Immunotherapy: Basic science and preclinical studies

Establishment of Reference Standards for Vector Copy Number Assay & CAR Expression

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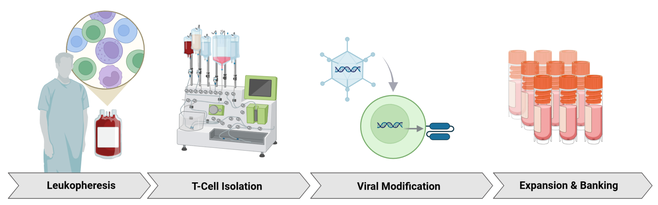
Abstract

Genetically engineered cell therapies represent a groundbreaking approach to treating diseases such as cancer, where traditional methods have been ineffective. These therapies use patient or healthy donor cells modified using viral or nonviral approaches. Cells modified with lentiviruses are assessed for vector copy number (VCN), a critical parameter which measures the dose of transgene to help assess their safety and efficacy. We had previously reported a universal ddPCR based method for accurate measurement of VCN in lentivirus-modified cells. This assay was further tested against the National Institute of Science and Technology (NIST) VCN genomic DNA standards isolated from Jurkat cells with known integration copies per cell.

In this study, we generated HT1080 expressing CD19 CAR as a reference standard for our VCN assay as well as a control for flow cytometry-based assessment of CAR expression. HT1080 cells were transduced with a CD19 CAR lentivirus and the bulk, modified cells were sorted into single cells for clonal isolation and expansion. Resulting clones were assessed for expression of the CD19 CAR and further molecular analysis performed to determine integrated vector copy number. Clones with VCN ranging from 1-4 were positively identified, aliquoted, and characterized for use as a reference standard. Research cell banks were assessed for stability of expression and vector copy number over long-term culture. Genomic DNA isolated from clones with known copy number was used as a reference for the VCN assay and cells were used as a positive control for flow-based assessment of CAR expression. These reference standards serve as valuable tools for assay qualification and validation while providing a relevant system for training and tech transferring methods across different teams.

Introduction

CAR-T cell manufacturing involves the genetic modification of isolated T cells and subsequent expression of chimeric antigen receptors (CARs). In viral modification methods, specific quantities of viral vector added per cell are defined through the multiplicity of infection (MOI). MOI is carefully addressed to achieve high transduction efficiency of the CAR transgene without generating high VCN. Due to the increased risk of oncogenesis, the Food and Drug Administration (FDA) recommends that the VCN remains less than five copies per genome. Thus, creating a highly controlled and qualified VCN assay is ideal for the assessment of virally manufactured CAR-T cells.



Materials and Methods

Lentiviral Transduction

HT1080 cells were transduced with internally generated lentivirus containing a CD-19 CAR construct (LV-CAR) at an MOI of 5. Cells were allowed to recover for 7-days post-transduction in DMEM Complete Medium +20% FBS before single-cell sorting.

Single Cell Cloning

Bulk transduced cells were sorted into single cells for isolation and expansion using the NX One (Nodexus). Cells were verified for clonality and monitored for expansion using the Incucyte™ (Sartorius).

Cell Banking

Clonal populations were expanded utilizing CellBIND Polystyrene CellSTACK™ (Corning, Catalog 3311) to create a cell bank consisting of 5E+06 cells/mL aliquots in DMEM Complete Medium + 10% DMSO. Cell viability and vector copy number of the cell bank for each clone was assessed.

Reference Standard Lot

To generate a VCN reference standard lot, genomic DNA was extracted from one vial of the cell bank using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Catalog K182002). 5 µg of gDNA was aliquoted per lot.

Analytics

For ddPCR assessment of VCN, gDNA was added to the appropriate supermix for duplex reactions. TaqMan gene expression assay mixes were custom ordered for the lenti-specific and housekeeping targets. Samples were assessed by the QX One (BioRad). For flow cytometry (Attune™ CytPix™, Thermo Fisher Scientific), harvested HT1080 cells were stained with antibody that targeted CAR+ surface antigens, PE-Cyanine7 Labeled Anti-V5 Tag Monoclonal Antibody TCM5 (25-6796-42, eBioscience™).

$$\frac{LV\ target\ concentration}{Housekeeping\ target\ concentration} x2 = VCN$$

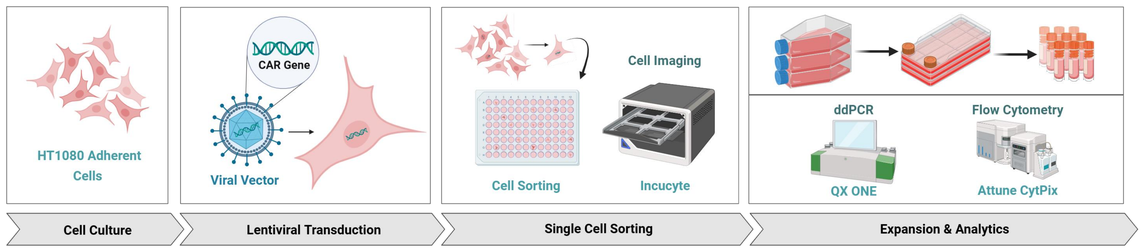


Figure 1: Graphical Abstract of VCN Reference Standard Generation. Establishment of VCN standards began with the transduction of HT1080 adherent cells with a lentivirus vector containing the CAR construct of interest. Cells with a range of viral integrations were generated and sorted into a 96-well plate utilizing the NX ONE instrument. The plate was immediately loaded into the Incucyte™ to assess clonality and monitor expansion. During expansion, analytics were performed to establish a profile for each clone, including VCN by ddPCR and a CAR expression panel by flow cytometry. A high cell density for adherent cell culture was achieved by utilizing CellSTACKs™. Cells were then frozen in 10% DMSO to establish the standard cell bank. Thawed cells were assessed for CAR expression by flow cytometry and VCN after gDNA extraction.

Results

Clonal Expansion

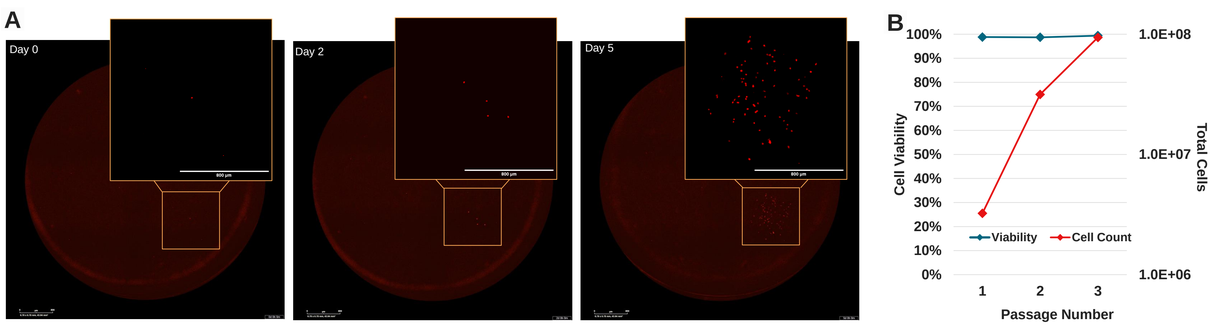


Figure 2. Monitoring Clonal Growth A) Representative images from a single well of a 96-well plate containing cells sorted by the NX ONE, scale bar, 800 µm. Cells were stained with Incucyte™ Nuclight Rapid Red Dye for Nuclear Labelling of Live Cells for easier visualization of clonal cells and imaged in the Incucyte™ (Days 0, 2, and 5 shown). Wells with clonal populations were monitored for growth and expanded to adherent vessels, as necessary. **B)** Transduced HT1080 cell viability and populations were measured bi-weekly during scale-up using the NucleoCounter™ NC-3000 and Solution 13 AO-DAPI Staining Reagent. Cells were thawed from vials created at the conclusion of the Incucyte™ monitoring phase. Cells were banked after the third passage. Population growth increased rapidly during expansion while maintaining high viability (>95%).

Establishing Clonal Standards for VCN & Flow Cytometry

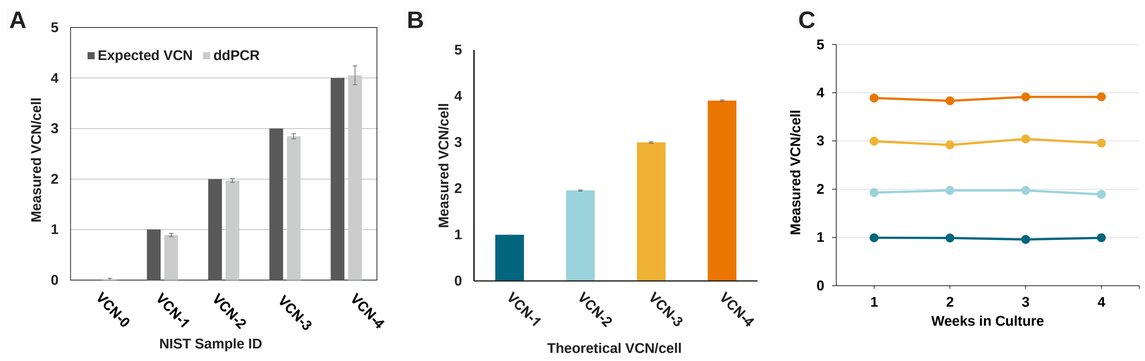


Figure 3. VCN Reference Standard Establishment by ddPCR. A) Universal ddPCR assay accuracy was assessed during a NIST interlab study involving blinded samples ranging from VCN 0 to 4 (He et al., 2025, preprint). B) The universal ddPCR assay was then used to assess HT1080 LV-CAR clones. One clone with a VCN of 1-4 was chosen to expand and generate a cell bank. C) During the stability study, each of the banked standards were kept in culture for at a minimum of four weeks to ensure the maintenance of the viral integration. Data represented as mean +/- standard error of n=3 assays. All runs passed ddPCR assay criteria including 1) <3 copies/µL in negative template control wells and 2) within 70-130% recovery of an established ddPCR positive plasmid control (Kandell et al., 2023).

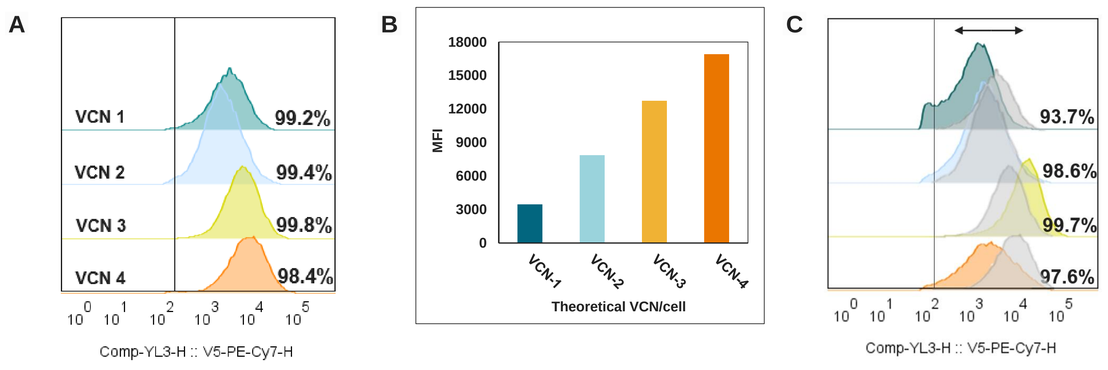


Figure 4. VCN Reference Standard Establishment by Flow Cytometry. A/B) Initial Assessment of Clonal VCN Standards. To ensure clonality and establish a baseline for CAR expression, HT1080 clones were assessed by flow cytometry. MFI: Mean Fluorescent Intensity. C) Assessment of Banked VCN Standards. After expansion and banking of cells, CAR populations were assessed and compared with initial screen (grey). Gates were set utilizing the following controls: fluorescence minus one (FMO), non-transduced HT1080 (VCN-0), and unstained cells.

Utilizing Reference Standards – Bridging Study

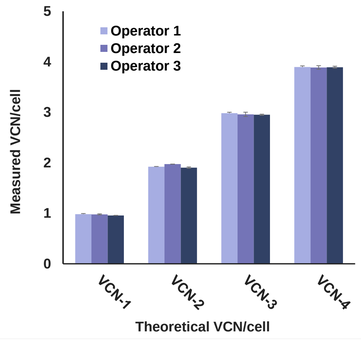


Figure 5. Precision of VCN Reference Standard Lot. From the clonal cell bank, gDNA was extracted, aliquoted, and stored as a reference standard lot. Three independent operators assessed the lot utilizing the universal VCN assay. Data represented as mean +/- standard error of n=3 assays. All runs passed ddPCR assay criteria including 1) <3 copies/µL in negative template control wells and 2) within 70-130% recovery of an established ddPCR positive plasmid control (Kandell et al., 2023).

Conclusion

In this study, clonal LV-CAR modified HT1080 cells were successfully generated to serve as reference standards for the VCN by ddPCR assay. During the screening process, clones were selected based on live cell monitoring (Figure 2), as well as MFI and CAR expression profiles during flow cytometry (Figure 3A/B).

Following the successful implementation of the VCN by ddPCR assay utilizing NIST standards (Figure 1A), LV-CAR HT1080 clones were assessed for VCN over multiple runs (Figure 3B), multiple weeks in culture (Figure 3C), and multiple operators (Figure 5). The stability study results indicate that the reference standards are both renewable as a cell bank as well as a stable control for the VCN assay (Figure 3C).

CAR integration frequency and location is random during viral modification. Thus, expression of the CAR complex may vary between clones and must be considered once selecting a cell-based standard (Figure 4C).

Taken together, the results from this study successfully establish reference standard generation for the universal VCN by ddPCR assay & CAR expression by flow cytometry.

Future Directions

Additional testing by next-generation sequencing (NGS) is ongoing and will be used to orthogonally confirm the vector integration copy number of banked clones. This data will further ensure the specificity of the reference standards.

Furthermore, all banked reference standards will be passaged and sampled for an additional four-week period to iterate confidence in cell bank renewability and VCN stability. Samples will be assessed by ddPCR, ensuring that the vector is stably expressed throughout multiple passages of the reference standards.

Currently, the universal VCN by ddPCR assay utilizes a plasmid positive control to ensure accuracy and linearity (Kandell et al., 2023). Moving forward, these reference standards will be integrated into the assay workflow, allowing for an additional control that is cell-based and similar to the sample material. The range of known vector integration copy numbers provided by these standards will enable future unknown samples to be quantified with greater confidence.

References

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[3] Paugh, B.S., Reference standards for accurate validation and optimization of assays that determine integrated lentiviral vector copy number in transduced cells. Scientific Reports Rep. 2021 Jan 11.

Acknowledgements

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