Quality standards

Consultation

British Pharmacopoeia public consultation for guidance for replication competent virus assays

Consultation period 2 December 2024 to 28 February 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This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore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

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[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 two sets of guidance to support ATMP development across a wide range of organisations, laboratory settings, and therapy types. As such, the guidance is product-agnostic and does not provide a step-by-step protocol, nor constitute a prerequisite for product acceptance, but instead offers measures to ensure the production of robust, comparable, and reproducible data within and across organisations.

In the scope of gene therapy and viral vector development, the guidance on replication competent virus assays is thought to be very helpful. This guidance serves as a resource for ensuring the safety, efficacy, and reproducibility of viral vector products, pivotal in both research and clinical applications.

The guidance provides detailed recommendations regarding the sampling requirements necessary to achieve a reliable probability of detection for lenti and adeno-associated virus types. This is particularly important as the accuracy of these detection methods directly impacts the quality and safety of the viral vectors used in therapeutic applications. Proper sampling ensures that any replication competent viruses (RCVs) are identified promptly, minimizing the risk of unintended consequences in clinical settings.

Additionally, the guidance outlines best practices for the available methods used in replication competency testing. These best practices are designed to help laboratories achieve consistent and dependable results, thereby ensuring that the viral vectors meet the stringent safety standards required for therapeutic use. By following these recommendations, organizations can standardize their testing protocols, leading to improved reliability and reproducibility of results across different settings.

The guidance also addresses the considerations involved in deciding whether to utilize a Contract Development and Manufacturing Organization (CDMO) or to develop assays in-house. This decision is significant, as it can affect the timelines, costs, and quality control processes of the viral vector production. The guidance highlights the benefits and potential drawbacks of each approach, thereby helping users make informed decisions that align with their operational capacities and strategic goals.

Moreover, the guidance includes mock replication competent lentivirus (RCL) test results, which serve as practical examples to illustrate the importance of rigorous testing protocols. These examples provide valuable insights into the types of considerations required when testing starting materials and during the manufacturing process. By understanding these considerations, users can better navigate the complexities of viral vector testing and ensure the integrity of their products.

This guidance is thought to be of use to researchers, developers, and manufacturers involved in the field of gene therapy. Understanding the nuances of sampling requirements and detection probabilities is vital for maintaining the safety and efficacy of viral vector products. By adhering to the outlined best practices, organizations can enhance the reliability of their testing processes, ultimately contributing to the advancement of gene therapy and improving patient outcomes.

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 Replication competent virus assays draft guidance

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1. Abbreviations

Acronym	Definition
AAV	Adeno-Associated Virus
AdV	Adenovirus
ATMP	Advanced Therapy Medicinal Product
BP	British Pharmacopoeia
CAR	Chimeric Antigen Receptor
CHMP	Committee for Medicinal Products for Human Use
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CPMV	Cowpea Mosaic Virus
CT	Clinical Trial
ddPCR	Droplet digital PCR
DIN	Drug Identification Number
LoD	Limit of Detection
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EC	European Commission
ECACC	European Collection of Animal Cell Cultures
ELISA	Enzyme-Linked Immunosorbent Assay
ELLA	Enzyme-Linked Lectin Assay
EMA	European Medicines Agency
EoP	End of Production
EU	European Union
FDA	Food and Drug Administration
HCP	Host Cell Protein
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
HTLV	Human T-lymphotropic Virus
HTS	High Throughput Screening
ICH	International Council for Harmonisation
ISO	International Organization for Standardization
ISSN	International Standard Serial Number
ITR	Inverted Terminal Repeat
LTR	Long Terminal Repeat
LV	Lentivirus
LVV	Lentiviral Vector

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Acronym	Definition
MAH	Marketing Authorisation Holder
MCB	Master Cell Bank
MHRA	Medicines and Healthcare products Regulatory Agency
MOI	Multiplicity of Infection
MSB	Master Seed Bank
MVS	Master Virus Seed
NGS	Next Generation Sequencing
PBS	Phosphate-Buffered Solution
PCR	Polymerase Chain Reaction
PERT	Product Enhanced Reverse Transcriptase
PSI	Packaging Signal
QFPERT	Quantitative Fluorescent Product Enhanced Reverse Transcriptase
QPCR	Quantitative Polymerase Chain Reaction
QPERT	Quantitative Product Enhanced Reverse Transcriptase
RCAAV	Replication Competent Adeno-Associated Virus
RCL	Replication Competent Lentivirus
RCR	Replication Competent Retrovirus
RCV	Replication Competent Virus
RNA	Ribonucleic Acid
RRE	Rev-Responsive Element
RT	Reverse Transcriptase
RV	Retrovirus
SIV	Simian Immunodeficiency Virus
TEM	Transmission Electron Microscopy
TU	Transducing Units
USA	United States of America
VSV	Vesicular Stomatitis Virus
VSVG	Vesicular Stomatitis Virus G Protein
WBC	White Blood Cell
WSB	Working Seed Bank
WVS	Working Virus Seed

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2. Introduction

Replication competency is a key safety consideration when viral vectors are used as, or in the manufacturing of, Advanced Therapy Medicinal Products (ATMP). Safety considerations and testing requirements vary considerably for the different types of viral vector used. This guidance aims to provide non-mandatory recommendations for replication competency testing for the major types of viral vector used in ATMPs, e.g., Adeno-Associated Virus (AAV) and Lentivirus (LV), and to share industry best practices.

EMA and FDA recommend in addition to the testing for replication competent virus (RCV) throughout the entire manufacture process, the follow-up testing of patients for RCV. However, patient testing is out of scope of this guide and relevant other guidelines should be consulted. The focus of this guidance is on the vector manufacturing testing (master & working cell banks, vector supernatant, end of production cells and product) and the *ex vivo* product testing.

2.1 General principles

All methods used for detection of replication-competent virus should be qualified as fit-for-purpose and validated in accordance with the principles of ICH Q2 (R2). In this context, the test for replication-competent virus is defined as a purity/impurity test and should be validated for, at minimum, specificity and limit of detection. Additional performance characteristics should be demonstrated if the method serves as a quantitative test. Development of appropriate assays is encouraged early in the product development process so that sufficient performance data can be accumulated in advance of product submission. Users are referred to ICH Q2 (R2) Annex 2 for illustrative examples of approaches to analytical procedure validation. Efforts in standardisation of analytics and reference materials for lentiviral vectors are ongoing.

The FDA, EMA and Committee for Medical Products for Human Use (CHMP) recommend testing 5% of clinical lot material and 1% or up to 10^8 end-of-production (EoP) cells. However, during test development statistical considerations impacting sensitivity should be included. For example, to detect a single RCV in 10^{10} vector particles, the test article used must contain a minimum of $>10^{10}$ vector particles. In practice, a higher quantity of vector particles should be inoculated to account for the presence or absence of particles at low concentrations following a Poisson distribution. Further details and examples are described in ICH Q5A(R2), Annex 2: *statistical considerations for assessing virus and virus reduction factors*.

When considering acceptable limits for sensitivity and level of replication-competent virus in a product, consideration should also be given to the intended dosage. For example, the potential risk of administration of replication-competent virus will be significantly different for a product intended to be used at 10^{12} virus particles per dose compared to a product used at 10^8 virus particles per dose, if the same limit of 1 RCV in 10^{10} vector particles is applied to both.

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41 ## 2.2 Existing guidance

42 *Table 1 The existing guidance from regulatory agencies, FDA and EMA can be found in the following documents.*

Organisation	Document Title
FDA Guidance for Industry (Jan 2020)	Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up
ICH (Jun 2009)	General Principles to Address Virus and Vector Shedding
ICH (Nov 2023)	ICH_Q2(R2)_Guideline_2023_1130.pdf - VALIDATION OF ANALYTICAL PROCEDURES
ICH (Nov 2023)	ICH_Q5A(R2)_Guideline_2023_1101.docx - VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN
EMA (Mar 2018)	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products
EMA (Feb 2018)	Guideline on safety and efficacy follow-up and risk management of Advanced Therapy Medicinal Products

43

44 ## 2.3 Rationale for Replication Competent Virus testing

45 The safety concerns for using viral vectors were confirmed when in the early 1990s in
46 a primate gene transfer study using retroviral vectors contaminated with replication-
47 competent virus, three of ten animals developed aggressive T cell lymphoproliferative
48 disease. To address the theoretical potential for recombination events during viral
49 vector manufacture which could result in replication competent virus (RCV) and
50 secondary malignancies the vector designs have been further developed.

51 For example, to increase the safety of lentiviral vectors modifications such as deletion
52 and splitting of viral genes across plasmids, minimisation of sequence homologies,
53 introduction of self-inactivating sequences, were carried out over the years resulting
54 in multiple generations of lentiviral vectors. The most commonly used third-generation
55 lentiviral vectors would require at least four recombination events to generate
56 replication competent lentivirus (RCL), but the likelihood of these events occurring
57 sequentially and in frame during vector production is exceedingly low.

58 There are several strategies to reduce the risk of replication competent AAV (RCAAV)
59 generation such as splitting the AAV rep and cap genes into two separate transcription
60 units within the same plasmid to reduce the likelihood of recombination resulting in the
61 generation of RCAAV.

62 To further minimise the recombination risk, ongoing improvements include reducing
63 homologous sequences between vector and helper sequences, dividing helper
64 functions across more plasmids, codon optimisation to reduce homology, and utilising
65 hybrid lentiviral vector systems, such as packaging from non-virulent SIV strains.

66 In line with these developments, so far only negative RCV test results have been
67 reported from clinical trials. This includes all cell products from 26 clinical trials,

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totalling 460 transduced cell products from 375 subjects and screening of 296 clinical trial participants, were negative for the presence of RCL.[1]

However, there remains a theoretical, but unlikely risk of recombination between vector components, or between vector components and endogenous viral sequences in cells resulting in RCV. Extensive long-term testing for replication competency on packaging cell lines, vector product, but also the genetically modified cellular product and patients is therefore still a regulatory requirement (e.g. by FDA and EMA & UK).

2.4 Decisions for use of CRO for ATMP manufacture and testing

When selecting a Contract Research Organisation (CRO) for cell and gene therapy testing, it is essential to consider several key factors that reflect both the CRO's capabilities and their alignment with industry standards.

The CRO should demonstrate a proven track record in cell and gene therapy testing, supported by relevant experience in handling similar projects. Testing capabilities are equally critical; the organisation should offer a comprehensive range of assays and testing methods. The ability to develop custom assays or transfer methods ensures flexibility to meet unique project requirements.

Evaluate the technology platforms utilised by the CRO, such as ddPCR and NGS, to determine how they enhance the organisation's testing capabilities. Alongside technology, robust quality assurance processes are required to ensure accurate, reliable, and consistent results throughout the testing cycle.

The CRO should be able to guide method selection at various stages of the product lifecycle, ensuring regulatory relevance. It is crucial that the CRO complies with all relevant regulatory standards, such as qualification and GMP, and holds the necessary certifications to maintain compliance. It is the MAH's responsibility to ensure compliance.

Effective project management practices are essential, particularly in terms of clear communication protocols, adherence to project timelines, and the ability to flexibly manage any changes that may arise. The CRO should also offer robust data management services, with clear and standardized reporting formats that facilitate interpretation of results.

The reputation of the CRO should be carefully evaluated. This includes reviewing available case studies, examining previous regulatory interactions, and seeking references where available to assess their reliability and performance of similar projects.

3. Sampling and Probability of Detection

The sampling and testing requirements for replication competent virus testing are divided into two categories: product and patient. According to the FDA Guidance for Industry titled "Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up"

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108 (January 2020), comprehensive guidelines are provided on the stages at which
109 material must be sampled and tested for both product and patient categories.

110 ## 3.1 Product testing

111 Replication-competent viruses (RCVs) can emerge at any stage of their manufacturing
112 process. The manipulation and expansion procedures involved in cell products offer
113 further opportunities for the amplification of any undetected RCVs present. The table
114 below outlines the FDA's recommended timing and frequency for product sampling
115 and testing, as specified in the guidance.

116 *Table 2 FDA recommended sampling requirements (recreated from Testing of Retroviral Vector-Based Human*
117 *Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up*
118 *(Jan 2020))*

Material to be Tested	**Frequency of Testing**	**Testing for Expected RCV** (Cells and Supernatant)[1]	**Testing for Ecotropic RCV** (Cells and Supernatant)
MCB Derived by transduction with ecotropic vector Derived by transfection of retroviral vector plasmid	One-time	Yes Yes	Yes N/A
Vector Harvest Material EoP cells Vector supernatant	Product release	Yes Yes	N/A N/A
***Ex vivo* Transduced Cells**	Product release	Yes, cells only OR archive[2]	N/A

119 *[1]RCV testing should be based on the type of vector envelopes used e.g. cell lines used.*

120 *[2]If an agreement is reached with the FDA to discontinue testing, archiving of samples for at least 6*
121 *months post product expiration is recommended.*

122 ## 3.2 Sampling for AAV

123 The impact of sample volume, concentration of drug substance vector, analytical
124 sensitivity of the method(s), downstream dilution/reduction process and the volume of
125 sample corresponding to a single human dose should all be considered when
126 designing an appropriate sampling strategy. Refer to ICH Q5A(R2), Annex 3:
127 '*Statistical Considerations for Assessing Virus and Virus Reduction Factors*'

128 Analytical methods currently available necessitate the consumption of the sample,
129 rendering it impossible to sample 100% of a process stage. Therefore, demonstrating
130 the complete absence of RCAAV is unattainable. When interpreting results,
131 considerations should include: i) the volume of the sample analysed, ii) any potential
132 dilution or reduction steps post-sampling, iii) the analytical sensitivity of the method,
133 and iv) the volume of sample corresponding to a single human dose. Reported results
134 may thus indicate '< 1 RCAAV per human dose', '< 1 RCAAV per 10^{12} vector particles',
135 or another suitable format.

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136 ### 3.3 Sampling for LV

137 In January 2020, the FDA updated its guidance for replication-competent retrovirus
138 and lentivirus (RCR/RCL) testing. The new guidelines, "Testing of Retroviral Vector-
139 Based Human Gene Therapy Products for Replication Competent Retrovirus During
140 Product Manufacture and Patient Follow-up," supersede the November 2006
141 recommendations. Key changes include:

142 - **Retroviral Vector Producer Cell Working Cell Bank (WBC):** RCR testing is
143 no longer necessary.
144 - ***Ex Vivo* Transduced Cell Products:** Must be tested for RCR regardless of
145 culture time.
146 - **Consistent Negative RCR Data:** Provision to support the elimination or
147 reduction of RCR testing on *ex vivo* transduced cells with consistent negative
148 results.
149 - **Vector Supernatant Testing:** Revised recommendation to test a sufficient
150 amount of vector to demonstrate less than 1 RCR per patient dose, as
151 applicable to the manufacturing practice.

152 ### 3.4 Retroviral vector producer cell testing

153 Stably transfected vector-producer cells are cells that have been genetically modified
154 to produce retroviral or lentiviral vectors. Recent recommendations state that both the
155 cells and the supernatant from the master cell bank (MCB) production should be tested
156 for RCR/RCL. Testing of the WCB derived from an MCB that tested negative for
157 RCR/RCL testing is no longer recommended.

158 RCR/RCL testing of the MCB does not apply to cells that are transiently transfected
159 with plasmids containing vector and transgene sequences. For instance, a HEK293
160 MCB intended for lentiviral vector production using transient transfection that contains
161 no vector-specific genetic modifications does not need to be tested for RCL.

162 ### 3.5 *Ex vivo* transduced cells

163 When cells are modified through retro- or lentiviral vector transduction (autologous or
164 allogeneic) the recommendation is that each lot of drug product to be tested for
165 RCR/RCL. Previous guidance stated that only *ex vivo* transduced cells cultured for
166 longer than 4 days should be tested. The time exception has been removed in the
167 2020 Guidance and all transduced cells regardless of the time in culture should be
168 tested.

169 The updated guidelines also state that accumulated manufacturing and clinical data
170 that demonstrates that the *ex vivo* transduced cell product is consistently RCR-
171 negative can be provided to the FDA to support the reduction or elimination of RCL
172 testing.

173 ### 3.6 Volume for vector supernatant testing

174 The most significant change from the 2020 Guidance is the recommendation on the
175 volume of vector supernatant to be tested in the RCR/RCL assay.

176 In all cases, the recommendation is to test at least 5% of the total supernatant by
177 amplification on a permissive cell line. However, this may not apply to all

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manufacturing processes. For instance, 5% volume in a 20-litre production process may lead to supply and testing set-up challenges.

For production volumes exceeding 6 litres, a sufficient amount of supernatant should be tested to ensure a 95% probability of detecting 1 RCR per dose equivalent. The 2020 FDA Guidance provides an equation in Annex 1 to calculate the required testing volume to meet this standard.

To calculate the volume of supernatant to be tested, independent of lot size, it is necessary to determine a dose equivalent using parameters for direct administration or cell transduction. Subsequently, the volume that demonstrates a 95% probability of detecting 1 RCR per dose must be determined.

For direct administration of the vector, the following steps should be followed:

1. Determine the maximum amount of vector to be administered at one time, measured in transducing units (TU).
2. Calculate the testing volume (Vt) using the formula provided:

$$V_t = -\left(\frac{1}{TU}\right) \ln{(1 - 0.95)}$$

This calculation ensures that the testing volume is adequate to achieve the required detection probability.

For vectors used in cell transduction, the volume to be tested must be calculated by using specific parameters. First, the Dose Equivalent must be determined, which involves the number of cells to be transduced, the multiplicity of infection (MOI), defined as transducing units per cell (TU/cell), and the vector titre in transducing units per millilitre (TU/mL). The Dose Equivalent is calculated using the following formula:

$$\text{Dose Equivalent} = (\text{Number of cells} * \frac{\text{MOI}}{\text{Titre}})$$

Once the Dose Equivalent is determined, the testing volume (Vt) can be calculated using the following formula:

$$V_t = -\left(\frac{1}{\text{Dose Equivalent}}\right) \ln{(1 - 0.95)}$$

An example of these calculations is provided in Annex 1 of the 2020 Guidance. The table below illustrates the differences in calculating volumes based on dose equivalence using a 10-litre production volume example.

Table 3 Example calculation illustrating the difference between volumes required based on dose equivalence.

Example – 10 Liter Vector Production Volume			
Cells to be Transduced	10^8	10^8	10^8

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Example – 10 Liter Vector Production Volume			
MOI [TU/Cell]	0.5	1	10
Vector Titre [TU/mL]	10^7	10^7	10^7
Dose Equivalent [mL]	5	10	100
Volume Tested 2020 Guidance [mL]	15	30	300

214

215 It is important to recognise that knowledge of the vector titre is essential for calculating
216 the volume to be tested. This requirement must be integrated into production sampling
217 plans for collecting material for RCR/RCL testing. Specifically, it is more appropriate
218 to test the purified product rather than the bulk harvest, as the titre is typically unknown
219 at the time of harvest. While the 2020 guidance does not explicitly define "vector
220 supernatant," it is clear that dose equivalent calculations necessitate a known titre for
221 the sample being tested.

222 For RCL pre-study, the number of replicate samples used in the RCR/RCL assay for
223 a specific sample is contingent upon the volume of the test article in which 1 RCR can
224 be consistently detected. It is important to emphasize that this must be calculated
225 using the testing volume formula provided in Annex I of the 2020 Guidance.

226

227
4. Method Validation

228 The detection of the replication-competent virus is categorised as a Limit Test for
229 Impurities in accordance with ICH Q2(R2). Therefore, validation of these testing
230 methods for RCV must assess performance characteristics including Specificity and
231 Detection Limit as a minimum requirement.

232 Specificity is a measure of test accuracy and the ability to determine the
233 appropriateness of the test system. In general, assays are performed by one operator
234 to demonstrate specificity and in all assay runs, negative controls must remain healthy
235 throughout the culture period with no evidence of cytopathic effects, but likewise
236 evidence of endpoint analysis (e.g. RT = reverse transcriptase, p24 = HIV p24 capsid
237 protein, P30 = gamma-retrovirus nucleocapsid protein) must be detected in all positive
238 controls. Both viral controls and non-viral controls are included in the test system.
239 Thus, endpoint results should show detection in virus-infected cell cultures at
240 concentrations at system suitability levels. Endpoint analysis results, e.g. RT activity
241 or p24, should not be detected in the supernatant harvested from infectivity negative
242 controls by RT assays or from non-viral controls.

243 Sensitivity or the Limit of Detection (LoD) describes the lowest concentration likely to
244 be reliably measured in the test system. LoD is challenged using mock spike samples
245 in triplicate e.g. 1, 0.1, 0.01 $TCID_{50}$/mL of positive control and examined by multiple
246 operators. Endpoint activity (e.g. RT or p24) is detected at virus concentrations on all
247 occasions at the lowest concentration tested. No evidence of RT activity or p24 should
248 be present in corresponding negative controls.

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5. Adeno-Associated Virus (AAV)

AAV is a small (20-30 nm) non-enveloped virus belonging to the *Parvoviridae* family, that packages linear single-stranded DNA. To date, at least 13 different AAV serotypes (AAV1-AAV13) have been identified and classified.[2] Each serotype can differ in several properties, such as tropism, transduction efficiency and immunogenicity.

AAV lacks the necessary genes for its own replication. The virus is therefore dependent on helper viruses to provide essential replication functions, for example, Adenovirus *E1, E2A, E4* and *VARNA* genes. AAV can persist as episomes for many (>10) years in non-dividing cells. There is therefore the potential for later reactivation of AAV should the cells become infected with a suitable helper virus such as AdV or HSV.

The AAV genome is ~ 4.7 kb. It is possible to remove almost the entire genome, except for the Inverted Terminal Repeats (ITR) which are essential for replication and packaging of the genome, to achieve a cargo gene capacity of ~5 kb. Sophisticated systems have been developed to expand the cargo capacity, such as overlapping, trans-splicing, hybrid & sequential homology directed repair methods.

Propagation of the virus can be achieved in several ways including transient transfection of AdV *E1A/B*-expressing cells with *rep* and *cap* genes, cargo gene and helper genes such as AdV *E2A, E4* and *VARNA*; stable transfection of cells with cargo plasmid and AAV *rep/cap* gene, followed by infection with AdV helper virus to initiate vector production. Systems that employ replication-competent AdV or HSV helper virus will have additional RCV risks due to the helper virus, in addition to the risk of replication-competent AAV. Baculovirus production systems have also been developed, avoiding the use of mammalian cells.

The presence of replication-competent AAV can be derived from several sources during the large-scale manufacture of recombinant AAV vectors. These sources can include but are not limited to non-homologous recombination events between AAV vector and helper virus DNA, contamination from helper viruses used for manufacturing, and/or adventitious viruses.

AAV is not known to be pathogenic in humans and requires the presence of wild-type adenovirus or herpesvirus as helper viruses to replicate. The potential for unintended immune responses against either AAV proteins or the products of non-target packaged plasmid or cellular DNA is, however, an issue as it may interfere with the intended therapeutic effect and/or introduce safety risks such as adverse events in patients. When working with AAV and helper virus, the probability of producing replication-competent adenovirus (RCA) or replication-competent AAV (RCAAV) increases in each successive amplification. The RCA can be produced when the adenoviral DNA recombines with *E1* containing genomic DNA in HEK293 cell, RCAAV can be generated using homologous or non-homologous recombination events between AAV sequences present in the vector and AAV *rep* and cap sequences that are present in the manufacturing procedure.[3 https://www.nature.com/articles/s41392-021-00487-6, 4]. The generated RCAAV can replicate in the presence of a helper virus, potentially leading

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to unintended infection and replication within host cells. The presence of RCAAVs in clinical-grade AAV vector preparations is a concern, as their behaviour in the host post-administration, especially in the presence of natural helper viruses, is not fully understood.

5.1 Available methods

While endpoint or quantitative PCR represents a highly sensitive and specific method for detection of AAV genomes, it will not discriminate between replication defective and replication-competent particles. The use of a cell-based assay that specifically confirms the presence of biologically active (i.e. replication competent) virus is, therefore, necessary.

An exemplar method is the co-culture of vector preparation with an appropriate helper virus. The test article is inoculated onto susceptible cells (e.g. HEK293 or HeLa) in culture along with helper virus such as adenovirus. The specific cell type used will be determined by the AAV serotype. The test may include one or more sub passages (to allow for amplification of any rcAAV present) of supernatant with heat-inactivation of the helper virus before inoculation onto fresh cells with and without helper virus to support RCAAV replication in the cell culture. A positive control of a chimeric AAV with the intended capsid in the presence of helper virus should also be included in the assay. Any RCAAV can then amplify and be detected by PCR targeting the AAV *rep* gene (or any other appropriate target). The background signal in the target-specific PCR is determined based on the appropriate control samples such as uninfected cells, cells infected with only helper virus and cells infected with AAV positive control in the absence of helper virus. Any target-specific PCR signal above the determined background indicates the presence of RCAAV. The above is provided as an example only and alternative or variant methods may also be applicable.[5]

As part of the RCAAV co-culture method development and validation it is recommended that the helper virus is procured from a suitable commercial biorepository and is used to generate master viral banks (MVB), working viral bank (WVB) and production banks to ensure a consistent supply of helper virus. The viral banks should be characterised for titre, sequence ID and microbial contaminants as a minimum. Stocks of replication competent AAV should also be generated to be used as a positive control within the co-culture method. The positive control could be a commercially available source of wild type virus or a specifically designed virus generated in-house. It is suggested to generate banks of the replication competent positive control before characterising (titre, sequence ID, sterility and microbial contaminants) and qualifying the control in a phase appropriate manner.

A platform or standard format of the RCAAV co-culture method can be established to analyse multiple AAV serotypes, a serotype specific positive control is then used to qualify the assay although AAV2 positive control has been shown to demonstrate assay suitability for serotypes other than AAV2. Matrix suitability and interference assessment studies are also recommended during method validation, serotype specific empty AAV particles may be used during these studies to investigate any potential sample inhibition.

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335 Commercially available kits are now available to detect the presence of RCV through
336 the use of droplet digital PCR (ddPCR) to detect and quantify viral replication genes,
337 potentially indicating an RCV. It is recommended to verify any positive results in cell
338 based co-culture method, however these kits can provide rapid and reliable indications
339 of RCAAV results. Another development for the analysis of RCAAV has been the use
340 of next generation sequencing (NGS) which is described in more detail in the future
341 technologies section.

342

343 ### 5.2 Recommendations and best practices

344 There are several strategies to mitigate the risk of RCAAV formation, one strategy is
345 promoter rearrangement by positioning the P5 promoter 3' of the cap gene when
346 considering vector design. An additional strategy is to incorporate introns into the
347 vector which exceed the AAV's packaging limit, this ensures that intact contigs cannot
348 be packaged and thereby reduces the production of RCAAV. Alternatively, the AAV rep
349 and cap genes can be split into two separate transcription units within the same
350 plasmid to reduce the likelihood of recombination resulting in the generation of RCAAV.

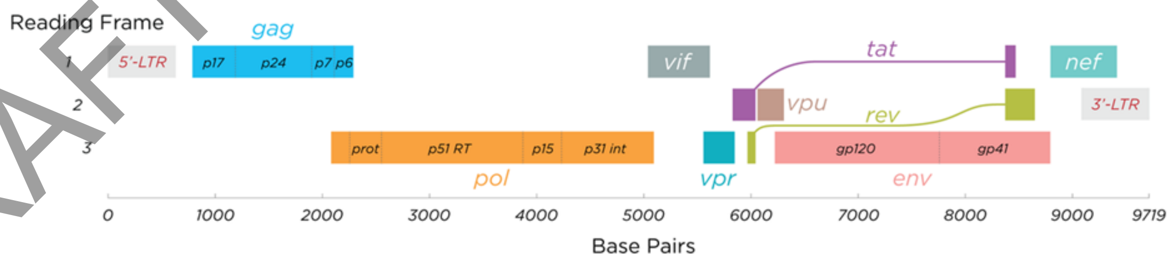
351

352 ## 6 Lentiviral Vectors (LVV)

353 Lentiviral vectors (LVV) are derived from the human immunodeficiency virus (HIV), a
354 member of the *Retroviradae* family, and are an efficient way to deliver genes to patient
355 cells as part of autologous *ex-vivo* cell and gene therapy treatment, such as CAR T-
356 cell therapies. Several cellular therapies have been licensed for medicinal use.
357 Lentiviral vectors have a broad tissue tropism, can stably integrate transgene(s) into
358 dividing and non-dividing cells and can deliver complex genetic elements. During cell
359 differentiation, they are less sensitive to gene silencing and have a safer integration
360 site profile than γ-retrovirus.

361 ### 6.1 LVV production system and safety features

362 HIV on which LVV are based has a genome that encodes nine proteins (see figure 1),
363 the structural proteins *gag*, *pol* and *env*, the regulatory proteins *tat* and *rev* as well as
364 the accessory proteins *vpu, vpr, vif* and *nef.*



365

366 *Figure 1 HIV-1 Genome.*

367

368 For the generation of LVV the HIV genome has been stripped down over time so that
369 the only sequences left are those required for the packing, transfer and integration of

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the gene of interest. These are either integrated in stable production cells lines or are transiently transfected in cell lines. Due to the toxicity of remaining HIV genes the establishment of stable transduced cell lines for LVV remains challenging and currently the best examples are using inducible expression systems (e.g with doxycycline). For the manufacture of LVV by transient transfection the most common method is the use of a third-generation, self-inactivating set of four plasmids and HEK293T cells. Earlier generations of LVV encoding plasmids have less developed safety features (see table 4) which results in a higher risk of recombination and, therefore, of generation of RCL. The production cells release the LVV particles into the cell culture supernatant which makes the downstream processing of the vector relatively easy. That includes clarification steps like filtration and steps for purification and concentration by a variety of methods (e.g. chromatography, tangential flow filtration) prior to storage of the aliquoted vector product.

Table 4 Characteristics of different generations of lentiviral vectors.

Vector generation	**1st**	**2nd**	**3rd**
Deleted HIV-1 genes	*env*	*env, vpu, vpr, vif, nef*	*env, vpu, vpr, vif, nef, tat*
Number of plasmids	3	3	4
Viral sequences in	*cis*	*cis*	*trans*
Viral genes on packaging plasmid/s	*gag, pol, tat, rev, vpu, vpr, vif, nef*	*gag, pol, tat, rev*	*gag, pol* *rev*
Viral gene on envelope plasmid	Frequently Vesicular Stomatitis Virus (VSV-G)		
Viral sequences in transgene plasmid	3' and 5'LTR, packaging signal (ψ)	3' and 5'LTR, packaging signal (ψ)	3' and 5'LTR, packaging signal (ψ), enhancer/promoter deleted in U3' region of 3'LTR (ΔU3), U3 region of 5' LTR replaced with cytomegalovirus (CMV) promoter

6.2 RCL testing methodology – Biological systems[6]

The predominant method for detecting replication-competent lentivirus (RCL) involves testing in a suitable permissive cell line through serial passages followed by a quantitative assay, such as PCR or ELISA.

Cell line C8166 (ECACC 88051601) is able to support and amplify an RCL infection over multiple passages. It is a clonal T cell line derived from the fusion of primary umbilical cord blood cells with an HTLV-1 producing line from an adult T cell leukaemia lymphoma patient and contains a defective delta retrovirus HTLV-1 genome.

For the testing C8166 cells should be inoculated with the test material or relevant control. The following day the cells should be harvested, washed and reseeded in fresh cell culture medium followed by incubation for several days. After several days these cells should be passaged five to eight times to amplify the RCL infection. It is

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397 recommended that one of the passages is a blind passage, i.e. filtered supernatant (to
398 retain cells but allow virus particles through e.g. 0.45 um) from the cell cultures is
399 transferred to fresh unmanipulated cell cultures, followed by several further cell
400 passages to minimise accidental transfer of vector particles together with the cells
401 during the cell manipulation. At each passage the cell cultures should be
402 microscopically examined for cytopathic effect (CPE). The supernatant harvested at
403 the later passages should be kept for testing for the presence of RCL by analytical
404 methods like QFPERT assay for reverse transcriptase, ELISA for p24 or VSV-G, PCR
405 for viral sequence detection.
406
407 As negative control, C8166 culture with just medium should be carried out expecting
408 the culture to remain healthy throughout the culture period with no evidence of CPE.
409 As a positive control for the assay wild-type HIV-1 or HIV-derived R8.71 should be
410 added to the control culture to confirm that the method being used has the sensitivity
411 to detect a single RCL in a sample. Additionally, to assess the impact of the test
412 material on the assay a spiked control condition should be set up by adding to the cell
413 culture the test material and positive control virus. The use of HIV-1 as control requires
414 that at least these parts of the assay are performed under Biosafety Level 3 conditions.
415
416 As the detection limit for the C8166 assay a single RCL in the background of $2.5x10^8$
417 transducing units of purified vector or $1.1x10^8$ TU of the unpurified vector can be
418 achieved which provides 99.5% confidence that less than 1 RCL per 10^9 TU of the
419 vector is present. That meets the FDA requirement for an RCR of 95% confidence
420 level equalling less than 1 RCR per 100 mL.
421

422 ### 6.3 RCL testing methodology – Analytical methods (PCR, ELISA)

423 The harvested supernatant from the cellular assay can be analysed by ELISAs
424 targeting the viral coat protein p24/p30 or spike protein e.g. VSV-G. These assays are
425 not very sensitive with a single RCL in $2.5x10^8$ TU being detectable.

426 A more common method for testing the supernatant collected from the different C8166
427 culture conditions is to use of QFPERT assay (quantitative fluorescent-product
428 enhanced reverse transcriptase). In this two-step assay, if reverse transcriptase is
429 present in a sample like in the positive and spike control the MS2 phage RNA template
430 is converted to cDNA and then amplified in a PCR step. For the PCR, a no template
431 control should be used to screen all RT-PCR reagents for the presence of
432 contaminations and medium negative control screening for air-borne contaminants. As
433 positive reverse transcriptase control, the PCR reaction should be spiked with different
434 amounts of reverse transcriptase enzyme to confirm the detection limit of the assay.
435 See Annex 1 for an example of the results of passaging C8166 infected with LVV and
436 analysed by QFPERT. Since cellular polymerase can mimic retroviral reverse
437 transcriptase activity there is a chance that amplification signals are interpretated as
438 false positive. Therefore, for the confirmation of the result an infectivity assay should
439 be carried out.

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440 Additionally, nucleic acids can be extracted from cells of the different passages of the
441 C8166 cell assay (including the control cell cultures) and used for qPCRs targeting
442 *VSV-G* envelope gene or *gag-pol* sequences to show the absence or at least decrease
443 of those over the passages. The testing and analysis of multiple PCR targets over
444 several passages, plasmid-specific sequences like antibiotic-resistance and vector-
445 specific targets like VSV-G and HIV-gag, combined, allows to come to even more
446 reliable conclusions about recombination events and likelihood for RCL. Standard
447 controls for PCRs should be included in the assay like nucleic acid extract controls
448 (e.g. by spiking samples with defined copy number of Cowpea mosaic virus DNA),
449 negative (e.g. negative control nucleic acid human DNA, no template) and positive
450 controls (e.g. appropriate viral target sequence, *CPMV*) to confirm the performance of
451 each PCR. Furthermore, the development of ddPCR methods for the detection of RCL
452 is progressing.

453

454 ### 6.4 LVV recommendations and best practices

455 For analysis of replication competent lentiviral vectors, it is recommended to perform
456 manufacture using HEK293T cells and a third generation self inactivating plasmid
457 system. The development of stable cell lines have made significant progress and are
458 also a viable option, if the licencing costs can be tolerated. As there is a theoretical
459 risk for the generation of RCL, whichever method is used for manufacturing, testing of
460 vector products and patients is still required. For that the preferred testing method is
461 the inoculation and passaging of C8166 cells using QF-PERT for analysis.

462

463 ## 7. Future Technologies

464 Cell-based methods can be time-intensive, often taking several days to weeks to
465 generate data. A new technology for detection of RCAAV is Next Generation
466 Sequencing (NGS) which is a large-scale sequencing technology. One strategy of
467 NGS known as long read sequencing enables searching for events caused by random
468 recombination, such as the generation of RCAAVs. NGS testing can therefore assist
469 in the rapid detection of RCAAVs in comparison to traditional cell culture techniques.
470 However, as previously described for PCR methods, the NGS technique alone is
471 unable to discriminate between replication defective and replication competent
472 particles therefore the use of the cell-based RCAAV test to confirm potential RCAAV
473 identified by NGS would be advised.

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476 ## Annex 1 RCL testing mock results - LVV

477

478 When testing lentiviral vector for the presence of RCL C8166 cell cultures were
479 inoculated in duplicate with the vector material (test sample) and appropriate controls
480 (negative, positive, spiked sample), respectively, to be passaged multiple times (P1-
481 P6). At each passage

482 - The cells were microscopically examined for cytopathic effect (see table 5 for
483 results),
484 - The harvested supernatant after the last passage (PP6) was analysed by PERT
485 (see table 6 and 8 for results). Usually, the test sample and negative control are
486 tested separately from positive samples which means separate assay controls
487 are carried out to determine the presence of virus (see table 7 and 9).

488 *Table 5 Cytopathic Effects by Microscopy.*

Sample	P1	P2	BP	PP3	P4	P5	P6	PP6
Negative Control 1	-	-	-	-	-	-	-	-
Negative Control 2	-	-	-	-	-	-	-	-
Test Sample 1	-	-	-	-	-	-	-	-
Test Sample 2	-	-	-	-	-	-	-	-
Spiked Test Sample (10 TU)	-	+	NA	-	+	NA	NA	NA
Positive Control 1 (10 TU)	-	+	NA	-	+	NA	NA	NA
Positive Control 2 (10 TU)	-	+	NA	-	+	NA	NA	NA

489 - no cytopathic effect observed
490 + cytopathic effect observed
491 P Passage
492 PP Post Passage
493 BP Blind Passage
494 NA Not Applicable
495 TU Transducing Units/mL
496
497
498
499
500
501
502
503
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505 *Table 6 PP6 Harvested Supernatant PERT Results.*

Sample	Mean C_T Value	Result
Negative Control 1	34.39	-
Negative Control 2	35.13	-
Test Sample 1	35.25	-
Test Sample 2	33.93	-
Negative Control Spiked with 1TU	31.35	+
Negative Control Spiked with 10TU	27.49	+

506 - No virus associated Reverse Transcriptase activity detected
507 The mean CT value was greater than that generated by the negative control
508 sample spiked with 1 infectious unit (IU) MLV.
509 + Positive Virus associated Reverse Transcriptase activity detected
510 The mean CT value was less than or equal to that generated by the negative
511 control sample spiked with 1 IU MLV.
512 C_T Cycle Threshold Value
513 PP Post Passage
514 TU Transducing Units/mL
515

516 *Table 7 PP6 PERT Assay Controls Results.*

Sample	Mean C_T Value	Result
No Template Control (Nuclease Free H_2O)	38.19	-
DMEM Control	34.72	-
Positive Control (10TU)	28.40	+
RT Positive Control – 10^{-2} units	17.21	+
RT Positive Control – 10^{-3} units	19.45	+
RT Positive Control – 10^{-4} units	22.74	+
RT Positive Control – 10^{-5} units	26.26	+
RT Positive Control – 10^{-6} units	29.89	+
RT Positive Control – 10^{-7} units	32.65	+

517 - Negative Mean C_T value greater than 10E-7 units RT
518 + Positive Mean C_T value less than or equal to 10E-7 units RT
519 TU Transducing Units/mL
520 DMEM Dulbecco's Modified Eagles Medium
521 RT Reverse Transcriptase
522 C_T Cycle Threshold Value
523
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530 *Table 8 PP3 Harvested Supernatant PERT Results.*

Sample	Mean C_T Value	Result
Spiked Test Sample (10TU)	19.39	+
Positive Control 1 (10TU)	19.16	+
Positive Control 2 (10TU)	19.20	+

531 + Positive Mean C_T value less than or equal to 10E-7 units RT
532 TU Transducing Units /mL
533 C_T Cycle Threshold Value
534

535 *Table 9 PP3 PERT Assay Controls Results.*

Sample	Mean C_T Value	Result
No Template Control (Nuclease Free H_2O)	38.56	-
DMEM Control	33.72	-
RT Positive Control – 10^{-2} units	12.34	+
RT Positive Control – 10^{-3} units	15.23	+
RT Positive Control – 10^{-4} units	18.18	+
RT Positive Control – 10^{-5} units	22.63	+
RT Positive Control – 10^{-6} units	26.40	+
RT Positive Control – 10^{-7} units	29.98	+

536 - Negative Mean C_T value greater than 10E-7 units RT
537 + Positive Mean C_T value less than or equal to 10E-7 units RT
538 TU Transducing Units / mL
539 DMEM Dulbecco's Modified Eagles Medium
540 RT Reverse Transcriptase
541 C_T Cycle Threshold Value

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