Countable Labs.

Early vector copy number determination and integration assessment for cell therapy using single-molecule PCR.

Soo-Mi Kweon[1], Chih-Hong Lou[1], Heather Stark[2], Michael Balamotis[2], Jeanette Mei[2], Emily Blair[2], Eleen Y. Shum[2]

[1] Carrigent, 668 Arrow Grand Circle Covina, CA 91722 USA
[2] Countable Labs, Inc. 1810 Embarcadero Rd. Suite 200 Palo Alto, California 94303

Abstract

Background: Vector copy number (VCN) is a critical analytical parameter in cell therapy development, directly influencing product safety and potency. Conventional VCN workflows often require extended culturing to distinguish stable genomic integration from transient episomal vector, delaying process optimization and clone selection. Sensitive and low-variability methods that enable earlier integration assessment are needed.

Methods: Genomic DNA from lentivirally transduced cell lines was analyzed using Countable PCR, a single-molecule PCR platform enabling absolute quantification without standard curves. Multiplex assays targeting a lentiviral regulatory element (WPRE) and a human reference gene (*RPP30*) were used to calculate VCN within a single reaction. Clonal isolates were evaluated across multiple passage timepoints using low DNA inputs (~12.5 ng per reaction, corresponding to ~1900 genome equivalents). Technical replicates (N=4—8) were performed to assess assay precision. Additional vector-associated targets were incorporated to evaluate multiplex expandability.

Results: VCN measurements were reproducible across passage timepoints with strong recovery of the human reference target, indicating high analytical sensitivity even at low DNA input. Across samples, VCN measurements exhibited low variability, with coefficients of variation consistently below 5% for most passages and modestly increased variability only at the lowest target concentrations. Multiplexed detection enabled simultaneous quantification of episomal and integrated vector species. Episomal signal was readily detected at early timepoints but declined below detection by week 2 post-transduction, while integrated signal stabilized and remained consistent across later passages. This enabled early confirmation of stable integration without extended culturing. Expanded multiplex assays successfully detected additional low-abundance vector-associated targets within the same reaction, demonstrating assay flexibility without compromising precision.

Conclusion: Single-molecule, multiplexed PCR provides precise and sensitive VCN determination while enabling early discrimination between episomal and integrated vector. This approach supports faster process optimization, earlier clone selection, and improved analytical confidence in cell therapy development workflows.

Background

Need for faster, low-variability VCN methods to accelerate clone selection.

Vector copy number (VCN) is a core safety/potency attribute for lentiviral gene-modified cell therapies and is used to support clone selection, process optimization, and lot release. However, early post-transduction measurements can be confounded by residual episomal/ unintegrated vector, and conventional workflows often wait for extended passaging/culture to ensure signals reflect stable genomic integration (**Figure 1**). This delay slows development decisions and increases cost, especially when screening many clones/conditions. A method that delivers early, low-variability, absolute VCN from low DNA input—while also distinguishing episomal vs integrated vector—would accelerate cell therapy development and improve analytical confidence.

Here we show that single-molecule, multiplexed PCR enables precise VCN quantification and earlier integration assessment without standard curves, supporting faster clone down-selection and process optimization.

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 chemistry enabled the implementation of VCN multiplex assays within 3 days from initial design.

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.

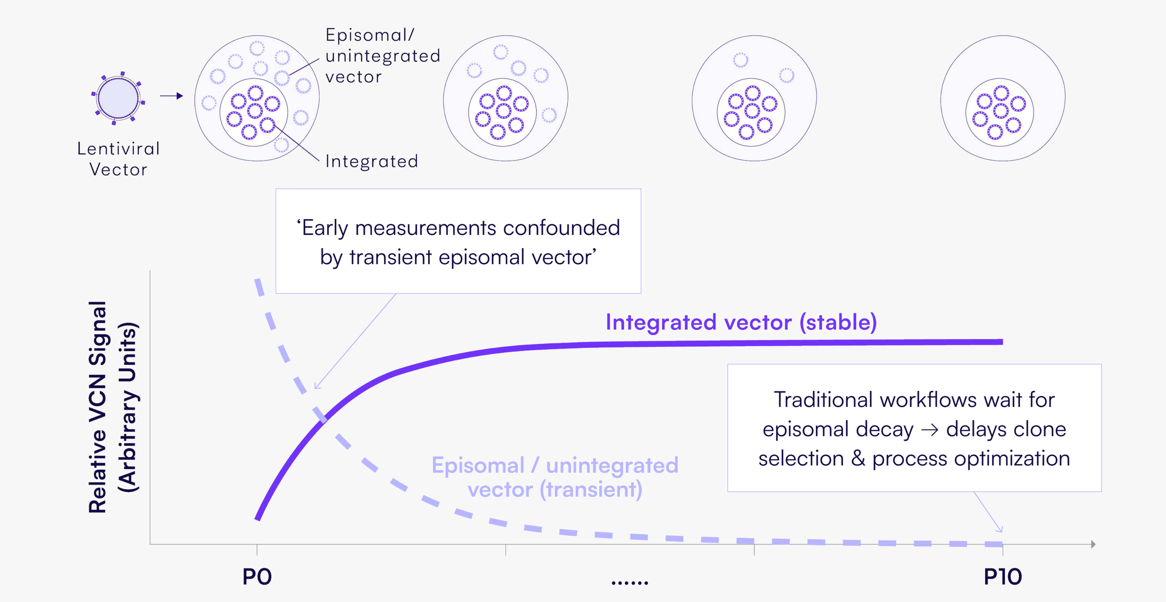


Figure 1. Challenge of early VCN measurement after lentiviral transduction. Immediately post-transduction, total vector signal reflects a mixture of transient episomal/ unintegrated vector (high early, decays over ~1—2 weeks) and stable integrated vector (rises and plateaus). This confounds early VCN readouts and is why traditional workflows often wait for episomal decay before making clone selection and process optimization decisions.

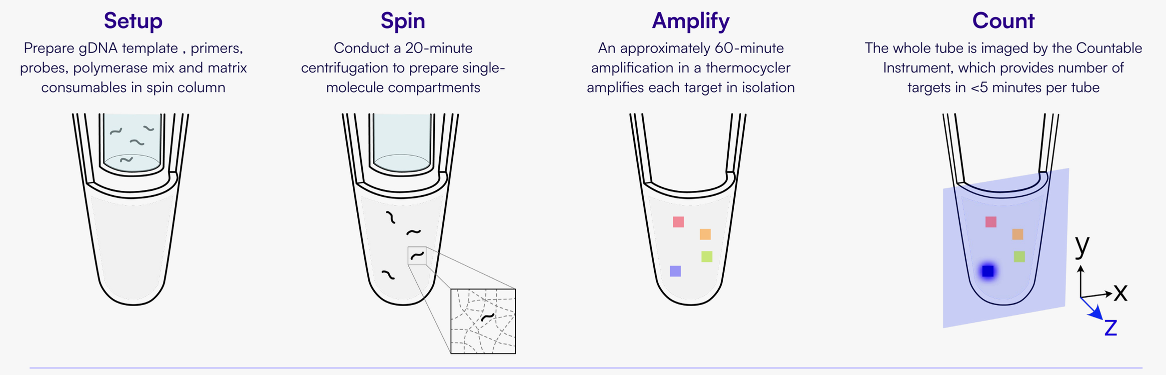


Figure 2. Schematic diagram of standard Countable PCR workflow.

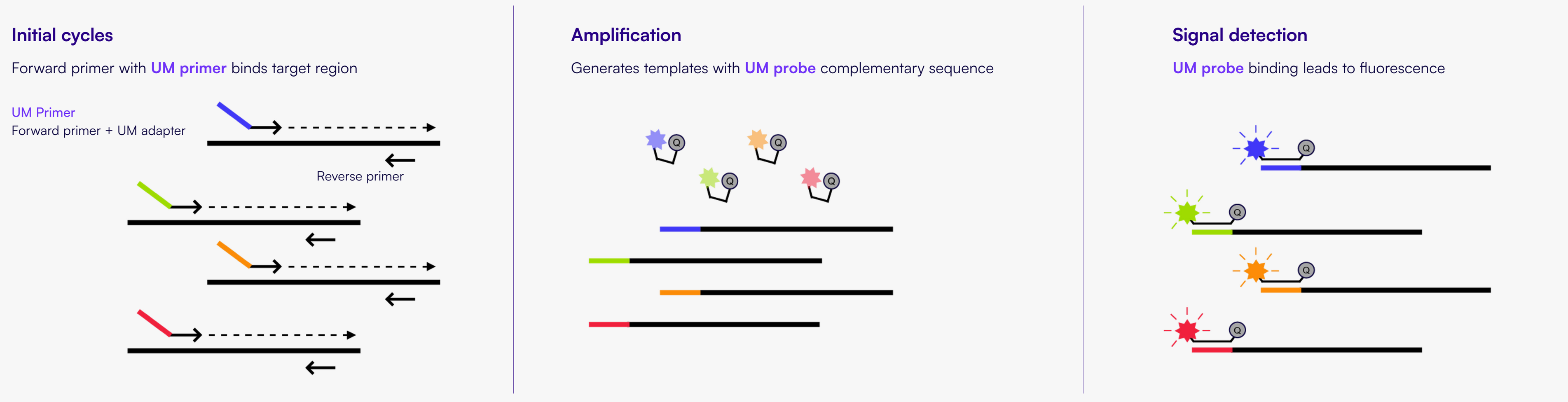


Figure 3. Universal Multiplex uses standard desalted oligos to develop targets for multiplex assays. Universal Multiplexing uses adapters on standard amplification primers to create a leading adapter. The complimentary sequence generated during amplification binds with UM probes, creating the detection signal for Countable light sheet imaging.

A 3-plex design that separates stable integration from transient episomal signal.

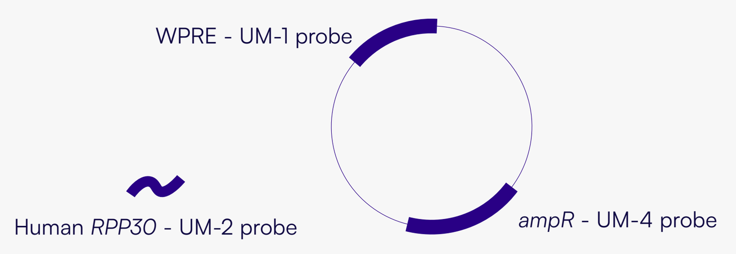


Figure 4. Three-plex Universal Multiplex assay for simultaneous VCN quantification and episomal vector discrimination. A single Countable PCR reaction co-detects WPRE (UM-1), present on both integrated and episomal vector; *ampR* (UM-4), a plasmid-backbone marker specific to episomal/unintegrated vector; and human *RPP30* (UM-2), a host genomic reference for VCN normalization.

Results

A 6-log dynamic range lets Countable PCR resolve targets even when their abundances differ by orders of magnitude.

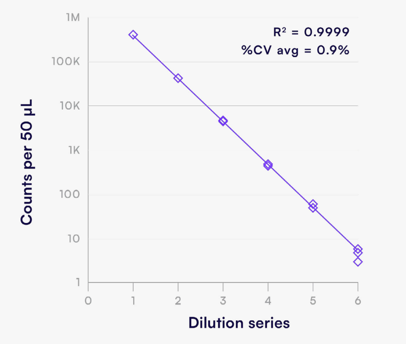


Figure 5. Six-log dynamic range of WPRE. Serial dilutions of a WPRE-containing lentiviral vector were quantified using Countable PCR. Counts per 50 µL are plotted against dilution series, demonstrating linearity. Inter-replicate variability was consistently low.

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.

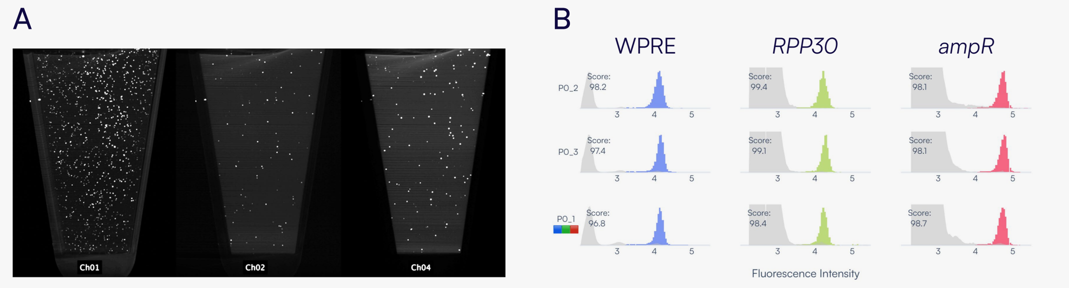
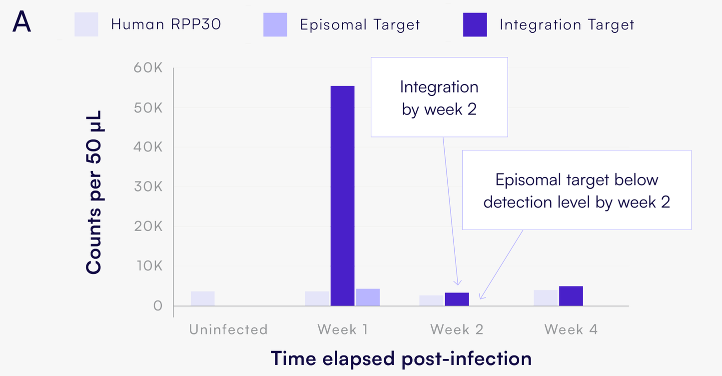


Figure 6. High signal-to-noise and automated positive calling across all three multiplex targets. (A) 3D light sheet images of a representative sample across three imaging channels (Ch01, Ch02, Ch04) show clearly resolved single-molecule positive compartments with low background. Because compartments are spatially fixed in the solid-state matrix, the same sample is interrogated repeatedly across channels with no loss of information. (B) Fluorescence intensity histograms for WPRE, *RPP30*, and *ampR* across three independent samples (P0_1, P0_2, P0_3).

Earlier integration confirmation + low-variability VCN in a single reaction.

A single Countable PCR 3-plex reaction simultaneously quantifies host reference (*RPP30*), total vector (WPRE), and episomal-only signal (*ampR*), enabling direct VCN calculation and integration assessment. The platform's 6-log dynamic range handles the very high early-timepoint vector signal that breaks conventional PCR assays, **while low DNA input (~12.5 ng / ~1,900 GE) and near-100% *RPP30* recovery confirm quantitative accuracy.** Episomal signal drops below detection by week 2, revealing stable integration weeks earlier than the conventional week-4 timepoint — accelerating clone selection and process optimization.



B

Sample (passage #)	DNA input	RPP30 counts (Total cells x 2)	WPRE counts (Total LV)	VCN	%CV	Episomal counts (AmpR)
WT (uninfected)	12.5ng (1900 genome equivalents)	3759	15	0	-	14
P0		3875	55371	**28.6**	**3.4%**	4356
P5		3034	3550	**2.3**	**4.3%**	72
P10		4183	5106	**2.4**	**2.9%**	95

Figure 7. Early episomal clearance and precise VCN determination from a single Countable PCR reaction. (A) Episomal profiling across passage timepoints. In a single 3-plex reaction, Countable PCR quantifies Human *RPP30* (host reference), Integration Target (WPRE), and Episomal Target (*ampR*). Integrated signal is detected early and remains stable from Week 1 through Week 4, while episomal signal drops below detection by Week 2 — enabling confirmation of stable integration two weeks earlier than the conventional Week-4 timepoint. (B) VCN determination with low variability. Using only ~12.5 ng DNA input (~1,900 genome equivalents), the assay achieves near-100% input recovery based on *RPP30* counts and resolves VCN across clones (P0, P5, P10) with %CV < 5%. VCN is calculated as WPRE / (*RPP30* / 2), accounting for the two *RPP30* copies per diploid human genome. Together, these data show Countable PCR enables sensitive, low-variability VCN measurement and earlier integration assessment than conventional workflows.

Conclusion

Countable PCR + Universal Multiplexing enables straightforward, true single-reaction VCN + episomal discrimination across high- and low-abundance targets (6-log dynamic range), without standard curves or Poisson correction.

A 6-log dynamic range + single-molecule multiplexing resolves high-abundance episomal vector and low-abundance integrated vector simultaneously, with %CV < 5% at ~12.5 ng DNA input.

Near-100% recovery from ~1,900 genome equivalents demonstrates quantitative accuracy at low DNA input — critical for early-timepoint and small-clone samples.

Together, these capabilities accelerate clone selection, process optimization, and analytical confidence in cell therapy development.

Learn more about how Countable PCR transforms cell and gene therapy analytical workflows.