APPLICATION NOTE

RainDance Technologies

Using EvaGreen® Dye on the RainDrop® Digital PCR System to Quantify NGS Libraries

Next Generation Sequencing (NGS) has rapidly grown to enable a wide range of research and genetic analysis. An important step in the sample preparation process is to confirm that libraries or sample pools are diluted to the optimal concentration for flow cell loading prior to Illumina sequencing. Overestimation of library concentration may result in a lower than desired cluster density. Furthermore, underestimation may result in higher than desired cluster density, which can lead to poor cluster resolution. Both scenarios result in suboptimal utilization of sequencing capacity.

Introduction

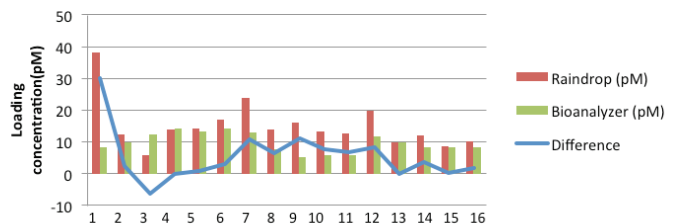
In the RainDrop Digital PCR System, samples are partitioned into millions of droplets and individual molecules are counted as positive or negative for marker(s), providing absolute single molecule quantitation and eliminating reliance on external calibration curves. The RainDrop System has fundamentally changed the performance of molecular assays by enabling digital answers for research applications including ultra-sensitive tumor allele detection, genotyping, copy number variation, gene expression, DNA methylation assessment, DNA quality control, and multiplexed detection of rare events. Most applications on the RainDrop utilize hydrolysis probes, such as TaqMan®. For details regarding a multiplex DNA QC assay, using hydrolysis-based probes, please refer to *Didelot A, et al, Multiplex Picoliter-Droplet Digital PCR for Quantitative Assessment of DNA Integrity in Clinical Samples, Clinical Chemistry 59:5, 2013*.

Here we present data showing the use of an intercalating-dye, EvaGreen (Biotium, P/N 31000), in a digital PCR assay to quantify sample concentration for improved NGS sample loading. This enables downstream savings in both NGS sequencing time and cost

Comparing the accuracy of RainDrop Digital PCR using an EvaGreen assay versus an Agilent Bioanalyzer to quantify NGS libraries

In this retrospective study done by GenomeScan/ServiceXS (www.genomescan.nl), the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to quantify library concentrations prior to loading onto a HiSeq (2000/2500) (Illumina, Inc. San Diego, CA). Subsequent to sequencing, the same samples were analyzed using a RainDrop System with EvaGreen qPCR chemistry. As shown in Figure 1, differences between the loading concentrations as determined by the Bioanalyzer and the RainDrop varied significantly. In retrospect,

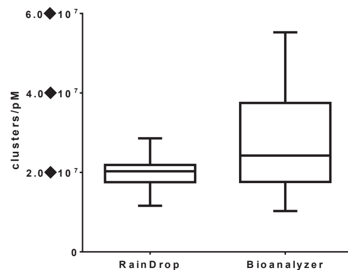
Figure 1.
Loading concentration (y axis) versus sample (x axis) showing differing concentrations when comparing a Bioanalyzer vs a RainDrop Digital PCR system using EvaGreen



this concentration variance resulted in either a total loss of a flow cell lane due to overloading (sample 1 below) or a drastically under loaded sample (sample 3 below). Both cases resulted in inefficient use of the flow cell and increased sequencing costs.

In addition, to enable the most efficient use of flow cells, samples are commonly multiplexed in a single lane. Moreover, to ensure uniform coverage across samples in a single lane it is imperative that samples are mixed at equimolar concentration. This is facilitated with the accurate determination of sample concentration. To this end, it is demonstrated here that the RainDrop provides much more accurate concentration readings, resulting in tighter clustering consistency (clusters/pM) (Figure 2). As a result, users are able to multiplex samples with a high degree of accuracy, thereby increasing the throughput per lane and decreasing per sample sequencing costs.

Figure 2.
The RainDrop system provides much more accurate clusters/pM quantitation than the Bioanalyzer, allowing users to multiplex in a single lane with greater confidence.



Using the RainDrop Digital PCR System to determine loading concentration, resulting in consistency of sequencing yield

A subsequent prospective study by GenomeScan/ServiceXS was conducted to determine if library quantification using the RainDrop with the EvaGreen assay could produce greater consistency in sequencing yields. Targeting an output of 60 gigabases per lane, multiple flow cells were loaded based on the results from the RainDrop quantification. Analysis of the sequencing yield for each of the 11 flow cells demonstrated that the RainDrop method showed significantly increased accuracy as compared to the Bioanalyzer method (Figure 3). In fact, RainDrop quantification provided a consistent output of sequencing data per Illumina flow cell, and high uniformity at the targeted sequencing yield of 60 gigabases was observed. Six of the libraries (labelled RNA-seq in Figure 3) were quantified using both the EvaGreen assay on the RainDrop and the BioAnalyzer method. In contrast to the accurate RainDrop quantification, Bioanalyzer results were highly variable and predicted values for flow cell loading that were off by as much as 3x in some samples.

Figure 3.
Samples were sequenced on a HiSeq 2500 with v4 SBS sequencing reagents. Flow cell loading was based on RainDrop concentration measurements. The blue bars represent the sequencing yields in Mb, the red line represents the loading concentration measured with the RainDrop, the green line represents the loading concentration determined by the Bioanalyzer (only available for 6 libraries).

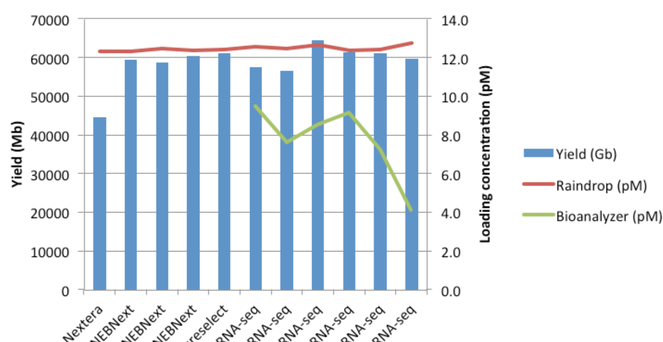
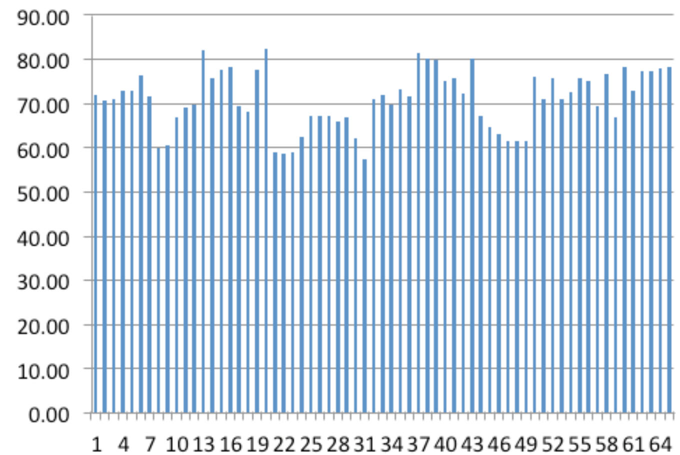


Figure 4.
The RainDrop system was used to target sample loading at 70 Gb (Y-axis). The average sample (X-axis) produced 70.97 Gb of sequencing data, with a %CV of 9.25 and standard deviation of 6.57 Gb.



Maximizing sequencing data per flow cell

In a final analysis, it was demonstrated that the increased accuracy and precision of the RainDrop method using EvaGreen quantification allowed GenomeScan/ServiceXS to target an optimal sample loading of 70 Gb for each run. Figure 4 shows the results from 64 samples using the RainDrop EvaGreen method as a library a library quantification tool. The average yield achieved was 70.97 Gb, with a standard deviation of 6.57 Gb and %CV of 9.25.

Conclusions

In conclusion, the RainDrop Digital PCR system using EvaGreen qPCR chemistry enables both an accurate and a reproducible quantification of NGS libraries prior to loading onto an Illumina HiSeq instrument. This consistency allows users to maximize their sequencing output per lane, saving time and per sample sequencing cost.

The RainDrop Digital PCR System is for Research Use Only; not for use in diagnostic procedures.

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