Quantifying Next Generation Sequencing Libraries using Digital PCR on the QuantStudio3D Platform

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Illumina Cluster Density

Blue boxes = total clusters
Green boxes = pass filter clusters
Qubit quantification + Fragment Analyzer size estimation $\rightarrow$ 2 nM stock

- Fine for most, standard libraries
- Quantitative PCR can be used
- **Digital PCR**
QuantStudio3D instrument

- Interrogated volume similar to real-time PCR (~16µl)
- Hexagonal packing enables 20,000 wells / 10mm² chip
- Each reaction well is isolated from its neighbors
Poisson Statistics describing random, quantized events
e.g. limiting dilution assays

\[ p(i, \lambda) = e^{-\lambda} \frac{\lambda^i}{i!} \]

\[ \lambda = -\ln P \]

\( \lambda = \text{molecules per rxn} \) and \( P = \text{fraction of negative} \)

**QS3D software does this calculation for you**
How digital PCR works on the QuantStudio™ 3D Chip

20,000 Reaction-wells

thermocycle
Taqman Assays for Illumina Libraries:

TruSeq DNA/RNA

5'-CAAGCAGAAGACGGCATACGAGATNNNNNNNTGACGTTACGGGACCTAGCTCTGCTCTCGATCTNNNNNNNNNNNNNNNAGATCGGAAGACGGCGTCGCTGTAGGTAGATCTCGGTGGTTCGGCTACATTT-3'

3'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNNNA

GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3'

Nextera

5'-CAAGCAGAAGACGGCATACGAGAT[i7]GCTCTGCTGGGCGGATCTGCTAAGACAGNNNNNNNNNNGCAGTCTTTTATACAGATCTCTCCGAGCCACGAGAC[i7]ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-AATGATACGGCGACCACCGAGATC[i5]TACACCGGTTCAGAGTTCTACAGTCCGACGATNNNNNNNNNNNNNTGGAATTCTCGGGTGCCAGGAACTCCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG-3'

TruSeq Small RNA

5'-CAAGCAGAAGACGGCATACGAGAT[i7]GCTCTGCTGGGCGGATCTGCTAAGACAGNNNNNNNNNNGCAGTCTTTTATACAGATCTCTCCGAGCCACGAGAC[i7]ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-AATGATACGGCGACCACCGAGATC[i5]TACACCGGTTCAGAGTTCTACAGTCCGACGATNNNNNNNNNNNNNTGGAATTCTCGGGTGCCAGGAACTCCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG-3'

Nextera

5'-CAAGCAGAAGACGGCATACGAGAT[i7]GCTCTGCTGGGCGGATCTGCTAAGACAGNNNNNNNNNNGCAGTCTTTTATACAGATCTCTCCGAGCCACGAGAC[i7]ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-AATGATACGGCGACCACCGAGATC[i5]TACACCGGTTCAGAGTTCTACAGTCCGACGATNNNNNNNNNNNNNTGGAATTCTCGGGTGCCAGGAACTCCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG-3'
TruSeq (FAM):

Small RNA library (VIC):

Nextera libraries (VIC):
Illumina sequencer cluster densities:

Blue boxes = total clusters
Green boxes = pass filter clusters

dPCR results (nM):

8.0  6.3  6.4  11.3  8.3

Stocks tested were nominally 10 nM
Yield from Sequencer: ~27 M reads
~5x lower than expected (130-180 M expected)

By dPCR: ~0.45 nM
(2 nM expected)
~4.4x lower than expected
The Dilution Factor

All Illumina Libraries are adjusted to **2 nM** based on Qubit quantification and MW estimation from fragment analyzer trace

2 nM = ~**1.2 Billion molecules/microliter**

**Digital PCR**

Dilute to <**800 molecules/microliter**

to run on the digital PCR chips

--this is ~**1.3 femtomolar**

Or, about a **1 to 1 million dilution**!
This dilution factor is problematic for this type of application

• Not so much of an issue for genomic DNA looking for CNV, etc.

• Human (mouse/canine/equine) DNA used at ~60 ng/chip
Internal Reference for dPCR:

Human RNaseP taqman assay from ABI......both FAM and VIC versions available

PCR amplify RNaseP locus from human DNA
Quantify (or otherwise) determine molarity, adjust to 1 nM

Dilute with 2 nM Illumina stock 1:1

Then, do a 1 to 1 million dilution (doesn’t have to be accurate!).

Divide copies/ul for test sample by copies/ul for the reference.
TruSeq genomic library (FAM) vs. RNaseP reference (VIC)

Ratio: 2.2  
FAM:VIC

VIC library ~1 nM, therefore FAM library ~2.2 nM
Using *Prospectively*:

**dPCR:**

VIC (library): 24 copies/ul
FAM (reference): 45 copies/ul
R (V/F): 0.53 ~0.53 nM
(stock is nominally 2 nM)

**MiSeq run:**

Ran at 3x concentration:

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<tr>
<th>Lane</th>
<th>Cluster Density (K/mm²)</th>
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<tr>
<th>Reads (M)</th>
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<td>14.28</td>
<td>13.48</td>
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Summary

• Developed Taqman primer/probe sets for the three major Illumina library types

• Developed an internal reference standard for use in digital PCR

• Retrospective testing of libraries correlate well with sequencing results

• Starting to use digital PCR **prospectively** in the QC protocol, especially for particularly challenging libraries

• Development of robust protocols ongoing
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