In-depth characterisation of a ddPCR-based platform method for vector copy number determination in lentivirally modified cells

Authors: Maria Michela Santamorena, Francesca Baiano, Arianna Spagliardi, Luca Benedan, Nicolò Sacchetti, Stefano Baila

Other resources

Introduction

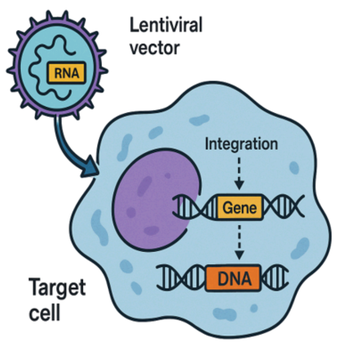
Accurate quantification of vector copy number (VCN) in genetically modified cells is a fundamental requirement in the development of lentiviral-based gene therapies. VCN serves as both a potency marker, indicative of transgene integration and expression potential, and a safety parameter, due to the direct correlation between high copy numbers and the risk of insertional mutagenesis. Regulatory expectations increasingly emphasize VCN analysis from the earliest stages of development to enable risk–benefit assessment and control of manufacturing consistency.

To address the need for a robust, cost-effective, and broadly applicable assay, we developed a platform-based approach for VCN determination. This strategy aims to provide a validated and transferable method applicable across different lentiviral vector constructs and compatible with a wide range of cellular substrates, including hematopoietic and non-hematopoietic cell types.

The assay was developed using droplet digital PCR (ddPCR), a technology that allows for absolute quantification without the need for standard curves and exhibits superior tolerance to PCR inhibitors compared to conventional qPCR. Method setup was performed using the WHO/NIBSC lentiviral reference material, and included an extensive troubleshooting and characterisation phase that enabled a deep understanding of assay performance parameters and limitations.

The Vector Copy Number

VCN refers to the number of vector genomes integrated into a target cell.
The assay typically measures the mean ratio between the copies of the transgene and the copies of an endogenous gene.



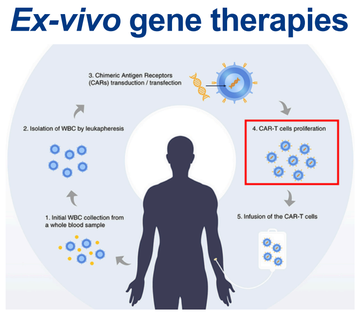
Efficacy:
More integrated copies can lead to increased and sustained expression of the therapeutic gene, which can enhance the product's effectiveness.

Safety:
High VCN increases the risk of insertional mutagenesis, which is the insertion of the viral DNA into or near an oncogene, potentially leading to cancer (oncogenesis). EMA and FDA recommend a justified VCN limit (e.g., less than five copies per genome for CAR T-cell products).

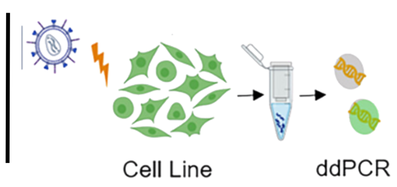
Applicability in both *ex-vivo* and *in-vivo* gene therapies

As VCN affects both potency and safety, it is a critical quality attribute in ex-vivo gene therapies, where it is mostly applied to characterize the cellular final product. A secondary application of the VCN test is in in-vivo gene therapies, where it is used as a readout for infectious titer determination of the viral vector.

Ex-vivo gene therapies



In-vivo gene therapies



What is a platform?

As VCN affects both potency and safety, it is a critical quality attribute in ex-vivo gene therapies, where it is mostly applied to characterize the cellular final product. A secondary application of the VCN test is in in-vivo gene therapies, where it is used as a readout for infectious titer determination of the viral vector.

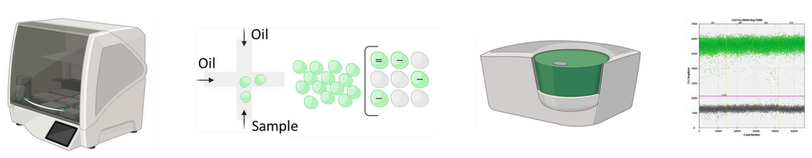
For ICHQ2 and Q14, a platform is an analytical procedure that is suitable to be applied to different products that share predefined similarities without significant change to its operational conditions.

The use of platforms can reduce overall development and validation effort, since it can be applied to multiple products, reducing time and material requirements

The platform approach allows to access qualified and validated analytical strategies since early clinical phases.

Eurofins platforms are available as plug-and-play methods that only require a minimal feasibility assessment.

Our ddPCR system



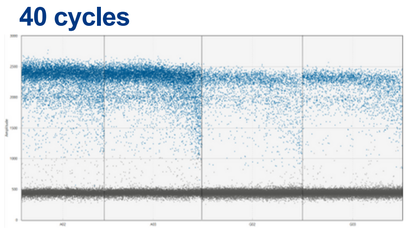
•DNA sample with target sequences is partitioned in up to 25,000 droplets where targets and background DNA are randomly distributed.
•Target sequences are amplified by end-point PCR in each droplet and positive droplets are counted to give precise quantification of target sequences in sample.
•Fully automated droplet generator eliminates user-to-user variability and generate consistent droplet counts across users and plates, enabling you to fully exploit the precision of your ddPCR System.
•QX600 is able to detect up to 6 colors through which it is possible to simultaneously quantify up to 12 targets.

qPCR Vs ddPCR

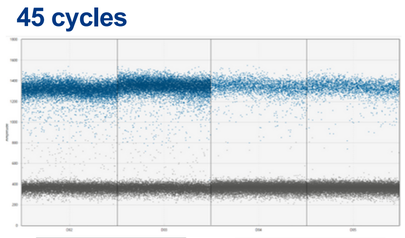
	qPCR	ddPCR
Principle	Measures fluorescence in real-time during amplification	Partitioning of the sample into approximately 20,000 droplets. PCR amplification occurs within each droplet.
Quantification Method	Relative or absolute quantification with a standard curve	Direct absolute quantification by counting positive droplets. Does not require a standard curve.
Sensitivity	High	Very high
PCR Efficiency	Influenced by the presence of inhibitors	Measurement is independent of PCR efficiency. Less susceptible to the presence of inhibitors.
Multiplexing	Yes, up to 2–4 targets (in red)	Yes, up to 12 targets (in red)
Dynamic Range	Wider	Narrower
Cost	Lower	Higher

Number of PCR cycles

40 cycles



45 cycles

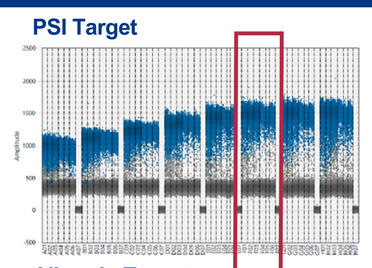


•To assess the impact of cycle number on signal clarity and quantification accuracy, reactions were performed using different amplification cycles.

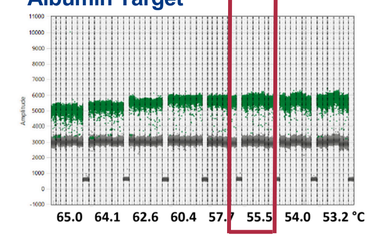
•The reaction performed with 45 cycles yielded a cleaner and more distinct fluorescence signal in ddPCR, improving droplet separation and quantification reliability compared to the lower-cycle reaction.

The Annealing Temperature

PSI Target



Albumin Target



•The PCR was set up considering two molecular targets: the PSI sequence, to detect the transgene, and albumin (ALB) as an endogenous reference gene.

•To optimise the PCR conditions, an annealing temperature range between 53°C and 65°C was tested for both targets.

•The PSI reaction was more sensitive to changes in annealing temperature compared to albumin.

The optimal annealing temperature was determined to be approximately 56°C

Singleplex Vs Multiplex

VCN 1

SINGLEPLEX	Min	Max
CV% PSI	0.58	1.51
CV% ALB	1.62	4.80
Recovery % PSI	103.23	105.42
Recovery % ALB	104.10	104.72
Recovery % VCN	98.71	101.30

MULTIPLEX	Min	Max
CV% PSI	3.90	4.13
CV% ALB	1.30	4.05
Recovery % PSI	94.55	97.66
Recovery % ALB	99.36	102.69
Recovery % VCN	92.05	98.31

•To verify the appropriateness of the multiplex approach, we analysed the same sample conditions in multiplex and in singleplex evaluating CV% and recovery % on both the targets.

•The experiment confirmed the robustness of the multiplex approach for both the DNA concentrations tested (25 and 50 ng/well) Results were confirmed also with a VCN of 8.76

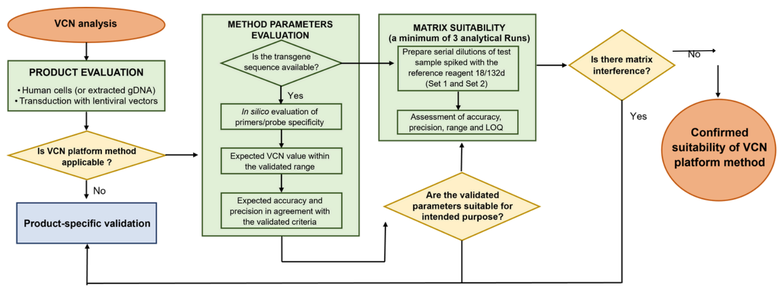
The Validation Parameters as per ICHQ2(R2)

SPECIFICITY	The specificity of a PCR assay is its ability to unequivocally recognise the target sequence and no other potentially related sequences. Is demonstrated in silico (e.g., BLAST) and using relevant background matrix controls.
ACCURACY	The accuracy describes the closeness of individual measures to the true value of the analyte and is determined with six independent runs conducted by two different operators.
REPEATIBILITY	Repeatability expresses the precision of the measurements taken under the same operating conditions over a short interval of time and it is assessed with a minimum of nine determinations.
INTERMEDIATE PRECISION	Intermediated precision refers to the intra-laboratory consistency across different days and between different operators. Is determined with six independent runs.
LINEARITY	The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. Tested in a minimum of five serial dilutions.
LOQ	The limit of quantitation (LOQ) of an analytical procedure is the lowest amount of sample analyte that can be quantitatively determined with suitable precision and accuracy.
REPORTABLE RANGE	The range of quantification of an analytical procedure is the interval between the lowest and the highest results in which the analytical procedure results linear, accurate and precise.
ROBUSTNESS	The robustness is a measure of the method capacity to meet the expected performance criteria despite deliberate variations of identified critical analytical procedure parameters.

Method Validation

Parameter	*Results*
Accuracy	70%≤ Recovery% PSI ≤130%
	70%≤ Recovery% ALB ≤130%
	70%≤ Recovery% VCN ≤130%
Repeatability	CV% PSI ≤ 30%
	CV% ALB ≤ 30%
	CV% VCN ≤ 30%
Intermediated precision	CV% PSI ≤ 30%
	CV% ALB ≤ 30%
	CV% VCN ≤ 30%
Linearity	R^2 PSI ≥ 0.98
	R^2 ALB ≥ 0.98
Reportable range	VCN 0.0 – 8.76
LOQ	46.7 copies/µL

Platform Application



Flowchart describing the analytical steps for the method parameter and matrix suitability evaluation.

Conclusions

•The method underwent a comprehensive validation in accordance with ICH Q2(R2) and new European Pharmacopoeia 3186 monograph. The following performance characteristics were assessed: repeatability, intermediate precision, intra- and inter-assay accuracy, linearity, and robustness under variable experimental conditions.

•The validation confirmed the method's suitability for VCN quantification in compliance with regulatory requirements for both preclinical and clinical phases.

•Despite its successful validation, the process reveled some limitations inherent to available reference standards. Current and future work will focus on investigating these aspects further to explore possible strategies for mitigation and refinement.

•This platform method represents a significant tool for a streamlined and GMP compliant VCN assessment in gene therapy development.

eurofins | Cell & Gene Therapy

Eurofins Biolab Srl
Via Bruno Buozzi, 2 Vimodrone | Italy

EurofinsBiolab@bpt.eurofinseu.com | www.eurofins.it/biopharma
consultancyitaly@bpt.eurofinseu.com | www.eurofins.it/consultancy-services