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Conference Report

Standards and Metrology for Viral Vectors as Molecular Tools: Outcomes from a CCQM Workshop

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Abstract: Viral vectors are agents enabling gene transfer and genome editing and have widespread utility across the healthcare and biotechnology sectors. In January 2023, the International Bureau for Weights and Measures' Consultative Committee for Amount of Substance (CCQM) held a workshop on Metrology for Viral systems as molecular tools. The workshop brought together international leaders from across regulatory, industry, government science, and metrology sectors to better understand key challenges for the community: Exploring current limitations in the measurement of virus-derived, virus-based, and virus-like systems in terms of quantification and characterisation; surveying the state-of-the-art in analytical methods and reference material provision for these entities; and initiating a dialog for the strategic development and implementation of suitable standardisation approaches for this sector. This article presents the workshop background and rationale, presentation summaries, conclusions, and recommendations.

Keywords: viral vectors; metrology; standards; measurement; analytical methods; characterisation; physical titre methods; functional titre methods; quantification; reference materials

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1. Introduction

Viral systems, including viral vectors derived from native viruses, can be utilised as molecular tools to facilitate the efficient transfer of nucleic acid sequences (i.e., DNA, RNA, siRNA, etc.) into cells to modify their function and achieve a desired clinical effect. As such, they are key components in 'the genomics toolbox', and improving their characterisation to enable their robust manufacture is a subject of intense interest to multiple arms

of biotechnology. Despite considerable societal and commercial potential, there remain challenges to realising the full potential of these technologies, including enhancing product quality (safety and efficacy), managing regulatory scrutiny, and improving the provision and efficiency of manufacturing capacity worldwide [1,2]. More specifically, difficulties in the purification of viral vectors alongside the removal of cellular impurities, a lack of standardisation due to differences in viral capsid variants, and inherent batch-to-batch variation of products can impact production [3,4]. Alongside these manufacturing challenges is a need for better vector quality assessment through standardised, and potentially quantitative, methods. These can help improve scrutiny by critical quality attributes (CQAs) and increase yield production and safety advantages, such as enabling accurately defined lower dose concentrations through the consistent production of more potent vectors or the ability to manage patient immune responses under treatment.

Comparability of measurement with agreed metrological traceability is achieved through the development of standardised reference measurement systems through reference measurement procedures and, where appropriate, certified reference materials (CRMs) with known quantities of analytes with high purity and stability. These products are qualified and distributed through a network of metrological laboratories, allowing more precise and accurate estimation of values with uncertainty estimates for routine laboratory measurements through an unbroken chain of calibrations. In order to realise quantitative measurements, we need to define the measurand—a quantity intended for measurement—in clear and unambiguous terms. Yet, this is extremely challenging for biological macromolecules and larger entitiessuch as cells or related subcellular structures, due to their complexity, dynamic properties, inherent variability, and limited stability. Furthermore, refinement of analytical methods is equally challenging, where even subtle variation in reagents due to stability or availability may yield non-comparable results of the same analyte with the same method, or where different analytical methods are used that measure related but separate phenomena.

Considering these challenges and the well-placed position of viral vector biotechnologies as transformative to healthcare and the wider bioeconomy, the Consultative Committee for Amount of Substance, Metrology in Chemistry and Biology (CCQM) of the International Bureau of Weights and Measures (BIPM) held a workshop in January 2023 with the following objectives; to survey the state-of-the-art in analytical methods and reference materials for viral vector quantification and characterisation; to collate and explore understanding of the measurement of viral vector entities and related components with integration to the SI system where possible; to consider a strategy for the international metrology community to start delivering measurement services for a wide variety of viral vector product stakeholders; and to consider wider challenges and opportunities for collaboration across sectors (government, regulatory, industry and academic) and applications (clinical, biotechnology and research). The workshop touched on many topics and guidance to instances in the following text is given in Table 1.

The opening workshop session, “Background Challenges and Opportunities”, was intended as scene setting and presented a series of regulatory and industry perspectives. Dr Francis Galaway (MHRA, UK) outlined gene therapy products from the point of view of the UK regulator (MHRA), which adopts a risk-benefit approach to their licensing. These are classified as advanced therapy medicinal products (ATMPs) where the mechanism of action (MoA) is related to a recombinant nucleic acid that changes gene expression in a cell. A clear understanding of MoA is required to enter clinical trials, where an increasing understanding of drug and manufacturing processes is expected through characterisation testing. This provides knowledge of product heterogeneity and CQAs, which can be controlled using a set of measures demonstrable through validated analytical methods during authorization.

During the long development phase and post-authorization, it is typical that changes to the manufacturing process occur. Analytical methods should develop in parallel and must be able to maintain control of product attributes. New facilities may be added, and

dramatic changes in scale may occur, requiring analytical methods suited to manufacturing rather than research. Strong applications will adopt appropriate analytical methods early in development and plan for change. The most common objection to a marketing authorization on quality grounds is failure to demonstrate comparability. The regulator requires assurance that the manufacturing process can consistently produce a commercial product comparable to that used in the pivotal clinical trial. A strong comparability study uses in-process monitoring, release testing, and stability data and will include well-designed potency assays that reflect the MoA and are able to detect meaningful changes in the product. With sufficient product knowledge, appreciable differences between clinical trial material and commercial products can be justified.

Table 1. Topics covered by presenter and section.

General Topics	**Presenter (Initials)/Section**
Product development and manufacturing aspects	FG, IS, WL, UH, JHB, ASK, LW, IEK, General Discussion
Characterisation. Physical/Biochemical	WL, EK, ASW, RB, TEC, S-YC, LL, H-JH
Characterisation. Function/Activity	UH, LL, TC, S-YC, LW
Bioinformatics and Data analysis	RB, TEC, S-YC
Documentary Standards and Harmonization	ASK, JHB, SK
Quality Control	FG, WL, EK, ASW, ASK, RB, LL, H-JH, SK, General Discussion
Vaccine development	LW, JHB, IEK
Metrology	
Inter-lab studies	EK, H-JH, SK
Strategy and general aspects	WL, H-JH, AB, IEK, General Discussion, Conclusions
Purities, Impurities	WL, ASK, RB, LL
Reference Materials	EK, RB, AB, H-JH, IEK, SK
Entities	
Virus-like particles	AB, IEK
AAV	EK, ASW, TEC, LL
LV	IS, UH, EK, H-JH
Methods	
Electron microscopies	TEC, IEK
Scanning probe microscopies and related	S-YC
Flow cytometry	LW
Immunoassays	EK
Quantitative PCR, Digital PCR	EK, ASW, H-JH
High-throughput Sequencing	WL, ASK, RB
Mass-based techniques	LL, AB, IEK

For gene therapies using viral systems, there are three key areas that must satisfy the regulator: the risk of in vivo recombination potential must be characterised, the risk of oncogenic insertions requires extensive study and minimization strategies, and the presence of replication-competent viruses must be characterised using a test capable of detecting a single particle per dose of medicinal product. The early establishment of in-house reference standards and reagents is encouraged, which help with bridging studies and managing changes during development.

Isobel Searing (Oxford Biomedica, UK) considered key CQAs and analytical methods for the characterisation of lentiviral vectors (LVs), as well as highlighting key challenges. The challenging requirements for analytical methods capable of providing appropriate levels of understanding of both vector attributes and process performance for complex products, like LV-based gene therapeutics, require specialist knowledge, techniques, and equipment. Variability between methods and expression of data in different units provides a challenge when detecting, for instance, the amount of host–cell protein (HCP) present within the viral vector product as a measure of residual contaminants from the manufacturing process or the analysis of the infectious titre of the lentiviral vectors through transduction of a permissible cell line to allow calculation of dosages of the vector product and assessment of product stability over its shelf life. The utilisation of modern analytics, such as mass spectrometry, next-generation sequencing (NGS), and HPLC-based vector quantification, may offer greater levels of understanding but still require exhaustive validation.

The importance of considering any inherent laboratory method variations between test centres, particularly for more complex multi-step biological assays that test products manufactured at multiple sites, should also be considered carefully. Key factors might include the choice of cell line or primer/probe sequence selection and assurance over the availability of critical reagents with unchanged specifications over time. Some of these challenges can be resolved by specific techniques or instrumentation, for example, employing droplet digital polymerase chain reaction (ddPCR) technology to overcome variability attributed to the preparation of a calibration standard. A particular challenge is the finite size of any batch of antibodies and the variation between batches due to their polyclonal nature. However, strategies for moving away from commercial kits to reduce demand for antibody stocks are likely to require cross-over studies. The inclusion of well-characterised reference standards has a crucial role to play in controlling consistency and the comparison of data.

Dr Wilson Li (Cell and Gene Therapy Catapult, UK) presented challenges and opportunities for adenovirus-associated viral vector (AAV) characterization and how analytics can be applied to the control of manufacturing processes. The importance of AAVs to the gene therapeutics sector was highlighted given their application in circa 80% of worldwide clinical trials in 2021; over 90% in UK/Europe, 80% in the US, and 60% in Asia [5]. The levels of drug substances and impurities can change throughout manufacturing, and there is a key role for analytics in process control, characterisation, and drug release. CQAs of importance to AAV characterisation were described, alongside measurements including capsid titre, genome titre, biological titre, infectious units, and transduction units. The importance of a comprehensive impurity assessment within the bioprocess was also discussed. For each highlighted CQA, the analytical technologies available to both research and development and quality control (QC) laboratories were described, such as the more conventional use of AUC and the increasingly popular use of mass photometry to assess the AAV empty/full particle ratio.

Specific challenges and strategies for AAV characterisation were outlined, including the use of hybrid serotypes of varying capsid structure as well as the importance of checking the presence and position of inverted terminal repeat (ITR) target sites, as these can vary by assay. Again, opportunities for how the metrology community could help were expressed, such as the availability of well-characterised reference materials (RMs), greater access and participation in interlaboratory comparison studies, and the general need for more reference methods of value to bench-mark in-house assays.

Dr Ulrike Herbrand (Charles River Laboratories, Germany) concluded the first session by presenting on challenges during the development of fit-for-purpose bioactivity assays as part of mandatory release and stability testing for large molecules, protein therapeutics, and ATMP products. Bioassays are never platform assays or off-the-shelf assays but instead require custom setup and transfer protocols to support clinical studies and clinical-to-commercial lot release and stability testing. Regulations require that fit-for-purpose bioassays be quantitative and stability-indicating, have established and documented accu-

racy, sensitivity, specificity, and reproducibility, be amenable to validation processes, and have pre-defined acceptance and rejection criteria. It was also recommended that bioassay design start as early as possible within the development programme.

The measurement of bioactivity for gene therapies can be affected by batch variability, transduction efficiency, gene vectors, cell culture conditions, batch size, and the shelf life of both RMs and control materials. Even when MoA is well reflected within the method design, it is often the case that high variability within the chosen method can lead to significant limitations for proving drug stability. Developers should also be aware that minor modifications to a development or ATMP production process can likely change requirements or parameters for bioassay testing. Therefore, both the design of the experimental approach and the provision of statistical support are useful recommendations for the early identification of robust parameters of relevance to MoA, as well as greatly increasing efficiency. Surrogate bioactivity markers identified from the characterisation stage can also be considered for release tests, provided appropriate bridging studies are applied.

Sessions 2 and 3 covered both physical and functional titre measurement for viral vectors by molecular, mass, particle, and imaging-based methods.

Dr Edward Kwee (NIST, USA) reviewed physical titre methods to quantify virus concentration by genome and capsid titre measurement and how these methods can be used to complement infectious titre measurement. Published values for materials are commonly misleading due to vendors mixing physical and functional titre measurements and making comparisons of titre and quality challenging. The development of relevant viral vector RMs and their associated physical titre measurement/value assignment would better enable the comparison of products and the calibration of more product-specific RMs.

Particle concentration measurement by capsid enzyme-linked immunosorbent assay (ELISA) and vector genome titre measurement by quantitative PCR (qPCR) were discussed for available AAV2 [6] and AAV8 [7] RMs. These measurements were used to infer the purity of the full capsid content and measure short-term stability. Reference was made to demonstrating optical density measurements as a measure of viral DNA for the long-term stability of the Ad5 reference material after long-term storage [7]. Developments in the field were outlined, including an LV RM that will utilise p24 ELISA measurements for particle concentration analysis. Assignment of physical titre values to RMs will require community involvement through interlaboratory studies to determine consensus measurements alongside the development and deployment of advanced techniques.

Dr Alexandra Whale (NML at LGC, UK) gave an overview of digital PCR (dPCR) technology, its advantages, and appropriate statistical analysis (Poisson). There are multiple points during production where quantification of viral targets by dPCR can support characterisation. These include: (1) determining the transfection efficiency of the viral vector components into the packaging cells, (2) quantifying the viral titre and quality assessment of vectors that have the correctly packaged genome, (3) quantifying packaging cell contamination or remnant plasmids from the production phase, (4) determining the transduction efficiency into the host–cell, (5) determining the integration efficiency in the host genomes for vectors that integrate, and (6) quantification of the expression of the gene cargo.

Through an AAV2 model, she demonstrated how dPCR could be used to quantify viral titre for AAV. Here, one of the plasmids used to transfect into the packaging cells, pTransfer, encodes the full single-stranded DNA (ssDNA) AAV2 genome between two ITRs with an enhanced green fluorescent protein (eGFP) reporter gene as the gene cargo. Assays were designed to target eGFP and ITRs. A third assay was designed for the PolyA signal. Linearised pTransfer was used to optimise the dPCR assays and to confirm no bias in copy number based on the location of the target. Concordance in the copy number of all regions of the pTransfer was observed. Further work demonstrated that the dPCR assay could be used to count the number of ITRs on either single plasmid molecules or when enzymatically separated. Proof-of-principle was demonstrated with the analysis of extracted ssDNA from AAV2 particles for identification of differences between production batches.

Dr Arifa Khan (US FDA/CBER, USA) presented on next-generation sequencing (NGS) for the detection of extraneous viruses and viral impurities. Extraneous viruses are a safety concern in biologics. These concerns include: (1) adventitious viruses that can be introduced during manufacturing due to the use of animal-derived raw materials such as cell–substrate and culture reagents, as well as through personnel, equipment, and facilities; (2) endogenous retroviruses that can be present in a latent state in cell–substrates but are active in some; (3) viruses associated with the manufacturing process, such as replication-competent viruses by de novo generation and helper viruses that are used with some viral vectors.

The quality of a product can be influenced by product-related viral impurities, for example, viral nucleic acids from disrupted cells, sequences non-specifically encapsulated in viral vector particles, and plasmids used in transductions. Dr Khan described strategies used to mitigate risk and assays recommended for adventitious virus detection, including their limitations compared to the use of NGS, which resulted in the identification of porcine circovirus type 1 in the licensed Rotarix vaccines [8] and the discovery of a novel rhabdovirus in an Sf9 cell line, used for baculovirus-expressed products [9,10].

With this background, Dr Khan introduced various applications of NGS for improving the viral safety of biologics: testing to mitigate the risk of adventitious virus introduction; monitoring the absence of extraneous viruses during production; detection and characterisation of extraneous viral sequences in the final product; and characterisation of viral vector sequences. She described the WHO International Reference Reagents for adventitious virus detection available from CBER for NGS qualification and validation studies [10]. Dr Khan described the ongoing efforts in the Advanced Virus Detection Technologies Interest Group [11] by regulatory agencies, industry, and contract research organisations to use NGS as a broad and sensitive method for supplementing or replacing the currently used adventitious virus assays. She also described collaborative studies for evaluating different NGS platforms and technologies, as well as the development of virus reference reagents for cell-based biologics. Dr Khan also indicated to participants that genetically engineered viral vectors and viral vector-derived products can undergo virus clearance were in the scope of the updated Q5A(R2) of the International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use [12].

Dr Ravneet Bhuller (MHRA, UK) presented on biological standardisation for assurance in NGS analyses. This talk covered the potential value and challenges of applying NGS in the QC of viral vectors as therapies, with examples of NGS-based international studies conducted by the MHRA for human cancer, the microbiome, and virus detection and characterisation. The use of NGS allows the verification of product identity as well as the detection of contaminating viral DNA, e.g., as adventitious agents. It can also detect the presence of structural variants at the binding sites. The complexity of the process, however, involving multiple wet-lab and bioinformatic steps, means that interpreting results is not straightforward. Further, international collaborative studies have demonstrated that NGS bioinformatic pipelines and results can be significantly variable, attributed to the wide range of wet-lab methods available. Since inaccurate NGS-based diagnostic results can have serious implications for quality, there is a need for better methods as well as harmonisation of guidance across laboratories. She suggested that RMs represent one solution that can be applied to the standardisation and validation of NGS.

Dr Thomas Cleveland (NIST, USA) gave a presentation on DNA content analysis of AAVs by cryogenic electron microscopy (CryoEM) to determine the fraction of AAV particles that are correctly loaded with DNA cargo. The workflow outlined was adapted from single-particle analysis methods within the RELION framework [13]. Briefly, this consisted of (1) motion correction and CTF estimation; (2) particle picking; (3) 2D classification to eliminate incorrectly picked particles; (4) initial model generation; (5) 3D refinement; (6) 3D classification; and (7) DNA content analysis performed on each 3D class. Since viral capsid structures are essentially the same for all particles, 3D classification serves to bin viral particles by DNA content, e.g., full, empty, and partially full 3D classes. This is determined

using 3D masks that specify the capsid and interior (DNA-loaded) regions of the AAV in the CryoEM density map. A filling metric can then be defined as the ratio of the average CryoEM density of the interior to the average density of the capsid. "Full" particles were classified above an empirically determined threshold.

Several aspects of method optimisation were discussed, including the freezing method, selection of grid type, sample concentration, microscopy magnification, and resolution settings. For data analysis, different particle picking procedures were examined, such as blob picking and crYOLO (version 1.8.4) [14], and the reproducibility of the computational workflow was assessed. A total of 2500–12,000 viral particles were analysed per sample. Particles from each sample were divided into three subsets, and the standard deviation of the full fraction across subsets was less than 11% of the mean of the subsets in the worst case. This variance reflects the uncertainty of the computational procedure and is much greater than the basic counting uncertainty. However, the dependence of the classification uncertainty on the number of particles was not examined systematically in this study. Finally, the linearity of the full fraction of AAV samples with known mixing ratios was examined.

Dr Sang-Yoon Cho (Park Systems Corp., Republic of Korea) presented on "Nanometrology for viral biological systems using scanning probe microscopy". Atomic force microscopy (AFM) and related technologies allow 3D imaging and measurement of mechanical properties at resolutions able to characterise viral sample surfaces. Various scanning probe microscopy (SPM) techniques have been developed to study sample structures and functions, with four major areas for biomedical AFM applications: (1) The measurement of force between interacting molecules, the most common being protein-protein, DNA-protein, and cell–cell interaction, as well as Single-Molecule Force Spectroscopy, which can measure a single protein's 3D structural forces. (2) Use as a nano-manipulation device where biomolecular nanolithography technologies use unique probes to control the pattern of proteins or nucleotides on a biological sample surface. (3) Observing dynamic changes in biological matter using a function that can be measured in a liquid phase with a probe. (4) Observing the topography of biological samples at high resolution using various probes.

Scanning ion conductance microscopy (SICM) offers direct, non-contact, single-cell resolution of the surface morphology of soft biological materials in liquid for the study of living cellular properties, including cell division, fusion, and other fundamental physiological phenomena. SICM uses ionic current as a feedback signal, with detection through the nano-sized opening of the glass pipette. Photo-induced force microscopy (PiFM) also enables new possibilities for chemical identification at the nanometer level. These technologies are expected to bring about radical changes in biomedical research and inspection fields, including viral research.

Dr Luise Luckau (NML at LGC, UK) described the contribution of mass spectrometry for protein analysis to characterise viral vector products by a range of CQAs, including identity, potency, and purity. An example of initial work was given using recombinantly produced AAV8 material expressed in HEK293T with analysis of capsid-specific proteins VP1, VP2, and VP3. Using top-down intact protein analysis by LC-MS, the molecular weight of all three VP proteins could be determined very precisely compared to the theoretical masses. Results showed that, due to high mass accuracy, AAV serotypes can be clearly identified and distinguished by differing amino acid sequences and thus molecular weights of the VP proteins. Additionally, liquid chromatography–mass spectrometry (LC-MS)-based bottom-up peptide mapping experiments were applied to the AAV8 material to gain more in-depth knowledge regarding protein sequence and potential post-translational modifications (PTMs). For that purpose, proteins were proteolyzed specifically to smaller peptides using enzymes such as trypsin prior to analysis via LC-MS and LC-MS/MS (tandem mass spectrometry). Based on the specific masses of intact peptide and peptide fragments, protein sequences can be confirmed unequivocally, and PTMs can be localised. Different AAV production processes can affect the PTM profiles of the AAV products. As such, PTM analysis of VP proteins is of particular interest in understanding the biologi-

cal differences between AAV serotypes and the impact of PTMs on viral infectivity, cell targeting, and host response.

A fully quantitative method for VP proteins based on isotope dilution-mass spectrometry can therefore provide an accurate, SI traceable capsid titre for an AAV preparation. Exact quantities of viral RNA and protein content can further help to evaluate the quality and purity of an AAV product as well as identify and characterise HCPs. An analysis of AAV8 material by LC-MS showed a large and dominant number of HCPs, such as histones and ribosomal proteins. In conclusion, mass spectrometry is a powerful tool to characterise viral vector products regarding identity, potency, and purity based on protein analysis and shows great potential to investigate PTMs and their impact on CQAs.

Dr Lili Wang (NIST, USA) discussed the characterisation of nanometer-sized gene delivery systems using flow cytometry. Recently, in vivo generation of CAR-T cells has become a reality as a therapeutic platform for cancer treatment using gene delivery systems [15]. This advancement requires gene delivery systems to be fully characterised as safe for use. Currently, several gene delivery modalities are utilised including viral-based systems [16,17], i.e., adenovirus, AAV, γ-retrovirus, and lentivirus, and non-viral systems such as extracellular vesicles [18,19] (EVs) and lipid nanoparticles (LNPs) [20]. These gene delivery vehicles have different characteristics, such as particle size, genome content, and structure. Measurements of both physical and functional properties are therefore vital. Physical properties include particle concentration, genome copy number, and structure integrity. Measurement of transducing titre via gene integration assay, potency via transgene expression, and functional/phenotypic changes provide more information than traditional plaque assays when trying to fully understand the functions of gene delivery systems.

Among single EV analysis tools, flow cytometry is a more versatile technique because it can interrogate multiple characteristics, including size, concentration, composition, and biological activity. Dr Wang demonstrated that flow cytometry is capable of confidently measuring 40 nm unlabelled polystyrene nanoparticles, 50 nm unlabelled silica nanoparticles, and ~30 nm green-fluorescent nanoparticles [21,22]. In addition, light scattering from a 405 nm laser or fluorescence detection was able to determine the distribution, concentration, and composition of EVs using membrane dyes and fluorescently labelled antibodies recognising surface receptor tetraspanin.

Developing broadly protective vaccines and determining functional titres of neutralising antibodies against new virus variants is vital to continuously combating a pandemic. The team developed pseudovirus neutralisation assays that measure neutralisation in BSL-2 laboratories by both live cell imaging and flow cytometry. Briefly, serial dilutions of convalescent serum samples are incubated with VSV-ΔG pseudotype particles expressing the original SARS-CoV-2 spike protein with a GFP reporter for one hour. The mixture then incubates with HEK293-hACE2-TMPRSS2-mCherry target cells for 16 h. Live cell imaging is performed using GFP fluorescence to monitor infection, enabling quantification of infection and neutralisation dynamics. After imaging, cells are processed and analysed via flow cytometry, enabling rapid, high-throughput assessment of neutralization. These pseudovirus neutralisation assays were validated using patient serum samples in comparison with a surrogate bead-based assay [22], also developed by NIST, and have been used to support the development of anti-SARS-CoV-2 monoclonal antibody-based standards for harmonising various serology assays in an interlaboratory study led by NIST and the Centers for Disease Control and Prevention (US).

The final session, "Standardization and Control", opened with Dr Julian Braybrook (NML at LGC, UK) giving a presentation on the current status of international gene system documentary standards initiatives. A brief overview of the different approaches for delivering nucleic acids to target cells was given before focusing on the advantages and disadvantages of common viral vectors (LV, AAV) as efficient tools for gene delivery. An example of a typical viral vector workflow was described, as well as current and next-generation analytical techniques (i.e., precise, fast, high-throughput) that are increasingly being used for accurate characterisation of viral vector CQAs. Although certain techniques

are becoming well-established for the evaluation of particular CQAs, each method will have specific performance requirements and characterisation capabilities, and it will be important not to lose potential characterisation knowledge through the perceived recommendation of any single method during standardisation or harmonisation efforts. He also discussed the fact that, since successful vaccine development for COVID-19, we are now more familiar with mRNA being used as a therapeutic agent to prevent and treat various diseases, and this requires safe, effective, and stable delivery systems that protect the RNA from degradation to allow cellular uptake and subsequent mRNA release in vivo. He completed his presentation by considering the role of consistent, transparent, and targeted international documentary standards, in particular current proposals for a series of (initially three) international gene delivery systems documentary standards covering vocabulary, viral vector titre, and LNPs.

Drug and gene carriers are important tools for applications ranging from gene therapy and vaccine development to virus diagnostics.

Dr Andrea Briones (NPL, UK) outlined the increasing interest in synthetic virus-like particles (VLPs) with a proven ability to deliver the cargo of interest into human cells without cytotoxicity or a pathogenic bias, supported by the need for SI-traceable reference materials to benchmark the performance of commercial gene carriers and technologies.

The compositional analysis of a VLP constituent developed as a prospective SI-traceable reference material was shown [23]. The purity profile of the polypeptide VLP material had been evaluated to the highest metrological order using a mass-balance approach. This is the first gene therapy and drug delivery measurement comparison that will provide data for intracellular delivery traceable to the SI-unit. Further to this, the material allows comparability of the physicochemical properties of virus-like systems and addresses the need for more globalised measurements in the fields of gene therapy and drug delivery. The work underpins further methodology developments aiming at SI-traceable analysis and quantification of genetic material taken up by human cells.

Dr Hua-Jun He (NIST, USA) presented the preliminary results from a NIST interlaboratory study on integrated lentiviral vector copy number (VCN) measurements. As an effective measure to reduce the genotoxic and tumorigenic potential caused by uncontrolled integration, the US Food and Drug Administration recommends that the integrated lentiviral VCN should be less than 5 copies per cell. Reference or control materials are essential for accurately measuring VCN, a CQA for the safety and efficacy of gene-modified cellular products [24]. The NIST test materials comprise five human genomic DNAs extracted from clonal cell lines with 1, 2, 3, and 4 copies of the integrated provirus and the parental Jurkat cell line, or fixed cell samples from these clonal cell lines.

A total of twelve laboratories from four countries participated in this study. The methods used in VCN measurements are as follows: qPCR (four laboratories), dPCR (seven laboratories using droplets, two of them also using chamber dPCR), NGS (targeted amplicon NGS, targeted locus amplification NGS, and single cell NGS), and molecular combing. All laboratories were able to identify the five blinded VCN samples by either qPCR, dPCR, or NGS assays. In all cases, no integrated LV was identified in the control parental sample. As expected, greater variability in VCN results was shown in qPCR compared to dPCR methods. All three NGS methods reported correct VCN copies. Interestingly, one integration site in the VCN 4 sample reported by all three methods was different from the site originally published [25]. Additionally, some discrepancies were found in these integration sites among labs. Future work is planned to achieve consensus values for VCN and integration sites, especially through bioinformatics analysis for NGS data. The preliminary results from this study highlight the needs and utilities of VCN reference materials/standards to ensure quality assurance.

Dr Ibolya Kepiro (NPL, UK) presented the most recent progress in the development of SI-traceable calibrants for high-resolution microscopy. Their use for the calibration of different instrument modalities, from optical and electron to atomic force microscopes, and the implementation of the calibrants in clinics. The structural, morphological, and ultra-

structural analysis of biological materials, specimens, and biopsies with nanoscale accuracy is of particular importance for differential diagnostics, pharmaceuticals development and emerging technologies in cell therapies and engineering biology. Measurements to support such analysis are typically performed using high-resolution microscopy. A challenge has been providing quantitative metrics that are reproducible across different laboratories and instruments. With all the information available to us, progress towards finding effective solutions to this challenge has been hampered by the lack of biologically relevant reference materials and measurement procedures.

As an exemplar, reference materials based on prototype vaccines, standardised procedures, and guidelines to help benchmark the performance attributes of commercial vaccines were discussed. Nanoparticulate vaccines, e.g., virus-derived and virus-like, can show variability in physicochemical and biological properties, whilst manufacturers must take particular care to ensure performance consistency for vaccine products from development to batch release. Electron microscopy is a technique of choice that is used for assessing the morphological purity and structural consistency of vaccine candidates, but without reference standards, the comparability of commercial candidates is deemed impossible, which hinders commercialisation and subsequently an effective pandemic response.

Dr Sarah Kempster (MHRA, UK) discussed establishing CQAs for AAV vector preparations in the context of characterising a candidate reference material. Physical reference materials for viral vectors support the harmonisation of testing between laboratories and enable the comparability of gene therapies. There are currently limited reference materials for AAVs and multiple analytics that are continually being developed for this rapidly advancing field. These analytics are employed to determine the various CQAs of a product to ensure product quality. AAV serotypes for gene therapy vary, so there is also capsid diversity in products, and an understanding of the utility and appropriate selection of a reference material is critical.

To gain a deeper understanding of the performance requirements of a MHRA reference material, AAV2-GFP, the materials were distributed and tested by participants using a variety of analytical assays. Initial data from this interlaboratory comparison were presented, demonstrating a range of assays available, including qPCR and dPCR, ELISA, transmission electron microscopy, and dynamic light scattering, for supporting various aspects of AAV characterisation in relation to CQA assessment. By applying the principles of biological standardisation and expressing samples relative to a reference standard, the variability of results from different laboratories can be reduced. In conclusion, the use of a reference material can support decisions as to which measurements correlate with CQAs and assure quality.

2. General Discussion

The workshop gathered insights from a range of professionals involved in the development, manufacture, regulation, and validation of virus-derived, virus-like, and virus-based products, including gene therapies and vaccines, within a developing analytical, metrology and standards landscape. A key hurdle in the drug development pathway is establishing the potency and safety of a product, which should be reflective of the MoA, through the application of a range of physiochemical and biological assays that correlate to product CQAs. Recognition of appropriate CQAs through a risk-based approach is a vital element of success and is exemplified by starting as a comprehensive series of tests that maximise characterisation data, progressively narrowing to more specific tests upon licensing. A sufficient demonstration of product comparability is an acknowledged major hurdle to licensing due to product complexity. This is often compounded by less well-established best practices, a lack of successful case studies, or limited knowledge within the broader scientific community. It will be important to consider these factors in any strategy for standardisation. Particularly, do we need RMs that are more representative of real-world samples (commutable), better analytical support, or a clearer demonstration of industrial application and interest, and which should be prioritised?

Various analytical methods were presented, covering common sources of variability and strategies for control. Keeping parameters as constant as possible, supporting the availability of common analytical platforms, as well as the appropriate use of orthogonal methods, to verify measurements, were all considered advantageous. Proprietary assay development and a relative lack of information concerning kits, reagents, and processes were also highlighted as commonly encountered problems. Commercial pressures for efficiency savings were noted, with careful investment needed in the right areas to ensure success. Solutions that may be advantageous include open access RM specifications and reference methods with built-in quality data.

Several techniques were discussed from the point of view of specific pre-analytical and analytical or instrument qualification considerations. Examples include sample preparation and instrument cantilever choice for SPM, microfluidic considerations and instrument cleaning, manufacturer technical support for viral flow cytometry, and careful post-imaging process steps to assess capsid fullness for CryoEM. Certain methods are more platform/vendor-specific than others, with knock-on consequences and recommended strategies. For example, for NGS analysis, different available platforms have different characteristics and data outputs, so it would be advantageous to have access to several different platforms validated with reference viral stocks within varied sample matrices, particularly in situations with a high cellular background. Likewise, downstream, the use of different bioinformatics algorithms will give different results, necessitating best practices such as the use of a triplicate reference sample distributed to different pipelines. The use of TRUTH datasets [26], such as Genome in a Bottle [27,28] can also improve comparability. PCR used to validate NGS results was highlighted as an important strategy, as was the use of orthogonal methods such as infectivity assays.

With the adoption of highly dimensional, high-throughput technologies, the use of automated methods for picking standards and limiting any bias in such methods is becoming clear. In the case of particle image properties for capsid characterisation (full versus empty capsid), machine learning picking methods may be biased to pick completely full or empty particles versus partial-fill particles, necessitating multiple rounds of classification during the machine learning stage. The community should also properly assess the utility of artificial datasets alongside real datasets for machine learning applications. More work is needed to explore how these technologies can contribute to viral characterisation. Strategically, there may be cases where certain orthogonal techniques can be routinely combined with primary measurements to utilise associated advantages, such as speed and selectivity, as in the case of white light interferometry combined with AFM.

The current state of RM provision was considered, as were the forms and specifications future materials could and should take and which parties should take responsibility for their development, production, and maintenance. From a regulatory perspective, a target product specification, determined from the material that gave a good result in the pivotal clinical trial, should form the basis for an ideal reference material. Often, however, these materials are rare or may have been used up fully within the process, so developers should plan for the qualification of new reference materials with carefully applied bridging studies to link exhausted materials. The need for better guidance in the use of these existing primary (external) materials and how to bridge to secondary (in-house) materials was broadly acknowledged. Ideally, the community needs access to appropriate quantities of sufficiently stable external RMs (as representative as possible of the test items) with commercial availability for a period covering any complete development programme and effective bridging studies for new internal materials; this offers a challenge to the metrology community to consider the future development of either highly stable materials that retain a narrow set of key attributes or quantities for certain applications, i.e., a particular size measurement for a type of virus, or the production of RMs with broader properties and/or applications. These values should be tested as concordant across many analytical methods and link certain characteristics to secondary standards that are better suited for bio-analysis. Ultimately, better RMs will give more confidence in products to all parties, which in turn

drives data and knowledge sharing about a range of product types. Published results from publicly available materials will be very important for the community so that individuals can use the same materials and check their own results, often with the use of in-house pipelines. It was also clear that the development of reference methods might be an easier solution than developing RMs for biological assays.

Applying robust metrological principles to complex biological problems is indeed challenging, but we might be starting to see some progress, taking the case of VLPs fully synthesised and characterised. In such a manner, complexity could be built-in by developing robust standards and methods for concentration analysis, then progressing to purity analysis and calibration and measurement capability. In this progression, it will be essential to consider the ever-emerging techniques that enable more chemical, morphological, or functional studies, such as particle assembly, pathogenesis, or antimicrobial resistance. With more complex biology, we also need to extend our capabilities in deriving more statistically robust data sets for supporting machine learning and artificial intelligence.

The workshop also addressed the state of documentary standard provisions and strategies for the development of products covering viral vector analysis. Key features discussed included recognising the large application areas that viral vector products cover and the need to draw on translational experience from more mature related technical areas, wherever possible. Recognising specific standards needs early on was seen as challenging, due to their need to balance product specificity and complexity, be accommodating of state-of-the-art developments and advanced manufacturing environments, and recognise general concepts of value for many likely uses and types of products.

3. Conclusions and Recommendations

Several conclusions can be distilled from the workshop that should act as a basis for consideration of a strategy for standard development for viral vectors.

1. There is a need for greater physical stability of RMs for virus-derived, virus-based, or virus-like vectors. RMs should have sufficient stability to cover whole development programmes or situations where comparisons are needed across facilities or regions. These will be crucial to improving bridging studies where in-house RMs are used up or where there is a drift in product specification during manufacture.
2. The balance in determining generic versus specific material specifications should be shared between developers, regulators, and industry or government providers. More specific products with smaller patient pools are expected to lean towards developer-led initiatives, but these would be guided by common specifications allowing comparability.
3. There is a broad range of available analytical methods to support characterisation with different measurands, particularly for titre and purity assessments. A concerted harmonization effort for these methods and measurands and their correlative assessments is needed, in tandem with standardisation, which the metrology community is well placed to support.
4. Physical titre methods (capsid, genome, number concentration) should not be confused with infectious titre methods. This can lead to confusion if physical titre is used to aid a developer's understanding of how a product might act. Clearer documentary guidance on the terminology, properties, and appropriate use of these measurements between products and standards should be prioritised.
5. There are a number of emerging advanced methods that need more development to support knowledge of key uncertainties. These may combine a number of potentially important measurands of application to different CQAs for viral vectors, e.g., mass spectrometry enabling capsid protein purity/identity determination and confirming sequence and site-specific post-translation moderation; CryoEM for 3-D classification of viral particles and determination of state of filling and link to bioactivity; and flow cytometry adapted to count smaller particles and extracellular vesicles for neutralisation assays for infected cells.

The main recommendation as a next step to address these workshop conclusions is the establishment of a Task Force at CCQM with the proposed terms of reference:

- To prioritise, with external stakeholders, needs and gaps for reference measurement methods and standards, and/or best practice guidelines, for the quantification of gene delivery systems.
- To prioritise proposed comparison activities to be addressed by the international metrology community relating to the prioritised measurands of gene delivery systems.
- To document a calibration hierarchy for the prioritised measurands of gene delivery systems that would underpin the traceability of CMCs for the international metrology community through the BIPM KCDB and enable industry to demonstrate compliance with existing policies (i.e., GxP for cell and gene therapies).
- To develop knowledge transfer mechanisms supporting the metrology of gene delivery systems within the international metrology community and to user communities.

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List of Abbreviations

AAV	adenovirus associated viral vector
AFM	atomic force microscopy
ATMP	advanced therapy medicinal product
AUC	analytical ultracentrifugation
BIPM	Bureau International des Poids et Mesures
CBER	Center for Biologics Evaluation and Research
CCQM	Consultative Committee for Amount of Substance
CQA	critical quality attribute
CRM	certified reference material
CryoEM	cryogenic electron microscopy
crYOLO	Cryo-EM particle picking You Only Look Once (YOLO) object detection system
CTF	contrast transfer function
ddPCR	droplet digital polymerase chain reaction
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EVs	extracellular vesicles

GxP	Good Practice quality guidelines
HCP	Host–cell protein
ICH	International Council for Harmonisation
ITR	inverted terminal repeats
KCDB	Key Comparison Database (BIPM)
LC-MS	liquid chromatography–mass spectrometry
LNPs	lipid nanoparticles
LVs	lentiviral vectors
MHRA	Medicines & Healthcare products Regulatory Agency
MoA	mechanism of action
NGS	next generation sequencing
NIST	National Institute for Standards and Technology
NML	National Measurement Laboratory hosted at LGC Ltd.
NPL	National Physical Laboratory
PiFM	photo-induced force microscopy
PTMs	post-translational modifications
QC	quality control
qPCR	quantitative PCR
RELION	Regularised Likelihood Optimisation
RM	reference material
SICM	scanning ion conductance microscopy
SPM	scanning probe microscopy
ssDNA	single-stranded DNA
VCN	vector copy number
VLPs	virus-like particles
WHO	World Health Organization

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