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Biotechnology — Gene delivery systems —

Part 2: Quantification methods for viral vectors

Biotechnologie — Systèmes de transfert de gènes —

Partie 2: Méthodes de quantification des vecteurs viraux

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Foreword

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This document was prepared by Technical Committee ISO/TC 276, *Biotechnology*, Subcommittee SC 1, *Analytical methods*.

A list of all parts in the ISO 16921 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Modern biotechnology relies on the ability to manipulate genes and genomes in living systems. Gene delivery systems are foundational to genome engineering. Gene delivery technology is evolving rapidly with numerous types of gene delivery systems providing a comprehensive set of tools and capabilities for in vitro or in vivo targeted delivery.

The ISO 16921 series consists of multiple parts to provide common understanding, guides, analytical methods and, data reporting for characterizing these emerging biotechnology tools. ISO 16921-1 specifies Vocabulary related to gene delivery systems. This document (ISO 16921-2) focuses on quantification of one type of gene delivery system, viral vectors.

Viral vectors are engineered viruses for delivering the desired genetic payload into target cells. Viral vectors are powerful molecular biology tools and have been increasingly used in broad biotechnology applications and products. Various types of viral vectors are used as advanced gene therapies, as vaccines, and as critical reagents for cellular therapies. They have also been increasingly used in genome editing applications. Viral vector titer (titre) is central to all applications. Robust measurements for the quantification and reporting of viral vector titer are important for the industry. This document provides general guidance for viral vector titer as well as aspects of functional analysis including method selection, sample preparation, measurement, qualification and validation, data analysis and reporting.

Biotechnology — Gene delivery systems —

Part 2: Quantification methods for viral vectors

1 Scope

This document specifies minimum requirements for quantifying viral vectors in terms of physical titer and their associated activity. This document specifies key considerations for quantification methods for viral vector titer as well as activity, including method selection, measurement process, data analysis, and reporting.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16921-1, *Biotechnology — Gene Delivery Systems — Part 1: Vocabulary*

ISO 20395, *Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16921-1 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
direct particle counting
counting method in which one signal is (or several signals are) detected for each single event

Note 1 to entry: Each single event represents a single viral particle in an idealized measurement

3.2
indirect particle counting
counting method during which a signal (or a set of signals) is measured from a population of viral particles and that signal is then related to viral titer based on a measurement-specific mathematical model (e.g. calibration curve)

4 Concepts for viral vector titer

4.1 General viral vector concepts

Viral vectors are genetically engineered viruses designed to deliver nucleic acid into a cell. As such, viral vectors are almost always replication incompetent, so that no new viral vectors can be produced within the target cell.

NOTE 1 Viral vectors can be made to be replication competent in the presence of a helper virus or complementary replicative genes in a cell.

NOTE 2 Replication competent viral vectors are generally not used in therapeutic applications due to safety concerns.

A functional viral vector generally consists of three components:

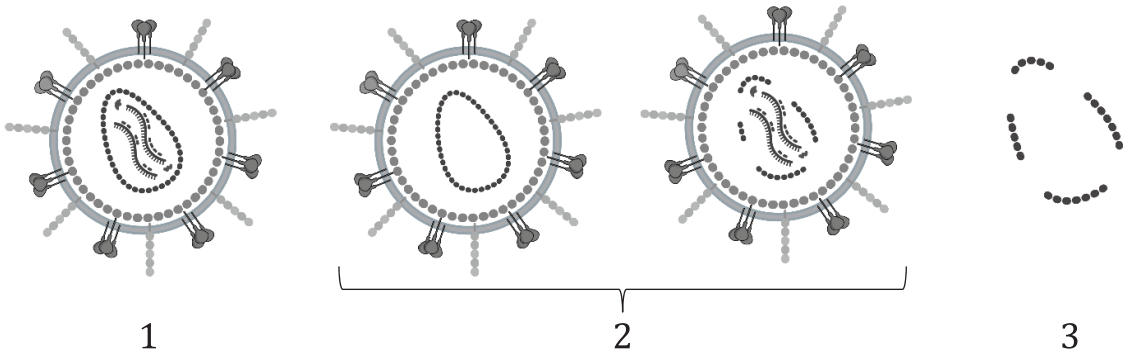
— the protein capsid with or without the envelope that encapsulates the payload, and defines the vector's tissue or cell tropism and antigen recognition;

— the gene of interest, which when expressed in cells, serves to confer a desired functional outcome; and

— the regulatory components including the combined enhancer, promoter, and auxiliary elements that control stable or transient somatic expression of the transgene as an episome or as a chromosomal integrant.

NOTE 3 Some viral vectors designs do not precisely follow the transgene and regulatory cassette descriptions stated above. Examples include those utilizing micro RNAs, guide RNAs, multiple transgenes/promoters, and dual vectors (which utilize in vivo splicing).

The process to engineer and manufacture viral vectors is complex and evolving. Current technologies generally produce a mixture of functional viral vectors that can deliver the appropriate payload into the target cells as well as non-functional viral vectors and free capsid proteins (Figure 1). Other potential impurities in the viral vector mixture include free DNA from the host cell or residual plasmid components, all of which may be encapsulated as well.



Key

1 functional particles
2 nonfunctional particles (empty and immature particles)
3 free capsid proteins

Figure 1 — Products from viral vector production that include functional particles, nonfunctional particles, and free capsid protein. Adapted from "Understanding viral titration- behind the science"[1].

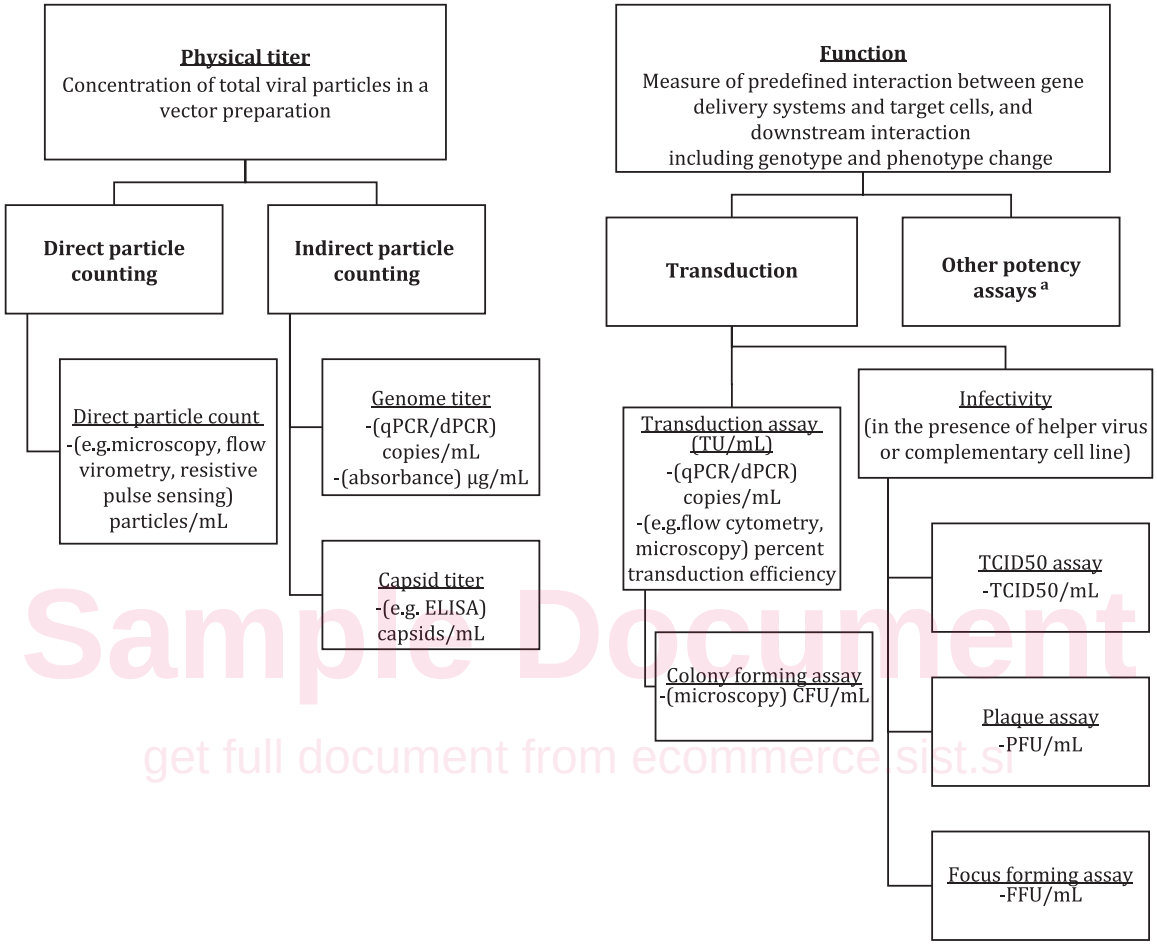
Non-functional viral vector particles do not have all the necessary components to complete the transduction process, express the intended protein, and result in the functional outcome. Non-functional viral vector particles include various types of defects, including for example:

— vectors with defective capsids that can affect its tissue or cell tropism and antigen recognition,

— vectors that contain missing or defective payloads,

— vectors that contain missing or defective regulatory components.

Viral vectors are characterized according to critical quality attributes, such as quantity, potency, safety, and identity. This document focuses on quantity. General methods for a) quantification of physical titer of viral vectors and b) functional characterization of viral vectors using cell-based measurements are shown in figure 2.



[a] Additional potency assays for titer quantification are often specific to the product and mechanism of action and is beyond the scope of this document.

Figure 2 — Analytical methods for quantification of viral vectors based on physical methods or their interactions with cells. This figure of quantification methods is not exhaustive and can be further developed.

Physical titer of viral vectors refers to the particle count, which is determined by direct particle counting or indirect particle counting methods.

Direct particle counting involves the recording of a signal or a set of signals from individual viral particles (3.1). Direct particle counting requires well-dispersed particles for optimal performance. The presence of debris and aggregated or agglomerated particle can lead to over- or underestimated virus titer. Whenever possible, a process should be established to prepare well- dispersed samples with minimized debris, aggregate, and agglomerate content.

Indirect particle counting (3.2) methods use a surrogate measure to evaluate the count. The accuracy of these methods depends on the accuracy of the measurement and the accuracy of a calibration curve, if a calibration curve is needed. Indirect particle counting methods include:

— the use of a calibration to estimate a count (e.g. fluorescence, qPCR, ELISA, or another method to back calculate count using a calibration curve);

— the measuring a component of the particle to infer the count (e.g. quantifying the total capsid protein concentration to estimate particle count).

Activity methods are used to measure the interaction between viral vector particles and target cells. Activity measures determine the concentration of functional particles in a viral vector preparation. Activity measurements can be further divided into transduction and other potency measures to gain insight into general transduction activity and product specific potency.

Transduction assays measure viral vector transgene entry into target cells for non-replication competent viral systems. Vector copy number per cell or transduction efficiency can be measured in the target cells. A dilution series of virus to cells is generally used to specify at which dilutions the multiplicity of infection to transduction efficiency is linear. The transduction units in the original virus preparation can be determined from the specified range of the dilution series.

Infectivity assays may include a viral vector, a helper virus, and a complementary cell line that enables the viral vector to replicate. Viral replication can result in target cells undergoing cytopathic effect. Replication and cytopathic effect can be visualized and quantified as plaques or foci in the cells. Viral vector replication in target cells enables quantification of virus activity and infectivity assay titer.

Potency assays measure transgene expression and transgene activity. Transduction assays can be considered one part of potency measures which can measure transgene insertion, integration, and expression. Additional potency measures beyond transduction and transgene expression are often production specific measures of transgene mechanism of action. Due to the product specific nature of such assays, use of potency assays for viral vector quantification are beyond the scope of this document.

See Table A.1 for attributes, biological properties, analytical methods, measurement principles, and units for the quantification of viral vector titer.

4.2 Physical titer measurements

4.2.1 General physical titer concepts

Physical titer refers to the total concentration of viral vectors or the concentration of a predefined, subset of viral vectors.

Physical titer of viral vectors shall be expressed as amount per volume (1/mL).

Physical titer can be determined via several analytical methods, including direct particle counting methods and indirect particle counting methods.

An appropriate measurement shall be selected to determine the total quantity or the quantity of a predefined, subset of viral vectors.

Assessment of the quality of the viral vectors may involve the use of two or more titers, such as filled to empty ratio.

See Annex B.2 for additional physical titer methods.

4.2.2 Direct viral vector titer measurement

Direct viral vector titer methods identify and enumerate single particles. Direct viral vector titer can be used for total viral vector enumeration or enumeration of particles of a specific predefined attribute.

Direct viral vector titer measurements can include electron microscopy-based counting, flow virometry, resistive pulse sensing, nanoparticle tracking analysis, and other methods capable of capturing single entities (i.e., viral vector particles).

Results of direct particle counting methods shall be reported in terms of particles/mL or specified units of volume.

Due to sample heterogeneity, direct viral vector counting methods can inadvertently count impurities, such as extracellular vesicles, as viral vectors. Counting methods targeting only the viral vectors within a sample may therefore be more appropriate. Combining methods specific for impurities, such as flow cytometry that targets extracellular vesicles, can also be used to correct direct particle count measurements to be more accurate for viral particles.

4.2.3 Indirect viral vector titer measurement

4.2.3.1 General indirect viral vector titer concepts

Indirect viral vector titer measurements include all methods to extrapolate the viral vector quantity not associated with enumeration.

Indirect methods generally involve the use of a calibration curve to determine quantity. These include methods that quantify a defined attribute of the viral vector, such as capsid protein quantification and genome quantification methods.

4.2.3.2 Capsid titer quantification

Capsid titer or capsid concentration is a measurement of protein quantification used to characterize viral vectors. An example of a method used to determine capsid titer is the enzyme-linked immunosorbent assay (ELISA). Other example methods for capsid titer can be found in Table A.1. ELISA capsid protein measurements reported as virus particles/mL is ambiguous to what the actual measurand is. Capsid titer shall be reported as capsids/mL. The specific capsid protein (e.g. p24 for lentivirus) being targeted shall be included in the measurement reporting. The method of determining the capsids/mL and associated calculations shall be documented. A standard curve shall be included for capsid titer determination using a well characterized and known concentration of the target protein.

NOTE 1 Capsid protein measurements measure free soluble capsid protein and encapsulated capsid protein as a quality measure of a viral vector preparation.

NOTE 2 ELISA measurements report absorbance values for the samples being measured. Depending on the viral vector, the absorbance values can be converted to mass and then to capsids/mL, or directly from absorbance values to capsids/mL.

The calculations to perform these conversions are based on literature assumptions and should be considered when evaluating the accuracy for capsid quantification. For example, p24 capsid quantification by ELISA relies on the assumption that there are approximately 2 000 molecules of p24 per lentivirus particle[2]. The accuracy of this estimate affects the calculated capsid titer.

4.2.3.3 Genome titer quantification

4.2.3.3.1 General genome titer quantification

Indirect physical titer methods for genome titer include absorbance, fluorescence, and qPCR/dPCR.

4.2.3.3.2 Absorbance

Absorbance can be used to measure total nucleic acid content of the viral vector. Absorbance at OD260 nm represents all nucleic acids species present in a sample (DNA, RNA, free nucleotides). Absorbance measurements are calculated as nucleic acid concentration and shall be reported as ng/µL or µg/mL. The

vector genome concentration can be determined from absorbance, the molecular mass of the viral vector, and the extinction coefficient as vector genome (vg)/mL.

Nucleic acid quantification using absorbance is impacted by impurities in the sample, such as proteins, and shall be considered when interpreting results (ISO 20395:2019, 5.2). In some cases, viral particles are lysed using a detergent, such as SDS, to denature proteins and release viral nucleic acid to allow for nucleic acid quantification. The absorbance of viral vectors at OD260 nm depends on the molecular mass of the vector DNA and the amount of capsid protein[3].

Acceptable purity for applying absorbance measurement can be assessed by inspecting the ratios of absorbance at different wavelengths that are altered by common contaminants. The purity of a particular viral vector preparation, such as AAV and AdV, can be determined based on the OD 260/280 nm ratio which accounts for the nucleic acid and the capsid protein content. These ratios can provide insight into the quality of the viral vector preparation.

NOTE It is possible that the OD 260/280 nm ratio is not appropriate for certain viral vectors, such as LVV. In addition, OD measurements can vary due to other factors such as pH and ionic strength.

4.2.3.3.3 Fluorescence

Similar to absorbance, fluorescence can be used to measure nucleic acid content of viral vectors. For the nucleic acid content to be quantified, fluorescent stains may be used.

NOTE 1 Common nucleic acid fluorescent stains include PicoGreen or GelGreen.[4,5]

A standard curve shall be used for fluorescent assays using a calibrant nucleic acid material. Examples of calibrants include plasmid DNA, genomic nucleic acid, and fragmented nucleic acid. The type of calibrant used (e.g. single-stranded DNA or double-stranded DNA) shall be taken into consideration based on the viral vector being analyzed.

Using a standard curve, fluorescent units are converted to nucleic acid concentration and shall be reported as ng/µL or µg/mL. Nucleic acid concentration can then be converted to vector genome concentration as vg/mL based on the molecular mass of the viral vector.

NOTE 2 The accuracy of the conversion from nucleic acid concentration to vector genome concentration depends on the sample purity.

4.2.3.3.4 Quantitative PCR (qPCR) and Digital PCR (dPCR) Methods

For viral vector titer determination, genome counting methods (i.e., dPCR) do not directly enumerate virus particles but rather targeted nucleic acid content and therefore is considered an indirect physical titer method.

ISO 20395 shall be referred to for information on qPCR and dPCR quality control, methodologies, and minimal information needed for reporting.

Specific primers that target a known sequence in the viral vector shall be optimized and tested for optimal parameters, including concentration, annealing temperature, and cycling parameters. A probe can be used to complement the primers to enhance the specificity of the assay. Reverse transcription-PCR (RT-PCR) is needed to convert RNA to cDNA for quantitation of RNA samples. The efficiency of the reverse transcriptase reaction shall be determined.

For qPCR, a calibration or standard curve of known concentration or copies of DNA shall be used to obtain DNA quantitation for an unknown sample. The efficiency of the PCR assay shall be determined based on the data from the standard curve and unknown samples. The DNA standard curve should match the unknown sample in terms of conformation and structure to not bias PCR efficiency between standard and sample. PCR efficiency is impacted by supercoiled or linear states and proximity of the amplicon to hairpins and other secondary structure in the DNA. Plasmids shall be linearized prior to performing qPCR.