Consultation

British Pharmacopoeia public consultation for revised vector copy number guidance

Consultation period 19 December 2024 to 19 March 2025

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, their product specificity, and the still-emerging technologies that support them. Publications such as the Advanced Therapies Manufacturing Taskforce Action Plan,[2] the Medicines Manufacturing Industry Partnership'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This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore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 BP'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 March 2020, has engaged with groups across the cell and gene therapy community to develop

[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/

non-mandatory guidance for key analytical technologies to ensure quality throughout the product lifecycle. The working party has developed 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.

This update consists of two parts:

1. Revision to the vector copy number guidance (highlighted in green in the main document)
2. Addition of validation plan annexes (1 to 4)

The validation plan outlines the essential steps required to validate a quantitative Polymerase Chain Reaction (qPCR) or digital PCR (dPCR) assays, with specific protocols and reports for two primary applications: determining the Adeno-associated virus (AAV) viral titre and Lentiviral (LV) viral copy number. The importance of this validation cannot be overstated, as it ensures the reliability, accuracy, and consistency of the assay results, which are critical for product quality and regulatory compliance.

AAV viral titre determination protocol assesses the concentration of viral particles in a sample, which is essential for accurate dosage calculations of AAV drug products. Validation plan includes assessment of accuracy, specificity, precision and range. For LV, the protocol focuses on determining the VCN by measuring the pro-viral DNA copies that contain the gene of interest per host cell genome, which is critical for the safety assessment of therapies using integrating vectors. Validation of this method also includes an assessment of accuracy, specificity, precision and range.

Validation plans are crucial for ensuring reliability, accuracy, and regulatory compliance in assay results, thereby guaranteeing product quality. They provide confidence in data, support risk management, and help identify and mitigate potential issues in the assay process. This comprehensive approach assures stakeholders of consistent, high-quality outcomes essential for both internal decision-making and external reporting.

3. How to contribute

The draft Replication competent virus assays best practice guidance will be posted online for public consultation for a period of two months. 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 the key methods for particle characterisation covered?
- 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.

Annex 1 Revised guidance for vector copy number and supporting validation annexes

Contents

1 Abbreviations

ATMP	Advanced Therapy Medicinal Products
AAV	Adeno-Associated Virus
BLAST	Basic Local Alignment Search Tool
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
Cq	Quantification Cycle
Ct*	Threshold Cycle (used for software that uses a set threshold for the Cq)
CV	Coefficient of Variance
dPCR	Digital Polymerase Chain Reaction**
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphates
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
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ISO	International organization of standardization
LCL	Lower Control Limits
ITR	Inverted Terminal Repeat
LTR	Long Terminal Repeat
LV	Lentivirus
MIQE	Minimum Information for Publication of Quantitative Digital PCR Experiments
MOI	Multiplicity of Infection

NHEJ	Non-Homologous End Joining
PCR	Polymerase Chain Reaction
Ph. Eur.	European Pharmacopeia
PPE	Personal Protective Equipment
qPCR	Quantitative Polymerase Chain Reaction (Kinetic/Real time PCR)
rAAV	Recombinant Adeno-Associated Virus
RNA	Ribonucleic Acid
RV	Retrovirus
SOP(s)	Standard Operating Procedure(s)
UCL	Upper Control Limits
UK	United Kingdom
US	United States of America
USP	United States Pharmacopeia
VCN	Vector Copy Number
Vg/mL	Vector Genomes per Milliliter
QC	Quality Control

*Ct (Cycle threshold) is commonly indicated for qPCR where software uses a threshold to decipher the cycle value, but as the threshold method is not universal, Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines suggests the use of Cq, due to the existence of methods other than Ct being used.

**comprises all forms of dPCR, including ddPCR™ or droplet digital PCR and plate based digital PCR

Part I AAV-VCN

2 Scope

This guidance addresses the use of polymerase chain reaction (PCR) based assays for genomic titre analysis in the context of AAV-based therapies and vector copy number (VCN) analysis in the context of lentiviral (LV) or retroviral (RV)-based therapies and will provide current best practice.

For the purposes of this part of the guidance, the terminology ~~vector copy number (VCN)~~genomic titre refers to PCR-based quantification of encapsidated genetic sequences in recombinant adeno-associated virus (rAAV) vector particles. rAAV vectors contain transgene sequences together with associated regulatory elements, flanked by inverted terminal repeats (ITRs). rAAVs are small, largely episomal, non-enveloped single-stranded DNA (ssDNA) viruses that belong to the Parvoviridae family. The unit applied to ~~VCN~~ genomic titre will be vector genome copies per millilitre (vg/mL).

~~Vector copy number in~~Genomic titre of rAAV particles is distinct from AAV infectious titre, transducing titre, total particle (capsid) titre or the numbers of replication-competent AAV in that it only represents copies of [encapsidated] vector genome in the test sample without providing any information on biological activity. AAV infectious titre, transduction titre, total particle (capsid) titre or replication-competent AAV are distinct measurement of different vector quality attributes and are not covered within this chapter.

The terminology of VCN in the context of lentiviral (LV) or retroviral (RV)-based therapies refers to the number of viral genome copies present in a given population of cells. Lentiviral and retroviral vectors have ability to integrate their genetic material into the host genome. PCR analysis of VCN is used to quantify the number of viral genomes per cell within the sample. This analysis provides information about the level of gene delivery achieved. The unit applied to VCN will be vector genome copies per host cell (copies/cell).

It is advisable to implement and optimise the most appropriate method early in development to allow for accurate and precise determination of ~~VCN~~genomic titre and 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~~genomic titre and 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 guidance includes a section that details how to manage assay changes.

~~This guidance 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 PCR 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 relevant regulatory guidelines.

3 Existing guidelines

The purpose of this section is to provide an overview of existing guidelines relating to genomic titre and VCN analysis, PCR, Advanced Therapy Medicinal Products (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
ICH	Q14	Analytical Procedure Development

ISO	ISO 20395:2019	Requirements for evaluating the performance of quantification methods for nucleic acid target sequences: qPCR and dPCR
MIQE	Clinical Chemistry, Volume 66, Issue 8, August 2020, Pages 1012–1029 Clin Chemistry, . 2009 Apr;55(4):611-22. doi: 10.1373/clinchem.2008.112797. Epub 2009 Feb 26.	The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020
MIQE		The MIQE guidelines: minimum information for publication of quantitative real-time 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)

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

4 Facility

Due to the extreme sensitivity of PCR, it is imperative to minimise the risk of contamination to areas designated to PCR. Target control material (i.e. Reference plasmid) should be diluted to an appropriate concentration before introduction to the PCR lab where possible.

Ideally the facility should be made up of at least 3 labs, the PCR reagent preparation (clean) lab should be under positive pressure, to avoid contamination entering the lab. The sample preparation lab should be under negative pressure to avoid contaminants escaping the lab. The PCR setup area/lab can be under neutral pressure.

Working practices must be implemented to ensure, or at least minimise the risk of contamination from PCR target sequences. This should include separate PPE in each area and practices to wipe down surfaces after use with an alkaline based solution (sodium hypochlorite/hydroxide).

PCR hoods should be used to maintain a laminar flow of clean air over the surface. Implementation of workflow and working practices should be carried out to minimise contamination, including changing lab coats and gloves, clean down of surfaces using nucleic acid degrading solutions such as sodium hypochlorite.

Due to the high sensitivity of PCR, it is imperative to minimise the risk of contamination in areas designated for PCR.

5 Methods for determination of genomic titre and VCN

Various methods can be used to assess the genomic titre and 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 (Table 3).

Table 3: The key differences between qPCR and dPCRS.

	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. As signal increases at each cycle, precision is relatively poor <2 fold change	Limited optimisation required to ensure good to excellent accuracy and precision, as quantification is absolute
Data collection	Real time	End point
Dynamic range	Very High >7 logs	Reduced 4-5.7 – logs (currently)
Data generation capacity	High throughput	Lower throughput with current platforms
Sample matrix	May be affected by complex sample matrices	Less affected by complex sample matrices

Determination of genomic titre for AAV is often used to define the target dose administered during toxicity or dose ranging studies and clinical trials. It 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 important that the method ~~used to determine VCN~~ is selected, developed, and optimised to provide both accurate and precise data. The information provided in this guidance 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.

5.1 Quantitative PCR

In the context of this chapter qPCR is a real-time quantification 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 fluorescent-tagged reporter probe(s) are designed to bind specifically to the target sequence, or a reporter dye that intercalates double stranded DNA. 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~~amplifies the amount of target sequence exponentially. During amplification fluorescence from the reporter dye increases with the amplification of the target sequence ~~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 analysed by appropriate software. The quantity of target sequence in a sample is directly proportional to the fluorescent signal and interpolated from the log regression formula of the calibration curve. This provides a relative quantification of target sequence in the test sample.

qPCR is sensitive, quantitative, 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 therefore important to ensure these parameters are well characterised and optimised. ~~Due to the wide dynamic range of qPCR, this method~~ qPCR is prone to a high level of imprecision of the quantitated result, as small changes in the Cq value have a great impact on the result. Assuming 100% PCR efficiency, each Cq is a 2-fold change. Therefore, if the method requires a precise result with <2-fold certainty of the result, the instrumentation must be validated to confirm that it can distinguish <2-fold difference and at a level of precision required for the method

Dilution of a reference standard to make the subsequent quantitated standards for each assay occasion, is a source of error and variability. To mitigate this issue, it is recommended to dilute the standards in bulk (preferrable with use of an automated liquid handler) then sub-aliquot this dilution series into single use microtiter well strips, that can be frozen and used on each assay occasion. An appropriate dilution buffer guarantying the stability over time should be considered (low TE + carrier or commercial number).

5.2 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 Poisson analysis is then used to quantify the concentration of target sequence in the test sample. dPCR does not rely on a standard curve and is less affected by amplification efficiency therefore generating an absolute quantification of target concentration.

5.3 General considerations

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.

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 those in close proximity to or within ITRs can be more complex in terms of primer / probe design due to their structure and high GC content. ITR can be more challenging to analyze using dPCR, as there are two copies that may no longer be completely linked. It should be noted that VCN quantification based on a single target may not equate to the presence of the full-length transgene and any associated regulatory elements (for example presence of the target sequence in a partially filled capsid). An assessment of vector genome integrity must therefore be considered separately.

The PCR assay can be product agnostic to allow development of a platform method by targeting elements, such as the promoter, common to different vector products. However, a product specific method may be desirable and could be a regulatory requirement. It is advised to seek guidance from the relevant regulatory agencies regarding the assay design in this respect.

Although the manufacturing process is likely to include an endonuclease step(s) some residual non-encapsidated plasmid or host cell DNA may still be present in final product. This contaminating DNA could contain complimentary sequence(s) to the target amplicon that would be amplified by the PCR reaction if not first removed. Methods for determination of genomic titre must therefore assess the requirement for inclusion and control of DNase steps for removal of any contaminating nucleic acid prior to extraction of the target DNA from the vector capsid.

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. In the case that the proteinase treatment step is omitted, adequate assessment is required to ensure that the denature efficiency and / or assay performance in the absence of proteinase K is adequate.

~~Requirement and control of these enzymatic steps are discussed within this guidance (See Section 4.5 and 4.8).~~

6 qPCR method development, sample preparation, and general considerations

6.1 Elution/Storage

It is imperative to mitigate against the potential degradation and plastic adhesion of the target controls and so it is not advised that these controls are simply stored in water. Both DNA and RNA are prone to enzymatic degradation. Aerosol, dust and surface contaminants can all introduce nucleases into the control sample and water will do little to inhibit their action. 1x TrisEDTA (10 mM Tris + 1 mM EDTA) buffer should be used for the storage of PCR template controls. Tris (Tris(hydroxymethyl)aminomethane): Tris acts as a buffering agent, maintaining a stable pH in the solution, this is important because DNA can be sensitive to changes in pH, which can lead to degradation or other changes in its structure. EDTA (Ethylenediaminetetraacetic acid): EDTA is a chelating agent that helps to sequester divalent cations, such as magnesium (Mg2+), which are necessary for the activity of certain enzymes that can degrade DNA, such as DNases. At 1x concentration this buffer does not show detrimental impact to PCR performance, but this can also be checked during development. On top of this, for long term storage, a carrier like polyA or salmon sperm should be considered to avoid adhesion to plastic.

DNA is relatively robust and can be stored at -20C, however it is recommended to store RNA at -80C. Repeated freeze/thaw cycles can have a detrimental impact on the stability and integrity of nucleic acids. Nucleic acids, such as DNA and RNA, are susceptible to degradation and structural changes caused by the formation of ice crystals during freezing and the subsequent melting process. These changes can lead to the loss of sample quality, reduced amplification efficiency, and inaccurate results in downstream applications such as PCR. To mitigate the effects of freeze/thaw cycles, it is recommended to aliquot the nucleic acid samples into single-use portions prior to freezing. By storing the samples in single-use aliquots, researchers can minimize the number of freeze/thaw cycles a sample undergoes, preserving its integrity and ensuring consistent and reliable results in subsequent analyses.

6.2 Linearised plasmid control / standard curve

A plasmid control is often used to generate the standard curve for qPCR, although use of other, suitable controls may be acceptable. Published data indicated that use of plasmid in its native, supercoiled form may overestimate genomic titre and VCN. Therefore, it is recommended to linearise a plasmid stock prior to use. This can be performed by a single suitable restriction site digestion.

The two most common methods for quantitating nucleic acids are spectrophotometry and fluorimetry, however users should be aware of the following shortfalls of each technique:

Spectrophotometry: Although cheap and widely accessible, this method is not specific to nucleic acids and is prone to inaccuracies from impurities such as contaminating proteins and phenol. In addition, it doesn't differentiate between RNA and DNA.

Fluorimetry: Although more expensive and less accessible than spectrophotometry, this technique is highly sensitive and specific. However, it is a relative method that requires the use of a well characterised DNA/RNA standard in order to quantitate the nucleic acid standard to be used in PCR.

dPCR itself may also be used to quantify the standards to be used in other PCR methods.

Variability associated with qPCR can often be attributed to the plasmid control. Therefore, the generation, handling, optimisation qualification and assigning of concentration of plasmid stocks need to be carefully controlled. The stock plasmid concentration should be determined using a robust method with good accuracy and precision, ideally from multiple individual analyses, each with a suitable number of replicates.

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 using the most accurate and precise methods available. 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 because introducing a new stock could create variation that may impact on assay performance. Consideration should be given to storing stock aliquots in separate locations to ensure a back-up supply in the event of a storage deviation. 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. Plasmid instability will affect the assay performance and data generated so the stability of all plasmid stocks, in particular the stability of low concentration or low volume stocks should be monitored. Stabiliser material can be added to preserve low concentration stocks but the impact of the stabiliser 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 events should be established, specified, and documented. Another option to consider during qPCR method development is the use of in-silico calibration curves which could alleviate issues associated with maintaining and using a physical standard curve. Although the diagnostic methods commonly use such methods, there are few examples in CMC and so such an option should be carefully considered with a robust data package for regulatory review.

6.3 Primers and probe

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 annealing temperature, the GC content and the concentration and amplicon size are important and can influence the slope of the standard curve. Longer amplicons will amplify less efficiently than shorter amplicons. Primers and probe should have demonstrated specificity to the target region only and not against any non-target DNA that could be present in the sample. 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 thought should be given to any additional qualification of primers, including lot to lot variability. Primer-dimers should 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 genomic titre and 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.

6.4 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 when using qPCR. For dPCR, the (2D) amplitude profile should not contain additional clouds pointing towards aspecificity. Assay validity criteria should be established for non-target signals.

6.5 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. pipette tips, tubes, and plates. The use of DNA / 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 accurate genomic titre and VCN quantification is not compromised by the loss of samples due to plastic adsorption. DNA, DNase and RNase-free consumables should also be used.

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

6.6 Sample suitability and preparation

Evaluation of genomic titre and VCN is likely to be required at different stages of product 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 as 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 genomic titre will vary depending on manufacturing stage from which the sample was obtained. Therefore, when developing a ~~VCN~~ genomic titre quantification 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.

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

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 may add to the assay variability and must be well controlled. 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 should be included.

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. The plasmid standard and / or other positive control material should be handled separately from the test sample to avoid cross-contamination. Appropriate no-template controls should be included to assess for contamination.

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

- Sample storage and the impact of freeze-thaw cycles.
- Sample dilution steps, including volumes and diluent for the preparation of samples and consistency with steps for standard curve preparation.
- Flexibility with regards to sample dilutions in cases where titres of samples are not as expected, such as additional dilutions for more concentrated material.
- Standardise mixing steps during preparation of samples and controls and plating of material to the reaction mix.
- Assay control preparation should be concurrent with sample preparation.
- Hold times between assay steps of digested samples or master mix solutions for example.
- 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.

6.7 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 genomic titre and 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.

6.8 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, therefore 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 be clearly 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 versus 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.

6.9 Assay controls

The 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. Some examples of commonly used controls are detailed Table 4. The controls used should be defined and justified on a case-by-case basis; for example, a specificity control may be excluded if specificity has been assessed as part of assay validation.

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 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 controls	Controls to monitor the performance of any sample pre-treatment steps / control for the activity of enzymes used in such steps. Examples include nuclease treatment to remove non-encapsidated DNA (control DNA of known concentration treated with or without nuclease to assess efficiency of digestion) and proteinase K digestion of capsids. Another example of a sample preparation control would be where DNA extraction is performed (control DNA spiked and assessed prior to and after

	extraction). Acceptance criteria should be established for each of these controls.
Positive control	Assay control containing ~~a defined amount~~ 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 material	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 but may be used to describe the material used for the calibration curve. For dPCR is can be used as a control to confirm the performance of the assay, not as a material for the calibration curve.
Spike control	Target DNA spiked at a known concentration to assess for matrix interference and / or binding of target DNA to consumables. Differences between pre/post spike values (once dilutions are factored in) may indicate the presence of PCR inhibitors from the matrix impacting PCR efficiency, resulting in inaccurate quantitation (underestimate).
Negative specificity control	Potential process contaminating DNA control material without the target sequence to monitor the specificity of the assay e.g. host cell DNA

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

6.9.1 Sample preparation controls

Extraction/ Purification controls: Where the method includes sample preparation to extract or purify the nucleic acid for absolute quantitation, it is important to understand the level of loss incurred by this process, and whether this loss is linear at different loads of starting material. The sample preparation process should be validated using pre-quantified controls (DNA/RNA). Such controls should be spiked in to lysed sample (to mitigate issues from enzymatic degradation) and % loss confirmed, due to the extraction process. The quantity of these spikes is known so that % loss through the sample preparation steps can be quantified. Depending on risk, extraction controls may also be run routinely as part of the assays System suitability criteria.

DNase control: This controls for the effectiveness of the DNase step. A DNA target (could be the plasmid used as reference standard) is spiked into blank sample matrix, at known quantities prior to the DNase digestion step. Following completion of the method the quantitated spike (pre-DNase) is compared to the spiked matrix to measure the effectiveness of the DNase step in the sample matrix. Criteria should be applied to the level of acceptable clearance for this step to be deemed effective.

Proteinase K Control: If used during the pre-PCR workflow, this control requires the supply of well characterised AAV reference standard where the full particle quantity has been established. The AAV standard is spiked into blank sample matrix and processed through the proteinase K step. The AAV standard pre and post proteinase K is then compared to monitor the effectiveness of this step in the sample matrix.

Qualitative Control

Qualitative controls are used to confirm correct functionality of the PCR reaction (external qualitative control or internal positive control). An external qualitative control consists of the target amplicon but carried out in a separate reaction to the samples with the assumption that functionality of the sample PCR's will behave in the same way as this control. A quantitative

value can be assigned to this control for trend analysis purposes, as well as a System Suitability test.

An internal positive control (IPC) is a template spiked into every reaction (added to the master mix) to confirm correct functionality of every reaction, mitigating false negatives at an individual reaction level. The IPC concentration should be optimised during development to ensure that this reaction does not out-compete the target reaction (typically 10 copies per reaction). In presence of target template, the target PCR outcompetes the IPC, however in its absence the IPC shows that the PCR would have worked had the target template been present. Optimal IPC strategy is the use of an amplicon with the same target primer binding sites but modified internally to distinguish the target from the IPC.

PCR reactions utilising intercalating or minor-groove binding dyes would have the IPC designed with a lower melting point than the target PCR amplicon. Following the elongation/extension step of every cycle a detection point would be added to detect any amplification from either the IPC or target at the elongation temperature (typically 70-75°C). A second detection point would also be added to every cycle set above the melting temperature (Tm) of the IPC but below that of the target amplicon. Amplification detected above the Tm of the IPC confirms positive amplification of the target PCR.

6.10 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 typical for qPCR, but tightening this criterion is recommended if within the capability of the assay (depending on the context of use).

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

6.11 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 and storage conditions where possible.
- 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, sample thawing, 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, including the ones in locked validated spreadsheets.
- 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.

6.12 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.

6.13 Control of critical reagents

Critical reagents are those, such as primers, probes, plasmid and 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.

6.14 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 equipment vendor and the level of qualification should reflect 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 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 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. Points to consider include audit trails, user access controls, e-signatures, and use of archive functionality.

6.15 Data interpretation, analysis, and reporting

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

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.

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 / or 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 Cq values of unknown test samples against the Cq values of the standard curve.

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

- Results for replicates and dilutions of a test sample are only considered valid where the Cq value falls within the range of the standard curve and that meet any additional pre-defined acceptance criteria.
- 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 double-stranded plasmid DNA standard curve 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 Cq 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.

The reporting and interpretation of any PCR result should be made in the context of the associated assay variability.

6.16 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, this allows for the generation of a robust genomic titre or 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.
- Inclusion of suitable controls and acceptance criteria.
- Careful control over liquid handling.
- Correct sample handling.
- Proper control and qualification of critical reagents.
- Appropriate replication strategy.
- Clear and consistent operating instructions
- Well trained operators.
- Correct operation and maintenance of equipment and data analysis.

Changes to operators, manufacturer / concentration and source of reagents / buffers, primer / probe sequence, consumables (tubes, plates, tips) and cycling conditions can all impact on assay performance and should be assessed in advance of implementation. Where methods are performed with more than one instrument consideration should be given to the criticality of the equipment in the analytical workflow and potential impact of instrument-to-instrument variability on the final readout.

Variance component / power 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.

7 Assay validation (qPCR)

As development progresses the regulatory requirements for assay validation will change. However, as described within this guidance determination of genomic titre is a critical assay for AAV toxicity studies, dose determination, and clinical studies. ~~VCN~~ Genomic titre 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. Phase appropriate validation may be termed qualification or validation in certain organisations at early phase, but the information contained in this document is valid whatever type of study is undertaken throughout the life cycle of the assay.

In terms of assay validation, the regulatory expectation for licensing is conformance to ICH Q2 ~~(R1)~~ and 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/qualified. 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

One example of the qPCR validation report and protocol for assessment of genomic AAV titre and one example of the qPCR validation protocol and report for assessment of LV VCN are presented as Attachment X.

7.1 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 incubation times, temperatures and hold times. 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 Cq 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.

7.2 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. During assay development specificity can be assessed using *in silico* tools such as Basic Local Alignment Search Tool (BLAST) to ensure no homology of the primer / probe binding sequence to other non-target DNA that could be present in the sample. During assay development and validation, specificity should be experimentally 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.

7.3 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 genomic titre 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 of 90% to 110% is standard for qPCR, but tightening this criterion is recommended if within the capability of the assay. 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.

7.4 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 +/- standard deviation of four of these replicates will be analysed as unknowns. Quantification 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 calculated by analysis of the unknown across the range. Typical limits of 70% to 130% of the nominal values are used to evaluate accuracy but wider limits can be used with appropriate justification. Other methods of inferring accuracy can be utilised with appropriate justification.

7.5 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.

7.6 Precision

7.6.1 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). It should be considered that precision should be evaluated across the whole method including any sample preparation prior to analysis by qPCR in order to report the precision of the reportable result.

7.6.2 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, quantification limit and repeatability obtained from a minimum of two operators over different days.

7.6.3 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.

8 dPCR considerations

The general principles for method development as described in Section 6 are also applicable to dPCR assays. The evaluation of a standard curve does not apply for dPCR assays. Instead, parameters around the partitions used to carry out the dPCR subreactions need to be evaluated. Independent of the chosen dPCR technology a minimum number of subreactions is required to infer the unknown test sample concentration through underlying statistical analysis (according to the dMIQE guidelines 10 000). 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.

8.1 Method development

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

- Cycling conditions (temperature profile, ramp rate, hold times) resulting in good separation between positive and negative partitions
- Consideration of restriction digestion to increase target accessibility, increase PCR efficiency or separate tandem copies (required for VCN).
- Optimisation of the required sample and control material dilutions are crucial for correct enumeration of test samples, as dPCR relies on the sample distribution following a binomial distribution function in which a part of the reaction volume does not contain the target sequence.
- ~~Stability of PCR reaction mix pre- and post-PCR~~Evaluation of the pre-PCR workflow: ~~evaluation of stability of reaction mixes during standard preparation times and under stressed conditions (e.g. longer hold times between steps of the method).~~ Often, for genome titer a batch is treated to remove free DNA and heat treatment to break up the viral capsid. ~~followed by Proteinase K digest to break up the viral capsid~~
- ~~Consistency of partition 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 resulting in robust thresholding and minimization of rain between the positive and negative droplets by optimization of the reaction parameters.
- Multiplexing: compare multiplex and singleplex performance ~~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 (e.g. overnight storage of digested samples prior to PCR amplification and endpoint readout) should be generated as part of method development and qualification.

Following optimisation 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.

8.2 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.~~

- Thresholding should be robust, by preference based on a robust automated algorithm or on a % RFU between the positive and the negative cloud.
- ~~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.~~
- A minimum number of accepted partitions should be established providing acceptable precision of the Poisson distribution.

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.
- ~~A minimum number of qualifying partitions should be established which is relevant to the used dPCR platform and provides acceptable accuracy of the Poisson distribution.~~
- ~~Limits in the number of copies detected per partition should be considered to ensure that enough negative partitions are present for all valid dPCR replicates for all samples.~~%CV threshold value should be assigned from valid dPCR replicates and dilutions per sample.

Typically, all samples should be run in triplicate with two out of three replicates meeting the pass criteria for a valid sample (although for dPCR replicates are not strictly necessary). Depending of the context of use, also merging of wells can be used to increase for example the LOD/LOQ. An appropriate number of dilutions based on assay development work should be chosen.

8.3 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 partitions and use of Poisson statistics. Typically, this 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 concentration falls within the dynamic range. ~~the count of positive reactions falls within the established range.~~
- Consider dilution factor from sample dilution, pre-treatments, and the mastermix generation, as appropriate.
- 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.

8.4 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 beginning any validation activities. dPCR assay validation should only be started once the method has been fully developed and proven 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 and would be implemented using change control procedures.

A description of analytical method validation parameters is given in Section 7, these also apply to dPCR. 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 only 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.

9 Implementation of method changes

9.1 Transition from qPCR to dPCR

Moving from one method to another to assess VCN may generate different titres 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 annealing temperature, ramp rates, elongation time, and elongation temperature need to be verified or optimised when changing from qPCR to dPCR.
- Addition of a restriction enzyme should be considered. For VCN it is a requirement to separate tandem copies.
- Dilution: dPCR relies on the presence of negative partitions; 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.
- It is not uncommon that dPCR and prior qPCR results do not give the same results. A correction factor could be considered.

9.2 Control of method changes

As the determination of genomic titre or 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 titre that may result from the method change. It can generate a very different titre 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 genomic titre or 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 and is required when operating according to 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.

10 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 prior to and following validation. Once the method has been developed and validated, it is anticipated that the performance of an 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 root cause analysis (RCA) 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).

In cases where assay standard curve is used, performance of the trending control will indicate the performance of the standard curve. Additional studies evaluating the performance of the standard curve can be considered to further ensure the overall assay quality if necessary.

10.1 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 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 affect method performance.

10.2 Trend analysis

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. Upper and lower control limits (UCL, LCL) for the target trending control are included in the chart. UCL and LCL become more defined the more occasions the assay is performed.

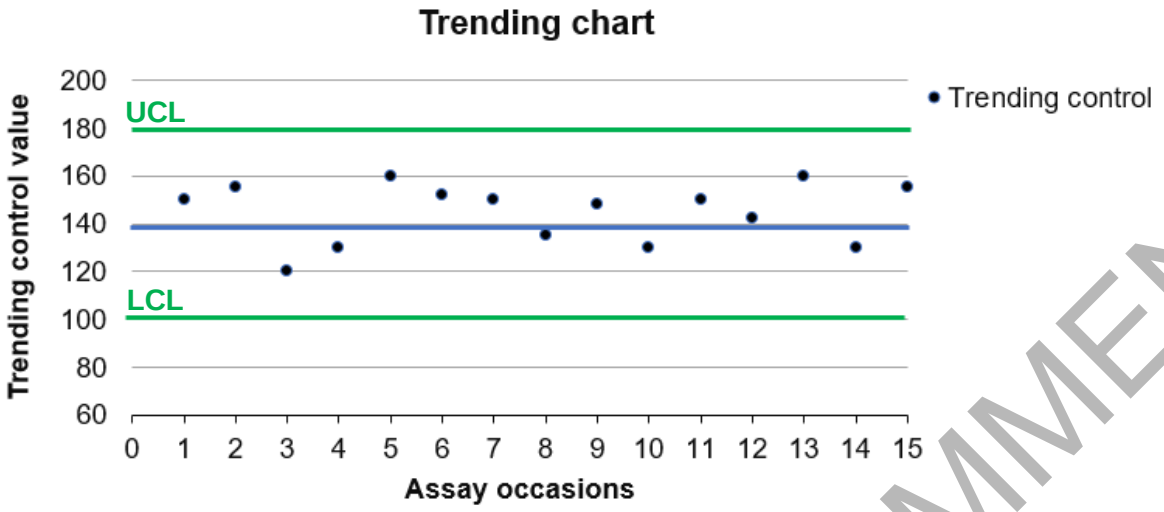


Figure 1. Exemplar trending chart to support trending analysis based on product specific positive control. The blue line represents the mean value and green lines represent UCL/LCL.

Part II LV-VCN

Part II 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~~genomic titre and VCN analysis are the same.

11 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 (EMA/CAT/GTWP/190186/2012).

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, at the moment, single cell VCN assays are outside the scope of this document.

12 Methods to determine VCN

VCN measures the number of pro-viral DNA copies per host cell genome and can be estimated by qPCR or dPCR. Table 3 in Section 5.2 summarises some key differences between qPCR and dPCR. Quantification can be performed using a standard curve or by using a duplex detection of the vector sequence normalized to the genome sequence. The accuracy of the quantification by standard curve is dependent on the efficiency of the primers, which can impact assay variability. The accuracy of the quantification by duplex is dependent on the lack of assay interference (See Section 13.6), and a standard curve containing both vector and genomic sequences should be evaluated. In both instances, it is extremely important to generate reliable reference material (See Section 13.2) that will last for different stages of development, qualification, and validation.

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.

13 Method development

13.1 Choice of a target sequence

Lentiviruses and retroviruses belong to the family of enveloped ribonucleic acid (RNA) viruses, Retroviridae. Once into the cell, the RNA is ~~retrotranscribed~~ reverse transcribed, and the complimentary DNA can integrate into the host genome. The region that integrates is the one in between the two long terminal repeats (LTRs). During integration, circular forms of the viral genomic DNA are generated by non-homologous end joining (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 give 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 one other. 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 6.3).

13.2 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 – genomic structural variations where entire sections of the genome are repeated 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.

13.3 DNA samples, from extraction to storage

The purity and quality of the genomic DNA (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. Should the purification not be an option, the dilution of the sample could be considered and may allow a simplified workflow for the analysis.

Integrity and concentrations should be monitored, based on electrophoretic analysis, fluorometric quantification, 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 significantly overestimate the amount of nucleic acid, as it cannot distinguish between single strand and double strand DNA. Therefore, fluorometric quantification may allow better estimation of the number of genomic copy number obtained. The quantification of the nucleic acid is recommended also when duplex is being used as mentioned earlier.

DNA is advised to be stored in single-use aliquots in suitable buffers 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 (See Section 6.2).

Sample dilution may be required prior to analysis to fit within the dynamic range of the qPCR or dPCR. 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.

13.4 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 the target sequence.~~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 (See Section 6.2). 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 9 LV integrations with an assigned unitage of LV copies/cell using a wide range of possible detection methods (TaqMan qPCR, SYBRGreen qPCR and digital PCR, WHO/BS/2019.2373).

13.5 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 BLAST 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, no additional aspecific populations should be obtained. ~~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 qMIQE or dMIQE guidelines. (See Section 6.3)

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. Platforms enable detection of ~~three~~ two ~~or five~~to seven different fluorophores. ~~Other platforms enable the detection of two fluorophores~~,Some platforms have the ability to ~~with the possibility~~ to perform higher multiplexing (up to 10-plex) combining the fluorophores in different ratios or at different concentrations.

13.6 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. This duplex should include 2 dilution curves (one of the target in a constant gDNA background, one the other way around). 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.

For dPCR, the concentrations should be the same between duplex and singleplex. For qPCR, the shift in Cq value should be low (ideally <0.5 Cq).

14 Additional considerations for method development

14.1 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. The dilution series should be within the context of use for VCN (for example from 10 copies per cell to 0.1). ~~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 and 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.

14.2 Dynamic range

Range of input template for which acceptable linearity (R^2 ≥ 0.980) and qPCR efficiency (between 90% and 110%) are standard, but tightening this criterion is recommended if within the capability of the assay. For a result to be valid, it needs to be in the validated range.

14.3 Sensitivity

Determination of the lowest amount of starting material that can be detected should be performed 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 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.

Annex 1 - dPCR and qPCR validation protocol for AAV genomic titre - worked example

READ THIS STATEMENT FIRST

The worked example for a validation protocol presented herein, is intended to support an understanding of the practical translation of expectations outlined in regulatory documents and guidelines. The objective is to assist readers having limited experience of undertaking assay validation activities with the development of their own internal procedures, processes, and protocol styles.

[Black italicised text provides some points for consideration with each section]

Blue text provides an exemplar in the context of a PCR assay aimed at determining AAV genomic titre (Vg/mL)

This document is not intended to present a gold standard to be copied or cloned. It is important readers develop their own protocols that are fit-for-purpose for the assay being developed. Any information and data presented herein is provided as an exemplar to illustrate possible content. It is not necessarily exhaustive of expectations, and where multiple approaches are potentially applicable (e.g. different statistical techniques / approaches) it is the reader's responsibility to ensure the correct methods are employed for their specific assay / validation plan design.

Readers with existing procedures and processes for assay validation activities are invited to reflect on the content but encouraged to continue using their own internally approved procedures, processes and documentation styles where they have been shown to be fit-for-purpose already.

Validation protocol

Validation plan for a PCR assay aimed at determining AAV genomic titre

Project code:	***XXXX***
Study title:	Validation plan for a PCR assay aimed at determining AAV genomic titre
Protocol number:	*XXX-XXXX*
Experimental start date:	*DD-MMM-YYYY*
Experimental end date:	*DD-MMM-YYYY*
Study locations: ***Address:***	
Experimental lead: ***Name:*** ***Signature:***	
Reviewed by: ***Name:*** ***Job Title:*** ***Signature:***	
Approved by: ***Name:*** ***Job title:*** ***Signature:***	

Amendments to version X.X of the validation plan

The following amendments were made to version X.X of the PCR assay validation plan to create this document:

1	***Change***	
	Reason for change	
2	***Change***	
	Reason for change	
3	***Change***	
	Reason for change	

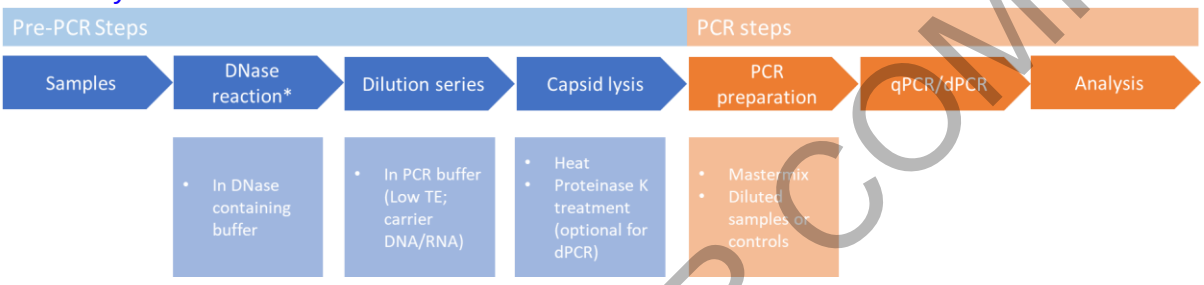
All changes were made at the beginning of validation activities.

Contents

1. Assay principle

[Describe in relevant sub-sections the principles underlying the assay validation. For example, the background to the assay (e.g. the assay purpose, what is being detected, why and/or how it is being detected); the objective of the assay (e.g. what it needs to demonstrate); the sources samples for performing the validation; and/or any other relevant considerations]

This assay is used for determination of genomic titre of AAV vectors by qPCR/dPCR at QC laboratory.



**not necessarily in this order*

1.1. Background

[Points to consider for inclusion in the background sub-section include sample source (e.g. clinical or manufacturing); the purpose of the assay (e.g. starting material characterisation, final product release testing or in-process controls testing to determine viral titre.)]

The validation plan will describe the procedures necessary to validate a qPCR or dPCR assay to determine the genome titre of final product samples. Genome titre will be determined by measuring the concentration of encapsidated AAV-vector genomes that contain the gene of interest. Determination of genome titer is a CQA for AAV drug product and is tested as part of batch release and stability.

This assay directly measures the concentration of encapsidated genomes of AAV vectors, that contain the gene of interest, by qPCR. Following Nuclease and Proteinase K treatment of diluted sample to remove non-encapsidated DNA and open the capsids, respectively, the test samples are serially diluted and then mixed with qPCR master mix, primers and probes. Dilutions of samples are subjected to qPCR and target sequences are amplified on a thermal cycler. By quantifying the amount of amplified DNA, the viral genome content can be estimated.

The assay has been qualified and is described in the report named 'Qualification of qPCR assay for quantification of AAV genomes'.

1.2. Objective

[Points to consider for inclusion in the objective sub-section include what the objective of the validation.]

The objective of this protocol is to validate analytical method SOP-X123, 'Determination of AAV genomic titre by qPCR or dPCR' used for determination of concentration of the gene of

interest in samples via qPCR or dPCR using sequence specific primers and probe. This assessment will include the following ICH Q2 parameters: specificity, range, accuracy and precision. Parameter values should be determined during the development phase, as per guidance set out in ICH Q14, ideally with use of an Analytical Target Profile (ATP) to ensure the method is fit for purpose, according to project requirements rather than simply applying method capabilities.

1.3. Scope

[Points to consider for the scope of the validation.]

The scope includes validation of the analytical method SOP-X123, 'Determination of AAV genomic titre by qPCR or dPCR' used for batch release and stability testing of [name] AAV drug product.

1.4. Responsibilities

[Define the roles and responsibilities of each team or department involved in the validation process.]

Role	Responsibility

2. ICH classification of assay

[Points to consider for inclusion in this sub-section of the document include reference to any current guidance documents used to support the validation protocol generation (e.g. ICH Q2) and if aspects of the guidance are not being followed then an explanation for any deviation must be recorded. For example, the intention may be to only validate certain assay characteristics (e.g. accuracy or linearity etc). at this point in development. It may also be pertinent to state the regulatory status of the assay.]

The validation of the assay will be carried out according to current ICH guidelines for the Validation of Analytical Procedures (ICH Q2) with respect to those assay performance characteristics detailed in Table 1.

Table 1. ICH recommendations for the characteristics to be assessed during validation of an analytical assay/procedure.

Performance characteristics	Assay validation requirements
Specificity	Yes
Range -Linearity	Yes
Accuracy	Yes
Precision - Repeatability - Intermediate Precision	 Yes Yes
Detection limit	For Information Only

Quantitative limit	For Information Only
Robustness	Yes

Robustness of the assay has been previously assessed and is documented in Report 'Qualification of qPCR assay for quantification of AAV genomes'.

3. Amendment and Deviation Reporting

[Points to consider for inclusion in this sub-section include how teams should manage and report deviations from the protocol, including deviations from pre-set acceptance criteria. Depending on the Quality system in place where the assay is being performed, reference to the related quality management procedures may be included.]

Any amendments / deviations to the protocol should be reported (this should include an assessment of the impact of the deviation to the study).

4. Materials and Methods

4.1. PCR Facilities

[Describe where the assay validation will be performed and characteristics of the PCR facility. Also describe controls used to prevent contamination between labs, such as dedicated lab coats/gloves within each lab, and wiping down of surfaces with nucleic acid degrading solution.]

The assay will be performed in the following locations:

1. PCR clean lab G10, to prepare the master mix.
2. PCR preparation lab G11, to prepare positive control and samples.
3. Pre-PCR lab G12, to pre-dilute the positive control.
4. PCR lab G13, to run the PCR reaction.

Separate lab coats will be used in each lab, with lab coats and gloves changed before moving from one lab to another. Surfaces will be cleaned with reagent X.

4.2. Sample sources

[Points to consider for inclusion in the sample source sub-section include a description of samples used in the assay validation and how they will be prepared for the protocol]. Also, if sample preparation for assay validation will vary compared to the actual samples analysed this should be described.]

Samples will be prepared by first treating the AAV vectors with DNase, in DNase reaction buffer. After incubation, the samples will be diluted in a 1:10 dilution series to the qPCR/dPCR ($1x10^5$ gc/mL and lower) working range in AAV dilution buffer. Inactivation of DNase and capsid lysis will happen in the appropriate dilutions ($1x10^5$ gc/mL and lower for dPCR) by heat inactivation and cooling down the samples slowly to 4°C.

4.3. Controls

[Points to consider for inclusion in this sub-section include a description of the controls (e.g. negative and specificity controls or quality controls) that will be used to support validation of the assay].

Controls described in Table 2 will be used in this validation study. Additional controls for consideration are described in Table 3.

Table 2. Controls used in validation study.

Control Type	Description	dPCR	qPCR
No-Template Control (NTC)	Buffer used for the process or water at PCR setup	☑	☑
Negative Specificity Control *1 (NSC1)*	(similar treated) producer cell line lysate/DNA	☑	☑
Negative Specificity Control *2 (NSC2)*	Environmental contaminating DNA	☑	☑
Reference Control	AAV / linearized plasmid/ synthetic construct that has been qualified. Useful for the calibration curve.	☑ *	☑
Positive Control	Previously qualified AAV / linearized plasmid/synthetic construct *at known copies (within quantitative range).*	☑**	☑

***** For dPCR this control will be used to generate the data on the dynamic range and linearity. However, the concentration from the dPCR data (and not from other characterisations) will be used to generate the data.

** For dPCR, this control will not be used for calculations.

Table 3. Additional controls for consideration.

Control Type	Description	dPCR	qPCR
Sample Negative Control	Negative control sample that is treated the same as the sample during sample preparation	☑	☑
DNase Control	Control to confirm effectiveness of DNase treatment	☑	☑
Proteinase K Control	Control for proteinase K treatment	☑ If applicable	☑
Extraction/Purification Control	Target DNA or reference sample, of known value, spiked at the start of sample preparation	☑*If applicable	☑If applicable
Spike Control	Target DNA spiked in at known copies to assess for matrix effects	þ*	þ

*Compare in the same experiment, not compared to known concentration.

4.4. Reagents and buffers

[List all the reagents required in relevant sub-sections, including variables such as reagent name, supplying company, catalogue numbers etc. The equipment used should also be detailed. Table 4 contains examples of reagents used in qPCR validation and Table 5 reagents used in dPCR validation study.]

Reagents will be used and prepared as per analytical method SOP-X123, 'Determination of AAV genomic titre by qPCR or dPCR'.

Table 4. Reagents required for a qPCR validation study.

Reagent	Company	Catalogue Number
Target PCR Primers	Company A	XX-XXX1
Target PCR Probe	Company A	XX-XXX2
Control gene Primers	Company A	XX-XXX3
Control gene Probe	Company A	XX-XXX4
qPCR Master mix	Company B	XX-XXX5
Molecular Grade Water	Company C	XX-XXX6
BSA (0.4%) (Optional)	Company D	XX-XXX7

Table 5. Reagents required for a dPCR validation study

Reagent	Company	Catalogue Number
Target PCR Primers	Company A	XX-XXX9
Target PCR Probe	Company A	XX-XXX10
Control gene Primers	Company A	XX-XXX11
Control gene Probe	Company A	XX-XXX12
dPCR Master mix	Company B	XX-XXX13
Molecular Grade Water	Company C	XX-XXX14
Restriction enzyme (optional)	Company D	XX-XXX15
Template DNA/cDNA	In-house	N/A

4.5. Equipment list

[Describe what equipment is used in this validation. Table 6 contains examples of equipment used in qPCR validation and Table 7 contains equipment used in dPCR validation study.]

Table 6. Equipment required for a qPCR study.

Equipment	Company	Serial Number
Automatic pipettes	Company E	X-ABC1

Real-time PCR system	Company F	X-ABC2
Plate centrifuge	Company G	X-ABC3
UV Hood	Company H	X-ABC4

Table 7. Equipment required for a dPCR study.

Equipment	Company	Serial Number
Automatic pipettes	Company E	X-ABC1
Droplet Generator (optional)	Company F	X-ABC2
Plate centrifuge	Company G	X-ABC3
UV Hood	Company H	X-ABC4
Plate sealer (optional)	Company F	X-ABC5
Thermal cycler*	Company F	X-ABC6
Plate reader*	Company F	X-ABC7

*Some suppliers have these 2 items combined as a single platform

4.6. Plasticware / Consumables

Table 8. Equipment required for a dPCR or qPCR study.

Plasticware	Company	Serial Number
Automatic pipette tips	Company I	X-ABC1
PCR plates	Company J	X-ABC2
Microcentrifuge tubes	Company K	X-ABC3
Plate seals	Company L	X-ABC4

[The use of DNA / protein resistant binding plastic consumables should be considered.]

5. Procedure

[Describe in relevant subsections the individual procedures being followed to evaluate the different assay performance characteristics. Where assay performance characteristics are not being evaluated, provide justification.]

5.1. General Instructions:

Analytical method will be performed as per SOP-X123, 'Determination of AAV genomic titre by qPCR or dPCR'.

All instruments/equipment that generate data used in this qualification will be within the calibration and maintenance period and have been qualified (i.e., IQ/OQ/PQ).

All experiments must satisfy the method system suitability criteria. Any analysis for which the results do not meet system suitability must be repeated as specified in the method. Experiments failing method system suitability criteria will be discussed between QC and QA representatives to determine if repeat is needed, justification or other options.

For dPCR, separation of positive and negative populations (Bright to Dim (BTD) ratio or peak resolution) should be optimized in the optimization phase. Strategic use of reference enzymes should be evaluated to obtain the good template accessibility and minimize rain. A minimum of 10,000 partitions will be recommended according to dMIQE guidelines. Upper and lower limits of detection should be carefully considered to allow accurate quantification.

The following assay performance criteria will be measured in this validation study:

5.2. Specificity

[Define Specificity in the context of the assay being performed - Where appropriate use the definition as provided by relevant guidelines being followed]

The specificity of a method is its ability to accurately and specifically detect the analyte in the presence of components that are expected to be present in the sample matrix.

5.2.1. Specificity assessment

[Define how the specificity assessment will be performed (e.g. this may be through use of the data generated from the linearity assessment), describe the specific type of to be detected, and the concentration range within which this detection should occur.]

Specificity will be assessed by comparing the number of viral vector copies in vector containing preparations, versus negative specificity controls and negative sample preparation controls that do not contain target transgene:

1. NSC1: sample buffer with 20 ng (untransfected) host cell DNA (restriction digested for dPCR)
2. NSC2: sample buffer with environmental contaminating DNA.

Samples will be analysed in triplicates (qPCR) or duplicates (dPCR), in 3 different runs.

5.2.2. Specificity results reporting

[Define how specificity will be reported and any statistical techniques employed]

Specificity will be reported as the mean copies/mL and 95% CI for the tested samples with and without the viral vector sequence (the negative specificity control). For dPCR a maximum number of positive accepted partitions can be set.

5.2.3. Acceptance criteria

[Define the acceptance criteria used to assess specificity]

qPCR: Reaction must be negative for at least two replicates of the negative specificity control 1 and 2 (NSC1 and NSC2).

dPCR: Reaction must be significantly different for at least two replicates of the negative specificity control 1 and 2 (NSC1 and NSC2) and the lowest preparation of the linearity assessment. For example, dPCR criteria can be set as follows:

NTC and NSC1/NSC2: target < 2 copies/3 wells

5.3. Range

5.3.1. Linearity

[Define Linearity – Where appropriate use the definition as provided by relevant guidelines being followed]

The definition of linearity is the ability to elicit test results that are directly, or by defined mathematical transformation, proportional to the concentration of analyte in the samples within a given range.

5.3.2. Linearity assessment

[Describe how the linearity will be assessed, including which attributes or characteristics of the sample are the focal point of assessment.

Considerations for:

1. *qPCR: theoretically a 10-log linearity could be reached*
2. *dPCR: the theoretical dynamic range will depend on the number of partitions.*

Systems with ~20 000 partitions will generate a theoretical dynamic range of 5 log. For systems with <10 000 partitions this becomes 4 log.

Section 4.3.1.1 describes approach used for qPCR linearity assessment and Section 4.3.1.2. for dPCR linearity assessment.]

5.3.3. qPCR linearity assessment

Linearity will be assessed by analysing a 1:10 dilution series of a DNase treated AAV viral vector in an AAV resuspension buffer or a linear reference standard curve nucleic acid, starting at $1x10^8$ – $1x10^{10}$ copies per reaction.

An example of a 10-fold dilution series is given in Table 9.

Table 9. 10-fold dilution series used in qPCR linearity assessment.

Dilution	Mix	Target copies/µL (assuming 1 µL will be measured in the final reaction)*
Dil 1	DNAse treated sample	$1x10^8$
Dil 2	180 µL buffer + 20 µL Dil 1	$1x10^7$

Dil 3	180 µL buffer + 20 µL Dil 2	$1x10^6$
Dil 4	180 µL buffer + 20 µL Dil 3	$1x10^5$
Dil 5	180 µL buffer + 20 µL Dil 4	$1x10^4$
Dil 6	180 µL buffer + 20 µL Dil 5	$1x10^3$
Dil 7	180 µL buffer + 20 µL Dil 6	$1x10^2$
Dil 8	180 µL buffer + 20 µL Dil 7	10
Dil 9	180 µL buffer + 20 µL Dil 8	1

As DNase treatment can be a factor for variation, replicates ideally should be also replicates of the DNase reaction.

The dilution series will be prepared and measured by at least 2 operators.

The dilution series will be repeated on different days.

If available, the dilution series will be measured on different equipment.

* take into account the measured volume. For a system with 50% dead volume, add for example 2 instead of 1 µL to the final reaction.

5.3.4. dPCR linearity assessment

Linearity will be assessed by analysing the concentration (copies/mL) of a dilution series of a DNase treated AAV viral vector in an AAV resuspension buffer or a reference control standard curve nucleic acid in a similar dilution buffer as the final product.

For dPCR a pre-dilution to ~a final concentration around 100 000 copies per well (for ~20K partition systems) should be made as the starting concentration. This can be estimated with spectrophotometry/fluorometry/previous experiments. For example, when adding a final volume of 1 µL to the dPCR, a pre-dilution to ~100 000 copies/µL should be considered.

To receive an 8-point dilution curve, dPCR requires a 1:5 dilution curve. An example is given in Table 10.

As DNase treatment can be a factor for variation, replicates ideally should be also replicates of the DNase reaction.

Table 10. dPCR dilution series used in the linearity assessment.

Dilution	**Mix**	**Target copies/µL (assuming 1 µL will be measured in the final reaction)***
Dil 1	DNAse treated sample	100 000
Dil 2	160 µL buffer + 40 µL Dil 1	20 000

Dil 3	160 µL buffer + 40 µL Dil 2	4000
Dil 4	160 µL buffer + 40 µL Dil 3	800
Dil 5	160 µL buffer + 40 µL Dil 4	160
Dil 6	160 µL buffer + 40 µL Dil 5	32
Dil 7	160 µL buffer + 40 µL Dil 6	6.4
Dil 8	160 µL buffer + 40 µL Dil 7	1.28

The dilution series will be prepared and measured by at least 2 operators.

The dilution series will be prepared repeated on different days.

If available, the dilution series will be measured on different equipment.

* take into account the measured volume. For a system with 50% dead volume, add for example 2 instead of 1 µL to the final reaction.

5.3.5. Linearity results reporting

[Describe how the linearity results will be reported and any statistical approaches used]

The coefficient of determination (R^2) will be measured for a mean of XXX replicates (in copies/ µl).

For qPCR the y-intercept and slope will be reported. From the slope, the amplification efficiency can be calculated using the formula = $-1+10^{(-1/slope)}$.

5.3.6. Acceptance criteria

[Define the acceptance criteria for the assessment]

For dPCR, r^2 must be ≥ 0.95 utilizing a linear curve of the reference control plotting expected values versus observed values. Linear range must include minimum 5 dilutions.

For qPCR also an amplification efficiency of 90-110% is required. R^2 must be ≥ 0.99 utilising a linear curve of the reference control or AAV sample within the linearity range. Linear range must include minimum 5 dilutions.

5.3.7. Range

[Define how the range will be assessed based on the results of different assay characteristics such as the linearity, accuracy, sensitivity and precision assessments]

The range assessment will use the data from the linearity assessment to define the range of assay where accuracy and precision criteria are satisfied.

5.3.8. Range results reporting

[Detail how the results will be reported, any statistical techniques employed]

The results are reported as %bias and %CV for the viral vector genome copies in each of the dilutions from the linearity assessment.

5.3.9. Acceptance criteria

[Define acceptance criteria]

The range (copies/reaction) will be accepted as the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) if the accuracy (% Bias) is ± 20.0% and the %CV is < 15.0% in each linearity and LLOQ sample and the R^2, generated utilizing a linear curve from the reference control within the linearity samples, is ≥ 0.98.

5.3.10. Quantification Limit (LoQ)

[Define Sensitivity and whether the aim is to determine the LoD and/or LoQ - Where appropriate use the definition as provided by relevant guidelines being followed.]

Lower Limit of Quantitation (LLOQ) – The lowest concentration of analyte in a sample that can be quantitatively determined with an acceptable degree of accuracy and precision.

Upper Limit of Quantitation (ULOQ) – The highest concentration of analyte in a sample that can be quantitatively determined with an acceptable degree of accuracy and precision.

5.3.11. LoQ assessment

[Determination of LoD and LoQ, should be performed according to…]

LLOQ will be designated based on the %CV and % bias of the reference control within the LLOQ/LOD samples.

ULOQ will be designated based on the %CV and %bias of the reference control within the ULOQ samples.

5.3.12. Acceptance Criteria

[Define acceptance criteria]

LLOQ - The lowest concentration (copies/reaction) where the %CV of the reference control and target gene is < 15.0%[1] for dPCR

LLOQ - The lowest concentration (copies/reaction) where the % bias of the reference control and target gene is within ± 20.0%[1] for qPCR

ULOQ - The highest concentration (copies/reaction) where the %CV of the reference control and target gene is < 15.0%[1] for dPCR

ULOQ - The highest concentration (copies/reaction) where the % bias of the reference control and target gene is within ± 20.0%[1] for qPCR

[1] Prior to setting any precision criteria in the method, the PCR instrument used should be verified to ensure that the instrument can accurately discriminate at that level *of precision* across the intended quantitative range*. All precision data should use quantitated values, use of Cq values is not acceptable.*

5.4. Accuracy

[Define accuracy – Where appropriate use the definition as provided by relevant guidelines being followed.]

Accuracy expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

5.4.1. Accuracy assessment

[Describe how the accuracy will be assessed, including which attributes or characteristics of the sample are the focal point of assessment. Provide details of reference standards used or how the "true" value is determined if a reference standard is not used. Accuracy will depend on the reference materials used. It is important to take into account the accuracy of the technique that was used to determine the amount of reference material. For dPCR, the dPCR method will often be more accurate than the method used to determine the reference material amount. Hence for dPCR accuracy assessment may not be suitable.]

Establish the ability of the test method to measure the closeness of agreement of reference standard (measured vs. expected values). Accuracy of sample concentrations will be established throughout the linear range of the assay. Measured concentrations will be compared to expected concentrations for each concentration by calculating the % Bias.

5.4.2. Accuracy results reporting

[Describe how the linearity results will be reported and any statistical approaches used]

Measured concentrations of reference standard will be compared to expected concentrations for each concentration by calculating the % Bias.

5.4.3. Acceptance criteria

[Define the acceptance criteria for the assessment]

% Bias is within ± 20.0% for reference control and analysed gene concentrations within the accuracy samples at each copies/reaction concentration in each experiment.

5.5. Precision

[Define Precision - Where appropriate use the definition as provided by relevant guidelines being followed]

Precision can be determined by repeat testing of sample. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision will be evaluated at two levels:

Repeatability or intra-assay precision: the variation between replicates of all dilutions within one assay run or occasion by the same operator.

Inter-assay variability (intermediate precision): Variation within a laboratory to include, tests performed on different days and by a different analyst.

5.5.1. Precision Assessment

[Detail the assessment approach for each of the types of precision being assessed (e.g. repeatability, intermediate precision or reproducibility), how the data will be generated, any statistical techniques employed.]

Intra-assay precision (repeatability) will be calculated as the percent coefficient of variation (%CV) for the viral sample and reference control run in the same experiment.

Inter-assay precision (intermediate precision) will be calculated as:

1. mean %CV on different days generated for the viral sample and reference control assessed at least three independent assay runs performed by one operator on different days,
2. mean %CV between operators generated for the viral sample and reference control assessed for at least three assay runs performed by two operators.

Samples and reference control will be run in triplicate.

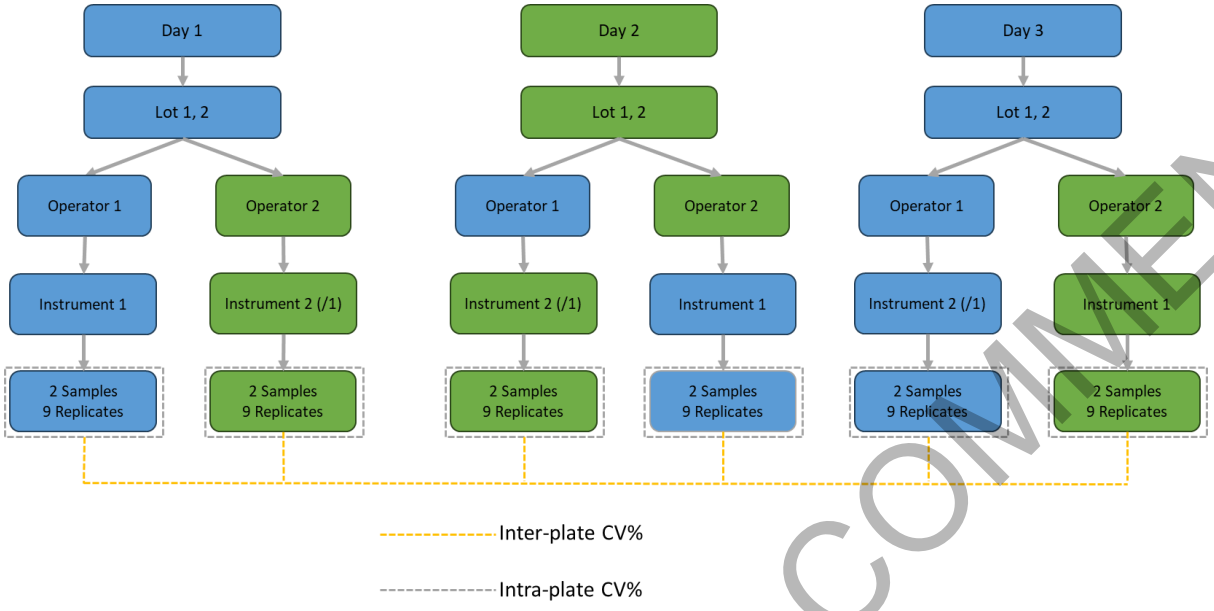


Figure 2: Example of a precision assessment *Yellow dotted lines are linking the samples for iner-plate CV%; Grey dotted lines for intraplate CV.*

5.5.2. Precision results reporting

[Detail how the results will be reported, any statistical techniques employed]

Intra-assay precision (repeatability) will be based on the results for all dilutions from a single run and will be reported as the mean, standard deviation, and coefficient of variance. For dPCR this will be reported as the merged copy number, 95% total confidence intervals (95% CI) and coefficient of variation.

Inter-assay variability (intermediate precision) will be based on:

1. ***Variability on different days* -** will be based on the results from 3 independent runs performed by a single operator on different days and will be reported as the mean, standard deviation, and coefficient of variance.
2. ***Variability as a function of operator/analyst* -** will be based on results from at least 3 runs performed by two operators and will be reported as the mean, standard deviation, and coefficient of variance.

5.5.3. Acceptance criteria

[Define acceptance criteria]

Intra-assay precision: %CV ≤15.0% for the viral sample and reference control tested in triplicate during the same experiment.

Inter-assay precision on different days: %CV ≤15.0% for the viral sample and reference control tested in three different experiments on different days by the same operator.

Inter-assay precision between operators: %CV ≤15.0% for the viral sample and reference control tested in three different experiments by two operators.

6. Reporting results

[Define how the results of the validation protocol will be reported, who has responsibility for producing the report, and how the raw data will be stored or maintained for future reference if needed. It may be valuable to include a summary of all acceptance criteria from the individual sections above as a summary for clarity.]

After implementation of each part of the validation data, it will be reviewed as per SOP-QC-0001, *QC Data Review Procedure*.

Verify that the concentrations and decimal places are correctly stated per Draft STM-00261 for every value.

Record the accuracy to one decimal place.

A summary report will be drafted that includes the following minimum information:

1. Summary of the procedure, as implemented.
2. Summary of equipment, materials and reagents used in the implementation.
3. Results.
4. Any deviations to the protocol or unexpected events, with investigation and impact if required.
5. Statement of method qualification status (pass/fail).

7. References

[Include any references and guidance used in the design of the validation plan and internal SOPs.

The following references were used for the design of this validation plan:

1. XYZ
2. XYZ

Annex 2 - dPCR and qPCR validation report for AAV Genomic titre - worked example

READ THIS STATEMENT FIRST

The worked example for a validation report presented herein, is intended to support an understanding of the practical translation of expectations outlined in regulatory documents and guidelines as presented in the exemplar report structure document. The objective is to assist readers having limited experience of undertaking assay validation activities with the development of their own internal procedures, processes and report styles.

[Black italicised text provides some points for consideration with each section]

Blue text provides an exemplar in the context of a PCR assay aimed at determining AAV genomic titre (Vg/mL)

This document is not intended to present a gold standard to be copied or cloned. It is important readers develop their own protocols that are fit-for-purpose for the assay being developed. Any information and data presented herein is provided as an exemplar to illustrate possible content. It is not necessarily exhaustive of expectations, and where multiple approaches are potentially applicable (e.g. different statistical techniques / approaches) it is the readers responsibility to ensure the correct methods are employed for their specific assay / validation plan design.
Readers with existing report styles for assay validation activities are invited to reflect on the content but encouraged to continue using their own internally approved procedures, processes and documentation styles where they have been shown to be fit-for-purpose already.

Validation report

PCR assay aimed at determining AAV genomic titre

Project code:	
Study title:	
Protocol number:	*XXX-XXXX*
Experimental start date:	*DD-MMM-YYYY*
Experimental end date:	*DD-MMM-YYYY*
Study locations: ***Address:***	
Experimental lead: ***Name:*** ***Signature:***	
Reviewed by: ***Name:*** ***Job title:*** ***Signature:***	
Approved by: ***Name:*** ***Job title:*** ***Signature:***	

Amendments to version X.X of the validation report

The following amendments were made to version X.X of the PCR assay validation report to create this document:

1	***Change***	
	Reason for change	
2	***Change***	
	Reason for change	
3	***Change***	
	Reason for change	

Contents

1 Assay principle

1.1 Background

[This section should briefly describe the background to the assay (e.g. the assay purpose, what is being detected, why and/or how it is being detected); the objective of the assay (e.g. what it needs to demonstrate); any important points for consideration the reader should take into account about the assay; and provide any cross-references to the validation protocol from which the report is based]

This report describes the procedures undertaken to validate the assay used for determination of genomic titre of AAV vectors by qPCR/dPCR at QC laboratory. Viral titre is determined by measuring the concentration of encapsidated AAV-vector genomes that contain the gene of interest. Validation of the method included assessment of accuracy, specificity, precision, linearity and range.

1.2 Assay Objective

[Recap the assay objective from the validation plan / protocol, reconfirming who the assay was performed by and where it was performed.]

This assay directly measures the concentration of encapsidated genomes of AAV vectors, that contain the gene of interest, by qPCR/dPCR. Following Nuclease and Proteinase K treatment of diluted sample to remove non-encapsidated DNA and open the capsids, respectively, the test samples are serially diluted and then mixed with qPCR/dPCR master mix, primers and probes. Dilutions of samples are subjected to qPCR/dPCR and target sequences are amplified on a thermal cycler. By quantifying the amount of amplified DNA, the viral genome content can be estimated.

Assay has been previously qualified which is described in Report 'Qualification of qPCR/dPCR assay for quantification of AAV genomes.

2 Amendments and deviations

[Describe any amendments or deviations to the approved validation plan / protocol on which the report is based and the potential impact of each, specifically noting any specific assay characteristics e.g. linearity, specificity etc. that may be affected.]

During validation no amendments and deviations from the SOP were noted.

3 Materials and methods

[Detail materials and methods used (e.g. a list of reagents and lot numbers to be used / instrument models / serial numbers), where relevant cross-reference to SOPs, the validation protocol or other relevant documents, detail correct version numbers etc. where applicable.]

Description of the methods and materials used are within SOP-XXX vX.X.

A detailed description of validation process is contained protocol XXX-XXXX vX.X "*Validation plan for…*".

The statistical analysis was conducted using XXX software, version Y.Y, following ZZZ methods. Thresholding methods were applied in accordance with the MIQE and DDES reporting guidelines. For validation, AAV lot XYZ samples were utilized.

4 Results

4.1 Assay specificity

4.1.1 Overview

[Provide brief overview of the assay specificity assessment (e.g. this may be through use of the data generated from the linearity assessment), and the concentration range within which this detection should occur, how the data was generated, any statistical techniques employed.]

Specificity was assessed by comparing the number of viral vector titre in vector containing preparations, versus negative specificity controls and negative sample preparation controls that did not contain vector:

1. NSC1: sample buffer with 20 ng (untransfected) host cell DNA (restriction digested for dPCR)
2. NSC2: sample buffer with environmental contaminating DNA.

NSC1 and NSC2 samples were analysed in triplicates (qPCR) or duplicates (dPCR), and specificity was assessed on 3 separate assay occasions by one analyst.

4.1.2 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol. Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

qPCR: Reaction must be negative for at least two replicates of the negative specificity control 1 and 2 (NSC1 and NSC2).

dPCR: Reaction must be negative for at least two replicates of the negative specificity control 1 and 2 (NSC1 and NSC2) and positive for the lowest preparation of the linearity assessment.

dPCR criteria were set as follows: NTC and NSC1/NSC2: target < 2 copies/3 wells

The specificity results are summarised in Table .

Table 1. Specificity Results qPCR

Sample	Assay Occasion 1	Assay Occasion 2	Assay Occasion 3	Pass/Fail
NSC1	3/3 Replicates negative	2/3 Replicates negative	3/3 Replicates negative	Pass

NSC2	2/3 Replicates negative	3/3 Replicates negative	3/3 Replicates negative	Pass

Table 2. Specificity Results dPCR

Sample	Pos partitions (2 wells)			Copies measured (2 wells)			Criterium	Pass/Fail
Experiment	1	2	3	1	2	3		
NSC1	0	2	1	0	2.4	0	<3	Pass
NSC2	1	0	0	1.2	0	0	<3	Pass

4.1.3 Summary

The assay demonstrates an acceptable level of specificity for assessment of AAV viral titre.

4.2 Assay range

4.2.1 Assay linearity and range

4.2.2 Overview

[Provide brief overview of the assay linearity and range assessment method, this could be paraphrased from the protocol document, include information on how dilution curves were set up including the calculations, the plate layout for the assay, any controls etc. Furthermore, describe how many times the procedure was repeated and by how many operators in order to support assessment of other assay characteristics].

4.2.2.1 qPCR linearity assessment

Linearity was assessed by measuring a 1:10 dilution series generated by at least two different operators on two different days. Sample used in the assessment was AAV lot XYZ.

For each dilution series, at least three replicates (DNAse-independent) were included. For each run linearity as R^2, slope/efficiency and y-intercept were reported.

Table 5. presents the dilution series used in the linearity assessment.

Table 5. Serial dilution scheme used in the linearity assessment.

Dilution	Mix	Target copies/µL
Dil 1	DNAse treated sample	$3x10^8$
Dil 2	180 µL buffer + 20 µL Dil 1	$3x10^7$
Dil 3	180 µL buffer + 20 µL Dil 2	$3x10^6$
Dil 4	180 µL buffer + 20 µL Dil 3	$3x10^5$
Dil 5	180 µL buffer + 20 µL Dil 4	$3x10^4$
Dil 6	180 µL buffer + 20 µL Dil 5	$3x10^3$
Dil 7	180 µL buffer + 20 µL Dil 6	$3x10^2$
Dil 8	180 µL buffer + 20 µL Dil 7	30

4.2.2.2 *dPCR linearity assessment*

Linearity was assessed by measuring a dilution series generated by at least 2 different operators during 2 different days. Sample used in the assessment was AAV lot XYZ.

For each dilution series, at least 3 replicates (DNAse-independent) were included. For each run linearity as R^2 was reported. Table 6 presents dPCR dilution series used in the linearity assessment.

Table 6. Serial dilution scheme used in the linearity assessment.

Dilution	Mix	Target copies/µL (assuming 1 µL will be measured in the final reaction)*
Dil 1	DNAse treated sample	100 000
Dil 2	160 µL buffer + 40 µL Dil 1	20 000
Dil 3	160 µL buffer + 40 µL Dil 2	4000
Dil 4	160 µL buffer + 40 µL Dil 3	800
Dil 5	160 µL buffer + 40 µL Dil 4	160
Dil 6	160 µL buffer + 40 µL Dil 5	32
Dil 7	160 µL buffer + 40 µL Dil 6	6.4
Dil 8	160 µL buffer + 40 µL Dil 7	1.28

*take into account the measured volume. For a system with 50% dead volume, add for example 2 instead of 1 µL to the final reaction.

4.2.3 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

For qPCR the following acceptance criteria must be met:

1. An amplification efficiency of 90-110% is required for each dilution.

2. R^2 must be ≥ 0.98 utilising a linear curve of the reference control or AAV sample within the linearity range.
3. Linear range must include a minimum of five dilutions.

For dPCR, the following acceptance criteria must be met:

1. R^2 must be ≥ 0.95 utilising a linear curve of the reference control plotting expected values versus observed values.
2. Linear range must include a minimum of five dilutions.

4.2.4 Results for linearity and range

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

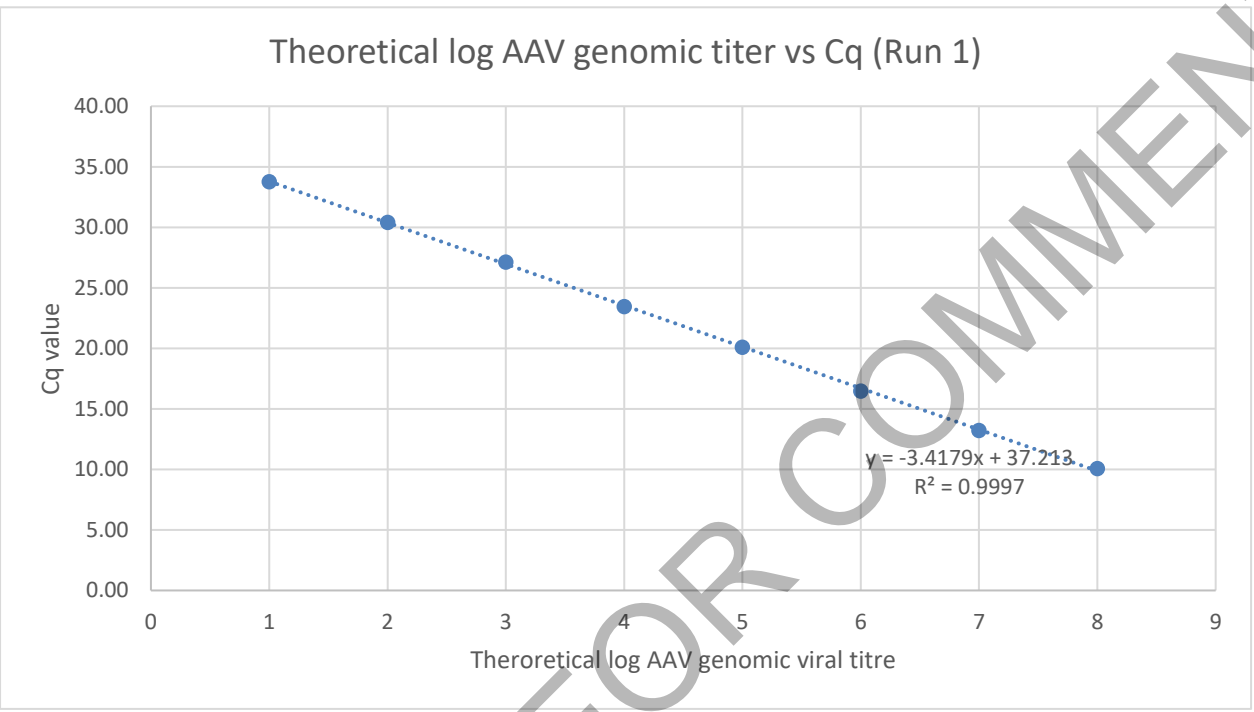
qPCR linearity assessment

Linear regression analysis was performed by plotting the logarithmic value of theoretical AAV titer and Cq value to determine R^2 value (Figure 1 and 2). Slope, intercept and efficiency were calculated for each for the runs (Table 7). From the slope, the amplification efficiency was calculated using the formula = $-1+10^{(-1/slope)}$.

Table 7. Results of the linearity assessment.

Run No.	Instrument	Analyst	Sample	Slope	Intercept	Efficiency	R^2	Pass/ Fail
						Acceptance criterion 90-110%	Acceptance criterion >0.98	
1	1	1	AAV lot XYZ	-3,4179	37,213	99%	0.9997	Pass
2	1	2	AAV lot XYZ	-3,4179	37,213	99%	0.9997	Pass

Figure 1. Example of a linear regression plot for Run 1



dPCR linearity assessment

Linear regression analysis was performed by plotting the logarithmic values of theoretical sample quantities per reaction against measured sample quantities per reaction to determine R^2 value (Figure 2 and Table 8).

Table 8. Results of the linearity assessment.

Run No.	Instrument	Analyst	Sample	R^2	Pass/ Fail
				Acceptance criterion >0.98	
1	1	1	AAV lot XYZ	0.9997	Pass

2	1	2	AAV lot XYZ	0.999	Pass

Figure 2. Example of a measured vs expected viral copies plot for Run 1

4.2.5 Summary

[State any final conclusions about linearity results]

The acceptance criteria were met and assay demonstrated an acceptable level of linearity.

4.3 Assay accuracy

4.3.1 dPCR and qPCR Accuracy Assessment

Accuracy was assessed by analysing the dilutions used from the linearity assessment. The samples used to create the dilutions were from AAV material of known concentration (as assessed by a separate technology with equivalent performance to dPCR). The acceptable accuracy for each dilution point was established to be within 20% of the expected concentration.

4.3.2 Accuracy Acceptance Criteria

For both dPCR and qPCR the measured concentrations of each dilution of the AAV sample must be within ± 20% of the expected concentration.

4.3.3 Accuracy Results

Table 3. qPCR Accuracy Results.

Dilution	Known Target copies/µL	Measured Target copies/µL	% difference
Dil 1	$3x10^8$	$3.15x10^8$	5.0
Dil 2	$3x10^7$	$2.97x10^7$	1.1
Dil 3	$3x10^6$	$3.03x10^6$	1.1
Dil 4	$3x10^5$	$2.99x10^5$	0.5
Dil 5	$3x10^4$	$3.0x10^4$	0
Dil 6	$3x10^3$	$3.1x10^3$	3
Dil 7	$3x10^2$	$2.9x10^2$	0.9
Dil 8	30	34	13

Table 4. dPCR Accuracy Results.

Dilution	Known Target copies/µL	Measured Target copies/µL	% difference
Dil 1	100 000	103 205	3.2
Dil 2	20 000	20240	1.2
Dil 3	4000	3968	0.2
Dil 4	800	807	0.9
Dil 5	160	151.8	5.1
Dil 6	32	33	4.6
Dil 7	6.4	5.7	11.0
Dil 8	1.28	1.5	15.2

4.3.4 Accuracy Summary

All samples fell within the required stated accuracy acceptance limits of ± 20% of the expected concentration.

4.4 Assay precision

4.4.1 Overview

[Provide brief overview of the assay precision assessment method for each of the types of precision being assessed (e.g. intra-assay, inter-assay / intermediate precision / reproducibility), how the data was generated, any statistical techniques employed.]

Precision was validated at two levels:

1. Intra-assay precision (repeatability) was calculated as the percent coefficient of variation (%CV) for the viral sample and reference control run in the same experiment.
2. Inter-assay precision (intermediate precision) was calculated as:
    a. mean %CV on different days generated for *the viral sample and reference control assessed in* at least three independent assay runs performed by one operator on different days,
    b. mean %CV between operators generated for the viral sample and reference control assessed for at least three assay runs performed by two operators.

Samples and reference control were run in triplicate.

4.4.2 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

For each of the precision assessments the assay will be considered precise if the CV ≤15% (Table 9).

Table 9. Precision acceptance criteria.

Assay Parameter	Acceptance criteria
%CV	≤15%

4.4.3 Intra-assay precision results

[Report the results, making it clear how the assay performed against the acceptance criteria and if it is considered to have passed or failed. Data obtained from qPCR would follow a similar format.]

Intra-assay precision was determined using dilutions outlined in the linearity study – which fall within the genomic range (Table 2) - for AAV genomic titers from the reference control.

For dPCR, the results are reported as the merged copies (per well or per µL), SD and %CV of the triplicate samples for each dilution (Table X). For qPCR the mean copies, SD and %CV are reported.

The repeatability is assessed by measuring the mean of the CV value at each dilution used. For the AAV sample 2 dilutions – within the linear range - were taken into account.

Table 10. Intra-assay precision results (dPCR example).

Dilution	AAV reference			AAV sample lot XYZ		
	Copies(well)	SD	%CV	Copies (well)	SD	%CV
1	110000	4400	4.0	95840	3162.72	3.3
2	23250	767.25	3.3	/	/	/
3	4658	237.558	5.1	/	/	/
4	883	65.342	7.4	769	76.9	10.0
5	180	16.74	9.3	/	/	/
Acceptance criteria (Mean CV)*	≤ 15%			≤ 15%		
Result (Mean CV)*	5.82%			6.12%		
Pass/Fail	Pass			Pass		

*Mean %CV = $100*(\text{mean } CV^2)^{1/2}$

Summary

The assay demonstrates an acceptable levels of intra-assay precision across the 5 highest dilutions of the qPCR/ dPCR data.

4.4.4 Inter-assay variability / intermediate precision

[Report the results, making it clear how the assay performed against the acceptance criteria and if it is considered to have passed or failed.]

4.4.4.1 Variability across different days

Inter-assay variability was determined using dilution 1 and 4 (Table 11) from 3 independent assay runs performed by a single operator over 2 days. Each dilution was run in triplicate and the reported results are the mean, SD and percentage CV of all nine replicates (triplicate samples from each of four assay runs).

The results are reported as in Table 11:

Table 11. Inter-assay variability of genome titer measurements (dPCR example).

Dilution	Reference sample			Sample lot XYZ		
	Mean	SD	%CV	Mean	SD	%CV
1	110000	4400	4.0	95840	3162.72	3.3
4	883	65.342	7.4	769	76.9	10.0
Acceptance criteria (CV)	≤ 15%			≤ 15%		
Result (CV)	5.8%			6.3%		
Pass/Fail	Pass			Pass		

4.4.4.2 Variability as a function of operator/analyst

Intermediate precision was determined using dilution 1 and 4 (Table 12) from six independent assay runs performed by two operators over 2 days. Each dilution was run in triplicate and the reported results are the mean, standard deviation, and percentage CV of all 18 replicates (triplicate samples from each of six assay runs).

Table 12. Inter-operator variability of genome titer measurements (dPCR example).

Dilution	Reference sample			Sample lot XYZ		
	Mean	SD	%CV	Mean	SD	%CV
1	110000	4400	4.0	95840	3162.72	3.3
4	883	65.342	7.4	769	76.9	10.0
Acceptance criteria (CV)	≤15%			≤15%		
Result (CV)	5.8%			6.3%		
Pass/Fail	Pass			Pass		

4.4.5 Summary

The assay demonstrates an acceptable levels of inter-assay precision (operators/days) of qPCR/dPCR assay.

5 Conclusion

[Present the overall conclusion for the study, summarising those characteristics of the assay validation protocol that passed and those that failed. Provide an overall statement as to whether the assay is fit-for-purpose based on the outcome of the results.]

Assay validation met the predefined acceptance criteria and has been successfully validated for GMP use.

Attribute	**Acceptance criteria**	**Results**	**Pass/ Fail**
Specificity	Reaction must be negative for at least two replicates of the negative specificity control 1 and 2 (NSC1 and NSC2).	Reaction was negative for at least two replicates of the negative specificity control 1 and 2 (NSC1 and NSC2) at 3 assay runs.	Pass
Range LLOQ ULOQ	The LLOQ and ULOQ are the highest and lowest AAV titer in the sample which can be measured with acceptable level of precision (CV% ≤ 15%) and accuracy (% bias = ± 20.0%).	Validated range of the assay is 30 – 3,23x10^8 copies/uL, with LLOQ being 30 copies/uL (CV% ≤12%, bias% ≤ +3,33%) and ULOQ being 3,23x10^8 copies/uL (CV% ≤7%, bias% ≤ +7,62%).	Pass
Linearity	-Amplification efficiency 90-110% -R^2 ≥ 0.98 for min. 5 dilutions	Run 1: -Amplification efficiency 99% -R^2 = 0.9997 Run 2: -Amplification efficiency 99% -R^2 = 0.9997	Pass
Accuracy	Measured concentrations of each dilution of the AAV sample must be within ± 20% of the expected concentration.	Results of 8 dilutions of the tested sample were within +13% of the expected concentration.	Pass
Precision - Repeatability	CV ≤ 15%	CV≤ 6.12% across 2 runs	Pass
Precision – Intermediate Precision	CV ≤ 15%	CV≤ 6.3% across 3 runs over 2 days by 1 operator	Pass

		CV≤ 6.3% across 6 runs over 2 days by 2 operators	
Robustness	Assessed during qualification of the method		

Table 13. Summary of the validation acceptance criteria (qPCR example)

The method has undergone successful validation and is now approved for implementation in a GMP setting.

6 Appendices

[Include any relevant supplementary data to support that presented in the main body of the report e.g. this may include individual Cq values for each qPCR experiment, or individual cps/reaction values for dPCR experiment]

Annex 3 - dPCR and qPCR validation protocol for LV VCN - worked example

READ THIS STATEMENT FIRST

The worked example for a validation protocol presented herein, is intended to support an understanding of the practical translation of expectations outlined in regulatory documents and guidelines. The objective is to assist readers having limited experience of undertaking assay validation activities with the development of their own internal procedures, processes, and protocol styles.

[Black italicised text provides some points for consideration with each section]

Blue text provides an exemplar in the context of a PCR assay aimed at determining LV Vector copy number or the number of pro-viral DNA copies per host cell genome (copies/cell)

This document is not intended to present a gold standard to be copied or cloned. It is important readers develop their own protocols that are fit-for-purpose for the assay being developed. Any information and data presented herein is provided as an exemplar to illustrate possible content. It is not necessarily exhaustive of expectations, and where multiple approaches are potentially applicable (e.g. different statistical techniques / approaches) it is the readers responsibility to ensure the correct methods are employed for their specific assay / validation plan design.

Readers with existing procedures and processes for assay validation activities are invited to reflect on the content but encouraged to continue using their own internally approved procedures, processes and documentation styles where they have been shown to be fit-for-purpose already.

Validation protocol

Validation plan for a PCR assay aimed at determining LV Vector copy number

Project code:	***XXXX***
Study title:	*Validation plan for a PCR assay aimed at determining LV Vector Copy Number*
Protocol number:	*XXX-XXXX*
Experimental start date:	*DD-MMM-YYYY*
Experimental end date:	*DD-MMM-YYYY*
Study locations: ***Address:***	
Experimental lead: ***Name:*** ***Signature:***	
Reviewed by: ***Name:*** ***Job Title:*** ***Signature:***	
Approved by: ***Name:*** ***Job title:*** ***Signature:***	

Amendments to version X.X of the validation plan

The following amendments were made to version X.X of the PCR assay validation plan to create this document:

1	***Change***	
	Reason for change	
2	***Change***	
	Reason for change	
3	***Change***	
	Reason for change	

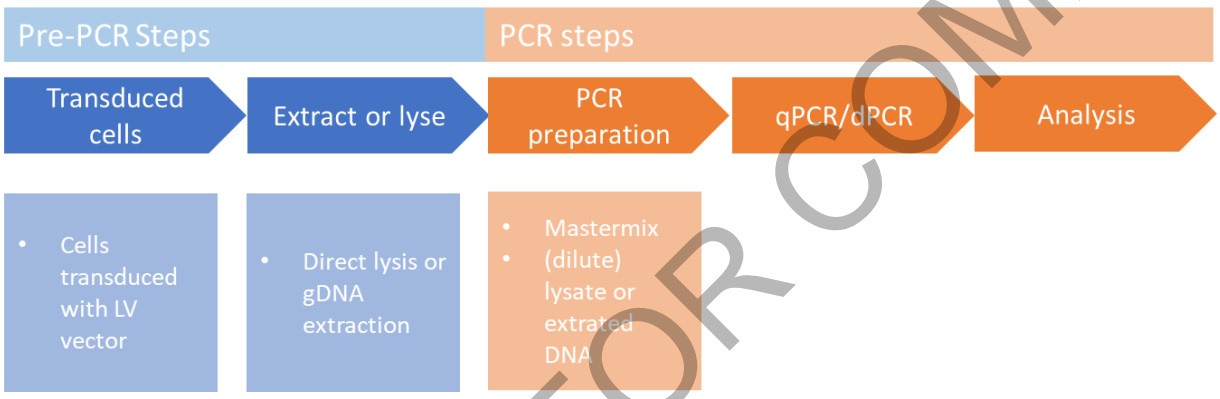
All changes were made at the beginning of validation activities.

Contents

1. Assay principle

[Describe in relevant sub-sections the principles underlying the assay validation. For example, the background to the assay (e.g. the assay purpose, what is being detected, why and/or how it is being detected); the objective of the assay (e.g. what it needs to demonstrate); the sources samples for performing the validation; and/or any other relevant considerations]

This assay is used for determination of LV Vector Copy Number (VCN) by qPCR/ dPCR at QC laboratory.



1.1. Background

[Points to consider for inclusion in the background sub-section include sample source (e.g. clinical or manufacturing); the purpose of the assay (e.g. starting material characterisation, final product release testing or in-process controls testing to determine viral titre.)]

The validation plan will describe the procedures necessary to validate a qPCR or dPCR assay to determine the LV Vector Copy Number of final product samples. VCN will be determined by measuring the pro-viral DNA copies, containing the gene of interest, per host cell genome.

This assay measures the concentration of pro-viral genomes that contain the gene of interest, normalised for a reference gene. This normalization will take into account the ploidy of the transduced cell type. Genomic DNA (gDNA) will be extracted and purified from transduced cells. Samples will be diluted to a suitable concentration and then mixed with q/dPCR mastermix. Target sequences will be amplified on a thermal cycler. The amount of amplified target and reference DNA will be quantified, and the estimated Vector Copy Number will be calculated.

1.2. Objective

[Points to consider for inclusion in the objective sub-section include what the objective of the validation.]

The objective of this protocol is to validate analytical method SOP-X123, 'Determination of LVV VCN by qPCR or dPCR' used for determination of pro-viral DNA copies, containing the gene of interest, per host cell genome via qPCR or dPCR using sequence specific primers and probe. This assessment will include the following ICH Q2 parameters: accuracy, specificity, precision, linearity and range. Parameter values should be determined during the development phase, as per guidance set out in ICH Q14, ideally with use of an Analytical

Target Profile (ATP) to ensure the method is fit for purpose, according to project requirements rather than simply applying method capabilities.

1.3. Scope

[Points to consider for the scope of the validation.]

The scope includes validation of the analytical method SOP-X123, 'Determination of LVV VCN by qPCR or dPCR' used for batch release and stability testing of [name] AAV drug product.

1.4. Responsibilities

[Define the roles and responsibilities of each team or department involved in the validation process.]

Role	Responsibility

2. ICH classification of assay

[Points to consider for inclusion in this sub-section of the document include reference to any guidance documents used to support the validation protocol generation (e.g. ICH Q2(R1)) and if aspects of the guidance are not being followed then an explanation for any deviation must be recorded. For example, the intention may be to only validate certain assay characteristics (e.g. accuracy or linearity etc. at this point in development. It may also be pertinent to state the regulatory status of the assay.]

The validation of the assay will be carried out according to ICH guidelines for the Validation of Analytical Procedures (ICH Q2) with respect to those assay performance characteristics detailed in Table 1.

Table 1. ICH recommendations for the characteristics to be assessed during validation of an analytical assay/procedure.

Performance characteristics	Functional Assay Validation requirements
Specificity	Yes
Range -Linearity	Yes
Accuracy	Yes
Precision - Repeatability - Intermediate Precision	 Yes Yes

Detection limit	For Information Only
Quantitative limit	For Information Only
Robustness	Yes

Robustness of the assay has been previously assessed and is documented in Report 'Qualification of qPCR assay for quantification of LVV VCN'.

3. Amendment and Deviation Reporting

[Points to consider for inclusion in this sub-section include how teams should manage and report deviations from the protocol, including deviations from pre-set acceptance criteria. Depending on the Quality system in place where the assay is being performed, reference to the related quality management procedures may be included.]

Any amendments / deviations to the protocol that have the potential to impact the integrity of the study should be reported (this should include an assessment of the impact of the deviation to the study).

4. Materials and Methods

4.1. PCR Facilities

[Describe where the assay validation will be performed and characteristics of the PCR facility. Also describe controls used to prevent contamination between labs, such as dedicated lab coats/gloves within each lab, and wiping down of surfaces with nucleic acid degrading solution.]

The assay will be performed in the following locations:

1. PCR clean lab G10, to prepare the master mix.
2. PCR preparation lab G11, to prepare positive control and samples.
3. Pre-PCR lab G12, to pre-dilute the positive control.
4. PCR lab G13, to run the PCR reaction.

Separate lab coats will be used in each lab, with lab coats and gloves changed before moving from one lab to another. Surfaces will be wiped down with DNase Away before and after use.

4.2. Sample sources

[Points to consider for inclusion in the sample source sub-section include a description of samples used in the assay validation and how they will be prepared for the protocol. Also, if sample preparation for assay validation will vary compared to the actual samples analysed this should be described.]

Two types of samples will be used in this validation. First consist of linearized plasmid DNA containing the LVV sequence and human DNA containing the endogenous human sequence. The second sample type includes cell-based samples – healthy donor cells transduced with LVV with a qualified VCN range.

4.3. Controls

[Points to consider for inclusion in this sub-section include a description of the controls (e.g. negative and specificity controls or quality controls) that will be used to support validation of the assay].

Controls described in Table 2 will be used in this validation study. Additional controls for consideration are described in Table 3.

Table 2. Controls to be considered in LV VCN validation study.

Control Type	Description	dPCR	qPCR
No-Template Control (NTC)	Buffer used for the process or water at PCR setup	☑	☑
Negative Specificity Control 1 (NSC1)	Non-transduced cell line.	☑	☑
Negative Specificity Control2 (NSC2)	Similar LV constructs that should not be detected.	☑ if suitable	☑ if suitable
Reference Control 1	Cell line spiked with known concentration of linearized plasmid.	☑ *	☑
Reference Control 2	Cell line transduced with LV at a known copy number (WHO controls).	☑ *	☑
Positive Control 1	Previously qualified LV transduced cell line at high VCN (10).	☑**	☑
Positive Control 2	Previously qualified susceptible cell DNA at VCN of 1.	☑**	☑

***** For dPCR, this control will be used to generate the data on the dynamic range and linearity. However, the concentration from the dPCR data (and not from other characterizations) will be used to generate the data. Prequalification of material is not necessary here.

** For dPCR, this control will not be used for calculations. It can be used to assess ease thresholding between positive and negative partitions. The high positive control should only be considered if relevant and should be compatible with the dynamic range.

Table 3. Additional controls for consideration.

Control Type	Description	dPCR	qPCR
Extraction/Purification Control	Target DNA or reference sample, of known value, spiked at the start of sample preparation	☑*If applicable	☑If applicable
Spike Control	Target DNA spiked in at known copies to assess for matrix effects	☑*Optional	☑Optional

*Compare in the same experiment, not compared to known concentration.

For LV based VCN methods the above controls should be considered for each of the two PCR reactions involved (LV/transgene and the susceptible host cells).

4.4. Reagents and Equipment

[List all the reagents required in relevant sub-sections, including variables such as reagent name, supplying company, catalogue numbers etc. The equipment used should also be detailed. Table 4 contains examples of reagents used in qPCR validation and Table 5 reagents used in dPCR validation study.]

4.5. Reagents and buffers

Reagents will be used and prepared as per analytical method SOP-X123, 'Determination of LVV VCN by qPCR or dPCR'.

Table 4. Reagents required for a qPCR validation study

Reagent	Company	Catalogue Number
Target PCR Primers	Company A	XX-XXX1
Target PCR Probe	Company A	XX-XXX2
Reference gene Primers	Company A	XX-XXX3
Reference gene Probe	Company A	XX-XXX4
qPCR Mastermix	Company B	XX-XXX5
Molecular Grade Water	Company C	XX-XXX6
BSA (0.4%) (Optional)	Company D	XX-XXX7
Template DNA/gDNA	In-house	N/A

The template DNA/gDNA could be prepared from transduced cells with a known transduction efficiency and was prepared in a dilution series to generate a standard curve:

1. Undiluted transduced cells (typically standardised to 100ng/µl)
2. 1:10 dilution of transduced cells (diluted in a pool of normal DNA to ensure overall DNA concentration remains stable)
3. 1:100 dilution as above
4. 1:1000 dilution as above
5. 1:10,000 dilution as above
6. 1:100,000 dilution as above

A no-template control will be included to assess contamination

Ideally, DNA/cDNA from non-transduced cells should be included as a control

Alternatively, serially diluted standards could be prepared from untransduced cells with a 1:5 spiked in dilution series of a gblock or linearized plasmid (cells at 20 ng/µL, see linearity section).

Table 5. Reagents required for a dPCR validation study

Reagent	Company	Catalogue Number
Target PCR Primers	Company A	XX-XXX9
Target PCR Probe	Company A	XX-XXX10
Reference gene Primers	Company A	XX-XXX11
Reference gene Probe	Company A	XX-XXX12
dPCR Mastermix	Company B	XX-XXX13
Molecular Grade Water	Company C	XX-XXX14
Restriction enzyme*	Company D	XX-XXX15
Template DNA/gDNA	In-house	N/A
If applicable: oils	Company E	XX-XXX16

Whilst a standard curve is not required for dPCR, it is advisable to assess linearity of the assay performance by running serially diluted transduced cells as described for qPCR.

* A common cutting restriction enzyme is strongly recommended for copy number assays to be able to separate tandem copies.

The reference DNA to assess the linearity and dynamic range could be prepared from untransduced cells with a 1:5 spiked in dilution series of a gblock or linearized plasmid (cells at fe 20 ng/µL, see linearity section).

4.6. Equipment list

[Describe what equipment is used in this validation. Table 6 contains examples of equipment used in qPCR validation and Table 7 contains equipment used in dPCR validation study.]

Table 6. Equipment required for a qPCR study

Equipment	Company	Serial Number
(Automatic) pipettes	Company E	X-ABC1
Real-time PCR system	Company F	X-ABC2
Plate centrifuge	Company G	X-ABC3
UV Hood	Company H	X-ABC4

Table 7. Equipment required for a dPCR study

Equipment	Company	Serial Number
(Automatic) pipettes	Company E	X-ABC1
Droplet Generator or Partitioning Device (optional)	Company F	X-ABC2
Plate centrifuge	Company G	X-ABC3
UV Hood	Company H	X-ABC4
Plate sealer (optional)	Company F	X-ABC5
Thermal cycler*	Company F	X-ABC6
Plate reader*	Company F	X-ABC7

*Some suppliers have these 2 items combined as a single platform

4.7. Plasticware

[Relevant plasticware used in the experiment. The use of DNA / protein resistant binding plastic consumables should be considered.]

Table 8. Equipment required for a dPCR or qPCR study

Plasticware	Company	Serial Number
(Automatic) pipette tips	Company I	X-ABC1
PCR plates / cartridges	Company J	X-ABC2
Microcentrifuge tubes	Company K	X-ABC3
Plate seals	Company L	X-ABC4

5. Procedure

[Describe in relevant subsections the individual procedures being followed to evaluate the different assay performance characteristics. Where assay performance characteristics are not being evaluated, provide justification.]

5.1. General Instruction

All instruments/equipment that generate data used in this qualification will be within calibration and maintenance period and have been qualified (i.e., IQ/OQ/PQ).

All experiments must satisfy the method system suitability criteria. Any analysis for which the results do not meet system suitability must be repeated as specified in the method. Experiments failing method system suitability criteria will be discussed between QC and QA representatives and determine if repeat is needed, justification or other options.

For dPCR, separation of positive and negative populations (Bright to Dim (BTD) ratio or peak resolution) should be optimized in the optimization phase. Strategic use of reference enzymes will be important to separate tandem copies and can also minimize rain. A minimum of 10 000 partitions will be recommended according to dMIQE guidelines. Upper limit of detection should be carefully considered to allow accurate quantification.

The following assay performance criteria will be measured in this validation study:

5.2. Specificity

[Define Specificity in the context of the assay being performed - Where appropriate use the definition as provided by relevant guidelines being followed]

The specificity of a method is its ability to accurately and specifically detect the analyte in the presence of components that are expected to be present in the sample matrix.

5.2.1. Specificity assessment

[Define how the specificity assessment will be performed (e.g. this may be through use of the data generated from the linearity assessment), describe the specific type of to be detected, and the concentration range within which this detection should occur.]

Specificity will be assessed by comparing the VCN of lowest samples of the linearity curve with viral vector/spike-in (RC or PC) versus samples containing non-transduced/non-spiked in cells (NSC1).If appropriate and suitable, specificity can also be assessed by comparing the VCN of samples with a related LV construct (NSC2) that should not be detected, to a non-transduced cell line (NSC1).

Samples will be analysed in triplicates. Data can come from the linearity assessment.

5.2.2. Specificity results reporting

[Define how specificity will be reported and any statistical techniques employed]

Specificity will be reported as the VCN/ genome for the NSC.

5.2.3. Acceptance criteria

[Define the acceptance criteria used to assess specificity]

The specificity of the assay is confirmed when the lowest VCN of the linearity assessment is significantly different from non-transduced cells at the same concentration.

For qPCR:

1. NTC must not exhibit amplification of LVV target sequence and human genomic target sequence in at least 3/3 wells.
2. Non-transduced samples (NSC1) must not exhibit amplification of LVV target sequence in at least 3/3 wells and must exhibit amplification of human genomic target sequence in 3/3 wells.
3. Transduced samples (PC or RC) must exhibit amplification of LVV target sequence in 3/3 wells and must exhibit amplification of human genomic target sequence in 3/3 wells.

For dPCR (example):

1. NTC:
    a. LVV target <2 copies/3 wells
    b. reference gene: <2 copies/3wells
2. Non-transduced samples (NSC1):
    a. LVV target sequence: <2 copies/3 wells
    b. Reference target sequence: ~copy number as PC/RC
3. Transduced samples (PC/RC):
    a. LVV target: ≥ 2 copies/3wells
    b. Reference target sequence: ~ copy number as NSC1

5.3. Range

5.3.1. Linearity

[Define Linearity – Where appropriate use the definition as provided by relevant guidelines being followed]

The definition of linearity is the ability to elicit test results that are directly, or by defined mathematical transformation, proportional to the concentration of analyte in the samples within a given range.

5.3.2. Linearity assessment

[Describe how the linearity will be assessed, including which attributes or characteristics of the sample are the focal point of assessment.

Considerations for:

1. *qPCR: theoretically a 10-log linearity could be reached*
2. *dPCR: the theoretical dynamic range will depend on the number of partitions. Systems with ~20 000 partitions will generate a theoretical dynamic range of 5 log. For systems with <10 000 partitions this becomes 4 log.*

Section 5.3.1.1 describes approach used for qPCR linearity assessment and Section 5.3.1.2. for dPCR linearity assessment.]

Linearity will be assessed by analysing a dilution series of target linearized plasmid DNA in a constant non-transduced cellular background of human genomic DNA.

Alternatively, dilution series of transduced cells diluted into a non-transduced cell population can be used.

Linearity and standard curves serve a different purpose in qPCR and dPCR. In qPCR the VCN calculation will depend on the standard curve, in dPCR this is not the case.

Ideally, also a dilution series of gDNA in a fixed concentration of the target sequence is made to account for possible variations.

5.3.3. qPCR linearity assessment

Linearity will be assessed by analysing a 1:10 dilution series of samples containing target linearised plasmid DNA in a constant non-transduced cellular background of human genomic DNA diluted in resuspension buffer, starting at $1x10^8$ – $1x10^{10}$ copies per reaction.

A negative specificity control will also be assessed, using non-transduced cells.

An example of a 10-fold dilution series is given in Table 9.

Table 9. 10-fold dilution series used in qPCR linearity assessment.

Dilution	Mix	Target copies/µL (assuming 1 µL will be measured in the final reaction)*
Dil 1	Sample	$1x10^8$
Dil 2	180 µL buffer + 20 µL Dil 1	$1x10^7$
Dil 3	180 µL buffer + 20 µL Dil 2	$1x10^6$
Dil 4	180 µL buffer + 20 µL Dil 3	$1x10^5$
Dil 5	180 µL buffer + 20 µL Dil 4	$1x10^4$
Dil 6	180 µL buffer + 20 µL Dil 5	$1x10^3$
Dil 7	180 µL buffer + 20 µL Dil 6	$1x10^2$
Dil 8	180 µL buffer + 20 µL Dil 7	10
Dil 9	180 µL buffer + 20 µL Dil 8	1

The dilution series will be prepared and measured by at least 2 operators.

The dilution series will be repeated on different days.

If available, the dilution series will be measured on different equipment.

5.3.4. dPCR linearity assessment

20 ng of cellular dDNA will be used as constant background

A 1:5 dilution series of the target DNA will be prepared and spiked into 20 ng of non-transduced cellular gDNA (~6060 copies for a diploid cell line) (Table 10). (If more appropriate, a smaller dilution series could be envisaged.)

20 ng gDNA from a non-transduced cells will be used as negative specificity control.

Table 10. dPCR dilution series

Dilution	**Mix**	**Estimated target copies/µL (assuming 1 µL will be measured in the final reaction)**	**Add gDNA for final 20 ng (µL added)**
Dil 1	DNAse treated sample	100 000*	
Dil 2	160 µL buffer + 40 µL Dil 1	20 000	
Dil 3	160 µL buffer + 40 µL Dil 2	4000	

Dil 4	160 µL buffer + 40 µL Dil 3	800	
Dil 5	160 µL buffer + 40 µL Dil 4	160	
Dil 6	160 µL buffer + 40 µL Dil 5	32	
Dil 7	160 µL buffer + 40 µL Dil 6	6.4	
Dil 8	160 µL buffer + 40 µL Dil 7	1.28 (theoretically too low for single well)	

* Could be necessary to adapt the highest copy number due to the system, assay or over/underestimation of the amount of DNA copies. A pilot experiment to quantify and estimate the linearized plasmid is recommended.

Figure 1 below illustrates the plate layout for the linearity study, black numbers show the amount of estimated target copies per well, and red numbers show the estimated amount (ng/copies) of cells per well. Estimated VCN is in green. This is only an estimation. Actual numbers will come from dPCR.

Dil1 100 000 20 ng/6060 33	Dil 1 100 000 20 ng/6060 33	Dil1 100 000 20 ng/6060 33	NSC1 0 20 ng/6060 0	NSC1 0 20 ng/6060 0	NSC1 0 20 ng/6060 0
Dil 2 20 000 20 ng/6060 6.6	Dil 2 20 000 20 ng/6060 6.6	Dil 2 20 000 20 ng/6060 6.6	NTC 0 0 0	NTC 0 0 0	NTC 0 0 0
Dil 3 4000 20 ng/6060 1.3	Dil 3 4000 20 ng/6060 1.3	Dil 3 4000 20 ng/6060 1.3			
Dil 4 800 20 ng/6060 0.26	Dil 4 800 20 ng/6060 0.26	Dil 4 800 20 ng/6060 0.26			
Dil 5 160 20 ng/6060 0.05	Dil 5 160 20 ng/6060 0.05	Dil 5 160 20 ng/6060 0.05			
Dil 6 32 20 ng/6060 0.01	Dil 6 32 20 ng/6060 0.01	Dil 6 32 20 ng/6060 0.01			
Dil 7 6.4 20 ng/6060 0.0025	Dil 7 6.4 20 ng/6060 0.0025	Dil 7 6.4 20 ng/6060 0.0025			
Dil 8 1.28 20 ng/6060 0.0004	Dil 8 1.28 20 ng/6060 0.0004	Dil 8 1.28 20 ng/6060 0.0004			

Figure 1. Example plate layout for the linearity study.

If appropriate, similar dilution series with higher and lower concentrations of non-transduced DNA can be taken along in the validation. Considerations are:

1. Upper limit of detection for dPCR. A diploid cell line at 150 ng (~45 000 copies) will maximum allow you a theoretical copy number of 5 copies/cell with a 20K partition system (VCN = target copies/reference copies *2).
2. Lower limit of detection for dPCR and qPCR. A diploid cell line at 1 ng (~300 copies) will allow you a theoretical copy number of 0.02 copies/cell irrespective of the system (VCN = target copies/reference copies *2).

The dilution series will be prepared and measured by at least a 2nd operator.

The dilution series will be prepared repeated on different days.

If available, the dilution series could be measured on different equipment.

5.3.5. Linearity results reporting

[Describe how the linearity results will be reported and any statistical approaches used]

Linearity will be reported as

Correlation coefficient of the regression line plotted for:

1. VCN or Vector Copy Number ((Target copies /Reference gene copies) * 2) at a constant concentration of gDNA
2. Slope, intercept and for qPCR efficiency should be calculated

Of note: the factor to calculate the Vector Copy Number will depend on the ploidy of the cells

5.3.6. Acceptance criteria

[Define the acceptance criteria for the assessment]

R2 for qPCR and dPCR should be >0.980. If lower, the presence of outliers should be investigated. If appropriate, the highest or lower points should be removed from the linear range, and therefore, from the validated range that can be used to analyse the results.

Efficiency should be between 90% and 110% for qPCR.

Results within the dilution curve should comply with the precision and accuracy criteria (see other points)

5.3.7. Range

[Define Range - Where appropriate use the definition as provided by relevant guidelines being followed]

The range assessment will use the data from the linearity assessment to define the range of assay where accuracy and precision criteria are satisfied.

Upper and lower limits should be set for the separate target sequences (LVV + reference) as well. These limits should be fit for purpose.

5.3.8. Range results reporting

[Detail how the results will be reported, any statistical techniques employed]

The results are reported as %bias and %CV for the VCN in each of the dilutions from the linearity assessment.

5.3.9. Acceptance criteria

[Define acceptance criteria]

The range (VCN) will be accepted as the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) if the accuracy (% Bias) is ± 20.0%[1] and the %CV is < 15.0%[1] in each linearity sample and the R^2, generated utilizing a linear curve within the linearity samples, is ≥ 0.98.

[1] Prior to setting any precision criteria in the method, the PCR instrument used should be verified to ensure that the instrument can accurately discriminate at that level across the intended quantitative range. All precision data should use quantitated values, use of Cq values is not acceptable.

5.4. Accuracy

[Define accuracy – Where appropriate use the definition as provided by relevant guidelines being followed.]

Accuracy expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

For dPCR, accuracy will not be suitable, as the dPCR method is more precise than most other qualification methods.

5.4.1. Accuracy assessment

[Describe how the accuracy will be assessed, including which attributes or characteristics of the sample are the focal point of assessment (e.g. all cell types or just one specific target cell type). Provide details of reference standards used or how the "true" value is determined if a reference standard is not used.]

Establish the ability of the test method to measure the closeness of agreement of linearized plasmid DNA spiked into human DNA (measured vs. expected values). Accuracy of sample concentrations will be established throughout the linear range of the assay. Measured concentrations will be compared to expected concentrations for each concentration by calculating the % Bias.

5.4.2. Accuracy results reporting

[Describe how the linearity results will be reported and any statistical approaches used]

Measured concentrations of samples containing linearized plasmid DNA spiked into human DNA will be compared to expected concentrations for each concentration by calculating the % Bias.

5.4.3. Acceptance criteria

[Define the acceptance criteria for the assessment]

% Bias is within ± 30.0% of theoretical concentration for samples containing linearized plasmid DNA spiked into human DNA in each experiment.

5.5. Precision

[Define Precision - Where appropriate use the definition as provided by relevant guidelines being followed]

Precision can be determined by repeat testing of sample. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision will be evaluated at two levels:

1. **Repeatability or intra-assay precision:** the variation between replicates of all dilutions within one assay run or occasion by the same operator.
2. **Inter-assay variability (intermediate precision)**: Variation within a laboratory to include, tests performed on different days and by a different analyst.

5.5.1. Precision Assessment

[Detail the assessment approach for each of the types of precision being assessed (e.g. repeatability, intermediate precision or reproducibility), how the data will be generated, any statistical techniques employed.]

Intra-assay precision (repeatability) will be calculated as the percent coefficient of variation (%CV) for the VCN of LVV transduced cell sample run in the same experiment.

Inter-assay precision (intermediate precision) will be calculated as:

1. mean %CV on different days generated for VCN of LVV transduced cell sample assessed at least three independent assay runs performed by one operator on different days,
2. mean %CV between operators generated for VCN of LVV transduced cell sample assessed for at least three assay runs performed by two operators.

Samples will be run in triplicate.

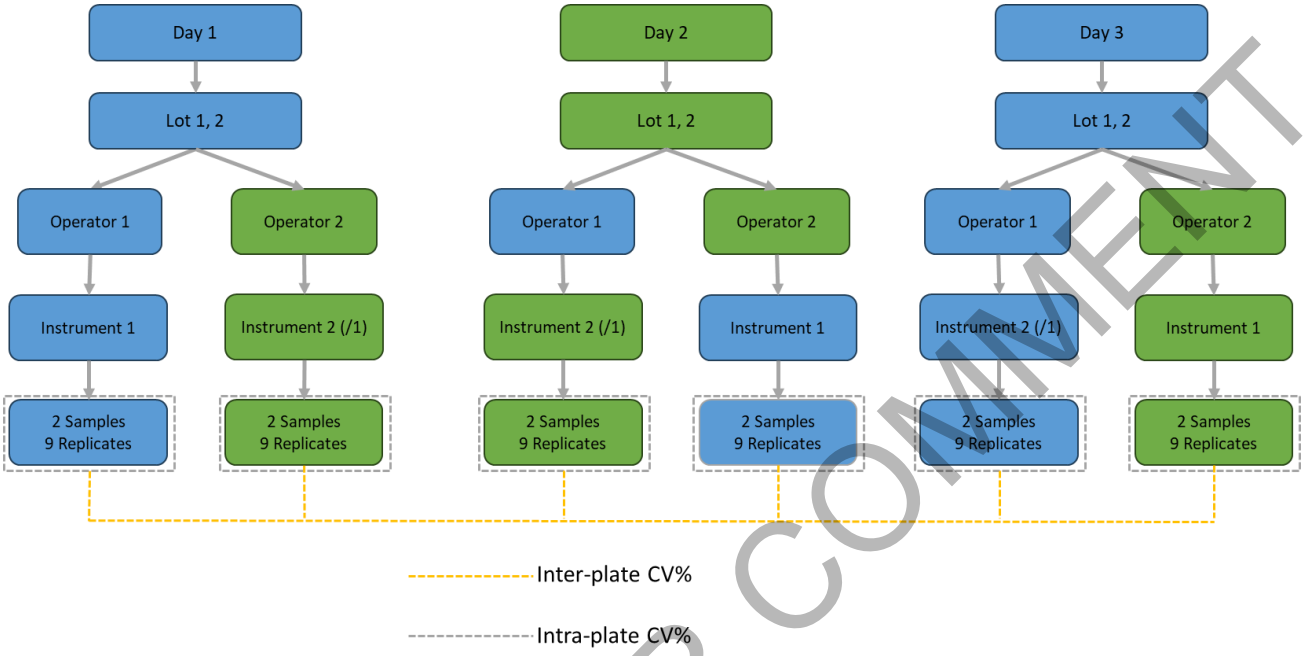


Figure 2: Example of a precision assessment *Yellow dotted lines are linking the samples for iner-plate CV%; Grey dotted lines for intraplate CV.*

5.5.2. Precision results reporting

[Detail how the results will be reported, any statistical techniques employed]

Intra-assay precision (repeatability) will be based on the results for all dilutions from a single run and will be reported as the mean, standard deviation, and coefficient of variance for qPCR. For dPCR this will be reported as the merged copy number, 95% total confidence intervals (95% CI)/SD and coefficient of variation.

Inter-assay variability (intermediate precision) will be based on:

1. ***Variability on different days* -** will be based on the results from 3 independent runs performed by a single operator on different days and will be reported as the mean, standard deviation/95% CI, and coefficient of variance.
2. ***Variability as a function of operator/analyst* -** will be based on results from at least 3 runs performed by two operators and will be reported as the mean, standard deviation/95%CI, and coefficient of variance.

5.5.3. Acceptance criteria

[Define acceptance criteria]

Intra-assay precision: %CV ≤25.0%[1] for the of LVV transduced cell sample tested in triplicate during the same experiment.

Inter-assay precision on different days: %CV ≤25.0%[1] for the of LVV transduced cell sample tested in three different experiments on different days by the same operator.

Inter-assay precision between operators: %CV ≤25.0%[1] for the of LVV transduced cell sample tested in three different experiments by two operators.

[1] Prior to setting any precision criteria in the method, the PCR instrument used should be verified to ensure that the instrument can accurately discriminate at that level across the intended quantitative range. All precision data should use quantitated values, use of Cq values is not acceptable.

6. Reporting results

[Define how the results of the validation protocol will be reported, who has responsibility for producing the report, and how the raw data will be stored or maintained for future reference if needed. It may be valuable to include a summary of all acceptance criteria from the individual sections above as a summary for clarity.]

After execution of each portion of the qualification data will be reviewed per SOP-QC-0001, *QC Data Review Procedure*.

Verify that the concentrations and decimal places are correctly stated per Draft STM-00261 for every value.

Record the accuracy to one decimal place.

A summary report will be drafted that includes the following minimum information:

1. Summary of the procedure, as executed.
2. Summary of equipment, materials and reagents used in the execution.
3. Results from the execution.
4. Any deviations to the protocol or unexpected events, with investigation and impact if required.
5. Statement of method qualification status (pass/fail).

7. References

[Include any references used in the design of the validation plan. Including internal SOPs etc]

The following references were used for the design of this validation plan:

1. XYZ
2. XYZ

Annex 4 - dPCR and qPCR validation *report for LV VCN - worked example*

READ THIS STATEMENT FIRST

The worked example for a validation report presented herein, is intended to support an understanding of the practical translation of expectations outlined in regulatory documents and guidelines as presented in the exemplar report structure document. The objective is to assist readers having limited experience of undertaking assay validation activities with the development of their own internal procedures, processes and report styles.

[Black italicised text provides some points for consideration with each section]

Blue text provides an exemplar in the context of a PCR assay aimed at determining LV Vector copy number or the number of pro-viral DNA copies per host cell genome (copies/cell)

This document is not intended to present a gold standard to be copied or cloned. It is important readers develop their own protocols that are fit-for-purpose for the assay being developed. Any information and data presented herein is provided as an exemplar to illustrate possible content. It is not necessarily exhaustive of expectations, and where multiple approaches are potentially applicable (e.g. different statistical techniques / approaches) it is the readers responsibility to ensure the correct methods are employed for their specific assay / validation plan design.

Readers with existing report styles for assay validation activities are invited to reflect on the content but encouraged to continue using their own internally approved procedures, processes and documentation styles where they have been shown to be fit-for-purpose

Validation report

PCR assay aimed at determining LV Vector copy number

Project code:	
Study title:	
Protocol number:	*XXX-XXXX*
Experimental start date:	*DD-MMM-YYYY*
Experimental end date:	*DD-MMM-YYYY*
Study locations: ***Address:***	
Experimental lead: ***Name:*** ***Signature:***	
Reviewed by: ***Name:*** ***Job title:*** ***Signature:***	
Approved by: ***Name:*** ***Job title:*** ***Signature:***	

Amendments to version X.X of the validation report

The following amendments were made to version X.X of the PCR assay validation report to create this document:

1	***Change***	
	Reason for change	
2	***Change***	
	Reason for change	
3	***Change***	
	Reason for change	

Contents

1 Principle

1.1 Background

[This section should briefly describe the background to the assay (e.g. the assay purpose, what is being detected, why and/or how it is being detected); the objective of the assay (e.g. what it needs to demonstrate); any important points for consideration the reader should take into account about the assay (e.g. … etc.); and provide any cross-references to the validation protocol from which the report is based]

This report describes the procedures undertaken to validate the assay used for determination of LV Viral Copy Number (VCN) by qPCR/ dPCR at QC laboratory. VCN was determined by measuring the pro-viral DNA copies, containing the gene of interest, per host cell genome. Validation of the method included assessment of accuracy, specificity, precision, linearity and range.

1.2 Assay Objective

[Recap the assay objective from the validation plan / protocol, reconfirming who the assay was performed by and where it was performed.]

This assay measures the concentration of pro-viral genomes that contain the gene of interest, normalized to a reference gene. This normalisation takes into account the ploidy of the transduced cell type. Genomic DNA (gDNA) is extracted and purified from transduced cells. Samples are diluted to a suitable concentration and then mixed with q/dPCR mastermix. Target sequences are amplified on a thermal cycler. The amount of amplified target and reference DNA is quantified and the estimated Vector Copy Number is calculated.

The assay has been previously qualified, which is described in Report 'Qualification of assay used for determination of LV Viral Copy Number (VCN) by qPCR/ dPCR'.

2 Amendments and deviations

[Describe any amendments or deviations to the approved validation plan / protocol on which the report is based and the potential impact of each, specifically noting any specific assay characteristics e.g. linearity, specificity etc. that may be affected.]

During validation no amendments and deviations from the SOP were noted.

3 Materials and methods

[Detail materials and methods used (e.g. a list of reagents and lot numbers to be used / instrument models / serial numbers), where relevant cross-reference to SOPs, the validation protocol or other relevant documents, detail correct version numbers etc. where applicable.]

Description of the methods and materials used are within SOP-XXX vX.X.

A detailed description of the validation process is contained in protocol XXX-XXXX vX.X "*Validation plan for…*".

VCN was calculated as follows:

$$\text{VCN} = \left(\frac{Target\ copies}{reference\ copies}\right) x2$$

Either this was done manually (qPCR) or by the dPCR software (dPCR).

Statistics were performed using (software XXX and methods YYY).

4 Results

4.1 Assay specificity

4.1.1 Overview

[Provide brief overview of the assay specificity assessment (e.g. this may be through use of the data generated from the linearity assessment), and the concentration range within which this detection should occur, how the data was generated, any statistical techniques employed.]

The specificity of a method is its ability to accurately and specifically detect the analyte in the presence of components that are expected to be present in the sample matrix.

Specificity was assessed by comparing the VCN of samples with viral vector/spike-in (RC or PC) versus samples containing non-transduced/non-spiked in cells (NSC1).

Samples were analysed in triplicates, and specificity was assessed on 3 separate assay occasions by one analyst.

4.1.2 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

The specificity of the assay is confirmed when the lowest VCN of the linearity assessment is significantly different from non-transduced cells at the same concentration.

For qPCR:

1. NTC must not exhibit amplification of LVV target sequence and human genomic target sequence in at least 2/3 wells.
2. Non-transduced samples (NSC1) must not exhibit amplification of LVV target sequence in at least 2/3 wells and must exhibit amplification of human genomic target sequence in 3/3 wells.
3. Transduced samples (PC or RC) must exhibit amplification of LVV target sequence in 3/3 wells and must exhibit amplification of human genomic target sequence in 3/3 wells.

For dPCR (example):

1. NTC:
    a. LVV target <2 copies/3 wells

b. reference gene: <2 copies/3wells

2. Non-transduced samples (NSC1):
   a. LVV target sequence: < 2 copies/3 wells
   b. Reference target sequence: ~copy number as PC/RC
3. Transduced samples (PC/RC):
   a. LVV target: ≥ 2 copies/3wells
   b. Reference target sequence: ~ copy number as NSC1

4.1.3 Results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

The specificity results are summarised in **Error! Reference source not found.**1.

Table 1. Specificity Results qPCR

Sample	Target	Assay Occasion 1	Assay Occasion 2	Assay Occasion 3	Pass/Fail
NTC	LVV	3/3 Replicates negative	2/3 Replicates negative	3/3 Replicates negative	Pass
	Reference	3/3 Replicates negative	2/3 Replicates negative	2/3 Replicates negative	Pass
NSC1 (Non-transduced cells)	LVV	3/3 Replicates negative	2/3 Replicates negative	3/3 Replicates negative	Pass
	Reference	3/3 Replicates positive	3/3 Replicates positive	3/3 Replicates positive	Pass
Transduced sample (PC)	LVV	3/3 Replicates positive	3/3 Replicates positive	3/3 Replicates positive	Pass
	Reference	3/3 Replicates positive	3/3 Replicates positive	3/3 Replicates positive	Pass

Table 2. Specificity Results dPCR

Sample	Target	Assay Occasion 1	Assay Occasion 2	Assay Occasion 3	Pass/Fail
NTC	LVV	3/3 Replicates negative	2/3 Replicates negative	3/3 Replicates negative	Pass
	Reference	3/3 Replicates negative	2/3 Replicates negative	2/3 Replicates negative	Pass
NSC1 (Non-transduced cells)	LVV	3/3 Replicates negative	2/3 Replicates negative	3/3 Replicates negative	Pass
	Reference	3/3 Replicates positive	3/3 Replicates positive	3/3 Replicates positive	Pass
Transduced sample (PC)	LVV	3/3 Replicates positive	3/3 Replicates positive	3/3 Replicates positive	Pass
	Reference	3/3 Replicates positive	3/3 Replicates positive	3/3 Replicates positive	Pass

4.1.4 Summary

The assay demonstrates an acceptable level of specificity for detection of AAV viral vector copies.

4.2 Assay range

4.2.1 Assay linearity

4.2.1.1 Overview

[Provide brief overview of the assay linearity assessment method, this could be paraphrased from the protocol document, include information on how dilution curves were set up including the calculations, the plate layout for the assay, any controls etc. Furthermore, describe how many times the procedure was repeated and by how many operators in order to support assessment of other assay characteristics].

4.2.1.2 qPCR linearity assessment

Linearity was assessed by analysing a 1:2 and 1:5 dilution series of a target linearised plasmid DNA in a constant non-transduced 50 ng cellular background of human genomic DNA

(plasmid sample lot XYZ), starting at $1.5x10^5$ copies per reaction. Table 5 presents the dilution series used in the linearity assessment.

Two runs were executed by two operators on different days in Laboratory A. Each dilution was analysed in triplicate.

Table 5. 10-fold dilution series used in qPCR linearity assessment.

Dilution	Mix	Target copies added	Add gDNA (µL)	Target VCN
Dil 1	Linearized plasmid XYZ in PCR buffer	$1.5x10^5$	4	40
Dil 2	100 µL buffer + 100 µL Dil 1	$7.6x10^4$	4	20
Dil 3	100 µL buffer + 100 µL Dil 2	$3.8x10^4$	4	10
Dil 4	100 µL buffer + 100 µL Dil 3	$1.9x10^4$	4	5
Dil 5	160 µL buffer + 40 µL Dil	$3.8x10^3$	4	1
Dil 6	160 µL buffer + 40 µL Dil 5	$7.6x10^2$	4	0.2
Dil 7	100 µL buffer + 100 µL Dil 6	$3.8x10^2$	4	0.1
Dil 8	100 µL buffer + 100 µL Dil 7	$1.9x10^2$	4	0.05
NSC1	buffer	0	4	0.00

Additionally, a curve with a dilution series of cells (1 µg to 6.25 ng cell line XYZ) into a constant linearized plasmid DNA background ($8x10^4$ cp Plasmid XYZ) was prepared and evaluated but will not be reported here.

4.2.1.3 dPCR linearity assessment

Linearity was assessed by analysing a 1:2 and 1:5 dilution series of a target linearized plasmid DNA in a constant non-transduced 50ng cellular background of human genomic DNA (plasmid sample lot XYZ), starting at $7.6x10^4$ copies per reaction. Table 6 presents the dilution series used in the linearity assessment. 20 ng gDNA from non-transduced cells were used as negative specificity control.

Two runs were executed by two operators on different days in Laboratory A. Each dilution was analysed in triplicate.

Table 6. dPCR dilution series

Dilution	Mix	Estimated target copies/µL (assuming 1 µL will be measured in the final reaction)	Add gDNA for final 20 ng (µL added)	Estimated VCN
Dil 1	Linearized plasmid XYZ in PCR buffer	$7.6x10^4$	4	20
Dil 2	100 µL buffer + 100 µL Dil 1	$3.8x10^4$	4	10
Dil 3	100 µL buffer + 100 µL Dil 2	$1.9x10^4$	4	5
Dil 4	160 µL buffer + 40 µL Dil 2	$7.6x10^3$	4	2
Dil 5	160 µL buffer + 40 µL Dil 3	$3.8x10^3$	4	1
Dil 6	160 µL buffer + 40 µL Dil 5	$7.6x10^2$	4	0.2
Dil 7	100 µL buffer + 100 µL Dil 6	$3.8x10^2$	4	0.1
Dil 8	100 µL buffer + 100 µL Dil 7	$1.9x10^2$	4	0.05
NSC1	Buffer	0	4	0.00

4.2.1.4 Acceptance criteria for qPCR and dPCR

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

R^2 for qPCR and dPCR should be >0.980. If lower, the presence of outliers should be investigated. If appropriate, the highest or lower points should be removed from the linear range, and therefore, from the validated range that can be used to analyse the results.

Efficiency should be between 90% and 110% for qPCR.

4.2.1.5 qPCR results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

Mean Cq values and dilution series of a target linearized plasmid DNA (plasmid sample lot XYZ) in a constant non-transduced cellular background of human genomic DNA (50 ng cell line XYZ), starting at $1.5x10^5$ copies per reaction. Linear regression analysis was performed by plotting the logarithmic value of theoretical VCN (+ theoretical copies per gene) against the

Cq value to determine R2 value (Figure 1). Slope, intercept and efficiency were calculated for each for the runs (Table 7, 8).

Table 7. Results of the linearity assessment for the target assay.

Run No.	Analyst	Sample	Slope	Intercept	Efficiency	R2
1	1	Plasmid lot XYZ *in gDNA cell line XYZ*	-3,2743	42.456	104%	0.9820
2	2	Plasmid lot XYZ *in gDNA cell line XYZ*	-3,4124	42,874	99%	0.9918

Table 8. Results of the linearity assessment for VCN:

Run No.	Analyst	Sample	R2
1	1	Plasmid lot XYZ in gDNA cell line XYZ	0.9994
2	2	Plasmid lot XYZ in gDNA cell line XYZ	0.9981

Figure 1. Linear regression plot for Run 1

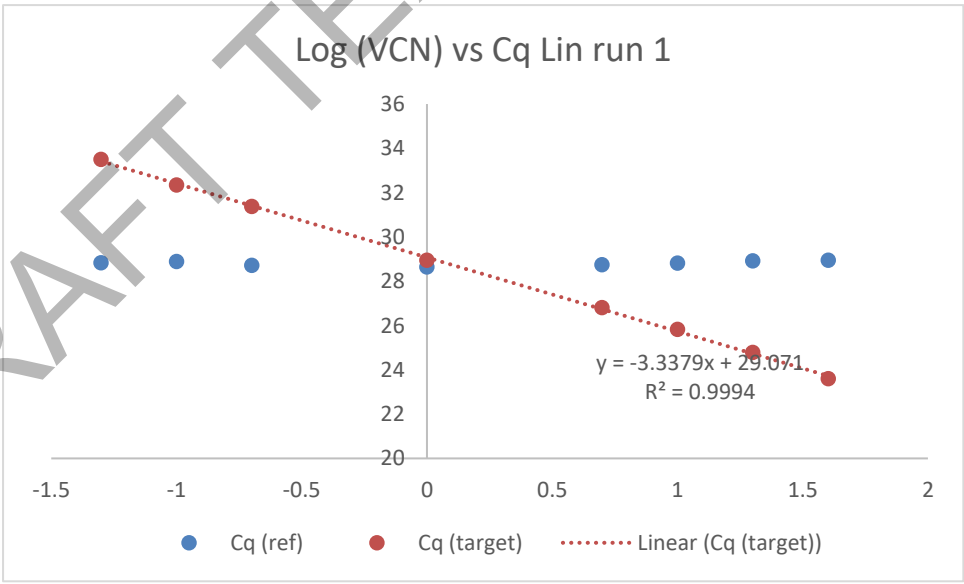
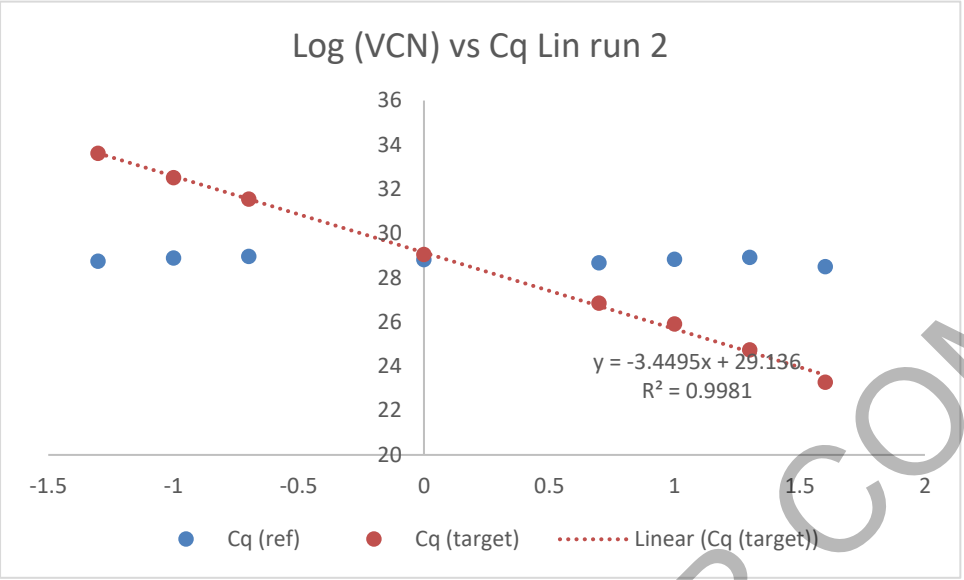


Figure 2. Linear regression plot for Run 2

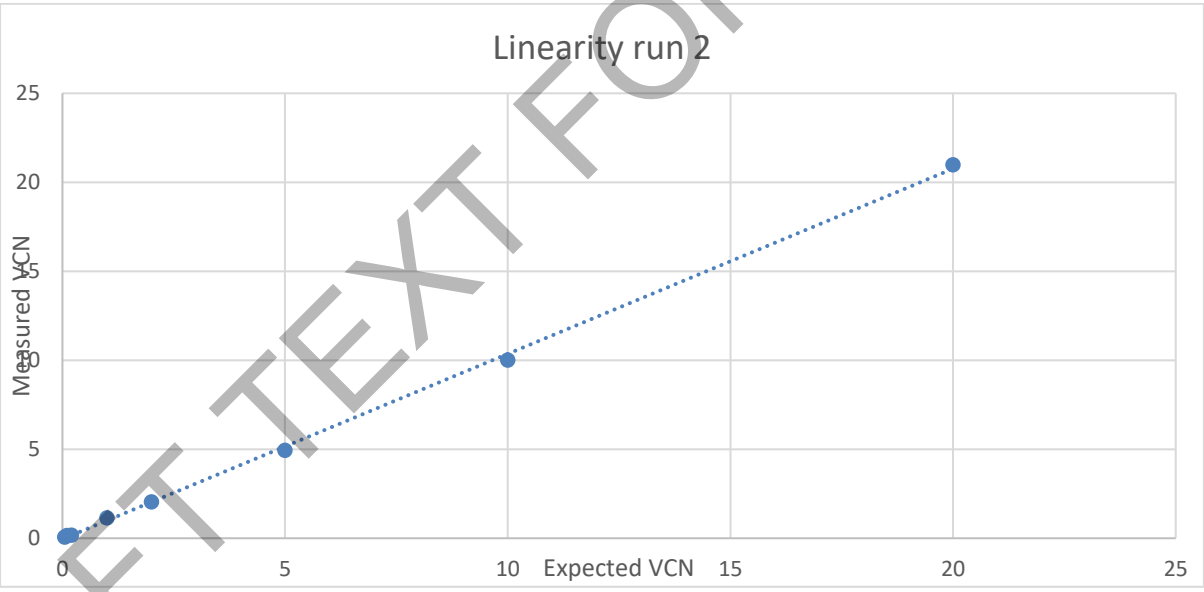


4.2.1.6 dPCR results

For dPCR expected vector copy number versus observed vector copy number was plotted.

Table 9. Results of the linearity assessment for VCN:

Run No.	Analyst	Sample	R2
1	1	Plasmid lot XYZ in gDNA cell line XYZ	01.00
2	2	Plasmid lot XYZ in gDNA cell line XYZ	0.915



Additionally, a fixed linearised plasmid concentration (plasmid XYZ at $3.8x10^4$ cp) versus a dilution of genomic DNA (350 ng to 2.7 ng) was tested, but results are not shown here.

4.2.1.7 Linearity summary

[State any final conclusions about linearity results]

The acceptance criteria were met, and assay demonstrated an acceptable level of linearity.

4.2.2 Assay range, LLOQ, ULOQ

[Provide brief overview of the assay range assessment methods utilised, this could be paraphrased from the protocol document.]

The range assessment used the data from the linearity assessment to define the range of the assay where accuracy and precision criteria are satisfied.

4.2.2.1 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

The range (VCN) will be accepted as the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) if the following criteria are met:

1. The accuracy (% Bias) is ±20.0% (dPCR – only possible if prior dPCR assessment or based on 3 dilutions) and the %CV is <20.0% in each linearity and LLOQ/ULOQ sample
2. The R^2, generated utilizing a linear curve from within the linearity samples, is ≥ 0.98.

4.2.2.2 Assay range results

[Report the results (including any relevant tables of data, graphs, statistical analysis performed etc.) state how the assay performed against the acceptance criteria and if the assay characteristic is considered to have passed or failed against the acceptance criteria.]

qPCR/dPCR range:

The acceptable range for the assay is based on the linearity studies. The LLOQ and ULOQ are the highest and lowest VCN in the sample which can be measured with acceptable level of precision and accuracy. From the data generated (Table 6) the validated range of the assay is 0.1 to 10 copies/cell.

The two highest dilutions and the lowest dilution were excluded from the linearity assessment (marked in red) due to being outliers and not meeting accuracy and precision acceptance criteria.

Table 10. Summarised assessment of the range of the assay (qPCR/dPCR example)

Sample	Dilution number	Av. Expected VCN (copies/cell)	Av. Measured VCN (copies/cell)	Bias (%)	Precision (%CV)
plasmid lot XYZ in DNA background	1	40	28	30	70,0%

plasmid lot XYZ in DNA background	2	20	15.0	25	50,0%
plasmid lot XYZ in DNA background	3	10	9.02	10	19,8%
plasmid lot XYZ in DNA background	4	5	4,40	12	12,0%
plasmid lot XYZ in DNA background	5	1	1.02	2	8,0%
plasmid lot XYZ in DNA background	6	0.2	0.23	3	15,0%
plasmid lot XYZ in DNA background	7	0.1	0,12	2	18,0%
plasmid lot XYZ in DNA background	8	0.05	0,07	40	25,0%

4.2.2.3 Summary

From the data generated (Table 6) the validated range of the assay is 0.1 - 10.0 copies/cell with LLOQ being 0.1 copies/cell and ULOQ being 10 copies/cell.

4.3 Assay accuracy

4.3.1 dPCR and qPCR Accuracy Assessment

Accuracy was assessed by analysing the dilutions used from the linearity assessment for qPCR (see section 4.4 Range assessment). (For dPCR this can only be done if the sample has been assessed with dPCR before.)

Additionally, samples from reference material of known concentration with high, middle and low copy number (as assessed by a separate technology with equivalent performance to dPCR: Positive control 1 (PC1), Positive control 2 (PC2) and positive control 3 (PC3) were tested as described in the accuracy assessment. The acceptable accuracy for each dilution point was established to be within 20% of the expected concentration.

4.3.2 Accuracy Acceptance Criteria

For both dPCR and qPCR the measured VCN of each dilution of the reference standard must be within ± 50% (qPCR) or 20% (qPCR) of the expected VCN.

Assay Parameter	Acceptance criteria
CV	≤ 50% (qPCR) or ≤ 20% (dPCR)

4.3.3 Accuracy Results

Table 3. qPCR Accuracy Results.

Sample	Expected copy number	Observed copy number	% difference	Pass/Fail
PC1	10	8.95	10.5	Pass
PC2	5	4.40	12	Pass
PC3	1	1.20	20	Pass
Acceptance criteria mean %difference	≤ 50%			

Table 4*. dPCR Accuracy Results.*

Sample	Expected copy number	Observed copy number	% difference	Pass/Fail
PC1	10	9.90	1%	Pass
PC2	5	5.12	2%	Pass
PC3	1	1.02	2%	Pass
Acceptance criteria mean %difference	≤ 20%			
Result %CV	1.7%			
Pass/Fail	Pass			

4.3.4 Accuracy Summary

All tested reference samples fell within the required stated accuracy acceptance limits for both qPCR and dPCR assays. Assay precision

4.4 Precision

[Provide brief overview of the assay precision assessment method for each of the types of precision being assessed (e.g. intra-assay, inter-assay / intermediate precision / reproducibility), how the data was generated, any statistical techniques employed.]

Precision was determined by repeat testing of sample. Precision was evaluated at two levels:

1. **Repeatability or intra-assay precision:** the variation between replicates of all dilutions within one assay run or occasion by the same operator
2. **Inter-assay variability (intermediate precision):** Variation within a laboratory to include tests performed on different days and by a different analyst. Inter-assay precision was calculated as:
    a. mean %CV on different days generated for VCN assessed at least three independent assay runs performed by one operator on different days.
    b. mean %CV between operators generated for VCN assessed for at least three assay runs performed by two operators. Samples were run in triplicate. Data came from the linearity assessment (results in section Y 'range assessment') and from PC2 and PC3. Data were reported as mean or median VCN and %CV.

4.4.1 Acceptance criteria

[Re-iterate the acceptance criteria for the assessment, this should match that defined in the protocol.]

For each of the precision assessments the assay will be considered precise if the CV ≤20% (Table 11).

Table 11. Precision acceptance criteria.

Assay Parameter	Acceptance criteria
CV	≤20%

4.4.2 Combined assay precision results

[Report the results, making it clear how the assay performed against the acceptance criteria and if it is considered to have passed or failed.]

Assay precision was determined using the 8 dilutions outlined in the linearity study for VCN from a single run (run one) performed by a single operator, results are outlined in Section Y, 'range assessment'.

Assay precision was further assessed using the transduced controls (50 ng) at known vector copy number PC1, PC2 and PC3. Results are shown in Table 12-14.

Table 12. Repeatability assessment of VCN measurements

Sample	VCN		
	Mean	SD	%CV
PC1	9.9	0.62	6.3
PC2	5.12	0.30	5.9
PC3	1.02	0.05	5
Acceptance criteria (CV)	≤ 20%		
Pass/Fail	Pass		

Table 13. Intermediate precision assessment of VCN measurements (1 operator over several days).

Sample	VCN		
	Mean	SD	%CV
PC1	9.9	0.70	7.1
PC2	5.12	0.25	4.9
PC3	1.02	0.08	7.8
Acceptance criteria (CV)	≤ 20%		
Pass/Fail	Pass		

Table 14. Intermediate precision between operators of VCN measurements.

Sample	VCN		
	Mean	SD	%CV
PC1	9.9	0.77	7.8
PC2	5.12	0.28	5.5
PC3	1.02	0.04	3.9

Acceptance criteria (CV)	≤ 20%
Pass/Fail	Pass

4.4.3 Summary

The assay demonstrates an acceptable level of repeatability and intermediate precision across the tested VCN samples.

5 Conclusion

[Present the overall conclusion for the study, summarising those characteristics of the assay validation protocol that passed and those that failed. Provide an overall statement as to whether the assay is fit-for-purpose based on the outcome of the results.]

Assay validation met the predefined acceptance criteria and has been successfully validated for GMP use.

Table 15. Summary of the validation acceptance criteria (qPCR example)

Attribute	Acceptance criteria	Results	Pass/ Fail
Specificity	NTC must not exhibit amplification of LVV target sequence and human genomic target sequence in at least 2/3 wells. Non-transduced samples (NSC1) must not exhibit amplification of LVV target sequence in at least 2/3 wells and must exhibit amplification of human genomic target sequence in 3/3 wells. Transduced samples (PC or RC) must exhibit amplification of LVV target sequence in 3/3 wells and must exhibit amplification of human genomic target sequence in 3/3 wells.	NTC did not exhibit amplification of LVV target sequence and human genomic target sequence in at least 2/3 wells over 3 runs. Non-transduced samples (NSC1) did not exhibit amplification of LVV target sequence in at least 2/3 wells and did exhibit amplification of human genomic target sequence in 3/3 wells over 3 runs. Transduced samples (PC or RC) did exhibit amplification of LVV target sequence in 3/3 wells and did exhibit amplification of human genomic target sequence in 3/3 wells over 3 runs.	Pass

Range LLOQ ULOQ	The LLOQ and ULOQ are the highest and lowest AAV titer in the sample which can be measured with acceptable level of precision (CV% ≤ 20%) and accuracy (% bias = ± 20.0%).	Validated range of the assay is 0,3-7,3 copies/cell, with LLOQ being 0,3 copies/cell (CV% ≤13%, bias% ≤ +0%) and ULOQ being 7,3 copies/cell (CV% ≤5%, bias% ≤ +4,29%).	Pass
Linearity	-Amplification efficiency 90-110% -R^2 ≥ 0.98 for min. 5 dilutions	Run 1: -Amplification efficiency 104% -R^2 = 0.9820 Run 2: -Amplification efficiency 99% -R^2 = 0.9918	Pass
Accuracy	Measured VCN of each dilution of the LVV sample must be within ± 20% of the expected VCN.	Results of 8 dilutions of the tested sample were within +20% of the expected VCN.	Pass
Precision - Repeatability	CV ≤ 20%	CV≤ 6.3%	Pass
Precision – Intermediate Precision	CV ≤ 20%	CV≤ 7.8% across 3 runs over 5 days by 1 operator CV≤ 7.8% across 6 runs over 2 days by 2 operators	Pass
Robustness	Assessed during qualification of the method		

6 Appendices

[Include any relevant supplementary data to support that presented in the main body of the report e.g. this may include individual Cq values for each qPCR experiment, or individual cps/reaction values for dPCR experiment]