Accurate NGS library quantification using nanoplate digital PCR

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Introduction

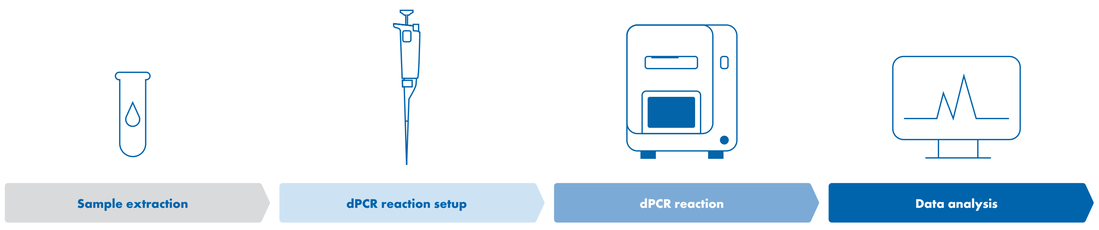
Accurate quantification of next-generation sequencing (NGS) libraries is a prerequisite for optimal yield on Illumina® NGS platforms. Incorrect quantitation will result in over- or under-saturation of the flow cell and uneven yield of individual libraries in a pooled library. In the worst case, the sequencing run must be repeated, resulting in additional costs.

Higher accuracy and absolute quantification of target molecules by digital PCR (dPCR) allows much more accurate quantification of these libraries and thus more efficient use of NGS flow cells. Here, we focus on the quantification of NGS libraries by digital PCR on the QIAcuity® dPCR System. We present the QIAcuity dPCR workflow for quantification of Illumina NGS libraries using a library-specific primer assay in combination with the QIAcuity EG PCR Kit.

Using Illumina libraries and synthetic libraries, we demonstrate the power of dPCR on the QIAcuity for quantification of NGS libraries compared to standard methods. Finally, we present a protocol for accurate quantification of Illumina NGS libraries using the QIAcuity dPCR system, regardless of their average fragment length and composition.

Absolute quantification of Illumina NGS libraries in less than 2 hours

Workflow for quantification of NGS libraries using Illumina sequencing primers P5 and P7 in combination with the QIAcuity EG PCR Kit in a digital PCR reaction on the QIAcuity instrument. The NGS libraries are diluted prior to quantification using dPCR. This is a critical step, and the use of low-bind tubes is recommended.



- DNA dilution buffer (NADB, TE)
- Recommended dilution factor: 10–5 to 10–7 depending on the library concentration
- Use 4 µL diluted library in the dPCR reaction

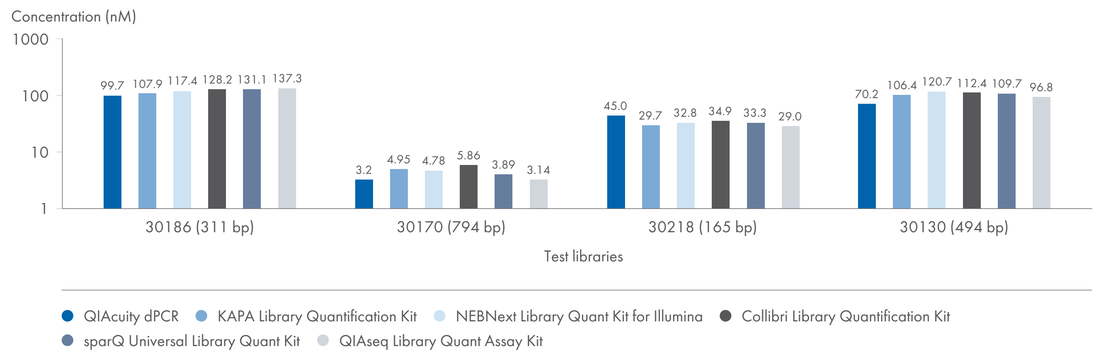
- EG assay with Illumina P5 and P7 primer:
  Illumina forward:
  5'-AAT GAT ACG GCG ACC GA-3'
  Illumina reverse:
  5'-CAA GCA GAA GAC GGC ATA CGA-3'
  Use 0.4 µM final concentration for each primer in the dPCR reaction!
- QIAcuity EG PCR Kit

- QIAcuity dPCR instrument
- Cycling protocol:
  - 2 min 95°C
  - 40 cycles (15s 95°C, 15s 55°C, 15s 72°C)
  - 5 min 40°C

- QIAcuity Software Suite

Comparison with qPCR solutions

The dPCR protocol was applied to four test libraries. Quantification was performed using several commercially available qPCR NGS library quantification kits, including the gold-standard KAPA kit. The test libraries comprise four different library types, such as a QIAseq® miRNA library with very short library fragments of on average 165 bp but also a QIAseq 16S/ITS library with long fragments of on average 794 bp. For each of the kits, the manufacturers recommended materials and protocols were followed. The quantification results are comparable with moderate differences between dPCR and qPCR for the two libraries with the very short and the very long library fragments.

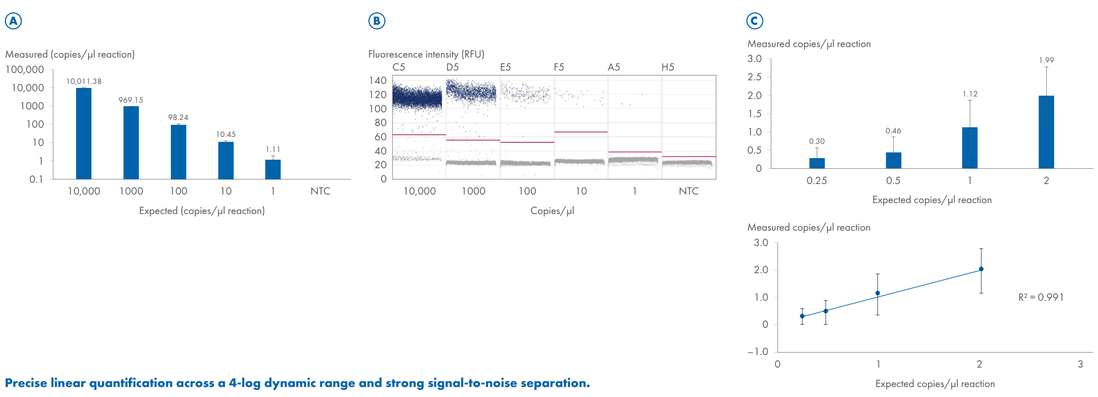


Estimated concentrations (nM) of four test libraries based on the measurements using primer assays in dPCR and five different qPCR NGS library quantification kits.

Precise and linear quantification across a 4-log dynamic range

To evaluate the dynamic range of quantification for the 96-well nanoplate a 4-log dilution series of test library 30170 was quantified. The bar graph (A) depicts the average quantification results with error bars representing the SD of 12 replicates each. The mean measured concentration hardly differs from the expected across the complete dynamic range of 4 log levels from 1 copy/µL reaction to 10,000 copies/µl reaction. Further, the standard deviation is small underlining the precision of linear quantification across this range. The 1D scatterplot (B) of individual partitions for a representative well for each of the dilutions illustrates the strong signal to noise separation.

To confirm a lower limit of linear quantification of 1 copy/µL, we quantified the test libraries at concentrations below 1 copy/µL. 48 replicates were run on a 96 well nanoplate to capture the SD at these low concentrations. As shown in figure (C), the average copy number reflects the expected concentration. Furthermore, the measured concentrations follow a linear regression with an R^2 >0.99. The lower limit of linear quantification of 1 copy/µL reaction for NGS libraries is confirmed by the data.



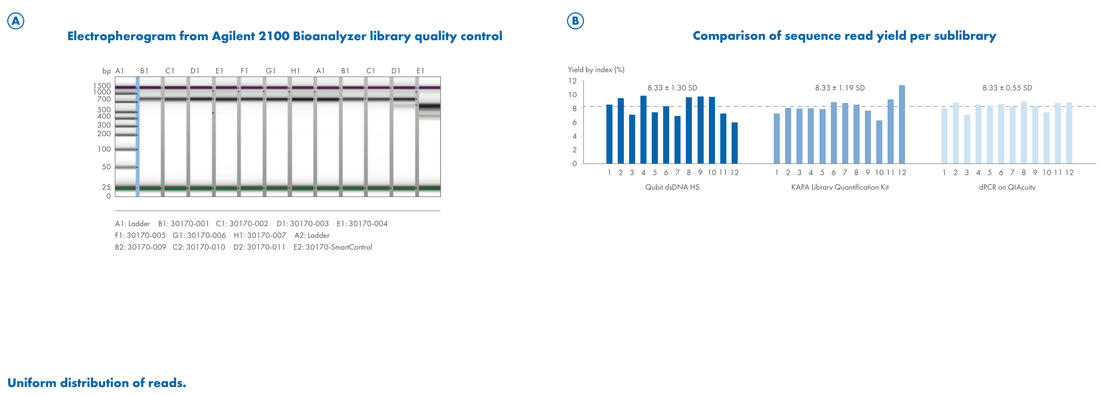
Precise linear quantification across a 4-log dynamic range and strong signal-to-noise separation.

Superior uniformity for library pooling

How can quantification with digital PCR can improve the accuracy of library pooling?

The sub-libraries of a library pool were quantified before pooling and compared to the yield of sequencing reads after NGS on a MiSeq® instrument. The library pool consisted of 12 sub-libraries with a 22-fold concentration range from approximately 2 to 50 nM. The electropherogram (A) illustrates the results of a Bioanalyzer run for each of the sub-libraries.

Three methods were used for quantification: Qubit® HS, KAPA qPCR and dPCR on the QIAcuity. Libraries were pooled equally based on the quantification results obtained and sequenced on a MiSeq instrument. The bar graph (B) illustrates that quantification with digital PCR provides much more accurate equimolar pooling compared to Qubit and qPCR, as the SD of dPCR is significantly lower at 0.55 across the 12 libraries compared to KAPA library quantification.



Uniform distribution of reads.

Conclusions

Digital PCR on the QIAcuity combined with Illumina sequencing primers and the QIAcuity EG Mastermix offers four key features:

- **Accuracy:** An accurate absolute quantification of 'clusterable' library fragments unbiased by amplification efficiencies
- **Precision:** Precise quantification enables superior uniformity for library pooling
- **Speed:** The workflow delivers quantification results in less than 2 hours
- **Compatibility:** All types of Illumina libraries can be quantified due to the usage of universal primers for P5 and P7

Sample to Insight