APPLICATION NOTE

Reducing Costs and Time of SARS-CoV-2 Variant Detection by Incorporating Normalization Beads into an NGS Workflow

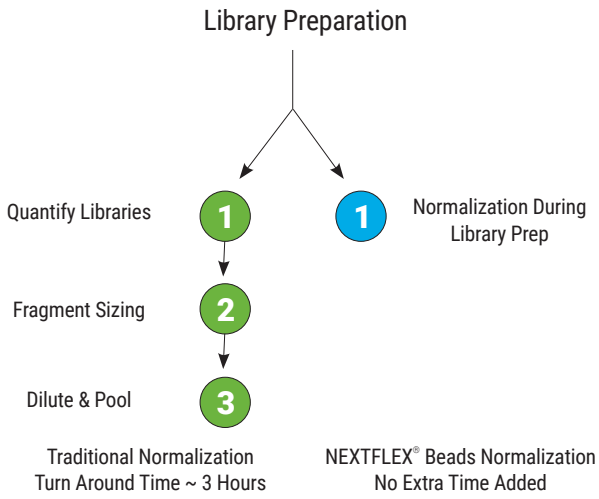
NEXTFLEX® Variant-Seq™ SARS-CoV-2 Kit v2

A streamlined workflow for faster turnaround times in high throughput labs.

Introduction

Library normalization is one of the most important steps to ensure optimal clustering and data quality. It traditionally involves three steps: quantifying libraries with a Thermo Fisher® Scientific Qubit® fluorometer, sizing fragments with LabChip® GX Touch™ nucleic acid analyzer or similar and diluting the libraries to an equimolar concentration.

In practice, traditional normalization protocols add up to 3 hours to the time it takes to construct libraries from 96 samples. This can have a big impact in the turn-around time, especially for high-throughput applications such as SARS-CoV-2 variant detection.



One of the challenges of normalization for the detection SARS-CoV-2 variants is the highly variable yields obtained in the library preparation, which are direct consequence of the range of viral loads (as indicated by their respective Ct values) that appear in the SARS-CoV-2 PCR positive samples. PerkinElmer's bead-based normalization provides a convenient alternative to this traditional method of normalization, delivering a faster workflow and significant cost savings per sample.

This note demonstrates the relative normalization of SARS-CoV2-PCR-positive nasopharyngeal swabs using our NEXTFLEX® Normalization Beads VS. Along with the introduction of bead-based normalization, additional changes were made to the NEXTFLEX® Variant-Seq™ SARS-CoV-2 Kit v2 protocol to increase library yield concentrations and increase the assay sensitivity to provide results from lower viral load samples.

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Methods

In a first set of experiments, the performance of the normalization beads was established using high template quality control samples. To do so, the amplicon material obtained after post-PCR purification was pooled and quantified using the Thermo Fisher® Scientific Qubit® BR dsDNA kit. Bulk pooled amplicon material was added to the fragmentation step in different mass amounts to mimic the input anticipated from low SARS-CoV-2 viral load to high viral PCR positive samples. A total of 15 libraries were made with inputs into fragmentation ranging from 25 ng to 2,400 ng. This range of input concentrations represents a typical range of input from SARS-CoV2-PCR-positive nasopharyngeal samples with Ct values from ~15 (high viral load) to ~40 (low viral load).

Table 1: *Range of input concentrations used to mimic SARS-CoV-2 PCR-positive nasopharyngeal samples*

Library #	Input (ng)
1	25
2	50
3	100
4	200
5	400
6	600
7	800
8	1000
9	1200
10	1400
11	1600
12	1800
13	2000
14	2200
15	2400

In a second set of experiments, 55 SARS-CoV2-PCR-positive PCR positive samples were processed through the above NEXTFLEX® Variant-Seq™ SARS-CoV-2 Kit v2 protocol. Samples were assayed using the PerkinElmer® New Coronavirus Nucleic Acid Detection Kit and had a minimum Ct detection value of 15.56 and maximum Ct detection value of 39.27 for the N region qPCR target. The same RNA extracts were used as input for the NEXTFLEX® Variant-Seq™ SARS-CoV-2 Kit v2.In all cases the final libraries were purified at the end using the NEXTFLEX® Normalization Beads VS. 5 µL of each normalized library was then added into a single pooled library, diluted to 10 pM, denatured and sequenced on an Illumina® MiSeq® instrument at 1x36 bp, targeting a minimum of 1 million clusters per library.

FastQ files were uploaded to the CosmosID® SARS-CoV-2 Strain Typing Analysis Portal for analysis. Data was also run through the Illumina® DRAGEN COVID Lineage App for comparison. SARS-CoV-2 genome coverage was also reviewed visually with the Integrative Genomics Viewer software (IGV). Detection of at least 90 out of 98 amplicons at a minimum of 10X coverage per base across the covered genomic region (>92%) was required to consider preparation successful.

Results

Mass & Cluster Normalization of the Post-PCR Control Samples

All 15 libraries (100%) normalized +/- 1.4-fold by mass, assessed using Qubit® for each individual sample and by flow cell clustering on the Illumina® MiSeq® instrument (Figure 1). Analysis of sequencing data in all samples passed the QC criteria described in the previous section.

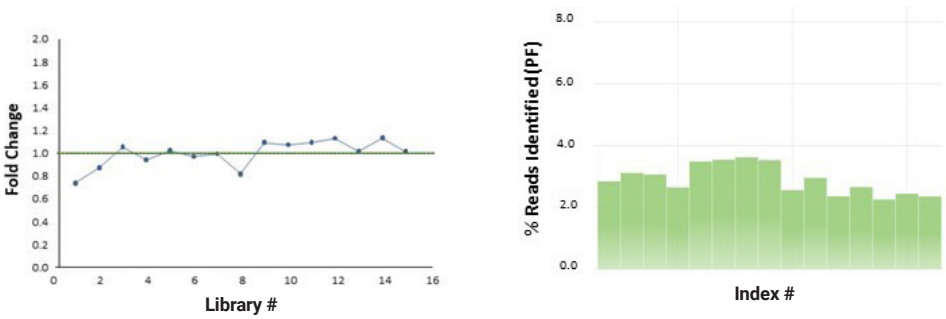

Figure 1: *Mass and cluster normalization with pooled samples of high-quality control template*

These results indicate, when using the same high-quality RNA as starting material for the Variant-Seq v2 workflow, normalization works across roughly 2 orders of magnitude of input material into the fragmentation step.

Effective Normalization of COVID-19 Positive Nasopharyngeal Samples

The results from SARS-CoV-2 PCR-positive samples show the same close correlation between mass and clustering normalization (Table 2).

Library #	N Gene Ct	Mass Ratio	Cluster Ratio
1	15.56	1.04	0.89
2	16.75	0.99	0.74
3	17.04	1.07	0.89
4	17.23	1.04	0.62
5	17.72	0.99	0.82
6	18.07	0.92	1.07
7	18.13	0.93	0.9
8	18.27	0.77	1.03
9	18.6	0.9	0.79
10	18.65	0.91	1.05
11	19.01	1.08	0.95
12	19.59	0.84	1.28
13	19.73	0.92	0.93
14	20.14	0.85	1.22
15	20.43	1.03	1.08
16	20.95	1.2	0.89
17	22.13	0.99	1.24
18	22.17	1.01	1.17
19	22.18	1.03	1.05
20	22.23	0.92	1.05
21	22.59	0.96	1.04
22	23.66	1.03	0.93
23	23.95	1.12	0.88
24	24.01	0.81	1.29
25	24.13	0.79	0.98
26	24.4	1.02	1.04
27	25.65	1.09	1.15
28	26.4	0.92	0.67
29	26.53	1.08	1.12
30	26.94	1.11	1.03
31	26.94	0.88	1.14
32	27.4	0.92	1.03
33	27.58	1.17	1.26
34	27.88	1.11	0.96
35	28.5	0.89	1.43
36	28.89	1.15	1.42
37	30.38	1.18	1.25
38	32.35	1.34	0.84
39	33.45	1.11	1.1
40	33.48	0.97	0.95
41	34.26	1.17	0.98
42	35	1.03	1.31
43	36.04	1.08	0.59
44	36.77	1.26	1.11
45	36.85	0.94	0.95
46	37.08	0.89	1.04
47	37.56	0.93	0.95
48	38.74	1.2	0.8
49	38.78	1.09	1.35
50	38.8	1.15	1.01
51	38.88	0.96	0.93
52	38.91	1.01	1.01
53	38.93	0.98	1.59
54	39.15	1.1	1.18
55	39.27	1.22	1.05

Table 2: *Mass and Clustering ratios obtained with PCR positive nasopharyngeal samples*

Of the SARS-CoV-2 PCR-positive nasopharyngeal samples tested, 36 out of the 55 samples had a Ct value ≤ 28 for the N gene target.

- 36 out of 36 (100%) normalized +/- 1.4-fold by mass.
- 34 out of 36 (94%) normalized +/- 1.4-fold by clustering.

Of the samples tested, 39 out of the 58 samples had a Ct value ≤ 32 for the N gene target,

- 39 out of 39 (100%) normalized +/- 1.4-fold by mass.
- 35 out of 39 (90%) normalized +/- 1.4-fold by clustering.

Of the remaining 16 samples, with Ct > 32 for the N gene target,

- 16 out of 16 (100%) normalized +/- 1.4-fold by mass.
- 14 out of 16 (87.5%) normalized +/- 1.4-fold by clustering.

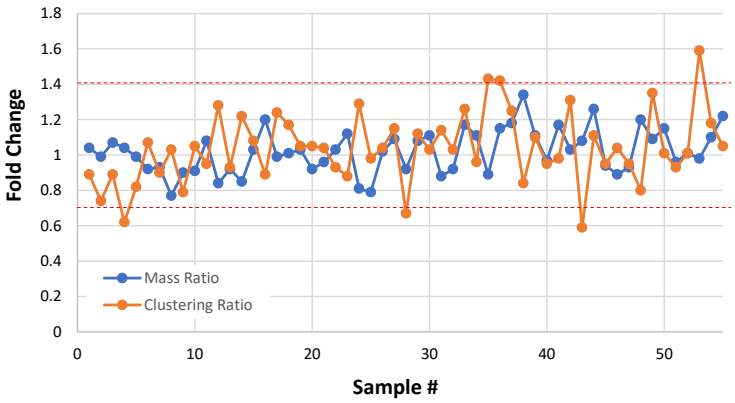

Figure 2: *Representation of mass and clustering ratios with from low Ct (left) to high Ct (right)*

Since there is no clear trend towards higher Ct/lower viral load and lower yield or cluster density (Figure 2), this the data suggests that the outliers for mass and clustering are most likely caused by quality of input RNA material, e.g. fragmented viral genomes. This hypothesis is further demonstrated by the observed 100% correlation of normalization by mass and clustering seen in the high-quality control material. In all cases where normalization by clustering was effective, consistent genome coverage was observed. The samples with poorer genome coverage also exhibited lower cluster density, but not lower mass or higher Ct values.

Conclusions

NEXTFLEX® Variant-Seq™ SARS-CoV-2 kit V2 effectively generates sequencing data sufficient to make reliable variant calls using RNA extractions with Ct detection values up to 32 with ≥ 90% alignment to the SARS-CoV-2 genome. Incorporation of NEXTFLEX® Normalization Beads VS successfully normalizes libraries from samples with Ct detection values ≤ 32 within +/- 1.4-fold by mass (100%) and even clustering (90%), allowing the ability to extend the upper Ct detection value specification to ≤ 32 with this version of the NEXTFLEX® Variant-Seq™ SARS-CoV-2 kit. This enables increased sample inclusion and higher throughput sequencing without the need for the traditional laborious and time intensive manual library quantification and pooling steps. This workflow is also more automatable because the same volume of each purified sample is added to the final pool. This workflow is completely automated on the Sciclone® G3 NGSx iQ™, Sciclone® G3 NGSx, and Zephyr® G3 NGS workstations.

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