Automated NGS Quantification, Normalization and Pooling Using the OT-2

Written by
Matthew Akana, MS[1], Kinnari Watson, PhD[1]
[1]Opentrons Labworks, Inc.

ABSTRACT

The Opentrons® OT-2 effectively performs qPCR quantification of NGS libraries, maintaining high precision while reducing hands-on time. Two methods of sample normalization were tested: individually for each sample or pooled with enzymatic normalization.

Key Findings

- The OT-2 achieved low coefficient of variation (CV) values for both methods, while the pooled workflow was more streamlined.
- Precise and accurate automated pipetting was demonstrated by comparing Ct values across dilution replicates performed on reference kit standards.

INTRODUCTION

Accurate and reproducible quantification of DNA is essential to achieving consistent NGS sequencing results. It is important to measure both total quantity of DNA and the distribution of fragment sizes to achieve an optimal cluster density on the Illumina® flow cell. Both factors impact data quality and total sequencing output. Among the common methods of DNA quantification, qPCR has the highest sensitivity. Unlike fluorescence or spectrophotometry, qPCR is uniquely capable of selectively quantifying only the full-length library fragments that will form clusters via primers that anneal to Illumina p5 and p7 sequences.

However, qPCR setup is time intensive, and results are highly sensitive to small differences in pipetting. Automating NGS quantification provides a streamlined approach to getting reproducible, high-precision results while minimizing hands-on time. The Opentrons OT-2 automated liquid handling platform is capable of highly accurate pipetting and can be customized to automate a broad selection of NGS workflows. Here we demonstrate the capabilities of the OT-2 in automating NGS quantification and normalization in preparation for Illumina sequencing.

Two studies were performed to evaluate two methods of qPCR normalization during the NGS workflow. The "standard" method, where each sample is treated independently, and the enzymatic method, where samples are pooled and treated with Normalase™. The Normalase enzymatic normalization supersedes bead-based clean-up, while also substantially reducing the number of qPCR reactions performed. Performance of the OT-2 was measured in terms of cycle threshold (Ct) values, coefficient of variation (CV), and time needed to complete the preparation.

The OT-2 was used to effectively prepare NGS libraries for qPCR using both normalization methods. The Normalase method was easier to implement in the automated workflow, saving 1 hour overall and 20 minutes of hands-on time compared to standard normalization. Both procedures demonstrated good Ct values compared to reference kit standards and low CV values.

MATERIALS AND METHODS

NGS libraries of 24 isolated DNA samples were quantified using qPCR and pooled in preparation for Illumina sequencing using two different automated workflows with the Opentrons OT-2. **Figure 1** outlines the steps of two methods used.

In both methods, the concentration of isolated DNA samples was measured using the Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Assay Kit (cat. no. P11496) and Invitrogen Qubit™ 4 fluorometer. Following measurement, the samples were normalized by dilution

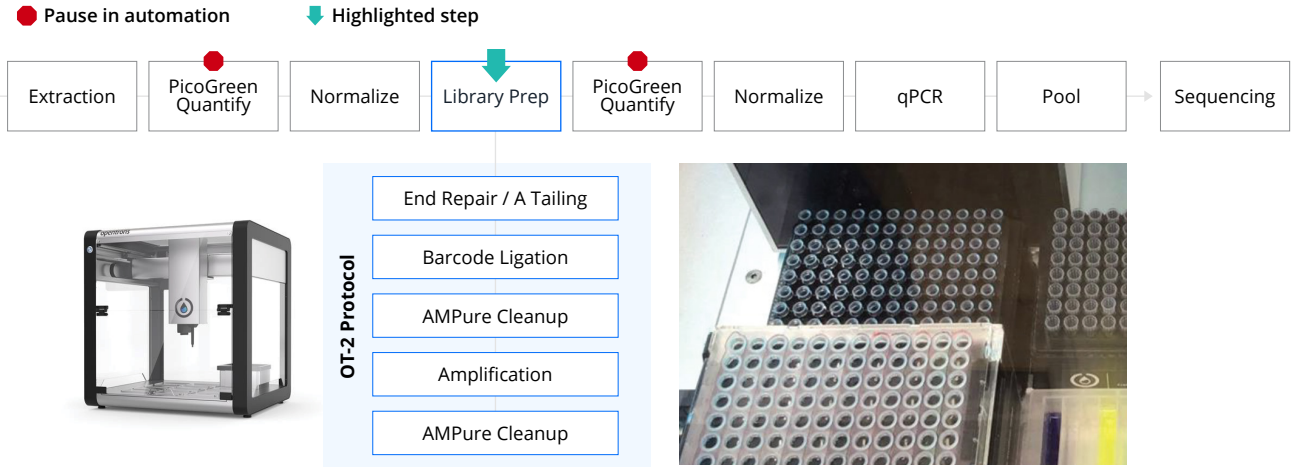
to a target concentration of 6 ng/µL using a single P200 pipette tip.

For the standard workflow the samples then underwent an automated NGS library preparation using enzymatic fragmentation and AMPure™ clean-up **(Figure 1)**. Following library preparation, the concentration of each sample was measured again. Each sample was then normalized to a target concentration of 1 ng/µL. To increase precision this dilution was accomplished with repeated aliquots. After normalization, qPCR was performed using the Roche™ KAPA Library Quantification Kit (cat. no. KK4824) and the LightCycler® 480 Instrument II. The samples were then pooled in final preparation for sequencing.

The Normalase workflow repeated the initial step of the standard workflow but utilized the IDT® xGen™ Normalase Module to enzymatically perform the final normalization. Following adapter ligation in the NGS library preparation procedure, Normalase PCR primers were used to amplify the libraries and condition them for downstream Normalase enzymology, yielding a concentration of ~12nM. The libraries were then individually incubated with the Normalase I master mix

Standard method workflow

Overview of the NGS Quantification on the OT-2



Normalase method workflow

Overview of the NGS Quantification on the OT-2

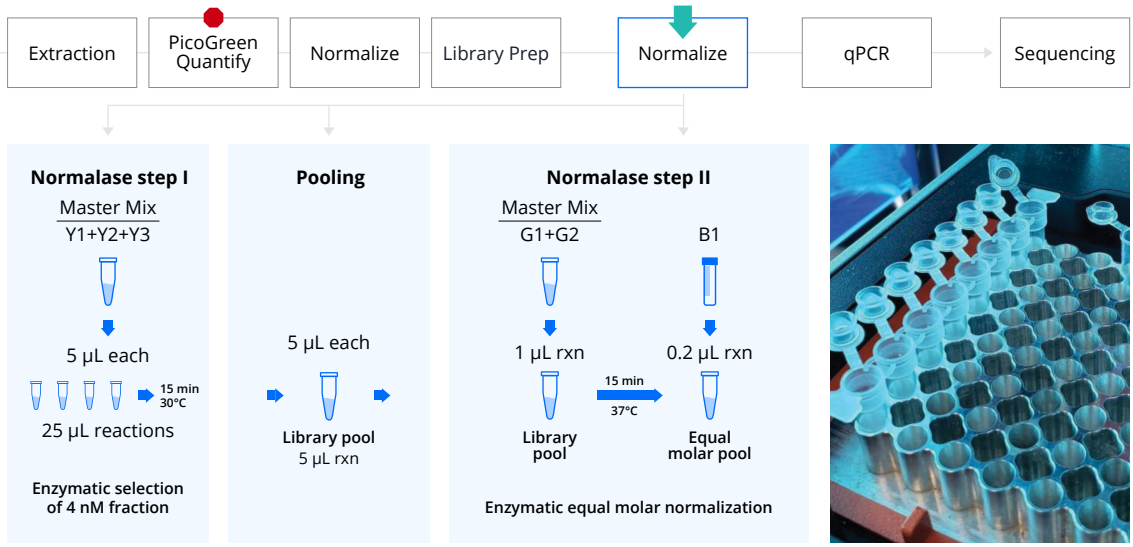


Figure 1: Image of two workflows. The Normalase method workflow follows the same steps as the standard method workflow through the library preparation step, then replaces the second standard normalization step with an enzymatic normalization process.

for 15 minutes at 30°C to enzymatically normalize the libraries to a concentration of 4 nM. After Normalase I, the libraries were pooled and incubated with the Normalase II master mix for 15 minutes at 37°C to enzymatically normalize the pooled library to the target concentration of 1 ng/µL. The concentration of this pooled library was then measured with qPCR in preparation for sequencing.

The Workflow Layout

The layout of the Opentrons OT-2 Platform includes the modules, labware, and DNA Prep reagents **(Figure 2)**. For both workflows, the OT-2, P300 Multichannel Pipette, Magnetic Module, Temperature Module, and Thermocycler Module were used. Other materials included the Fluorometer: Invitrogen Qubit 4 fluorometer, dsDNA Assay Kit: Invitrogen Quant-iT PicoGreen dsDNA Assay Kits and dsDNA Reagents, spectrophotometer, qPCR Quantification Kit: Roche KAPA Library Quantification Kits — Complete Kit (Universal), and qPCR Plate Reader: LightCycler 480 Instrument II.

RESULTS

The OT-2 Constructs Highly Uniform Libraries

During the standard normalization workflow each sample was individually normalized down to a target concentration of 6 ng/µL for NGS library preparation and 1 ng/µL prior to qPCR. The objective was to get all samples as close to the target concentration as possible and demonstrate a low coefficient of variation (CV). Results were measured using PicoGreen on a Qubit™ 4 fluorometer.

When performing dilutions from the same input library the OT-2 achieved an average concentration of 5.81 ng/µL for the 6 ng/µL target, and 1.10 ng/µL for the 1 ng/µL target with CV values of 3.4% and 9.4% respectively **(Table 1)**, demonstrating highly accurate pipetting for standard normalization. The P200 tip loaded onto a multichannel pipette still performed well while dealing with small volumes that may be considered at the edge of its range.

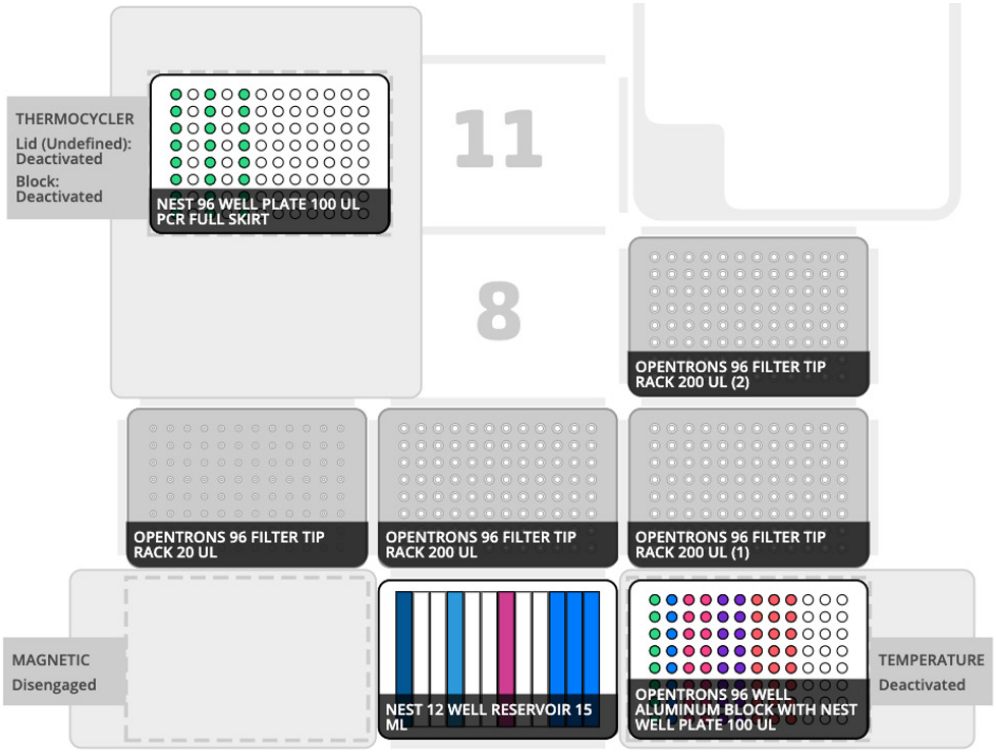


Figure 2: Opentrons OT-2 deck layout equipped with modules, labware, and DNA Prep reagents.

	Target = 6 ng/µL			Target = 1 ng/µL	
	Input	Normalized		Input	Normalized
	8.14	5.67		8.14	1.17
	9.94	6.06		9.94	0.98
	16.8	5.88		16.8	0.91
	11.1	5.86		11.1	1.10
	7.28	5.61		7.28	1.14
	10.3	5.58		10.3	1.21
	9.11	5.7		9.11	1.09
	7.99	6.09		7.99	1.16
Average	10.08	5.81		10.08	1.10
CV	29.8%	3.4%		29.8%	9.4%

Table 1: Standard Normalization with the OT-2. Normalized samples showed average concentrations close to the target with low CVs, demonstrating high pipetting accuracy by the OT-2.

The OT-2 Performed Accurate Dilution of Samples for qPCR

To test the ability of the OT-2 to perform accurate dilution of samples, qPCR was performed on 10 replicates each of two reference standards diluted at a series of 10-fold increments. Kit #1 concentrations were 20 to 2.0×10^{-4} pM and Kit #2 concentrations were 10 to 0.1 pM **(Figure 3)**.

It is expected that the Ct values should increase linearly inverse to concentration of the samples qPCR was performed on with the error bars in Ct values reflecting the precision of dilution across the 10 samples. As demonstrated in the error bars for both experiments **(Figure 3)**, the OT-2 provided highly precise pipette handling. For experiments with Kits #1 and #2, the error bars for Ct values begin to increase at 1000-fold and 100-fold dilutions respectively **(Figure 3)**.

The OT-2 Achieved Good Sample Balance with Normalization PCR

The Normalase workflow samples were pooled and normalized enzymatically with Normalase rather than normalized individually. After qPCR the pooled sample was then directly run through Illumina sequencing and assessed for sample balance and CV across samples.

The OT-2 achieved a CV of 16% and 11% in the xGen MC kit and the Roche® KAPA HyperPlus™ kit respectively **(Figure 4)**. These numbers are comparable to manual performance, though enzymatic normalization saved time.

Enzymatic Normalization Saved Significant Total and Hands-on Time

The total duration of the procedure using each method (standard individual quantification or pooling and enzymatic normalization) was compared using 24 samples for each. Standard individual normalization required 2 dilutions and 3 replicates each for 144 total reactions, taking ~3 hours including 30 minutes of hands-on time

qPCR Setup on the OT-2

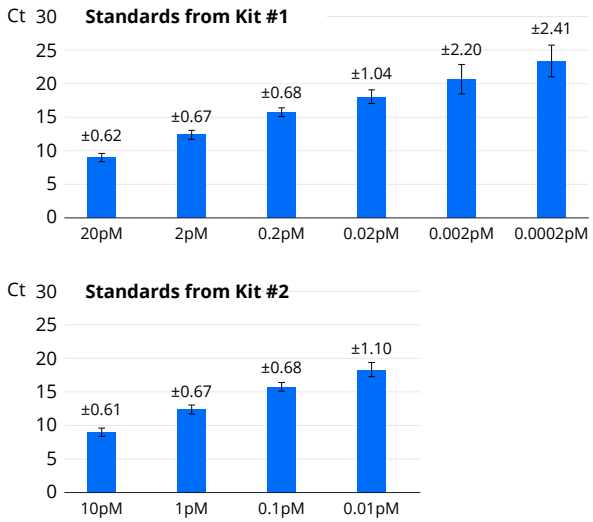


Figure 3: qPCR Ct values as a function of sample dilution. Ct values increased linearly with successive dilution while maintaining small error bars, demonstrating high precision pipetting by the OT-2.

Sample Balance: Normalase

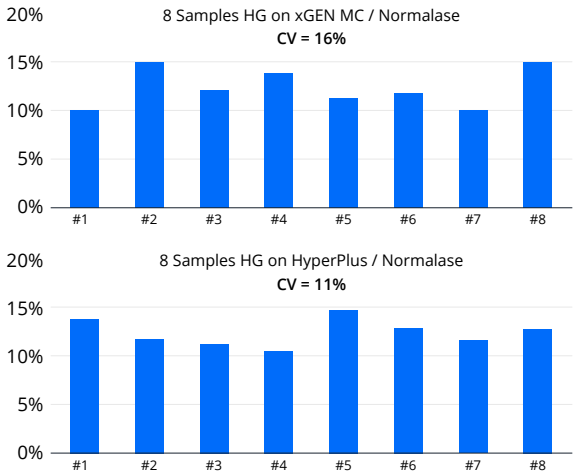


Figure 4: Pooled Enzymatic Normalization. Enzymatic normalization achieved a sample balance consistent with manual protocols while saving time.

(Table 2). Pooled enzymatic normalization also required 2 dilutions and 3 replicates but of only 1 pooled sample for a total of 6 reactions, taking ~2 hours with 15 minutes of hands-on time **(Table 2)**. The Normalase workflow streamlines the process, offering similar CV values with less hands-on time, and qPCR on fewer samples.

CONCLUSION

The OT-2 effectively performed quantification and normalization with two methods: individually, for each sample, or pooled, with enzymatic normalization. With standard, individual normalization, the OT-2 achieved low CV values and faithful recapitulation of reference standard concentrations **(Figure 3)**. Using pooled enzymatic normalization, the OT-2 delivered CV values similar to that of manual performance **(Figure 4)**. The pooled enzymatic reaction saved total and hands-on time by streamlining the second half of the workflow and reducing the number of total reactions run, reducing the number of qPCR reactions for a 24-sample library from 144 samples to just 6 samples **(Table 2)**.

The OT-2 effectively automated NGS quantification using both standard and enzymatic normalization methods. With the Normalase workflow the OT-2 can further streamline NGS quantification while improving sequencing sample balance and reducing the overall number of qPCR reactions.

Overall using the OT-2 for NGS quantification reduces hands-on time, tedious pipetting, and errors due to operator variability or human error. Because small aliquots are highly sensitive to human error, operator variability may lead to inconsistent results between repetitions of the same protocol. With final NGS output being dependent on accurate DNA quantification, the consistency provided by the OT-2 not only improves the results from qPCR but the productivity of the entire sequencing pipeline.

The precise and accurate pipetting demonstrated in this application combined with a highly flexible protocol setup indicate that the OT-2 can extend the benefits discussed here to other related procedures -providing more consistent results across your workflows and freeing up substantial time and energy across your research team.

24 SAMPLES STANDARD NORMALIZATION WORKFLOW			
	OT-2	**Hands-on**	**Thermo**
Quantification	64 min	15 min	
Normalization			
qPCR	45 min	5 min	45 min
Pooling		5 min	
Loading		5 min	

24 Samples x 2 Dilutions x 3 Replicates = 144 Reactions
~3 Hours

24 SAMPLES NORMALASE WORKFLOW			
	OT-2	**Hands-on**	**Thermo**
Normalase	9 min	5 min	30 min
qPCR	23 min	5 min	45 min
Pooling			
Loading		5 min	

1 Sample x 2 Dilutions x 3 Replicates = 6 Reactions
~2 Hours

Table 2: Standard Individual Normalization vs Pooled Normalase. Pooled enzymatic normalization streamlined the workflow, saving total and hands-on time, with fewer reactions.

qPCR SETUP TIPS
1. Use a broad range of concentrations for first qPCR then lower range after the procedure is well established
2. Use larger volumes to stay in good precision range
3. Use technical and dilution replicates
4. Save run libraries to use as future controls
5. Aliquot standards and use only 2-3 times
6. Dilute in 0.05% Tween to avoid loss
7. Know the fragment size to gauge performance
8. Include melting curves to watch for primer dimers
9. PicoGreen concentration of 1 ng/uL is ~STD2

Table 3: qPCR Setup Tips. Additional methods to achieve high performance qPCR preparation with the OT-2.