Countable Labs.

Validation of Countable PCR reproducibility across technical replicates, users, and systems

Achieve low CV values — indicating low variability and high reproducibility — for robust and more statistically significant data

Introduction

Precise DNA quantification is crucial for applications such as cell and gene therapy manufacturing, copy number determination, rare event detection, and general quantification of gene targets. Although digital PCR is widely used for DNA quantification, it has technical limitations that hinder low-variability results, typically yielding a coefficient of variation (CV) of around 10% [1]. These include:

01. Reliance on Poisson correction, which depends on consistent and accurate partition volumes to calculate copy number reliably [2, 3, 4].
02. A limited number of partitions, which begin to saturate at target counts typically near 100,000 molecules. As the target molecule counts approach the upper limit of the dynamic range, an increasing number of partitions contain multiple molecules, leading to reduced measurement resolution and accuracy [5].
03. Manual thresholding, which introduces user-dependent bias and reduces reproducibility.

Countable PCR partitions individual target molecules within a 3D gel-like matrix, enabling direct counting across four channels up to 1 million counts. With over 30 million partitions ensuring single occupancy, the need for Poisson correction is eliminated, and the dependence on partition consistency and high occupancy correction is removed. In addition, Countable PCR's light sheet imaging and automated counting algorithm allow for high-throughput processing with reduced variability between replicates and minimal user-dependent error.

Here, we demonstrate the reproducibility of Countable PCR across a six-log dynamic range and different users and instruments. These results highlight the power of direct counting to deliver low-variability data and consistent performance, which is essential for cross-validation and reliable assay development at multiple sites.

Materials & Methods

Measurement variability across a six-log dynamic range

Four short (<200 bp) synthetic double-stranded DNA templates, derived from four human genes (*RPP30*, *JAK2*, *RAD51*, and *MET*), were prepared and serially diluted to prepare counts between 10 and 1,000,000 molecules. A pool of TaqMan assays for *RPP30*, *JAK2*, *RAD51*, and *MET* were prepared for Ch01, Ch02, Ch03, and Ch04, respectively (**Table 1**).

Table 1. Probes and primers for reproducibility validation

Assay	Name	Sequence	Final reaction concentration (nM)
RPP30	Forward primer	AGATTTGGACCTGCGAGCG	200
	Reverse primer	GAGCGGCTGTCTCCACAAGT	200
	Probe	/56-FAM/TTCTGACCT/ZEN/GAAGGCTCTGCGCG/3IABkFQ/	100
	Probe additive	TTCAGGTCAGAA/3IABkFQ/	300
JAK2	Forward primer	AAGCTTTCTCACAAGCATTTGGTTT	200
	Reverse primer	AGGCATTAGAAAGCCTGTAGTTTTACTT	200
	Probe	/5HEX/CTCCACAG+A+C+A+CA+TAC/3IABkFQ/	100
	Probe additive	GTCTGTGGAG/3IABkFQ/	300
RAD51	Forward primer	TTGGTGACTTTTGCCCATATTA	200
	Reverse primer	GGTTGTGGTCAACAAAATACGT	200
	Probe	/5Alex594N/CCTGCTATAGTA+T+CATGGAACGAGG/3IAbRQSp/	100
	Probe additive	ATGGAACGAGG/3IAbRQSp/	300
MET	Forward primer	CAATGTGAGATGTCTCCAGCAT	200
	Reverse primer	GGGAACTGATGTGACTTACCCTA	200
	Probe	/5ATTO647NN/CGGACCCAATCATGAGCACTG/3IAbRQSp/	100
	Probe additive	ATTGGGTCCG/3IAbRQSp/	300

The pool of synthetic templates, ranging from 10 to 1,000,000 molecules, was added to a series of multiplex PCR reactions containing the pool of TaqMan assays (**Table 2**).

Table 2. Four-plex Countable PCR reaction setup

Reagent	Catalog #	Amount per 50µL	Final Concentration
Nuclease-free water	N/A	Up to 50 µL	N/A
4X Countable PCR Mix	KT0004	12.5 µL	1X
50X Primer and probe set (per target)	N/A	1 µL per target 4 µL total	1X
Synthetic template (per target)	N/A	5 µL per target 20 µL total	Variable

Eight replicates of each dilution were quantified with Countable PCR and used to calculate the CV at each target molecule input per channel. Thermal cycling was performed as described in **Table 3**.

Table 3. Thermal Cycling protocol for the *RPP30*, *JAK2*, *RAD51*, *MET* assays

Cycle	Step	Tempurature (°C)	Time	Ramp Rate
1	Initial denaturation	95	2 min	2 °C/s
40	Denaturation	95	20 sec	
	Annealing	60	1 min	
1	Store	8	∞	

Measurement variability across users and instruments

The Countable Control Assay Kit (KT0009) was used to prepare Countable PCR reactions with 100,000 counts per reaction across four channels. The kit includes primers that utilize the Universal Multiplexing chemistry to quantify four different synthetic DNA templates provided in the kit. Three users set up 12 reactions, and all 36 reactions were each quantified on three separate Countable systems. Reaction setup and thermal cycling were performed according to the Countable user guide.

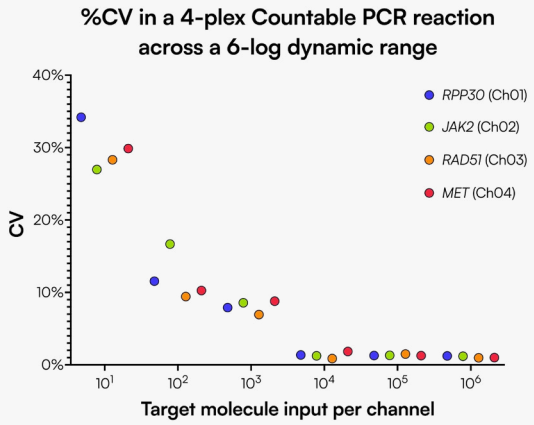

Figure 1. Measurement variability in a four-plex Countable PCR reaction across a six-log dynamic range. The CV of eight technical replicates is plotted against the target molecule input per channel in a series of four-plex reactions targeting a pooled mixture of double-stranded DNA targets *RPP30* (blue), *JAK2* (green), *RAD51* (orange), and *MET* (red). For 10,000 counts and above, the CV for measurements in all four channels is below 2%.

Results

Countable PCR attains low CV across a six-log dynamic range in a four-plex reaction

We performed Countable PCR on a series of four-plex reactions containing pooled double-stranded DNA targets *RPP30*, *JAK2*, *RAD51*, and *MET*, corresponding to Ch01, Ch02, Ch03, and Ch04, respectively. The pooled templates were serially diluted to produce final counts for each channel ranging from 10 to 1 million, and the CV at each concentration was calculated from eight technical replicates (**Figure 1**). Measurements taken at 1000 counts had a CV under 10%, and measurements taken at 10,000 counts and above had a CV under 2% across all channels.

Countable PCR achieves low CV across different users and instruments

To assess measurement variability across different users and instruments, three users ran 12 reactions with the Countable Control Assay Kit and imaged each reaction on three different imagers (**Table 4**). Within users, the CV was very low, typically less than 1%, averaging 0.92% across all users and channels. Between users, the CV averaged 2.42% across all channels. Between three different instruments, the CV averaged 0.62% across all channels and users. These results demonstrate strong concordance of data across users and Countable PCR systems, supporting their use in cross-functional workflows and cross-site collaborations.

Table 4. The Countable system is highly reproducible between technical replicates within each user, across instruments, and between users.

User	n	CV (■ Ch01)	CV (■ Ch02)	CV (■ Ch03)	CV (■ Ch04)
1	12	0.72%	0.95%	0.71%	0.57%
2	12	1.03%	0.78%	0.80%	1.54%
3	36	0.79%	0.92%	1.18%	1.07%
All users	36	2.15%	2.60%	2.57%	2.36%
Inter-instrument	95	0.57%	0.58%	0.61%	0.71%

Conclusion

Countable PCR delivers results with exceptionally low variability across technical replicates, users, and systems across a wide dynamic range. Specifically, this study demonstrates:

- Exceptional precision among technical replicates, with CVs below 1% for measurements around 100,000 counts.
- High reproducibility among users, with CVs below 3%.
- High reproducibility across instruments, with a CV of 0.65% across three systems.

These highly reproducible results highlight Countable PCR's robustness as a platform for quantifying gene targets at multiple points within a workflow and for cross-operator or cross-site experiment validation.

References

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