A fluorescence-based method for accurate molar quantification of NGS libraries in minutes.

Ashesh A. Saraiya[1], Chaitali Parikh[1], Bin Li[1], and Joe Don Heath[1]
[1] Tecan Genomics, Inc., Redwood City, CA

INTRODUCTION

Accurate quantification of NGS libraries is critical for a successful sequencing run. Currently used methods of quantification are time-consuming, costly, and can be highly variable. We have developed NuQuant®, a novel method to accurately quantify NGS libraries, that can be performed with a simple fluorescent measurement. NuQuant is compatible with the common red fluorescence excitation/emission filter set (650/670 nm), making it compatible with a wide range of bench top fluorometers and fluorescent plate readers. We have developed a custom library quantification application for the Qubit fluorometer that directly provides the molar concentration of a library. Utilizing this application, we have demonstrated that NuQuant has excellent reproducibility across users from multiple sites. We now demonstrate the compatibility of NuQuant with standard fluorescence plate readers, enabling quantification of libraries in a high-throughput fashion. We have tested NuQuant on a variety of commonly used plate readers such as the Tecan Infinite® 200 Pro and the Promega GloMax. Libraries in a 96-well format can be measured in a matter of minutes, without the need for sample dilution. Molar concentration of libraries was easily determined by utilizing a standard curve. We tested libraries with various input from 10 ng to 500 ng and insert size from 200 bp to 500 bp, and found good agreement between NuQuant values and a qPCR based quantification method. Most importantly, we observe good correlation between NuQuant library concentration and total number of sequenced reads. In conclusion, scientists with access to commonly used fluorescent plate readers can now use NuQuant to achieve rapid and cost-effective quantification of NGS libraries, generating highly uniform sequence reads in multiplex runs.

NuQuant provides accurate quantification of library molarity

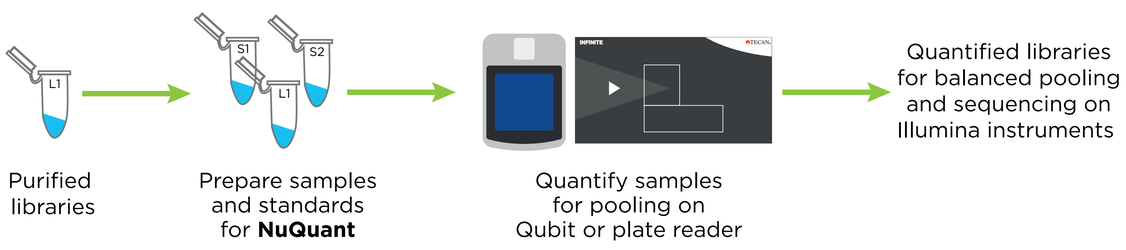


Figure 1: Library quantification workflow with NuQuant. After the final bead purification step of the Celero™ DNA-Seq or Universal Plus mRNA-Seq with NuQuant library preparation kits, the libraries and standards are diluted in the NuQuant buffer. The standards and libraries are assayed on fluorometers such as Qubit or other compatible fluorescence plate readers. The molar concentration of each library is directly measured for use in pooling. The pooled libraries are ready for sequencing on Illumina NGS instruments.

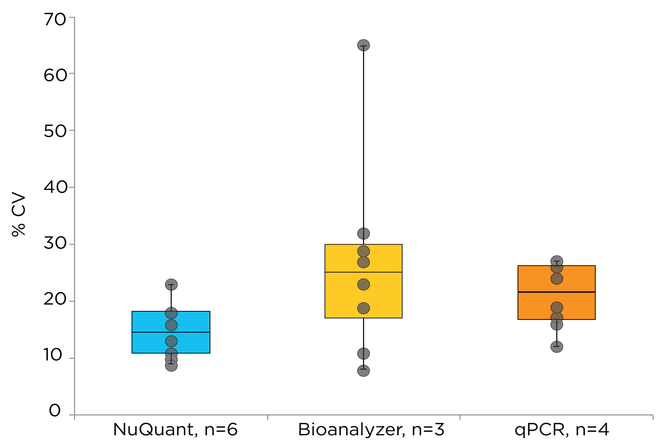


Figure 2: NuQuant has the lowest variability for library quantitation. A set of eight libraries was distributed to six different users at multiple institutions. Users were asked to use NuQuant and their own preferred method of library quantitation (number denoted by n). For each library, and each quantification method, the percent coefficient of variation (% CV) of molar concentration was calculated. NuQuant produced the lowest variation, followed by qPCR. Bioanalyzer gave the highest variation.

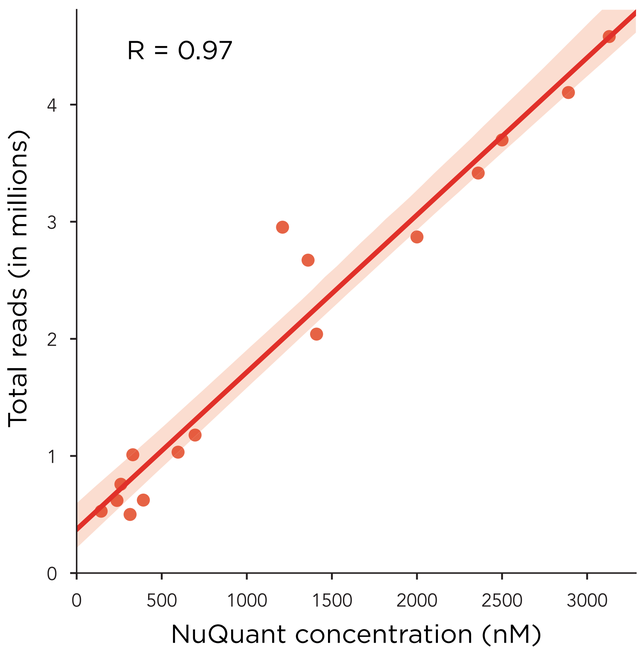


Figure 3: NuQuant accurately measures library molarity. Two users prepared eight libraries each from 10 ng of genomic DNA sheared to 200 or 300 bp using a Covaris system and amplified for 10 or 13 cycles to produce both lower and higher library concentrations. Purified libraries were quantified in duplicate by NuQuant, then equal volumes of all libraries were pooled and sequenced on one lane of an Illumina sequencer. The number of reads for each sample highly correlated with the NuQuant concentration demonstrating the accuracy of NuQuant.

High-throughput library quantification with NuQuant

Generate 24 unique libraries with the Celero DNA-Seq kit

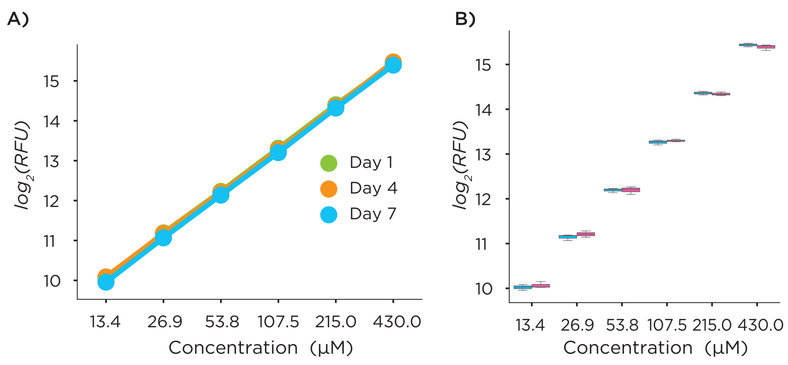
Aliquot in 96-well PCR plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			Library 1	Library 9	Library 17				Standard	Standard	Standard
B	Standard (430 nM)	Standard (430 nM)		Library 2	Library 10	Library 18			Standard	Standard		Standard
C	Standard (215 nM)	Standard (215 nM)		Library 3	Library 11	Library 19			Standard	Standard	Standard	Standard
D	Standard (107.5 nM)	Standard (107.5 nM)		Library 4	Library 12	Library 20				Standard		Standard
E	Standard (53.8 nM)	Standard (53.8 nM)		Library 5	Library 13	Library 21			Standard	Standard	Standard	Standard
F	Standard (26.9 nM)	Standard (26.9 nM)		Library 6	Library 14	Library 22			Standard	Standard		Standard
G	Standard (13.4 nM)	Standard (13.4 nM)		Library 7	Library 15	Library 23				Standard	Standard	Standard
H				Library 8	Library 16	Library 24			Standard	Standard		Standard

Measure fluorescence

Figure 4: Experimental design. Libraries generated with the Celero DNA-Seq kit were aliquoted into a 96-well black full-skirted PCR plate. The NuQuant standard was used to generate a standard curve and test for edge effects. The plate was sealed and NuQuant fluorescence was measured using the Tecan Infinite 200 PRO F Nano+ on days 1, 4, and 7.

NuQuant stability



C)

	9	10	11	12
A	49	6283	5450	6775
B	6040	6173	35	5682
C	6323	5285	7023	6680
D	37	6808	35	7267
E	6651	5480	5714	6841
F	6738	6088	35	6790
G	36	6925	6349	6808
H	6415	6295	35	6738

	Avg. RFU	% CV
Edge wells	6502	3.5%
Internal wells	6277	

Figure 5: NuQuant is consistent over time and between plates. The NuQuant standard was used to generate a standard curve and aliquoted into two plates. NuQuant measurements are presented in log_2 of relative fluorescent units (RFU). **A)** Measurement of the NuQuant standard on days 1, 4, and 7 provided consistent results demonstrating that the quantification is stable even after multiple freeze/thaw cycles. **B)** The NuQuant standard curve was added to a second PCR plate and concentration was measured over time. The standard curve between the two plates is similar indicating the consistency of measurement. This also indicates that values from a standard curve can be applied to other plates. **C)** Replicates of the NuQuant standard were added to selected wells. Analysis of the fluorescence demonstrated no difference between samples on the edge of the plate (red text) versus samples in center of the plate. Additionally, blank wells (gray) had a consistently low fluorescence indicating minimal cross-talk across the plate.

Library molarity (nM) over Days 1, 4, and 7

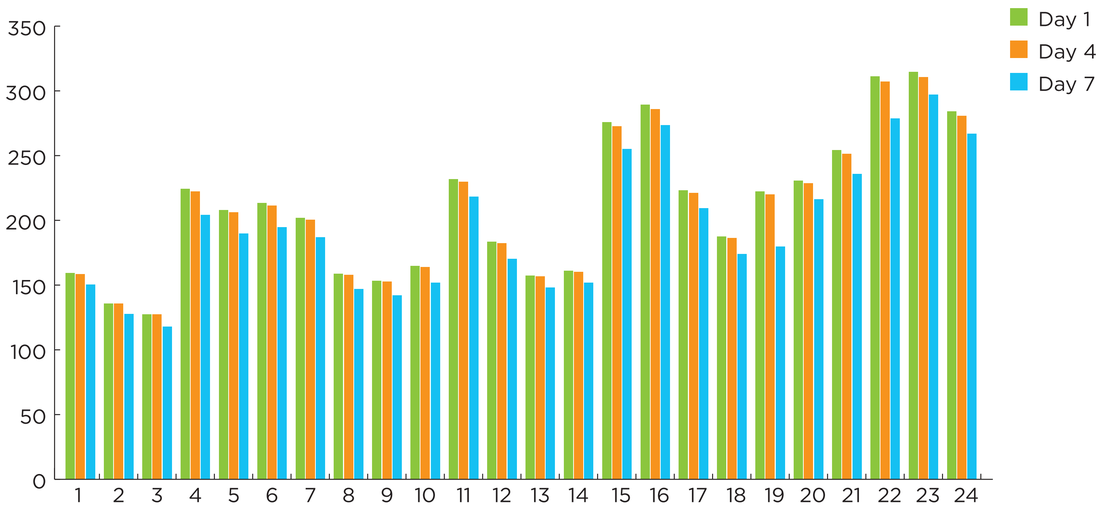


Figure 6: Consistent library quantification. The total recovered volume from twenty-four libraries were aliquoted into a 96-well, eliminating loss of sample due to standard QC methods. Library molarity was measured with NuQuant on days 1, 4, and 7. Four µl of each library was taken from each well on day 4 for sequencing. The day 7 fluorescent readings were normalized to account for lower sample volumes. The % CV between the three measurements is 5.6%. The library molarity is consistent even after multiple freeze/thaw cycles.

CONCLUSIONS

- NuQuant provides a simple method to calculate library molarity eliminating the time and cost associated with current methods.
- Accurate molar concentration can be determined in minutes with a Qubit or a standard plate reader for high-throughput quantification of NGS libraries (tested with Tecan Infinite F Nano, Infinite M Plex, Promega GloMax)
- Use of 96-well PCR plates and a plate reader allows direct library quantification without the sample loss observed with other QC methods

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