Countable Labs.

AAVnerGene
Together We Find The Cures

Rapid assessment of AAV genome integrity using multiplexed PCR with single-molecule detection.

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Background

Need for accurate, reproducible AAV genome quality assessment

AAV genome integrity is a critical quality attribute directly influencing therapeutic efficacy. During AAV production, multiple forms of incomplete or aberrant genomes may arise, including partially packaged genomes, snap-back structures, and residual genomic impurities from producer plasmids. Detecting these impurities alongside genome integrity is essential for regulatory compliance and process development. Traditional methods such as qPCR and droplet digital PCR assess titer at individual genomic regions but require separate reactions per target, making simultaneous multi-region integrity analysis impractical. Long-read sequencing provides comprehensive genome characterization but is costly and slow for routine QC applications. A method enabling rapid, multiplexed quantification of of titer, genome integrity, and residual DNA detection from direct viral input would streamline AAV process development and quality control.

Methods

Introduction to Countable PCR.

Countable PCR is a single-molecule PCR platform with ~30 million compartments per reaction—providing far deeper partitioning than ddPCR to enable accurate, high-precision multiplex quantification in one well.

- Massive partitioning (~30M compartments) → supports a 6-log dynamic range, so very high and very low abundance targets can be measured together.
- Single-molecule multiplexing (Poisson-free) → co-quantifies episomal + integrated + host reference in the same reaction without Poisson correction or sample splitting.
- Direct absolute counts with low variability → no standard curves or dilution series, delivering typically <5% CV and improved cross-run consistency.

Universal Multiplexing enables 3-day multiplex assay development for AAV genome integrity assessment.

Universal Multiplexing (UM) paired with Countable PCR accelerates assay development by separating target-specific amplification from universal probe detection, making high-plex designs fast to build and easy to expand.

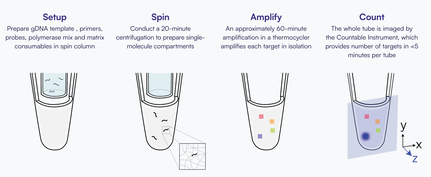


Figure 1. Schematic diagram of standard Countable PCR workflow.

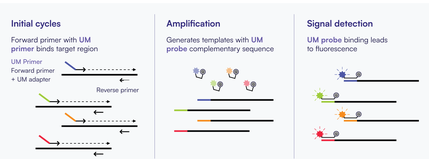


Figure 2. Universal Multiplex uses standard desalted oligos to develop targets for multiplex assays. Universal Multiplexing uses adapters on standard amplification primers to create a leading adapter. The complimentary sequence generated during amplification binds with UM probes, creating the detection signal for Countable light sheet imaging.

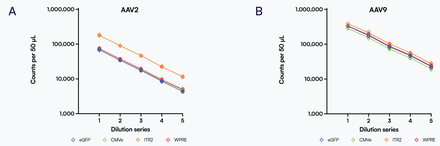
Methods, cont.

A 4-plex design enabling simultaneous titer quantification and genome integrity assessment.

Figure 3. Four-plex Universal Multiplex assay for simultaneous AAV titer quantification and genome integrity assessment. A single Countable PCR reaction detects ITR (UM-3), CMVe (UM-2), *eGFP* (UM-1), and WPRE (UM-4). Truncated or aberrant genome species are distinguished from intact genomes as a result of the Universal Multiplexing assay design.

Results

A 5-point dilution series confirms linearity and reproducibility across AAV2 and AAV9 serotypes.



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Target	AAV2 R^2	AAV9 R^2
eGFP	0.9966	0.9967
CMVe	0.9979	0.9970
ITR2	0.9981	0.9973
WPRE	0.9983	0.9963

Figure 4. Four-plex Countable PCR demonstrates strong linearity across a 5-point dilution series for AAV2 and AAV9. Counts per 50 µL shown against dilution series for *eGFP*, CMVe, ITR2, and WPRE for (A) AAV2 and (B) AAV9. ITR2 shows elevated counts consistent with known non-specific amplification and was excluded from titer calculations. (C) R^2 values confirm strong linearity across all targets and both serotypes (n=3).

Signal quality you can see: visual QC + ID-score—based automated positive calling.

Countable PCR images ~30 million spatially fixed compartments in 3D using sequential laser excitation and light sheet microscopy, capturing a clean optical signature per compartment across channels. The analysis software then uses a Reference Sample to learn what a true positive looks like and automatically calls positives across every sample — eliminating manual thresholding. Each sample receives an ID score reflecting signal-to-background discrimination, providing a built-in QC metric for assay performance.

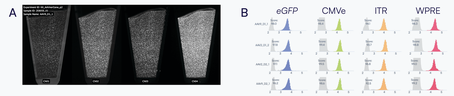

Figure 5. High signal-to-noise and automated positive calling across all four targets. (A) 3D light sheet images across four channels (Ch01-Ch04) show clearly resolved single-molecule positive compartments with low background; compartments are spatially fixed in the matrix, enabling repeated interrogation across channels with no loss of information. (B) Fluorescence intensity histograms for each target across two serotypes and two dilutions demonstrate consistent signal quality across samples.

Consistent titer and simultaneous genome integrity from a single Countable PCR reaction.

A single Countable PCR 4-plex reaction simultaneously quantifies AAV titer across three genomic targets (eGFP, CMV, WPRE) and the ITR, while linkage analysis on the same molecules reports genome integrity. Linkage is the fraction of molecules in which all targets co-occur on the same DNA strand, providing a direct readout of full-length genome content rather than bulk target abundance. AAV2 titer closely matches the reported stock concentration. Despite similar molecular counts across individual targets, linkage analysis reveals that only 26.0% of AAV2 and 48.2% of AAV9 genomes carry eGFP, CMV, and WPRE on the same molecule, demonstrating that titer alone does not capture genome integrity.

Target	AAV2 titer (GC/mL)	AAV9 titer (GC/mL)
eGFP	1.38E13	7.05E13
CMVe	1.44E13	6.08E13
WPRE	1.53E13	7.31E13
Configuration	CMVe eGFP WPRE	
% Linkage	26.0%	48.2%

Figure 6. Single Countable PCR reaction enables simultaneous AAV titer quantification and genome integrity assessment. Titer (GC/mL) calculated from *eGFP*, CMV, and WPRE targets across AAV2 and AAV9 serotypes shows consistent quantification across all three regions. % Linkage reports the fraction of molecules in which *eGFP*, CMV, and WPRE all co-occur on the same DNA strand, a single-molecule measure of full-length genome content. Linkage values well below 100% indicate that a substantial fraction of packaged genomes are truncated or fragmented, information not accessible from titer alone.

Results, cont.

Countable PCR enables comprehensive AAV quality assessment across genome integrity and residual DNA detection.

To demonstrate the breadth of Countable PCR, we assessed genome integrity and residual DNA across two single-plasmid AAV production systems from AAVnerGene, AAVone1.0 and AAVone2.1, and benchmarked them against a conventional tri-plasmid (AAVtri) system (Figure 7A). The AAVone platform consolidates the adenovirus helper genes (*E2A, E4orf6, VA RNA*), AAV packaging genes (rep, cap), and the transgene cassette into a single compact plasmid, simplifying manufacturing and reducing batch-to-batch variability (Figure 7B).

We designed 2 complementary 4-plex assays mapped across the AAVone construct (Figure 7B): a genome integrity panel (*ITR2, CMV, eGFP, WPRE*), and an R-ITR impurity panel (*ITR2, VA, Cap, Rep*). To capture all particle classes, empty, full, and heavy capsids were collected after a single round of CsCl ultracentrifugation and analyzed in parallel (Figure 7C). Linkage analysis across these panels reports the fraction of molecules carrying each element, providing a direct readout of intact genomes and residual plasmid backbone contamination in the same workflow (Figure 7D).

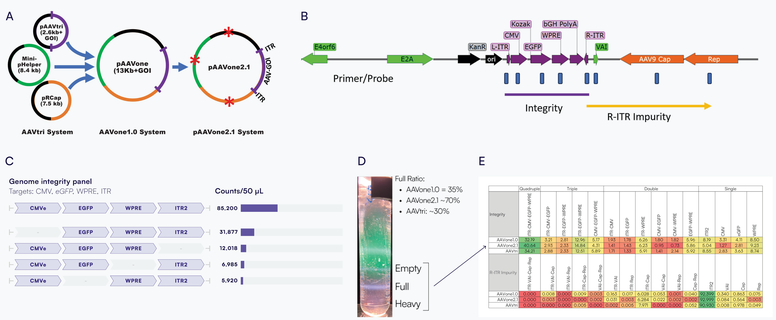

Figure 7. Countable PCR enables AAV genome integrity and impurity assessment across production systems. (A) Schematics of three AAV production systems: the conventional tri-plasmid AAVtri, the consolidated single-plasmid AAVone1.0 (pAAVone, 13kb + GOI), and the optimized AAVone2.1. (B) Map of the AAVone construct with primer/probe locations for the three 4-plex Countable PCR panels: a genome integrity panel spanning *CMV, eGFP, WPRE,* and *ITR2* (between L-ITR and R-ITR), and L-ITR / R-ITR backbone impurity panels flanking the cassette. (C) Schematic of the genome integrity readout: each row represents a distinct molecular species defined by which targets co-occur on the same DNA strand. Quadruple-positive molecules (CMV-*eGFP*-WPRE-ITR2) represent fully intact, ITR-flanked transgene cassettes, while triple-positive species lacking one element indicate truncations at specific positions in the genome. Counts and % linkage quantify the abundance of each species. (D) All AAV particles, including empty, full, and heavy particles, are collected for analysis after one-round of CsCl ultracentrifuge. (E) Linkage-based quantification of genome integrity (top) and R-ITR backbone impurities (bottom) across AAVone1.0, AAVone2.1, and AAVtri, reported as the fraction of total counts carrying each element combination. Quadruple, triple, double, and single linkage columns reveal the spectrum from full-length intact genomes to fragmented species, while impurity panels reveal reverse-packaging of plasmid sequence beyond the ITR boundaries.

Conclusion

- Countable PCR + Universal Multiplexing enables straightforward AAV titer quantification, genome integrity assessment, and residual DNA detection, providing comprehensive quality characterization from a single workflow.
- Existing qPCR primer designs can be adapted into a working 4-plex assay within 2—3 days, with no standard curves or Poisson correction required.Near-100% recovery from ~1,900 genome equivalents demonstrates quantitative accuracy at low DNA input — critical for early-timepoint and small-clone samples.
- Direct viral input delivers the highest count recovery and titer accuracy, and the 6-log dynamic range accommodates samples across a wide concentration range with %CV < 5%.
- Together, these capabilities accelerate AAV process development, batch release testing, and analytical confidence in gene therapy manufacturing.

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