British Pharmacopoeia

Consultation

British Pharmacopoeia public consultation for guidance on the application of vector copy number quantification for the cell and gene therapy community. Part I: Adeno-associated virus. Part II: Lentivirus and Retrovirus.

Consultation period 22nd July 2021 to 27th September 2021

MHRA

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1. Patients, standards, and innovation

The quality of a medicine is critical to ensuring its safety and efficacy, and therefore the medicine's suitability for patients. Pharmacopoeial standards are part of an interlinked system, together with good practice guidelines and regulatory assessment, that form a foundation to ensuring medicines are of an acceptable quality. Additionally, standards have a place in supporting and enabling innovation through the availability of consistent and widely applicable quality requirements. Innovation in the field of medicines and healthcare has the potential to support patients throughout the world to live longer, healthier, and happier lives.

In recognition of the increasingly important role of biological medicines to healthcare worldwide, the Medicines and Healthcare products Regulatory Agency (MHRA) has developed and implemented a Strategy for pharmacopoeial public quality standards for biological medicines.[1] This strategy, adopted following consultation with stakeholders, laid out a vision of working collaboratively to explore and develop new standard setting approaches for biological medicines. It included a commitment to investigate and take forward standard setting opportunities for innovative Advanced Therapy Medicinal Products (ATMPs).

ATMPs have the potential to be transformative to patients and healthcare globally. However, development, characterisation, and production of these innovative medicines is challenging due to their high complexity and the still-emerging technologies that support them. Publications such as the Advanced Therapies Manufacturing Taskforce Action Plan,[2] the MMIP's Manufacturing Vision for UK Pharma[3] and stakeholder feedback have emphasised the important role that standards can have in the development of these medicines. This includes a focus on the value of widely applicable standards that could support knowledge building and facilitate analytics and characterisation.

This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore contribute to the quality assurance of innovative medicines for patients.

The MHRA and British Pharmacopoeia would like to recognise and thank the numerous experts in the British Pharmacopoeia's Working Party for ATMPs that have contributed to the development of this text. The work has been supported by a joint-staff secondment scheme between the BP and the UK's Cell and Gene Therapy Catapult.[4]

2. The draft document

As part of the MHRA strategy for the creation of pharmacopoeial public quality standards for biological medicines, the British Pharmacopoeia Working Party for ATMPs, established in

[1] https://www.gov.uk/government/consultations/strategy-for-pharmacopoeial-public-quality-standards-for-biological-medicines

[2] http://www.abpi.org.uk/publications/advanced-therapies-manufacturing-action-plan/

[3] https://www.abpi.org.uk/publications/manufacturing-vision-for-uk-pharma-future-proofing-the-uk-through-an-aligned-technology-and-innovation-road-map/

[4] https://ct.catapult.org.uk/

March 2020, has engaged with groups across the cell and gene therapy community to develop non-mandatory standardisation guidelines for key analytical technologies to ensure quality throughout the product lifecycle. The working party has developed two sets of guidance to support ATMP development across a wide range of organisations, laboratory settings, and therapy types. As such, the guidance is product-agnostic and does not provide a step-by-step protocol, nor constitute a prerequisite for product acceptance, but instead offers measures to ensure the production of robust, comparable, and reproducible data within and across organisations.

The first guideline focussed on the application of flow cytometry within the cell and gene therapy community. Flow cytometry is an analytical technique which uses a combination of lasers, fluidics, and detectors to characterise the properties of a population of cells or particles. The second guideline, which this consultation is focussed on, is a similar document related to the application of polymerase chain reaction techniques to vector copy number quantification which is likely to be split into two parts; one for non-integrating viruses such as adeno-associated viruses focussing on quantitation of encapsidated genetic sequences, and one for integrating viruses such as lentiviruses / retroviruses focussing on number of viral vector copies per host genome. The documents are intended to be useful for people operating within research and development, clinical trials, Good Manufacturing Practice (GMP) regulated environments, and academia.

Following external collaboration to identify requirements, both the flow cytometry and vector copy number guidelines include information related to method development, assay validation, reference materials, and controls used. Exemplar validation protocols and validation reports with anonymised real data, thought to be particularly useful to the end user are also included. The guidelines have been written by experts in flow cytometry and gene therapy for the cell and gene therapy community and have been reviewed by MHRA and NIBSC regulatory experts.

The draft vector copy number guideline is included as Annex 1 to this document.

3. How to contribute

The vector copy number guideline will be posted online for public consultation for a period of six weeks. During this time, we are asking stakeholders to complete and return the response document, available on our website, to BiolStandards@mhra.gov.uk.

When reviewing the guidance, you may want to consider the following points:

- Do you agree with the technical recommendations made in the document?
- Are there any aspects which you think are missing from the document?
- Is there any terminology within the document that you think needs to be more clearly defined?
- Is the document understandable and are recommendations clear and unambiguous?
- Could the format / style of the guidance be improved?

In addition to the request for technical comments, the response form includes more general questions around the value of the guidelines and other work within the area of ATMPs where standards and standardisation could add value. This information will be used to help the BP to understand and prioritise future work related to ATMPs.

4. Confidentiality and Freedom of Information

Information we receive, including personal information, may be published or disclosed in accordance with the access to information regimes (primarily the Freedom of Information Act 2000 (FOIA), the Data Protection Act 1998 (DPA) and the Environmental Information Regulations 2004).

Please let us know if you would like any information you provide to be treated in confidence, and please indicate any commercial sensitivities. We will maintain that confidence and resist disclosure under the access to information regimes where possible and in compliance with our legal obligations. We will also consult you and seek your views before any information you provided is disclosed.

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Annex 1. Guidance on the application of vector copy number quantification for the cell and gene therapy community. Part I: Adeno-associated virus. Part II: Lentivirus and Retrovirus.

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0. Abbreviations

ATMP	Advanced Therapy Medicinal Products
AAV	Adeno-Associated Virus
BMR	Batch Manufacturing Record
BP	British Pharmacopeia
CAT	Committee for Advanced Therapies
cDNA	Complementary DNA
CGT	Cell and Gene Therapy
CMO	Contract Manufacturing Organisation
CNV	Copy Number Variations
CQA	Critical Quality Attribute
CRO	Contract Research Organisation
ct	Cycle threshold
CV	Coefficient of Variance
dPCR	Digital Polymerase Chain Reaction
DP	Drug Product
EMA	European Medicines Agency
EU	European Union
FDA	Food and Drug Administration
gDNA	Genomic DNA
GTMP	Gene Therapy Medicinal Product
GMP	Good Manufacturing Practice
ITR	Inverted Terminal Repeat
LTR	Long Terminal Repeat
LV	Lentivirus
MOI	Multiplicity of Infection
NHEJ	Non-Homologous End Joining
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
Ph. Eur	European Pharmacopeia
RV	Retrovirus
SOP(s)	Standard Operating Procedure(s)
UK	United Kingdom
US	United States of America
USP	United States Pharmacopeia

VCN	Vector Copy Number
QC	Quality Control

Part I AAV-VCN

1. Scope

For the purposes of this part of the guidance, the terminology vector copy number (VCN) refers to in vitro polymerase chain reaction (PCR)-based quantitation of encapsidated genetic sequences in recombinant adeno-associated virus (AAV) vector particles. AAVs are small, non-integrating, non-enveloped ssDNA viruses that belong to the Parvoviridae family. The units applied for VCN will be vector genomes per millilitre (vg/mL).

Vector copy number is distinct from AAV infectious titre, transducing titre, total particle titre or replication-competent AAV. These attributes are not covered within this chapter.

It is advisable to implement and optimise the most appropriate method early in development to allow for accurate and precise determination of VCN. This chapter provides guidance on how to develop and optimise a robust and acceptable PCR-based assay in different sample types for the determination of VCN. Details of how to control for assay performance and validate the assay are also described.

Wherever possible method changes should be avoided or minimised during clinical development to prevent the challenges that can occur from changing an assay and generating a different titre with a new assay format. However, it is recognised that there may be a need to change to a new method or new testing facility so this chapter includes a section that details how to manage assay changes.

The guidance within this framework addresses the use of VCN analysis in the context of AAV-based and lentiviral (LV) or retroviral (RV)-based therapies and will provide current best practice. The adoption of this guidance is envisioned to help promote standardisation of the technique within the cell and gene therapy community. Several aspects of the framework are applicable to all users and should be given due consideration irrespective of the context in which the assay is being developed; their purpose is primarily to facilitate reproducible, high-quality data generation. In the context where the assay is intended for use as part of a human medicine's development program, then it is important to recognise that it will need to conform to pertinent regulatory guidelines.

2. Existing guidelines

The purpose of this section is to provide an overview of existing guidelines relating to VCN, PCR, ATMPs or other topics relevant to this chapter. This section is not intended to be an exhaustive/comprehensive list of all guidance and ATMP developers should familiarise themselves with regulatory guidelines from the relevant competent authority. Guidelines can be divided into those associated with defining quality attributes for specific product types and starting materials (Table 1) or those associated with ensuring that products (and assays) are consistently produced and controlled according to the appropriate quality standards (Table 2).

Table 1: Guidance documents defining expectations relating to product characterisation for ATMPs.

**Guidance in draft.*

Organisation	Reference	Title
EMA	EMA/CAT/80183/2014	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products
EMA	EMA/CAT/852602/2018*	Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials*

Ph. Eur.	2.6.21 Appendix XIV L	Nucleic Acid Amplification Techniques
FDA	-	Guidance for Industry: Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)
USP	Chapter <1046>	Cellular and Tissue-Based Products guidelines.
USP	Chapter <1047>	Gene Therapy Products
EMA	EMEA/CHMP/GTWP/587488	Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors

Table 2. Guidance document defining standardised procedures to ensure product quality standards.

Agency	**Reference**	**Title**
EMA	EudraLex Vol 4.	Good Manufacturing Practice specific to ATMP
ICH	Q2(R1)	Validation of analytical procedures: text and methodology
ICH	Q6B	Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
ISO	ISO 20395:2019	Requirements for evaluating the performance of quantification methods for nucleic acid target sequences: qPCR and dPCR
MIQE	Bustin SA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-622. doi:10.1373/clinchem.2008.112797	The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments
MIQE	Huggett JF, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin Chem. 2013;59(6):892-902. doi:10.1373/clinchem.2013.206375	The Digital MIQE Guidelines: Minimum information for publication of quantitative digital PCR experiments
FDA		Analytical Procedures and Methods Validation for Drugs and Biologics
IUPAC		Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report)

**Guidance in draft*

In the context of ATMP development, VCN quantification is used as an analytical tool for various stages of product characterisation such as pre-clinical development, clinical development, process characterisation / performance, and product release and stability. As such, VCN quantification should be conducted in accordance with GMP requirements as appropriate.

3. Methods for determination of VCN

Various methods can be used to assess the vector copy number with the most common being molecular determination based on polymerase chain reaction (PCR) – a quantitative method that amplifies a specific target within a genetic sequence. PCR methodology includes quantitative (qPCR) and digital (dPCR) with various similarities and differences existing between these two methods.

Determination of VCN for AAV is often used to define the target dose administered during toxicity, dose ranging studies and clinical trials and may also be used to determine input into other key assays to assess product quality such as infectious titre and potency. Therefore, when used for these purposes it is paramount that the method used to determine VCN is selected, developed, and optimised to provide both accurate and precise data. The information provided in this chapter is intended to give a detailed understanding of best practise for both methods. This guidance does not recommend one method over another and the user should determine the method best suited for their own requirements and capabilities.

3.1 Quantitative PCR

In the context of this chapter qPCR is a real-time quantitation of the amplified target region (amplicon) using fluorescence detection and a plasmid standard, although other standards may be used. Forward and reverse primers together with a fluorescent-tagged probe are designed to bind specifically to the target sequence. The test sample containing the target region is mixed with the primers and probe in a reaction mix that also contains heat-stable polymerase enzyme and deoxynucleoside triphosphates (dNTPs) in a suitable buffer. This mix then undergoes multiple rounds of thermal cycling which increases the amount of target sequence exponentially. During amplification fluorescence reporter dye is released which correlates with the amount of target sequence in the sample. Reactions are run in designated qPCR instruments and the collected data is analyzed by appropriate software. The quantity of target sequence in a sample is extrapolated from a fluorescent signal generated from a plasmid reference/standard curve. This provides a relative quantification of target sequence in the test sample.

qPCR is sensitive, quantitative, can be relatively cheap, and capable of achieving high throughput. Compared to dPCR, qPCR has an increased dynamic range and is simple to perform. However, the accuracy and precision of data obtained by qPCR depends on the selected standard, amplification efficiency, and performance of the standard curve. It is important to ensure these parameters are well characterised and optimised.

3.2 Digital PCR

dPCR is a newer technique that also utilises specific primers and a fluorescent probe generated against the target sequence in a similar reaction mix to qPCR. However, with dPCR the reaction mix is divided into thousands of individual partitions of equal size. The reactions are then amplified to an endpoint allowing the number of positive (fluorescent) and negative (non-fluorescent) partitions to be determined, providing a binary, digital readout. Statistical analysis is then used to quantitate the concentration of target sequence in the test sample. dPCR does not rely on a standard curve or multiple rounds of thermal cycling and is less affected by amplification efficiency therefore generating an absolute quantification of target concentration.

Both qPCR and dPCR can also be performed with DNA intercalating dyes rather than a probe, although the latter adds specificity. Either approach is acceptable.

Table 3 summarises the key differences between qPCR and dPCR.

Table 3: The key differences between qPCR and dPCR.

	qPCR	**dPCR**
Standard curve	Standard curve required	No standard curve required
Quantification	Relative	Absolute
Accuracy and precision	Significant optimisation required to ensure good accuracy and precision	Minimal optimisation required to ensure good accuracy and precision
Data collection	Real time	End point
Dynamic range	High	Reduced
Complexity and cost	Simple and relatively cheap	Complex method that can be more costly
Data generation capacity	High throughout	Low throughout
Sample matrix	May be affected by complex sample matrices	Less affected by complex sample matrices

For PCR based methods primers and probes may be directed against any part of the encapsidated sequence, whether this be the transgene, promoter, or other accessory sequences, although some regions such as close proximity to or within ITRs can be more complex in terms of primer / probe design due to their structure and high GC content.

Consideration needs to be given to non-encapsidated nucleic acid contaminants from the manufacturing process within the test sample that could contain complimentary sequence(s) to the target amplicon. These would be amplified by the PCR reaction if not first removed. Methods for determination of VCN must therefore assess steps for removal of any contaminating nucleic acid.

AAV viral vectors encapsidate the target nucleic acid within an outer viral protein capsid so an additional proteinase treatment may be required prior to performing the PCR step to release the encapsidated nucleic acid. For some AAV products the thermal cycling within the PCR step may be sufficient to break open the viral capsid and release the target DNA but this will depend on the product and sample as different AAV serotypes have different melting temperatures.

Requirement and control of these enzymatic steps are discussed within the chapter (See Section 4.5).

4. qPCR method development, sample preparation, and general considerations

4.1 Plasmid control

A plasmid control is often used to generate the standard curve for qPCR, although use of other, suitable controls is also acceptable. Variability associated with qPCR can often be attributed to the plasmid control. Therefore, the generation, handling, optimisation, and qualification of plasmid stocks need to be carefully controlled.

Where possible it is advisable to assign a single, high quality batch of plasmid as the assay control. This should be appropriately qualified for its purity, identity, integrity, quantity, and stability prior to release for use in the assay. A plasmid control does not need to be manufactured to GMP standards but manufacture according to a well-defined and controlled process will help ensure plasmid consistency and purity, whilst minimising lot to lot variability.

The longevity of an assigned plasmid stock should be optimised as introducing a new stock could create variation that may impact on assay performance. Qualification parameters should be established to allow for bridging between existing and new stocks.

Working stocks of plasmid may be generated from a master stock. Qualification of new working stocks of plasmid derived from the same master stock should be assessed by running multiple assays using both plasmid stocks in the same plate to compare data generated.

It is advisable to create and qualify single use aliquots of plasmid to avoid repetitive freeze-thaws and additional dilutions prior to use in each assay and to conserve material. Dilution of plasmid should be performed using a suitable diluent. The stability of all plasmid stocks, in particular the stability of low concentration or low volume stocks should be monitored. Plasmid instability will affect the assay performance and data generated. If stabilisers are used to preserve plasmid stocks, impact of the stabilisers on assay performance should be evaluated. If it is not possible to generate stable, single use aliquots of plasmid, the impact of freeze-thaw on larger aliquots should be assessed and maximum number of thawing should be established, specified, and documented.

Published data indicated that use of plasmid in its native, supercoiled form may overestimate VCN. Therefore, it is recommended to linearise a plasmid stock prior to use. This can be performed by a single suitable restriction site digestion.

4.2 Primers and probe

The principle components for qPCR are the primers, probe, and the reaction (master) mix.

Information on the nucleic acid sequence and other relevant construct information is required for the design of primers (and probe, where applicable).

When designing a pair of PCR primers, the amplicon size is important and can influence the slope of the standard curve. Larger amplicons will amplify less efficiently than smaller amplicons. Primers and probe should have specificity to the target region only. Consideration should be given to the synthesis, purification, and quality control of primers and probe. Manufacturers of primers and probes undertake relevant QC testing, but consideration should be given to any additional qualification of primers, including lot to lot variability. Primer-dimers should also be avoided.

When designing the primers and probes the choice of amplification region should be considered. If the assay is required to be product specific, it is advisable that the primers and probe are located within the transgene region or bridge between the transgene and associated regulatory elements such as the promoter to help assure specificity of VCN to vector product. A platform design to quantitate VCN for a range of products would require the primers and probes to be located in a region common to all constructs such as within a common promoter.

It is good practice to design multiple sets of primers/probe and test these (in combination with various suitable master mixes) to identify those that provide good and consistent amplification efficiency (95-105%).

4.3 Master mix

The master mix quality and components can impact on the qPCR performance and data generated. It is recommended to evaluate multiple types of master mixes for individual primer-probe sets during assay development to identify the best suited mix that demonstrates consistent amplification profiles for standards and test materials. Assessment of lot to lot variations and qualification of the master mixes and components should be considered. If a new master mix is introduced this should be appropriately qualified to ensure its suitability for the intended use.

For non-probe based PCR reactions using DNA intercalating dyes, melt curve analysis of master mixes is required to optimize PCR amplification of the target and to reduce background signals from non-amplicon molecules. Assay validity criteria are established for non-target signals.

4.4 Plasticware

Viral vectors are complex biological products comprising therapeutic nucleic acids within a viral protein capsid, both of which may bind to the surfaces of plastic consumables, e.g. tips, tubes, and plates. The use of DNA and protein resistant-binding plastic consumables should be considered and introduced early in assay development to reduce inter and intra-assay variabilities. Although some product formulations may contain excipients that can prevent products from binding to plastic, an assessment of each vector product and sample type should be performed on the chosen plasticware to ensure that the VCN quantitation is not compromised by the loss of samples due to plastic adsorption.

The choice of pipettes and the priming of tips during a set of qPCR reactions might also influence variability within the assay, so establishing methods to standardise these operations is advised.

4.5 Sample suitability and preparation

Evaluation of VCN is likely to be required at different stages of clinical development and throughout the manufacturing process using a range of sample types. For example, samples taken from early stages of the manufacturing process to assess process capability and performance are likely to contain a higher concentration of impurities such host cell DNA and host cell protein compared to final purified product. While the final product is expected to contain lower levels of impurities, excipients may be added which could interfere with PCR analysis. In addition, the expected concentration of VCN will vary depending on sample type. Therefore, when developing a VCN quantitation assay, it is important to consider the nature and purity of the sample(s) to be tested and the expected VCN concentration to ensure suitability and sensitivity of the method developed for each sample type or sample matrix.

Sample dilution and/or extraction may be required prior to analysis to remove interfering impurities. Any manipulation of the sample prior to the PCR step must be assessed for impact on the data generated. Crude samples may require spin down of cell debris and/or 0.45/0.2 um filtration. Buffer neutralisation or dialysis may be required for samples from in-process purification steps.

Additional sample preparation steps involving enzymes such as the addition of nuclease to remove contaminating nucleic acids from the manufacturing process and / or proteinase treatment to break open the capsids need to be considered. The inclusion of these steps will add to the assay variability. They must be well controlled if required, and include optimisation of enzyme concentration, suitable controls to assess enzymatic performance, and steps to ensure complete inactivation of the enzyme(s) once the reaction is complete.

However, enzymatic steps may not always be required. The impact of such treatment steps on each sample type should be assessed and compared with samples that have not undergone these treatments so that any potential changes in final titres determined can be evaluated and data used to determine and justify the requirement for such steps.

Additional points to consider for handling of samples during PCR preparation to help ensure consistency and minimize variation are listed below:

- Sample dilution steps including volumes and diluent for the preparation of samples and consistency with steps for standard curve preparation.
- Order of plating material onto reaction plates
- Steps to minimise manipulations or ensure ease of plating and time taken to plate material.
- Steps to control and minimise evaporation from the plate, which could potentially impact the reaction and evaluation of results.
- Standardise mixing steps during preparation of samples and controls and plating of material to the reaction mix.
- Sample storage and the impact of freeze-thaw cycles should be evaluated and controlled.
- Assay control preparation should be concurrent with samples preparation.
- Flexibility with regards to sample dilutions in cases where titres of samples are not as expected i.e. additional dilutions for more concentrated material.

4.6 Replicates and sample dilution

Careful consideration should be given to the number of replicates in the assay and requirement to assess different sample dilutions. It is often standard procedure to include two or more sample dilutions with each dilution tested in triplicate but for determination of VCN which requires very stringent assay accuracy and precision criteria to ensure confidence in the reported titre a greater number of replicates and sample dilutions may be required. It might also be required to perform the assay on more than one occasion using separate containers of test material (true replicates) and report the mean from all test occasions. True replicates should be used rather than pseudo / technical replicates (where replicates are generated from a single sample / dilution) where possible.

When assessing different dilutions and replicates, intra and inter-assay acceptance criteria should be established to ensure consistency in performance. This can include defining set %CV that should be attained between replicates and for separate dilutions. It is worthwhile considering the application of statistical analysis such as variance component analysis to the assay design to help define the optimal assay set up and to achieve the required accuracy and precision.

4.7 Preparation of dilutions, solutions, and mixing

Liquid handling, correct pipetting technique, preparation of dilutions, solutions and mixing can be a significant source of variation contributing to inconsistent and inaccurate assay performance, so it is critical that all these steps are carefully controlled.

The development of the assay requires assessment of appropriate dilution volumes Preparation of sample or control dilutions should clearly be documented to include the exact volumes to be used and order of manipulations for each dilution series. Specifying pipette size and type to be used for individual manipulations may also be helpful. Introducing a minimum volume to be pipetted will help prevent the introduction of variability and errors that can result from working with very low volumes.

Mixing of material generally includes pipetting or using a vortex mixer and should avoid generation of bubbles or foaming. Techniques should be applied that allow a good level of mixing without risk of shearing the DNA. Mixing of samples at all steps within the assay should be assessed in development and the requirements for mixing clearly defined in the final assay

set up (such as pipetting verses vortex mixing, number of repetitions if pipetting or time if vortex mixing).

When transferring samples, the order of sample transfer to plates should be consistently defined. Use of a plate map and other visual effects could be included in the assay protocol. The type of pipettes, their use and control (including routine calibration) and the priming of tips should be established during assay development so that these are standardised as early as possible.

4.8 Assay controls

Inclusion of appropriate controls and acceptance criteria around these controls can be an effective and often essential way to provide assurance over assay performance. Several assay controls should be included to ensure assay performance and assurance of data generated. See Table 4.

If samples undergo any enzyme treatment digest controls will be required. Other types of controls include no-template controls, assay positive control and a trending control. It might be necessary to assess other types of controls or materials, such as early manufacturing material, so that assay performance can be assessed using controls best suited to sample type.

Table 4. Commonly used assay controls used and their descriptions.

Control*	**Description**
Negative (no-template) control	Assay control containing all assay reagents and prepared alongside test samples but without inclusion of target (template) DNA for amplification to ensure absence of contaminants.
Sample preparation control	Control materials which have partially or not undergone any sample pre-treatment (e.g. nuclease and/or proteinase digestion) to monitor the performance of the pre-treatment steps.
Positive control	Assay control containing a defined amount of target DNA copies which will give rise to a pre-determined signal within the assay range when the assay is performed successfully.
Trending / performance control	Control that allows routine assay performance and trending to be monitored. Any material that gives a positive result within the range of the assay can be used.
Reference standard	Product specific material which has been qualified and is representative of the samples being tested. This is generally only utilized in relative assays where the result is reported relative to the control.

**The same material may serve as more than one of these controls (e.g. positive, trending, reference control may be the same material)*

4.9 System suitability and validity criteria

The use of appropriate system suitability and / or assay validity criteria are important elements in the control of an assay to provide assurance over the data generated. Some examples are included but individuals should consider what controls and criteria are most suitable for the intended assay use.

Assay validity criteria for the standard curve (covering ≥ 5 dilution points) is recommended to be more stringent compared to standard PCR where there is a requirement for increased accuracy and precision. For example, amplification efficiencies of 90-110% and slope R^2 >0.98 is standard for qPCR, but tightening this criterion is recommended if within the capability of the assay.

Introducing controls associated with assay replicates and dilutions as discussed above will provide additional assurance over assay performance. Criteria for trending controls can be established over qPCR development and verified or adapted at later stages.

Negative controls should be negative and positive controls positive within the defined range. Digest control acceptance criteria should be set to ensure optimal enzymatic activity.

4.10 Operational instructions, documentation, and testing

At all stages of development, clear written instructions or standard operating procedures should be prepared to detail exactly how testing should be performed and appropriate laboratory records should be used to capture all relevant information during execution of the test methods and data analysis. This is a regulatory requirement for testing of any material for clinical studies but is also good practice to implement a similar approach prior to clinical development. This can be written in the form of general operating instructions, laboratory manuals or SOPs and should provide sufficient detail such that another operator unfamiliar with the method can perform the testing and obtain equivalent data with minimal assistance.

Operational instructions should consider including the following:

- Brief description of the principle and purpose of the method / technology.
- Training / competence requirements for those performing the testing.
- Reagents and consumables to be used – specifying manufacturers and catalogue numbers where possible and storage conditions.
- Equipment to be used and calibration required.
- Test samples and controls.
- Step by step guide on how to perform each individual step of the procedure including details of how to perform calculations, dilutions, sample mixing, order of pipetting samples and controls, plate layout and operation of equipment. Images and flow diagrams can help clarify complex assays or steps.
- How to perform data analysis.

Prior to performing the procedures, operators should be suitably trained and have a good understanding of the method. During testing all key steps should be documented in laboratory test records or notebooks and consideration given to the following:

- Check box to confirm completion of individual steps, including date and time of execution.
- Details of all calculations.
- Details of reagents and materials used, including lot number, expiry, storage, and integrity.
- Details of incubation times, temperatures.
- Details of equipment used, including lot number and calibration status where applicable.
- Details of any deviations from the documented procedure and / or unusual or unexpected observations.
- Accurate record of all data generated and data analysis.

4.11 Operator training

Prior to performing any analytical testing operators should ensure they have been fully trained in the procedure. Training can include reading the procedure, observing the procedure being performed by others, performing the procedure under supervision, performing the procedure

unsupervised and suitable proof of understanding and competency. All stages of training should be documented including competency testing requirements. Re-training or refresher training should also be defined at suitable intervals to ensure operators remain fully competent, particularly if the method has not been performed for some time or is complex.

4.12 Control of critical reagents

Critical reagents are those, such as primers / probes, plasmid / positive controls, that may impact on assay performance if changed and should be defined for each individual assay. Critical reagents should be well characterised and controlled. This may include determination of stability over time at defined storage conditions and defined qualification parameters, where new lots or stocks of reagent are characterised side by side against an existing stock and accepted for use based on pre-defined acceptance criteria.

Short term assessment of reagent or reaction stability under normal preparation timeframes and steps can be helpful in establishing early assay preparation criteria and help form plans for robustness assessment later in assay development phases i.e. during validation activities. Use of passive reference dyes could be considered for normalisation of signals/baseline. Additional suggestions for control of some of these reagents have been provided in the sections above.

4.13 Instrument operation and control

Prior to use, the suitability of the instrumentation used in the execution of the test method needs to be confirmed. Typically, this is performed through a set of qualification steps starting with a qualification following the installation (IQ) followed by qualification of operation (OQ) and performance (PQ). Qualification steps are usually informed by the q/dPCR equipment vendor and the level of qualification should be commensurate with the stage of development.

Instruments can also be assessed to confirm if there are potential heat block deficiencies such as hot spots, which need to be avoided. Plans for regular assessment should be implemented.

Following equipment qualification, a program for maintenance should be established which ensures that the qPCR/dPCR instrument is routinely serviced. Routine calibration and maintenance programs should also be in place for other equipment used in the assay, such as but not limited to pipettes, heat blocks, and centrifuges.

Control of q/dPCR equipment and data analysis in a GMP environment will require the use of validated computer systems. The implementation, validation and use of such systems is beyond the scope of this guideline and appropriate guidance should be consulted to ensure a compliant system used in the execution of GMP testing. Examples of points to consider here would be audit trails, user access controls, e-signatures, and use of archive functionality.

4.14 Data interpretation, analysis, and reporting

There is a requirement that the initial data be captured in an unalterable format and that a chain of custody can be demonstrated for subsequent conversion for use in other analysis packages. This will allow for files to be acquired in the native format of the equipment, but also can allow conversion for specific analysis. The MHRA data integrity guidance document or other guidance specific to the relevant competent authority should be adhered to.

This section describes the steps to evaluate qPCR assay validity and data interpretation to determine the test results of unknown samples.

Upon completion of a qPCR assay the raw data will be initially generated by the software. The software may identify potential errors that need to be reviewed by the operator. The data is further analysed by application of a threshold value, either through automatic calculation or manual entry depending on the chosen approach for the test method.

Run data should be analysed to confirm the qPCR run is valid and all pre-determined acceptance criteria are met as defined during the method development and

validation. Depending on the nature of an established method, checks for assay validity may vary but the following checks are generally performed:

- Verify the linearity of the standard curve through calculation of the slope and correlation.
- Assay controls such as digest controls and sample negative / positive controls pass the defined acceptance criteria.
- Confirm plasmid DNA replicates and positive control replicates do not exceed the defined variability.

Once a qPCR run is considered valid the analysis of test samples is performed. In this step, unknown test samples are quantified through referencing the Ct values of unknown test samples against the Ct values of the standard curve.

Perform the calculation of unknown test sample according to the test method.

- Only include replicates and dilutions of a test sample for which the Ct value falls within the range of the standard curve and that meet any additional pre-defined acceptance criteria for replicates and / or dilutions.
- Consider dilution factor from sample dilutions, pre-treatments, and the mastermix generation, as applicable.
- If multiple replicates and dilutions are performed calculate the final test sample result per test method.
- Note that for single-stranded targets which are quantified against a plasmid DNA standard curve, such as AAV vectors, the result is multiplied by two as the first cycle in the PCR includes second-strand only for the test sample.
- Consider how to handle outliers by statistical tests or application of more general rules e.g. eliminate replicates >1 Ct from other replicates.

In case a PCR assay is performed using intercalating dye, it is required to confirm the melting temperature of the template and the presence of a single melt peak for each well containing plasmid DNA, positive control, or unknown test sample.

4.15 Minimising variation

There are many components of a PCR assay where variation can be introduced so it is important to understand and control these as much as possible early in development to allow for the generation of robust VCN method that ensures optimum accuracy and precision. Many examples of controlling variation have been discussed in the sections above, but in summary these include: optimised assay development, clear and consistent operating instructions, well trained operators, careful control over liquid handling, correct sample handling, proper control and qualification of critical reagents, inclusion of suitable controls and acceptance criteria, and correct operation and maintenance of equipment. Variance component analysis, careful design of experiments and other statistical tools may help identify the main sources of variation and define an optimal assay set up to achieve the required accuracy and precision.

5 Assay validation (qPCR)

As development progresses the regulatory requirements for assay validation will change. However, as described within this chapter determination of VCN is a critical assay for AAV toxicity studies, dose determination, and clinical studies. VCN is also used as the starting point for other key assays to assess product quality attributes. It is therefore recommended to optimise, qualify, and validate this assay as early in development as possible.

In terms of assay validation, the regulatory expectation for licensing is conformance to ICH Q2(R1), this document should be consulted prior to commencing any validation activities. Assay validation should only be initiated once the method has been fully developed and

demonstrated to be fit for purpose. Suitable acceptance/validity criteria based on prior assay performance will need to be defined prior to validation. Failure to meet these criteria will result in failure of the validation and will potentially require additional assay re-development. Any changes to the method post validation could require additional validation.

Note: it is advisable, if in doubt, to contact the regulatory authorities to ensure the proposals for assay validation are in conformance with regulatory requirements

5.1 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its' reliability during normal usage. Variable conditions might include different equipment, operators, and reagent lots. Robustness provides an estimate of experimental reproducibility with unavoidable error. Determination of assay robustness can initially be assessed from PCR primer and probe optimisation data generated during assay development. The Ct values observed at the different combinations of PCR primer and probe concentrations will be used to determine the significance of deliberate changes to essential reaction components on assay performance and reliability.

5.2 Specificity

The specificity of an assay is the capability of the assay to differentiate similar analytes or interference from matrix elements that could have a positive or negative effect on the assay. Specificity should be assessed using nucleic acids specific to and unrelated to the viral target(s) to demonstrate that positive signals arise only from the positive control or vector nucleic acid and do not arise from other non-specific nucleic acids. For example, genomic DNA from the host cell line used for production and other nucleic acid that may be present in the PCR materials, and reagents such a *E.coli* nucleic acids that may be present in residual levels in recombinant derived *Taq* DNA polymerases. In addition, reagents, materials, and internal controls used in the nucleic acid extraction systems being employed should be tested in the specificity assessment, if applicable.

5.3 Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample within a given range. For the establishment of linearity, a minimum of five concentrations is recommended. For example, the linearity and accuracy of a VCN assay could be determined from eight replicated reactions of eight ten-fold serial concentrations of positive control standard curve nucleic acid (10^8 to 10^1 copies per reaction). At least two operators using different batches of standard curve preparations, different equipment, different days can provide intermediate precision data on the test method.

The co-efficient of determination R^2 associated with the standard curve must be greater than or equal to 0.98. The R^2 (the square of the correlation co-efficient, r), y-intercept and slope will be reported for each generated standard curve. The qPCR efficiency should fall within pre-established limits determined in the validation. Generally, PCR efficiency can be determined from the slope of the standard curve constructed from serial dilutions of the target DNA. Typically, an amplification efficiency range of 90-110% (slope of -3.1 to -3.6) is considered desirable. Various factors can influence the slope of a standard curve, these include: the amplicon size (larger amplicons will amplify less efficiently than smaller amplicons), secondary structure, pipetting, and standard curve preparation.

5.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the accepted reference value and the found value. Accuracy can be assessed across the linear range of the standard curve, for example using the eight replicated ten-fold series standard curve reactions of known quantities vector nucleic acid (See Section 5.3). The mean value +/-

SD of four of these replicates will be analysed as unknowns. Quantitation of the unknown replicates will be achieved using the remaining four replicates as standards on the same qPCR plate, and the found values used to assess the accuracy. Accuracy can be then calculated by analysis of the unknown across the range.

5.5 Quantitative range

The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample, for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Using the same data set that is used to determine linearity, and accuracy, the quantitative range of the test can be determined with the data obtained from the unknown concentrations showing quantitation within ±2-fold of standard value.

5.6 Precision

5.6.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability can also be termed intra-assay precision. Repeatability should be assessed using a minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations / 3 replicates each). Repeatability can initially be determined using a minimum of six concentrations analysed using eight replicates. To further assess repeatability, 24 replicate reactions of vector nucleic acid at known copies, above and below the previously determined DL can be analysed (72 determinations).

5.6.2 Intermediate precision

Intermediate precision is expressed as the precision within laboratory variations determined over different days, different analysts, different equipment etc. Intermediate precision of mean, standard deviation and %CV can be reported for the determined range, QL and repeatability obtained from a minimum of two operators over different days. For a well-developed, optimised qPCR assay it should be possible to achieve intermediate precision of <15%.

5.6.3 Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardisation of methodology). Reproducibility can be assessed in the instance of a collaborative or assay transfer study between sites and/or laboratories.

An example of how validation data may be reported is presented in Annex 1. It is important to note that the values provided in these tables do not assume any required regulatory expectation and are for information only.

6. dPCR considerations

The general principles for method development as described in section 4 are also applicable to dPCR assays. The evaluation of a standard curve does not apply for dPCR assays. Instead parameters around the reaction chambers need to be evaluated. Independent of the chosen dPCR technology a minimum number of reactions is required to infer the unknown test sample concentration through underlying statistical analysis. Furthermore, the applied end-point detection relies on the clear separation of PCR positive and PCR negative reactions, which needs to be confirmed for each well containing samples.

Due to the absolute quantification of samples by dPCR, the selection, preparation, qualification and use of a plasmid or other control for relative quantification of test samples is not required.

6.1 Method development

The points described below should be considered for dPCR method development.

- Cycling conditions (ramp rate, temperature profile, hold times): Evaluate through confirmation of sample linearity; review intra- and inter-assay variability.
- dPCR relies on the sample distribution following a binominal distribution function in which a part of the reaction volume does not contain the target sequence; optimisation of the required sample and control material dilutions are crucial for correct enumeration of test samples.
- Stability of PCR reaction mix pre- and post-PCR: Evaluation of stability of reaction mixes during standard preparation times and under stressed conditions (e.g. longer hold times between steps of the method). Generally, a batch is treated to remove free DNA, followed by Proteinase K digest to break up the viral capsid, and then samples are partitioned prior to readout.
- Consistency of reaction volume. The consistency of the sample partitioning may need to be verified depending on the used dPCR platform.
- End-point detection: reliable classification/separation of positive and negative reactions, i.e. definition of sample threshold.
- Multiplexing: spike of non-relevant DNA after sample preparation to confirm the correct execution of test sample dilutions and correct performance of PCR and end-point analysis.
- Hold points: Data supporting hold points included in the procedure should be generated as part of method development and qualification.

Following optimization of new method parameters, acceptance criteria of controls need to be reviewed and adapted as required. In addition, new assay acceptance criteria may be needed to define parameters such as minimum reaction count or threshold definitions; as applicable to the chosen dPCR platform.

6.2 Assay and system suitability criteria

System suitability criteria that should be considered:

- The signals originating from a positive and negative reactions should be distinctly separated, consideration should be given to show exemplar plot of normal and abnormal separation.
- Threshold for total accepted positive signals in valid replicates should be set for each sample, as increasing the number of partitions will increase data precision.
- Acceptable percentage of positive signals for a negative control should be assigned based on scientific rationale.

Sample suitability criteria:

- If PCR readout is given in multiple fluorescent channels, different channels should be distinctly separated in accordance with a specific fluorescent channel.
- Limits in the number of copies detected per reaction chamber should be considered to ensure that enough negative reactions are present for all valid dPCR replicates for all samples.
- Percentage poisson error should be set up for all valid replicates of analysed samples.
- %CV threshold value should be assigned from valid dPCR replicates per sample.
- Percentage of accepted positive reactions accepted for tested samples should be assigned.

Typically, all samples should be run in triplicate with pass criteria assigned as two out of three replicates.

6.3 Assay reporting and data analysis

This section describes the steps to be considered in the evaluation of dPCR assays to confirm validity and determine the test result of unknown samples.

Run data should be analysed to confirm the dPCR run is valid and all pre-determined acceptance criteria are met as defined during the method development and validation. Depending on the nature of the established method, checks for assay validity may vary, the following checks are generally usually performed:

- Confirm the negative control meets the defined acceptance criteria.
- Assay controls such as digest controls and sample positive controls pass the defined acceptance criteria.

Once a dPCR run is considered valid the analysis of test samples is performed. In this step, unknown test samples are quantified based on the number of positive reactions and use of poisson statistics. Typically, this is calculation is performed by the software for each sample replicate.

Perform the calculation of unknown test sample according to the test method.

- Only include replicates and dilutions of a test sample for which the count of positive reactions falls within the established range.
- Consider dilution factor from sample dilution, pre-treatments, and the mastermix generation, as applicable.
- If multiple replicates and dilutions are performed calculate the final test sample result per test method.
- Consider how to handle outliers by statistical tests or the application of more general rules.

6.4 Method validation

As for qPCR method validation, the general principles of ICH regulatory guidance with respect to analytical method validation (ICH Q2(R1)) should be consulted prior to commencing any validation activities. dPCR assay validation should only be initiated once the method has been fully developed and demonstrated to be fit for purpose. Suitable acceptance/validity criteria based on prior assay performance will need to be defined prior to validation. Failure to meet these criteria will result in failure of the validation and will potentially require additional assay re-development. Any changes to the method post validation could require additional validation activities.

A description of analytical method validation parameters is given in section 5 which apply to dPCR also. However, as dPCR does not rely on a standard curve for sample quantification and uses end-point PCR, validation of these attributes does not apply. Instead, validation is performed with suitable test samples to determine precision, accuracy, and linearity.

The consistency of performing the sample partitioning into individual reaction chambers may need to be evaluated, depending on the chosen platform. Variations in this step may impact the assay robustness. In addition, the linearity of the method should be demonstrated by serial dilution of a test sample within the boundary of the underlying statistical approach.

7. Implementation of method changes

7.1 Transition from qPCR to dPCR

Moving from one method to another to assess VCN may generate different titers when assessing the same samples and this can have significant implications for a program that is already in the clinic as described in the control of method changes section below. However, if a transition from qPCR to dPCR is intended there are also technical points that should be considered, these are discussed below.

Implementation of a new method based on the same target sequence might require the re-evaluation of PCR reaction chemistry and conditions. Depending on the set up of the qPCR assay a re-design of the dPCR method may be more suitable than adapting a sub-optimal qPCR method to dPCR. As an example, the transition from a qPCR to dPCR assay may include the change from using DNA intercalating dyes for real-time monitoring of the PCR reaction to a probe-based dPCR method.

In addition to the points listed for qPCR and further considerations provided for dPCR method development, transitioning from qPCR to dPCR may require the optimisation of one or more of the following points:

- Master mix: Depending on the chosen platform specific reaction master mixes may be required which ensure the robust creation and stability of reaction chambers.
- Primer/probe concentration.
- PCR settings such as ramp rates, elongation time, and elongation temperature need to be verified or optimised when changing from qPCR to dPCR.
- Dilution: dPCR relies on the sample distribution following a binominal distribution function in which a part of the reaction volumes do not contain the target sequence; a change in the working range in the assay due to transition of the qPCR method to dPCR may require adjustment of the sample and control material dilutions.

7.2 Control of method changes

As the determination of VCN is a critical quality attribute, any planned or necessary changes to the method used to assess VCN at all stages of development should be carefully assessed for impact on assay performance and data generated before the change is implemented. Changes can include new lots or suppliers of critical reagents, changes to equipment, test location or test procedure.

Wherever possible it is advisable to avoid changes to the method used to assess VCN during clinical development but if this is unavoidable the change should be assessed before its introduction by performing bridging studies between the old and new methods. This should include assessing trending control and / or test samples on multiple occasions. Both methods should be used to gain a good understanding of any differences in titer that may result from the method change. It is not unexpected to generate a very different titer for VCN when assessing the same sample by qPCR and dPCR and if a product is already in clinical development when such a change is introduced this can have significant clinical and

regulatory consequences. A change in data generated for the VCN assay may require additional engagement with regulatory authorities.

In the context of GMP testing change control is a process for managing and controlling changes which have the potential to impact on quality, safety, or efficacy. It is a systematic approach used to ensure that changes to a system, process or procedure, e.g. analytical method, are introduced in a controlled and coordinated manner. Change control is often managed within a Quality Management System (QMS) and is required when operating according GMP as well as when changes are made to Chemistry Manufacturing and Control registered in regulatory submissions. The purpose is to ensure that no unnecessary changes are made, that all changes are documented, that the impact is minimal and that resources implementing the change are utilised effectively.

Change control can be described in the following steps:

- Identify the need for a change to be made.
- Initial evaluation where the change is reviewed, and benefits assessed.
- Detailed evaluation where the impact is on quality, safety, and efficacy.
- Decision on whether a formal change control is required or if change is already in the scope of standard operating procedures.
- Planning execution of the change.
- Change implementation.
- Review and archive.

8. Performance and trending

During assay development it is helpful to include a trending control to monitor assay performance and data generated and to help with investigations into anomalous data or failed assays. A trending control may also help to define assay acceptance criteria when method validation is performed and limits around the performance of this control can be set following validation. Once the method has been developed and validated, it is anticipated that the performance of analytical method remains unchanged while the method, and the execution of that method, remains unchanged. However, inclusion of a trending control in routine method execution may allow for the early identification of assay shift in data readout and therefore help address any issues with assay performance to avoid generation of subsequent invalid test results. The trending control can also be used to help assess the impact of planned method changes prior to implementation. It is worth mentioning that once trending is established changes can still be introduced based on route cause analysis and respective corrections implementation.

Samples used for trending analysis should be carefully considered and should be included in every assay run. The trending control can be any material that generates a positive and quantifiable result within the range of the assay. It is recommended to perform assay trending on qualified quantitative controls with clearly assigned target readout values and control limits indicating expected performance. Examples of controls are provided in Table 4. If acceptance criteria are not present, consideration should be given to record trending for information only until enough data points are collected to assure the stability and reliability of tested quantitative control(s).

8.1 Assigning target and control limits

Assigning assay target and control limits based on the performance of the trending control can be a valuable way to monitor assay performance. These control limits can be set based on a

defined number of valid runs and revised as more data becomes available or once the assay is validated revised based on the validated assay performance.

Once established, target values and control limits can be changed in such instances as:

- new batch of control has been generated at a different concentration. In this case appropriate bridging to the new control should be performed
- more representative data has been generated that can be used to make the values more robust.
- a controlled change is being made to the assay methodology which would be expected to effect method performance.

8.2. Generating trend analysis chart

Typically generating a trending chart requires the data to be normally distributed. Several charts can be used for trending the assay performance. An example of trending chart where assay occasion is plotted on the x-axis and the readout of trending control on the y-axis is shown in Figure 1 below. Upper and lower control limits (UCL, LCL) for the target trending control are included in the chart.

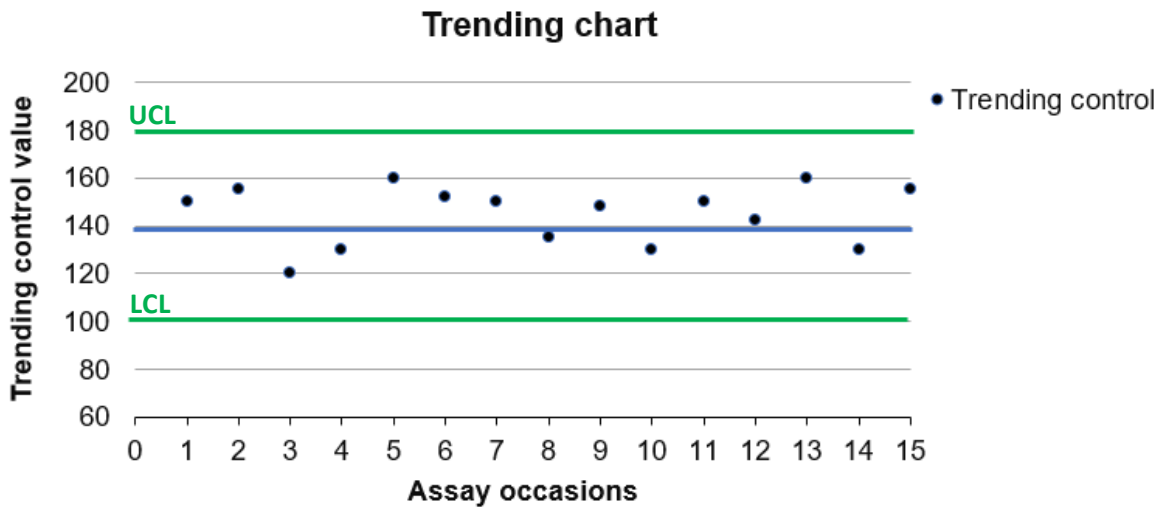

Figure 1. Exemplar trending chart to support trending analysis.

Part II LV-VCN

This section is included to detail the analysis of VCN with regards to LV or RV vectors where this analysis differs to that required for determination of AAV VCN. Part II of this document is intended to be read in combination with Part I. To avoid duplication of information Part I will be referred to where considerations for LV, RV, and AAV are the same.

9. Introduction

VCN in the context of integrating viruses, like LV and RV, is defined as the number of vector copies per host genome, although it does not provide information on whether the vector is integrated. The stochastic integration of the viral genome into the host genome carries some potential safety concerns, including the intrinsic risk of insertional mutagenesis which may occur when viral integration impairs the functionality of proto-oncogenes. An EMA reflection paper on management of clinical risks deriving from insertional mutagenesis highlighted the higher risk factor for insertional mutagenesis for high vector copy number products and recommends the assessment of the number of copies and integration profile. There are no official guidelines on the maximum number of copies that are considered to be safe.

VCN quantification can be used at various stages during any LV product development:

- Establishment of the manufacturing process, to standardise LV production and transduction and monitor consistency between production batches.
- Clinical dose determination, useful when administering the gene-modified cell therapy to the patient. Here the VCN is used to verify that the number of copies is not unreasonably high and is a useful tool to correlate possible side effects of the therapy later during the treatment.
- Patient safety follow-up, following the administration of the therapy to the patient, including engraftment of the transduced cells where necessary.

It is worth noting that bulk VCN analysis does not provide granularity on the heterogeneity of the population, it is an approximation that does not identify any individual cells or clones that may have a high VCN. The development of single cell VCN assays is a way to overcome these issues as it provides a more detailed analysis of heterogeneity of the whole population and allows for tighter control of the product safety by identifying the variability of VCN within the population. However, single cell VCN assays are outside the scope of this document.

10. Methods to determine VCN

10.1 qPCR

qPCR is typically used for bulk VCN analysis, using target-specific primers and fluorescent probes.

In qPCR, the accuracy of the quantification is dependent on the standard curve and the efficiency of the primers, both of which can impact assay variability. It is extremely important to generate reliable reference material (See Section 11.2) that would last for different rounds of development, qualification, and validation.

10.2 dPCR

Table 3 in Part I summarises some key differences between qPCR and dPCR.

The ideal VCN assay should include in the same reaction a target-specific probe to the vector used and a reference sequence that allows the normalisation of the number of copies per number of cells. Such a reaction is referred to as duplex. Should the reaction contain three or more targets, it will be referred to as triplex or high-order multiplexing. In cases where only one target assay can be utilised in the assay, the normalisation can be performed on parallel sample assayed for the reference gene, or even on µg of tissue/µL of blood, when follow-up studies are performed.

11. Method development

11.1 Choice of a target sequence

Lentiviruses and retroviruses belong to the family of enveloped RNA viruses, Retroviridae. Once into the cell, the RNA is retrotranscribed and the cDNA can integrate into the host genome. The region that integrates is the one in between the two LTRs. During integration, circular forms of the viral genomic DNA are generated by NHEJ of the full length linear viral DNA, creating 2-LTR circles or by homologous recombination between the two LTRs of the episomal viral DNA, creating 1-LTR. Therefore, these episomal DNAs do not integrate and persist in the cell until lost upon division. The final user should be aware that any analytical assay performed, within a few days of transduction, targeting the integrated region will also detect 1-LTR and 2-LTR circles. The cell line used can also have an impact on the vector copy number produced.

In addition, as different target-specific probes do result in slight variations due to the intrinsic characteristics of the target sequence, it would be ideal to design a number of sequences across the viral genome and cross-validate the results of one target assay with at least another one in. A design to quantitate VCN for a range of products would require the primers and probes to be in a region common to all constructs such as within a common promoter (See Section 4.2).

11.2 Reference target sequence

The choice of a reference sequence on a copy number reference gene is paramount and may vary depending on the clinical context. The quantification of a copy number reference gene allows the normalisation of the number of target copies for the number of cells analysed. Target-specific probe manufacturers provide information on the expected copy number for the targeted gene per diploid genome as part of the probe validation dataset. Regardless of the availability of such a dataset, it is recommended to test several copy number reference genes in different donors, preferably incorporating healthy and non-healthy donors, over several passages (to match the process, if it requires an expansion of the cells). The ideal reference gene maintains the same number of copies across different passages regardless of the comorbidities of the patients. In patients affected by cancer, where CNVs are recurrent, this aspect should be carefully considered. The peri-centromeric region of each chromosome is a good genomic location because it is less prone to CNVs. Like the target sequence, picking more than one gene allows for the generation of a more robust dataset.

11.3 DNA samples, from extraction to storage

The purity and quality of the gDNA used for VCN analysis is very important.

Several different methods can be used to lyse the sample; to eliminate proteins and lipids that might interfere with the amplification. Chemical, enzymatic, or physical lysis is usually performed, followed by the purification of the nucleic acid, generally using column-based or beads-based methods. A column-based approach preserves the purity and quality, it also allows the purification of large quantities, whereas bead-based methods might incur in the loss of the material. Bead-based methods could also lead to less homogeneous solution of nucleic acid The type of substrate should be considered too. When working with a suspension of cells any method can lead to high purity or quality. In solid tissues, the disaggregation protocol needs to be carefully optimised to ensure the DNA integrity is maintained. Commercial kits contain the instructions to process any type of samples, so it can be tailored to the type of starting material used. Phenol/chloroform and other systems where the lysate is not eliminated (e.g. in situ proteinase K) needs to be properly purified, as DNAses and PCR inhibitors might impair the success of the amplification. High purity ensures a lower variability between samples, even in dPCR, where the presence of inhibitors is less of a concern.

Integrity and concentrations should be monitored, based on electrophoretic analysis, fluorometric quantitation, and spectrophotometry. By spectrophotometry, the gDNA should display an absorbance ratio A260/A280 between 1.7 and 2.0. Lower ratios indicate the presence of residual salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate, while higher ratios may be indicative of RNA contamination. It is worth noting that the spectrophotometry will overestimate the amount of nucleic acid by 30%, as it cannot distinguish between single strand and double strand DNA. You do not need to quantify the nucleic acid if duplex is being used as mentioned earlier.

DNA is advised to be stored in single-use aliquots in 0.1 mM EDTA (in nuclease-free water) or TE buffer at -20°C or 80°C in single-use aliquots. For reference DNA, its stability should be monitored regularly to investigate the impact of freeze-thaw cycles.

Sample dilution may be required prior to analysis to fit within the dynamic range of the qPCR (7-logs) or dPCR (5-logs). Using a restriction digest enzyme may be useful to increase the signal-to-noise ratio, as the integration is stochastic, and two vector genomes could integrate close to each other. The user should use a restriction enzyme that does not cut the vector or the copy number reference gene.

11.4 Standard curve

The use of a standard curve is essential for the analysis of the samples by qPCR. The standard curve can be generated starting from a pure gDNA of a sample at known VCN or by using a plasmid that contains both the target gene and the reference gene. In the latter, the number of plasmid molecules is calculated to achieve a specific VCN, and the equivalent mass is spiked to background gDNA. Other options for generating the standard curve are also possible with suitable justification. The WHO has developed a panel of genomic DNA standards from a single cloned cell line containing 0, 1 or up to 10 of LV integration with an assigned unitage of LV copies/cell using a wide range of possible detection methods.

11.5 Primer design

General rules to design primers apply for both the target sequences and the copy number reference sequences. The primers of each target should be highly specific for the sequence and not bind to any other portion of the vector or host genome. During design, it is advisable to perform sequence alignment of the sequence on a reference genome to ensure the uniqueness of the assay. Open-source tools such as the Basic Local Alignment Search Tool can be used. During development, specificity should be assessed by the analysis of the melting curve when using DNA intercalating dyes. Specificity should be confirmed by the presence of a peak at the appropriate temperature and the absence of a primer-dimer peak at lower temperature. When using target-specific probes, the amplicons can be instead subjected to sequencing to verify the sequence. In dPCR, there are no methods to assess specificity of the intercalating dye-based amplification, unless the amplicon is recovered and sequenced, as it would be done with target-specific probe-based amplicons. For each assay, it is recommended to follow the MIQE guidelines. (See Section 4.2)

If performing a duplex, the use of two different fluorochromes will facilitate the combination of the two assays in the same reaction. Where more targets will be interrogated, the instrument being used will dictate the maximum number of fluorochromes that can be reliably detected. Some platforms enable detection of three or five different fluorophores. Other platforms enable the detection of two fluorophores, with the possibility to perform higher multiplexing (up to 10-plex) combining the fluorophores in different ratios.

12. Assays interference.

Where assays are to be used in duplex (vector assay and copy number reference gene), the linearity curve in individual assays should be compared to the curve obtained from the duplex. High correlations between these datasets ensure that:

- There are no unwanted interactions between primers and probes of different targets.
- The interaction is not dependent on the amount of starting material used.

If correlation is low or the slope of the linear regression of the individual assay and duplex do not overlap, the assays will need to be redesigned and tested again.

13. Considerations for reagents comparison

13.1 Linearity

A serial dilution of a single DNA source (e.g. purified gDNA from transduced cells or plasmid spiked into background gDNA) can be used. A 10-fold dilution series is recommended to cover most logs of the dynamic range; however, it can be reduced to 5-fold dilution series to suit the dynamic range of dPCR. The coefficient of determination (R^2) should be determined for every target value defines the correlation between the Cq and the dilution series.

R^2 should be ≥0.980. If lower, the user should investigate the presence of outliers, possibly given by pipetting variability at low amounts. The final user should consider removing those points from the linear range, and therefore, from the validated range that can be used to analyse the results.

13.2 Dynamic range

Range of input template for which acceptable linearity (R^2 ≥0.980) and efficiency (between 90% and 110%) are observed. For a result to be valid, it needs to be in the validated range.

13.3 Sensitivity

You will need to determine what is the lowest amount of starting material that can be detected i.e. the lowest point of the linearity curve that is within acceptable ranges of efficiency and linearity. Sensitivity is an important consideration, especially if the assay will be used for follow-up studies to evaluate the engraftment of the cells after infusion. Sensitivity should be tested by obtaining a dilution curve where 100% of the transduced material is diluted with non-transduced material in defined percentages to reach a 0.001% of transduced material in non-transduced material. Alternatively, if a reference material plasmid is available, the plasmid can be spiked in non-transduced background gDNA. At the end of the curve, the standard deviation of the technical replicates increases, as it is very difficult to evenly distribute the target molecules into each replicate well.