

Quantifying reverse transcription efficiency through single-molecule analysis with Countable PCR for enhanced NGS library preparation optimization.

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Abstract

Optimizing enzymatic reactions in next-generation sequencing (NGS) library preparation is critical for achieving high sensitivity and reproducibility, especially in low-input and single-cell workflows. However, conventional sequencing-based evaluation is expensive, time-consuming, and limit rapid iteration.

Here, we present Countable PCR, a quantitative single-molecule linkage analysis method that enables rapid and precise measurement of enzymatic efficiency without the need for sequencing. By counting and characterizing millions of individual DNA molecules in parallel, Countable PCR provides absolute quantification of molecular conversion outcomes. All measurements are performed within a single PCR, enabling fast, scalable assessment.

We demonstrate the use of Countable PCR to evaluate reverse transcriptase (RT) performance across a panel of commercially available enzymes. This method directly measures cDNA generation, positional bias along transcripts, and template-switching rates—key factors governing library complexity and bias. Single-molecule linkage analysis revealed differences in processivity and template-switching efficiency across RTs, whereas qPCR-based evaluation was inconclusive due to target-dependent amplification bias.

Beyond reverse transcription, the same framework using Countable PCR can be extended to assess other enzymatic steps central to NGS library preparation, such as ligation. By eliminating the need for sequencing during optimization, the use of Countable PCR significantly reduces experimental cost and turnaround time, eliminating complicated bioinformatics analysis, thereby accelerating NGS assay development cycles.

Introduction

A rapid and precise method for evaluating enzyme performance in NGS library preparation is currently lacking.

Optimization of enzymatic reactions in NGS library preparation is critical for maximizing yield and sensitivity, particularly in low-input, single-cell, and spatial transcriptomics workflows. Small differences in enzyme performance in molecular conversion efficiency can have a large impact on transcript detection sensitivity and overall assay reproducibility.

Traditionally, optimization relies on direct NGS readouts to quantify these effects, but sequencing-based evaluation is costly, time-consuming, and limits iteration speed during development (Table 1).

Introduction, cont.

Table 1. Comparison of NGS and Countable PCR for enzyme optimization in library preparation. While enzymatic optimization is essential for maximizing NGS library yield, its impact is difficult to assess using sequencing-based readouts, which are time-consuming and costly. On the contrary, Countable PCR enables rapid evaluation by directly counting molecules after enzymatic step and can determine whether multiple sequences reside on the same DNA molecule.

	NGS	Countable PCR
Workflow	A multi-step enzymatic workflow including reverse transcription, cDNA amplification, fragmentation, adapter ligation, and UMI-based quantification	Following reverse transcription, cDNA molecules are quantified directly
Direct measurement for DNA processing event?	No. The contribution of individual processing steps is difficult to assess because evaluation relies on downstream sequencing readouts	Yes. In addition to absolute counting, Countable PCR provides structural information, enabling determination of whether multiple DNA sequences coexist on the same molecule
Cost per sample	> \$1000	~\$10
Turn around time	Several days	Within several hours

Countable PCR offers single-molecule DNA counting with linkage analysis.

The Countable PCR platform generates ~ 30 million compartments within a single PCR tube through centrifugation, forming a clear gel-like matrix. These compartments can isolate up to one million template molecules, enabling independent amplification of individual molecules without co-occupancy.

As a result, unlike conventional digital PCR platforms—which are limited in dynamic range and rely on Poisson correction to estimate target copy numbers—the Countable system enables true single-molecule counting. This capability further allows bias-free PCR multiplexing.

In addition, by leveraging the static nature of our matrix, we can image the same compartments across multiple fluorescent channels to determine whether each compartment contains signals indicative of structural integrity. By designing an assay that targets different regions of the same DNA molecule, this approach provides direct information on the structural integrity of the DNA template.

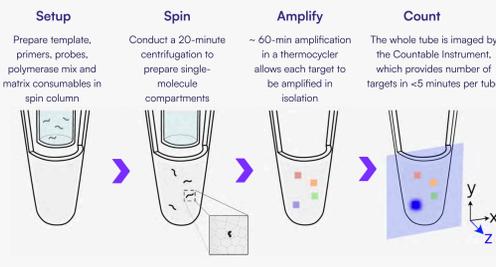


Figure 1. Schematic diagram of Countable PCR workflow in a single tube. In Countable PCR, target molecules are distributed across 30 million compartments in an optically clear matrix with simple centrifugation. After PCR, positive compartments emit a fluorescent signal and are quantified using 3D light sheet imaging.

Linkage analysis enables assessment of cDNA integrity through compartment interrogation.

We designed an assay targeting three regions of human *GAPDH* cDNA—the template-switching (TS) region, the 5' region, and the 3' region—using our proprietary Universal Multiplexing (UM) chemistry (Figure 2A). By attaching a UM probe sequence to each forward primer, this design enables efficient multiplex detection without the need for target-specific hydrolysis probes.

After cDNA is generated by reverse transcription, it is quantified using this assay, and linkage analysis is performed to determine whether each compartment contains signals indicative of integrity (Figure 3B). This approach distinguishes full-length from partial cDNA fragments and enables direct comparison of RT performance across commercially available RTs.

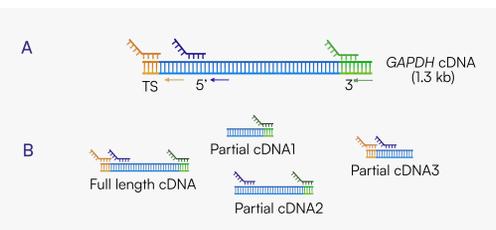


Figure 2. Universal multiplexed cDNA detection with linkage-based structural resolution. (A) Three regions of cDNA are simultaneously interrogated using UM chemistry, with UM probe sequences appended to forward primers. (B) Further linkage analysis enables differentiation of full-length cDNA molecules from partial products.

Results

The 3-plex assay was validated using a synthetic DNA template, and linkage analysis confirmed that most compartments contained signals indicative of integrity.

Before assessing RT efficiency using RNA targets, the 3-plex assay was validated using a synthetic DNA template containing all three target regions on the same molecule. Results from the 1-plex assay were consistent with those obtained under 3-plex conditions. Linkage analysis further confirmed that most compartments exhibited signals indicative of template integrity, demonstrating the presence of all three target regions within the same DNA molecule.

Countable PCR enables accurate, simultaneous quantification of multiple regions within cDNA, in contrast to qPCR, which suffers from amplification bias.

To evaluate RT processivity from the 3' to the 5' end and TS activity, reverse transcription was performed using oligo (dT) primers and a template-switching oligo (TSO) with human Jurkat RNA (400 ng) under manufacturer-recommended conditions. The resulting cDNA was 10-fold diluted and quantified (2 μ L input) using both the Countable PCR system and qPCR (QuantStudio 5) using the multiplexed assay above. Five commercially available RT enzymes were tested, and three input cDNA levels were analyzed on both platforms.

Figure 4 compares performance measured by Countable PCR and qPCR. In Countable PCR (Figure 4A), counts from the 3' end, 5' end, and TS region varied across RTs when using the same RNA input, enabling straightforward comparison of relative RT performance, including processivity and TS activity. TS counts may be elevated relative to 5'-end counts because the reverse primer used to amplify the TS region has homology to non-*GAPDH* transcripts that could have TS oligo appended.

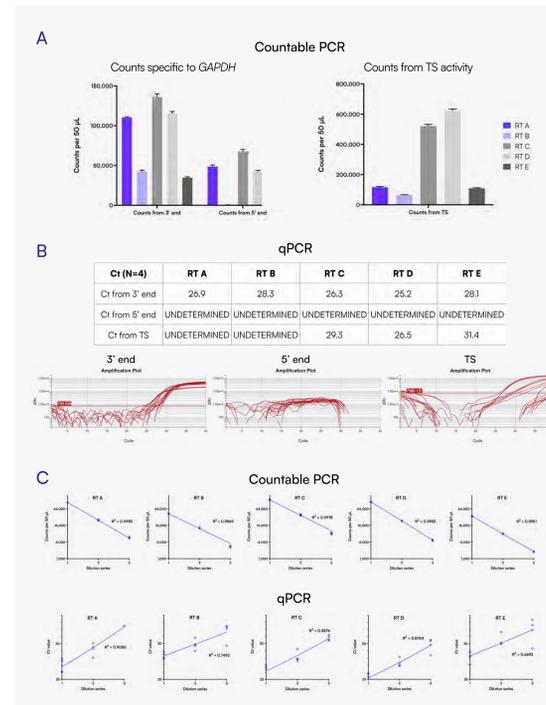


Figure 4. Comparison of Countable PCR and qPCR (N=4). (A) Using the cDNA input from each RT and 3-plex assay, RT-dependent differences in 3', 5', and TS counts were readily observed with Countable PCR. (B) In contrast, qPCR showed amplification primarily from the 3' end, while amplification from the 5' end was not successful due to amplification bias inherent to qPCR, making it challenging to compare RT efficiency with qPCR. (C) Even for the 3'-end target, Countable PCR showed more linear quantification ($R^2 > 0.9980$) compared to qPCR ($R^2 > 0.6690$) with low CV and showed expected counts change upon dilution.

Results, cont.

In contrast, when the same three targets were amplified together by qPCR (Figure 4B), amplification was observed primarily from the 3' end regardless of the RT used, making RT performance differences difficult to distinguish. This shows an inherent limitation of qPCR, where a dominant target or one with higher amplification efficiency can outcompete others in multiplex reactions. Moreover, even for the 3'-end target (Figure 4C), Countable PCR demonstrated substantially more linear quantification than qPCR ($R^2 > 0.9980$ vs. > 0.6690), with low CV and dilution-dependent changes in counts consistent with expected values.

Linkage analysis reveals differences in processivity and TS activity among RTs by assessing the signal indicative of integrity from multiple regions of same cDNA.

In addition to its efficient multiplexing capability, Countable PCR can determine whether signals originate from the same DNA molecule, enabling direct assessment of cDNA integrity (Figure 5A). Analysis of co-localized 5' and 3' signals within the same compartment reveals differences in RT processivity across enzymes (Figure 5B). To assess TS activity, we evaluated co-localization of signals from the 5' end, 3' end, and TS region (Figure 5C). The results are consistent with known RT properties, with higher TS activity observed for RTs known to exhibit TS activity. Based on analysis of the 1.3 kb *GAPDH* transcript, the best-performing commercial RT achieved approximately 30% full-length conversion and ~7% template-switching efficiency.

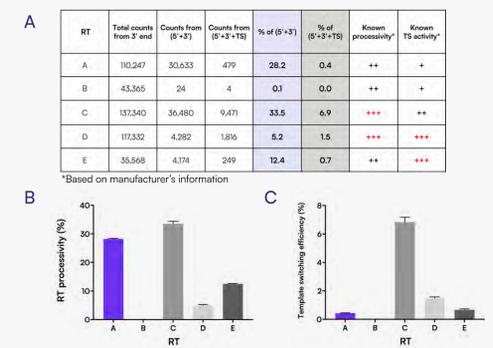


Figure 5. Linkage analysis enables direct assessment of cDNA integrity generated by different RTs. (A) Countable PCR enables molecule-level linkage analysis to assess cDNA integrity and RT performance. (B) Co-localization of 5' and 3' signals within the same compartment reveals RT processivity (5' + 3' counts divided by total 3' counts for each RT). (C) TS activity was assessed by co-localization of 5', 3', and TS signals (5' + 3' + TS counts divided by total 3' counts for each RT). Results are consistent with known RT properties, with higher TS activity observed for RTs with established TS capability.

This PCR-based single-molecule DNA linkage analysis is applicable to a wide range of DNA processing events, including ligation and gene editing.

Our linkage analysis can be applied to a wide range of DNA processing events, including the ligation of different DNA sequences or their separation through gene editing. These molecular events have traditionally been difficult to evaluate quantitatively, as assessment is typically limited to analysis of the final product. In contrast, our linkage approach provides a precise readout of whether specific sequences coexist within the same DNA molecule. This methodology has broad potential applications, not only in NGS library preparation, but also in gene cloning, genome editing, and gene and cell therapy workflows.



Figure 6. Linkage analysis enables quantitative assessment of DNA processing events. By determining whether specific sequences coexist within the same DNA molecule, linkage analysis provides a direct readout of molecular events such as sequence ligation or separation by gene editing.

Conclusion

We demonstrated that Countable PCR enables quantitative assessment of RT performance by providing molecule-level linkage analysis of cDNA integrity within a single PCR.

Countable PCR enables accurate, simultaneous quantification of multiple targets without gene-specific probes and avoids amplification bias compared with qPCR.

This approach can be further extended to evaluate a wide range of DNA processing events by determining whether specific sequences co-exist within the same DNA molecules.



Learn more about how Countable PCR transforms the assessment of enzymatic reaction.

Quantifying reverse transcription efficiency through single-molecule analysis with Countable PCR.