Countable Labs.

Simultaneous measurement of viral vector titer and genome integrity with long-read amplicons enabled by Countable PCR.

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Abstract

We introduce Countable PCR, a novel platform that enables transgene integrity and viral titer characterization, including long-read amplicons, within a single assay, with high sensitivity and low variability.

Current methodologies in the field present notable drawbacks for providing reliable viral titer measurements: quantitative, or real-time PCR (qPCR or rt-PCR, respectively) is too variable; digital PCR (dPCR) suffers from a narrow dynamic range, high Poisson errors, and arbitrary thresholding. Further, it is highly challenging to conduct both titer and genome integrity assessments in a single assay, as dPCR has partitioning limitations. Countable PCR overcomes these challenges by directly counting single molecules.

Here, we demonstrate the value of direct molecule counting by designing a novel multiplex assay that interrogates different regions of the gene-of-interest (GoI) or region-of-interest (RoI) to accurately quantify the ratio of partial vs. complete transgene while simultaneously measuring titer in the same sample. Unlike dPCR, this single-molecule approach prevents false co-occupancy of fragmented, unlinked targets, ensuring accurate genome integrity data. Complete transgene integration is further validated with the ability to analyze long-read amplicons, which we demonstrate here up to 2000 bp. Together, this work demonstrates how Countable PCR makes gene therapy viral characterization workflows more versatile and approachable compared to other methods.

Background

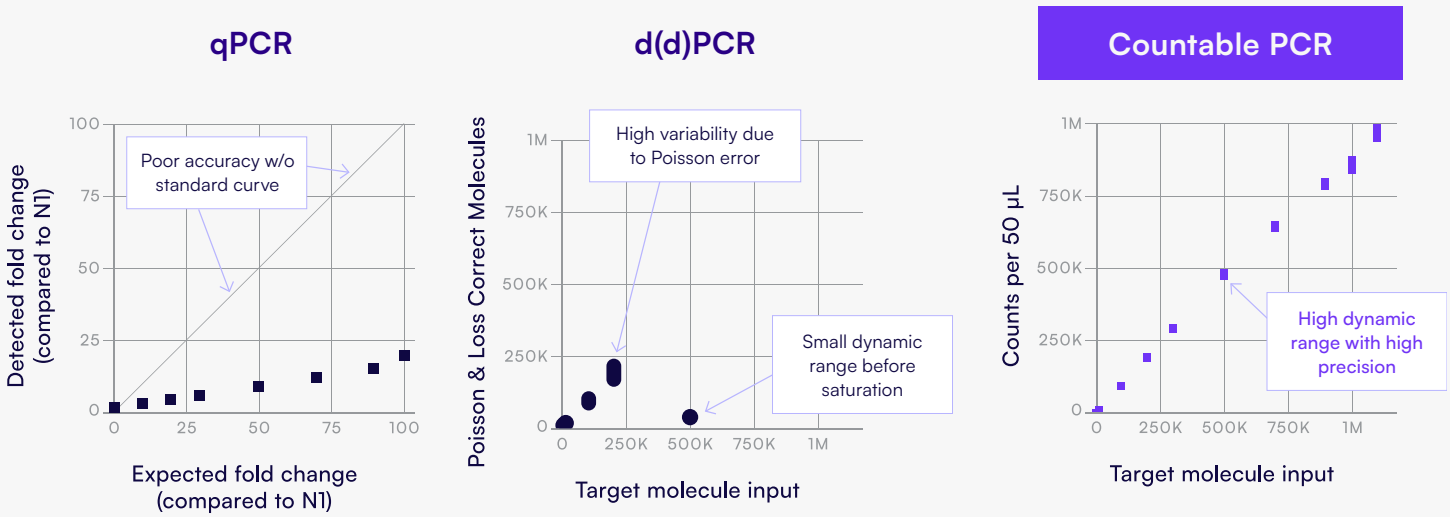
Single-molecule counting provides highly precise counts of amplicons across a broad dynamic range.

The key technology that powers Countable PCR is a structured gel matrix within a standard PCR tube that enables true single-occupancy suspension of individual gene-of-Interest (GoI) or region-of-interest (RoI) fragment(s). This overcomes many current limitations in the field:

- Reduce variability by avoiding Poisson statistics and manual thresholding
- Design multiplex and linkage analysis assays without interference from uneven amplification or co-occupancy
- Count molecules directly in sample tubes for precise, sensitive quantification
- Optimize faster with less trial-and-error
- Get accurate counts across a broad dynamic range

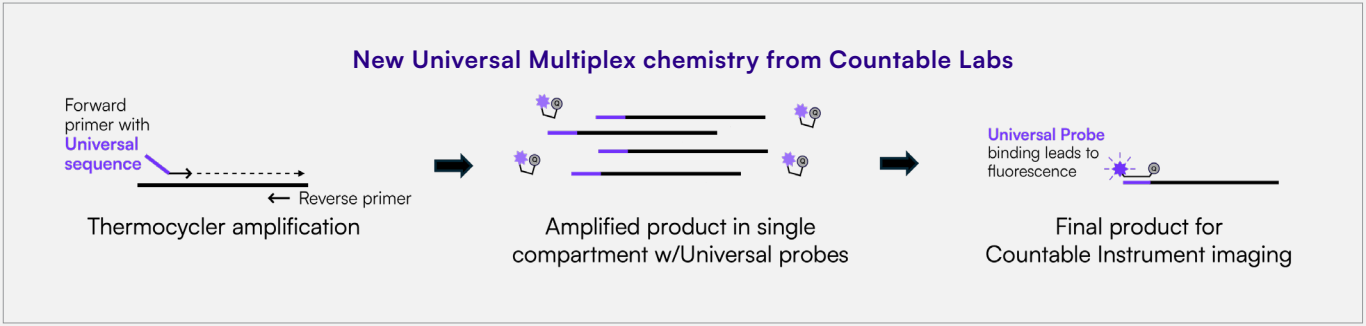
Countable PCR outperforms other PCR methods by combining high precision with broad dynamic range.

Figure 1. **Performance comparison of Countable PCR vs. qPCR and dPCR methods.** Data generated from same DNA samples to showcase platform dynamic range and variability.



Results

Universal Multiplexing simplifies assay development and reduces cost by using oligomeric primers instead of custom hydrolysis probes.



We designed ca. 100 bp amplicons targeting distinct transgene regions. Each forward primer included a universal adaptor for multiplex detection using the Universal Multiplex chemistry. This design enabled simultaneous measurement of titer and the ratio of partial vs. complete transgenes in a one-tube assay.

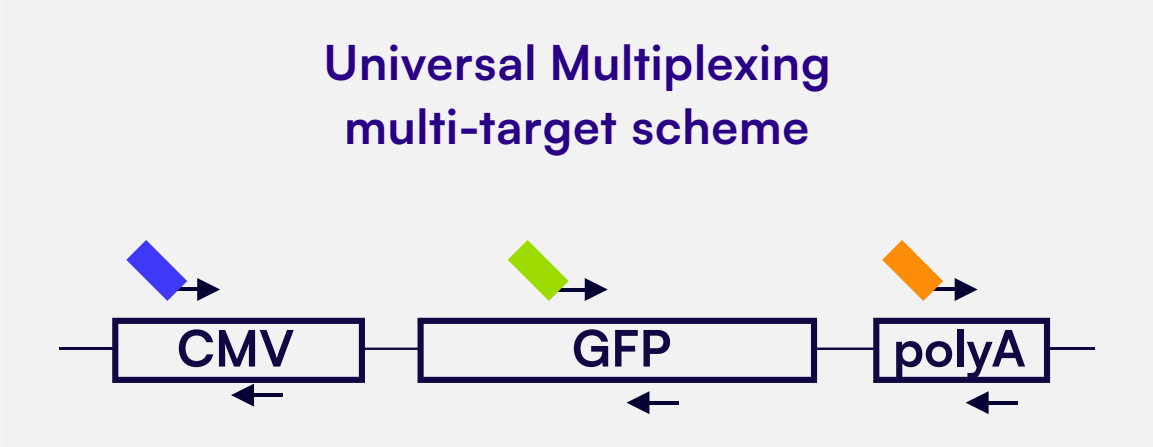
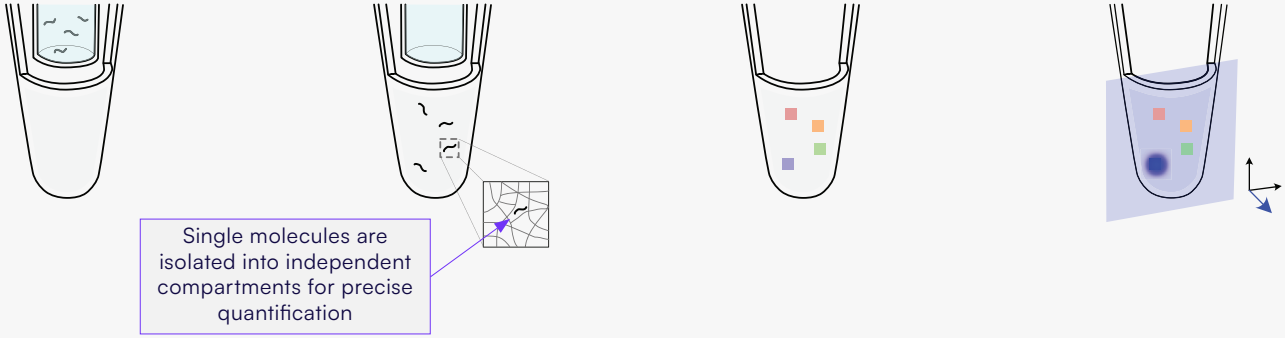


Figure 2. **Protocol**: Starting from 0.8 pg (ca. 500,000 molecules) of target AAV ssDNA, primers F/R at 04:20 molar ratio. UM probe kit from Countable PCR. PCR: 2-step 30 cycles, annealing at 60°C and 1 minute elongation time

Countable PCR sample preparation and analysis.



01. Setup

Prepare AAV ssDNA, primers, probes, polymerase mix and matrix consumables in spin column.

02. Spin

Conduct a 20-min centrifugation to prepare single-molecule compartments.

03. Amplify

An approximately 60 minute amplification in a thermocycler amplifies each target in isolation.

04. Count

The whole tube is imaged by the Countable Instrument, which provides the number of targets in your prep in <5 minutes per tube.

Figure 3. **Schematic diagram of standard Countable PCR workflow.**

Countable PCR sample preparation and analysis, continuted.

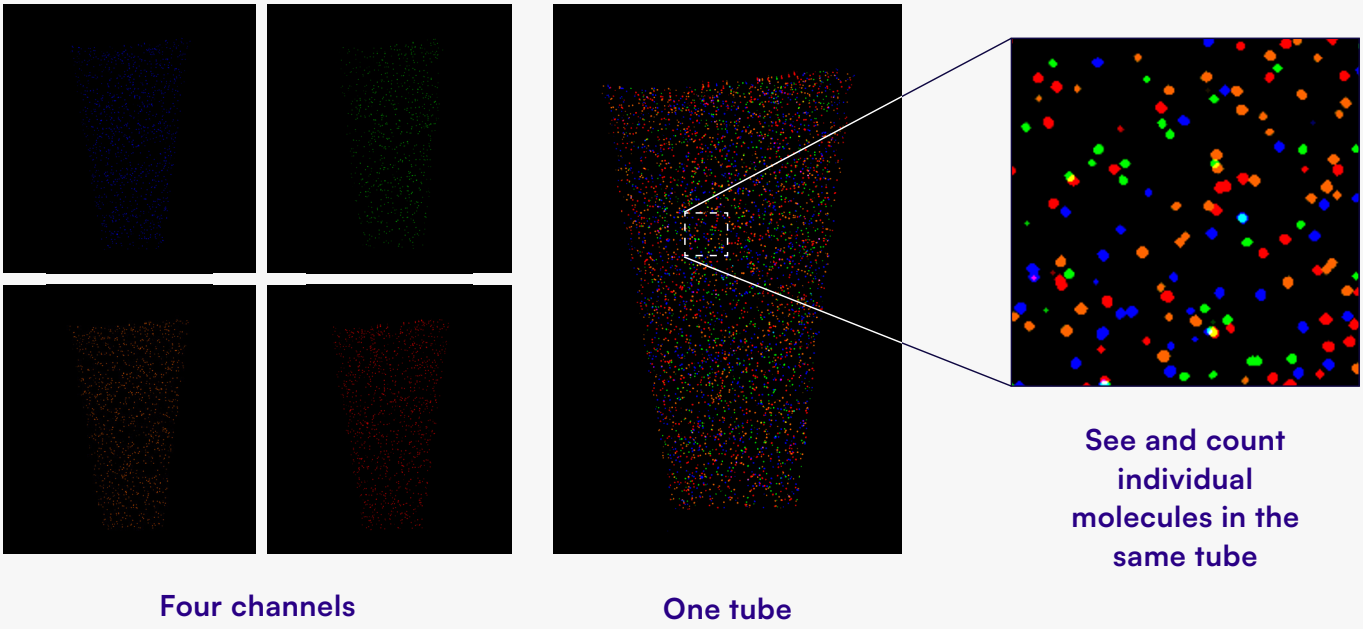
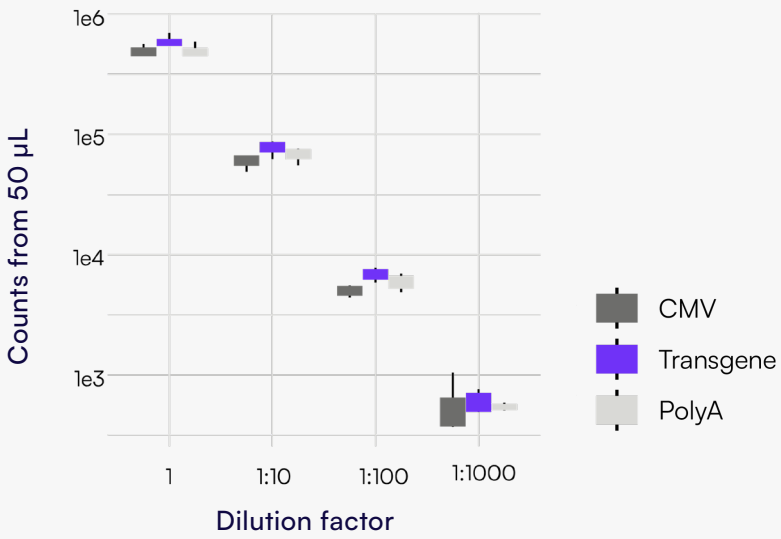


Figure 4. **Four-plex using Universal Multiplex on four different channels** (A) show the power of direct counting of molecules. One-tube preparation (B) allows for powerful direct visualization of different targets in the same prep (close-up, C).

Countable System. Quality indicators were reported via the Countable Control Analysis Report. Linkage analysis was done in beta mode on V0.10.0 software. Images were exported via the same software for representation purposes.

Countable PCR with Universal Multiplex scheme allows for multi-region titer determination for more accurate and versatile titer determination.

Universal multiplexing allows for inexpensive, easy-to-optimize titer determination across a broad dynamic range for multiple target regions within the vector genome.



Target	Channel	Dilution series R^2
CMV Enhancer	Ch01 (blue)	0.998
hrGFP	Ch02 (green)	0.998
SV40 polyA	Ch03 (orange)	0.998

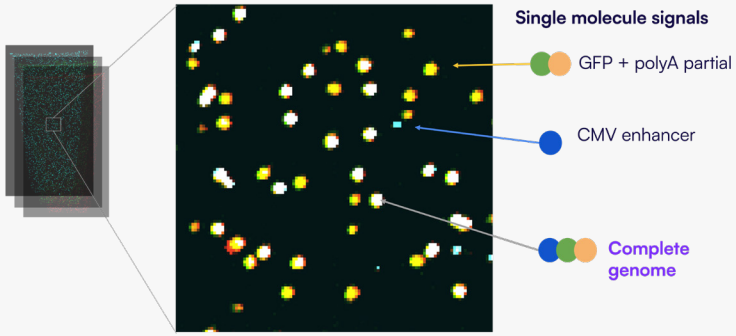
^Table 1. **R2 values for the linearity of signal along the dilution series.** Even with a broad signal range, linearity and quality remain strong. Dilution factor of 1 corresponds to 0.0008 ng of input ssDNA from AAV.

<Figure 5. **Results from a series of dilutions for each of three targeted areas of the transgene.** N= 4 replicates are reported.

Countable PCR enables precise linkage analysis of different regions of the transgene in the same experiment.

Prior to Countable PCR amplification and quantification, restriction enzymes were introduced between each target region, and complete target DNA was mixed with restriction-digested DNA to artificially introduce “partial” genomes for illustrative purposes.

Figure 6. **Representative images from a single reaction sample for linkage analysis.** The schematic reflects the visualization of compartments containing either partial or complete transgene.



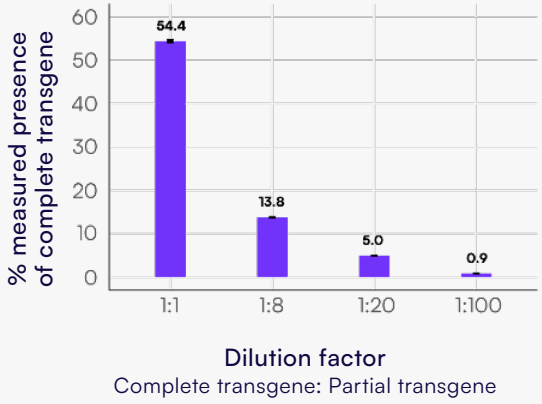


Figure 7. **Results of different ratios of complete vs artificially digested vector after analysis.** The x-axis shows the ratio of complete:partial transgene, while the y-axis shows the % of complete transgene in the sample determined from linkage analysis.

Countable PCR enables accurate molecule counting for long amplicons without optimization, demonstrating its versatility in designing complex experiments.

To demonstrate Universal Multiplexing's comparability to TaqMan© chemistry, as well as showcase proof-of-concept for long-amplicon interrogation of complete transgene, we generated progressively longer amplicons (ca. 150 bp to ca. 2000 bp) by shifting the reverse primer position downstream, while keeping the forward primer and TaqMan© probe constant; or by using the same F primer + adaptor for Universal Multiplex.

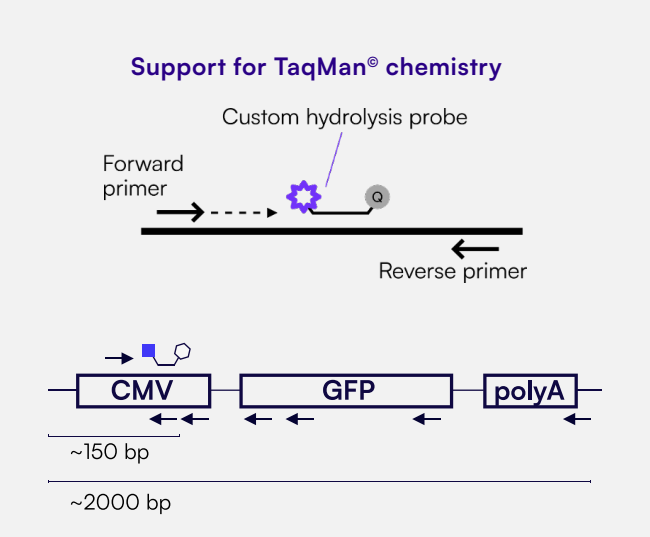
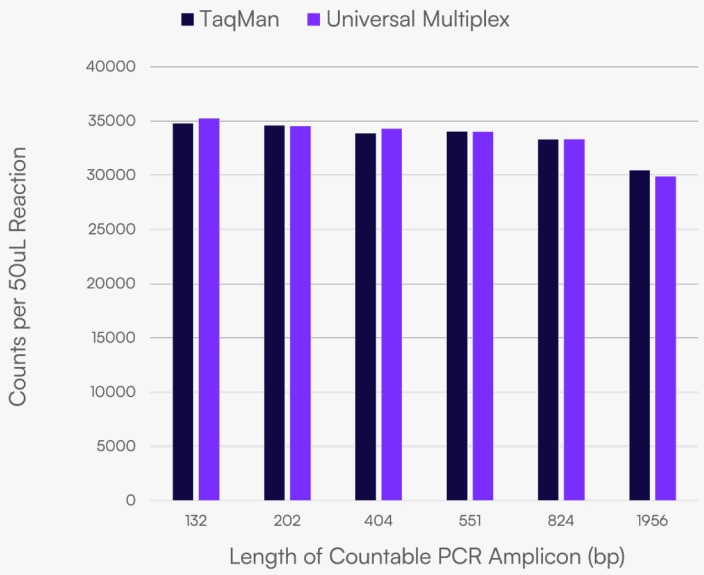


Figure 8. **Long-read protocol:** 0.5 pg (ca. 85,000 molecules) of target dsDNA, primers F/R/TM/PA at 10:10:05:15 molar ratio. TM© probe for FMA fluorophore. PCR: 2-step, 40 cycles, annealing at 60°C and 2 minute elongation time. Single replicates are reported.

Figure 9. Despite no assay-specific optimization, all amplicons produced results with good signal-to-noise ratio (SNR).

- Signal strength decreased with amplicon length but remained quantifiable
- Long-read targets (up to 1956 bp) yielded counts comparable to a typical 132 bp assay



These results confirm compatibility with long-amplicon designs to support complete transgene integrity assessments using full-length reads.

Conclusions

Countable PCR offers a new approach for cell & gene therapy researchers seeking to evaluate the genetic integrity of viral vectors. It combines single-molecule precision with a simple, flexible workflow that supports:

- Reproducible titer quantification across a broad dynamic range
- Linked-molecule analysis for assessing partial vs. complete transgene integration
- Fast, low-cost multiplex assay development for targeting multiple regions of the transgene using standard primers
- Compatibility with low to high genetic input amounts
- Reduced variability between users and replicates for improved data confidence

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