and needless waste due to limitations of manual pipetting volumes. Reduction of reaction volumes, combined with sub-microliter automated dispensing of reagents without consumable pipette tips, has the potential to provide significant advantages. Here, we describe the integration of several instruments, including the Labcyte Echo 525 acoustic liquid handler and the iSeq and NovaSeq Illumina sequencing platforms, to miniaturize and automate mNGS library preparation, significantly reducing the cost and the time required to prepare samples. Through the use of External RNA Controls Consortium (ERCC) spike-in RNAs, we demonstrated the fidelity of the miniaturized preparation to be equivalent to full volume reactions. Furthermore, detection of viral and microbial species from cell culture and patient samples was also maintained in the miniaturized libraries. For 384-well mNGS library preparations, we achieved a savings of over 80% in materials and reagents alone, and reduced preparation time by 90% compared to manual approaches, without compromising quality or representation within the library.

After library preparation, the next step before putting samples on the sequencer is to pool samples to be able to multiplex sequencing. Final concentrations of each sample will vary from sample to sample depending on various things like RNA input, library prep, and PCR cycles among other factors. It is difficult to manually quantify and pool uniformly 384 samples, so that is why we employ robots. To quickly and accurately pool hundreds of samples at a time, we employed a two-step method. Using the Labcyte Echo, equal volumes of each library are pooled together and sequenced on the Illumina iSeq or MiSeq sequencing platforms to determine the representation of each library in the total pool. Using this information, libraries were pooled using the Echo into a normalized pool for downstream sequencing on the Illumina NovaSeq.

Guidelines

Remember to always be mindful the dead volumes of Echo plates and maximum and minimum working volumes per source and destination plates when dispensing. These are vital for accurate dispensing.

Before start

Libraries should be in a 384-well Echo source plate at a volume of ≥21uL at the start of this protocol. Adjust if needed.

POOLING PREP

1 Transfer the libraries into a new 384 well Echo source plate. Skip if samples already complete. Make sure to dilute samples if needed to contain enough dead volume. Samples should be between 22-25uL to be able to dispense accurately using the Echo.

> Note
>
> Wells of the source plate containing samples should have a dead volume of 20uL for the Echo to be able to pool accurately.

EQUAL VOLUME POOLING

2 Using the Labcyte Echo, pool 0.5uL of each library into a 384-well destination plate.

Use a new 384 PCR plate, or a 96 well PCR plate, as the ***destination plate*** to dispense the appropriate number of samples into the necessary number of wells. Be sure to take into account of the maximum working volume per destination plate. For example, for a 384 well plate, do not dispense more than 12uL into each well (i.e. 500nL of 22 samples per well).

3 Manually combine the multiple wells of the pool into one single DNA lo-bind tube. Mix.

EQUAL VOLUME SEQUENCING

4 Sequence pool on an Illumina iSeq, or a low-throughput sequencer.

POOLING CALCULATIONS

5 Using the output from equal volume pooling, determine the approximate representation of each library in the total pool.

$$T = N \sum (\frac{1}{x})$$

x = percent of reads per sample
T = final volume of total pool
N = normalization factor

The sum of the inverse of the percent fraction of each sample from the iSeq run should give a normalization factor. This normalization factor will be used to determine the volume of each sample to be pooled.

Needed information:

1. ratio of reads of each sample to total number of reads (percent of reads) = x
2. desired total volume of pool necessary for sequencing submission = T (uL)

Use this information to solve for N (or the normalization factor).

Note

The normalization factor is an estimation that can be used to pool evenly. It can be adjusted for various reasons to fit the criteria per batch.

6 Use the normalization factor to determine the volume to pool for each sample.

EQUI-MOLAR POOLING

7 Use the Echo to dispense the original sequencing libraries to the final pool using the calculated volumes.

Use a new 384 PCR plate, or a 96 well PCR plate, as the destination plate to dispense the appropriate number of samples into the necessary number of wells. Take in account the maximum working volumes.

Note

Reminder: For 384, do not dispense more than 11uL into each well total.

8 Manually combine the multiple wells of the pool into one single DNA lo-bind tube. Mix.

9 Equimolar pool of libraries are ready to be sequenced on a high throughput sequencer like the Illumina HiSeq or NovaSeq.