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Review Article

Orthogonal approach and critical quality attributes for gene and cell therapy products

Ekaterina V. Melnikova[1], Marina A. Vodyakova[1], Nikita S. Pokrovsky[1], Vadim A. Merkulov[1]

1 Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation; 8/2 Petrovsky Blvd, Moscow 127051 Russia

Corresponding author: Ekaterina V. Melnikova (melnikovaev@expmed.ru)

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Abstract

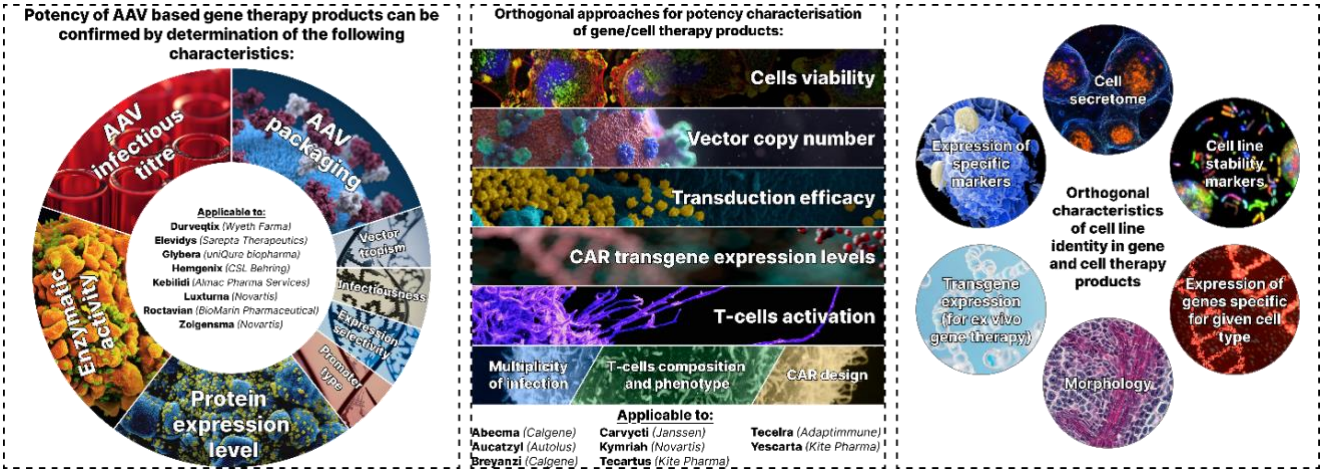
Introduction: Gene and cell therapy (GCT) products are revolutionizing medicine because they are made up of unique biological components like genetic material, viral vectors, and viable cells. However, their complexity necessitates rigorous quality control strategies to ensure efficacy, safety, and batch consistency. This manuscript explores the application of an orthogonal approach – employing multiple independent methods – to assess critical quality attributes, such as identity, potency, and purity of GCT products.

Materials and Methods: To achieve the aim of our work, we analyzed 15 GCT products for 11 different types of diseases and reports of multiple regulatory agencies.

Results: For cell-based therapies, identity is confirmed through genotypic, phenotypic, and morphological analyses, while potency is evaluated using functional assays tailored to the product's mechanism of action, such as cell viability, differentiation status, or cytokine secretion. Viral vector-based therapies require characterization of structural integrity, transgene expression, and the ratio of full to empty capsids, employing techniques like dynamic light scattering (DLS), PCR, and ELISA.

Conclusion: The paper highlights regulatory recommendations from the FDA, EMA, and WHO, emphasizing the need for validated assays during product release and stability testing. Case studies, including CAR-T cells and AAV-based therapies, illustrate the practical implementation of orthogonal methods. Challenges such as assay variability and the need for clinical correlation are discussed, underscoring the importance of assay development early in the product lifecycle. By integrating diverse analytical techniques, the orthogonal approach ensures comprehensive product characterization facilitating the translation of GCTs from research to clinical application.

Graphical Abstract



Keywords

orthogonal approach, quality attributes, quality control, cell therapy, gene therapy

Introduction

The composition of gene and cell therapy (GCT) products is fundamentally different from traditional medicines due to the presence of genetic material from plasmids, viruses carrying transgenes, cellular components, as well as physiologically active factors secreted by cells. On the one hand, the biological nature of active substances in GCT products ensures their unique characteristics. On the other hand, such products introduce an autonomous viable system into the human body, capable of persisting in the body for a long time, providing not only a positive therapeutic effect, but also causing such undesirable effects as deposition outside the target organs, off-target activity, or the development of tumors, which can pose serious risks for human health. Therefore, the development of GCT products, namely the characterization of the intermediate products, the active substance, the finished product, and subsequent production, including non-clinical and clinical studies and commercial use, requires a clear quality control strategy, especially with regard to critical quality attributes that may affect the efficacy, safety, batch-to-batch consistency, and stability of the final product. According to the recommendations of the leading regulatory agencies (Medicinal Products for Human Use 2008, 2016a; FDA 2011; CAT 2018, 2020, 2024; Salmikangas et al. 2023; CBER 2024a, 2024b), GCT products characterization studies need to utilize a wide range of orthogonal advanced techniques, including molecular, biological, and immunological testing. The orthogonal approach implies an integrated (synergetic) use of various methods for the assessment of a single quality attribute, allowing for a comprehensive characterization of the properties, composition (heterogeneity) of the active substance and finished GCT products. Orthogonal approach methodology can also be viewed as the use of independent assays to eliminate the possibility of obtaining false negative or false positive results.

Typically, functional critical quality attributes (not related to infectious safety) for GCT products are identity, potency and purity, as well as viability for cell therapy products (Committee for Medicinal Products for Human Use 2008, 2016a; FDA 2011; Council of the Eurasian Economic Comission 2016; CAT 2018, 2020).

The aim of the work was an assessment of orthogonal approach application practice for determination of gene and cell therapy critical quality attributes based on their composition, variability of their properties and biological origin.

Materials and Methods

We analyzed a number of reports and other regulatory documents of leading regulatory agencies across the globe, such as:

- Ministry of Food and Drug Safety, Republic of Korea (MFDS);
- Pharmeuropa (Ph. Eur.);
- The United States Pharmacopoeia (USP);
- The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH);
- World Health Organization (WHO);
- The United States Food and Drug Administration (FDA);
- The American National Standards Institute (ANSI);
- The Eurasian Economic Commission (ECC);
- Committee for Advanced Therapies (CAT);
- Center for Biologics Evaluation and Research (CBER);
- Ministry of Health of the Russian Federation (MoH);
- The National Institute of Health Sciences (NIHS).

Also during this work, we assessed reports on 15 GCT drug products (8 *in vivo* replacement therapy products and 7 *ex vivo* CAR-T products) for 11 different types of diseases.

All the regulatory documents that were used in this paper are available for public. We conducted additional research on the following resources: PubMed (https://pubmed.ncbi.nlm.nih.gov/); Frontiers (https://www.frontiersin.org); Biopreparations (https://www.biopreparations.ru); Elsevier (https://www.elsevier.com/); Nature (https://www.nature.com).

Results

Orthogonal approach for identity and purity characterization of gene and cell therapy products

The quality, efficacy, and safety of cell therapy products and *ex vivo* gene therapy are closely related to the quality and properties of their cellular components. The various combinations of cell/cell line properties allowing for unambiguous identification of the finished GCT product must be confirmed using an orthogonal approach to prove:

- that cells belong to a certain population, having a karyotype indicating genetic stability (and, therefore, a low risk of tumorigenic potential), and secreting certain factors;
- in the case of personalized autologous products – that cells were obtained from a specific person;
- in the case of allogeneic use, the identity of cell components must additionally be confirmed by determination of antigens of the major histocompatibility complex in order to prevent the rejection of the administered cells in the patient (Melnikova et al. 2019).

Thus, the orthogonal approach to identity testing of cell therapy products allows for cell component characterization at multiple levels – genotypic, phenotypic, and morphological. In the case of genetically modified cells, the identification should also cover the transgene (Fig. 1) (MoH 2017; Melnikova et al. 2019).

The leading regulatory agencies and the WHO recommend the following identity testing methods for cell therapy products and genetically modified cells (ICH 1998; WHO 2013; MFDS 2018; Pharmeuropa 34.3 2022; The American National Standards Institute oversees standards and conformity assessment activities in the United States 2022; Pokrovsky et al. 2024; USP 2024b, 2024c):

- flow cytometry,
- enzyme-linked immunosorbent assay (ELISA),
- fingerprinting (for example, short tandem repeat profiling (STR analysis)),
- molecular genetic methods,
- karyological chromosomal analysis, and others.

The identification of the target cell population by specific markers (phenotype) and by expression of specific genes and proteins can also serve as evidence of the product's potency. The identification of the cell karyotype indirectly characterizes the product's safety in terms of the genetic stability of cells, when assessing the risk of tumorigenic potential.

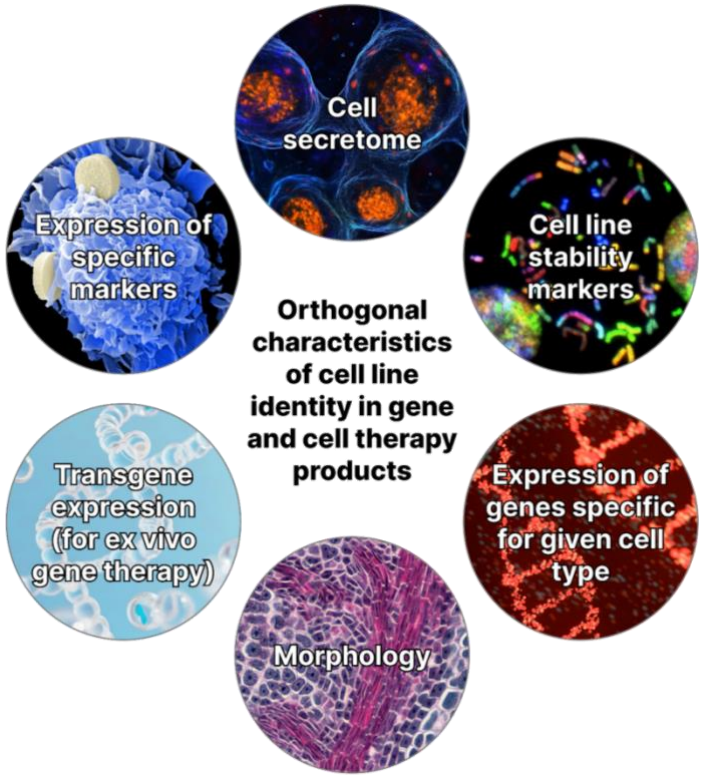


Figure 1. Characteristics that determine the authenticity of cells in cell therapy and *ex vivo* gene therapy products.

When carrying out identity testing for recombinant viral vectors, orthogonal methods are used to characterize both the viral construct (restriction analysis, transgene sequencing), and the transgene expression product (presence of the target protein and its structure) (EMA 2016; CHMP 2018b, 2018a, 2022b, 2022a; CHMP, CAT 2020). Thus, the characterization of viral vectors for the purpose of verifying their identity, activity and purity (with respect to related impurities) also requires the development of an integrated (orthogonal) approach allowing their comprehensive characterization to demonstrate efficacy and safety. For example, for recombinant adeno-associated viral vectors (rAAV), which are most often used in *in vivo* gene therapy products (EMA 2016; CHMP 2018b, 2018a, 2022b, 2022a; CHMP, CAT 2020), the following characteristics may affect critical quality attributes of the final product:

- titer of capsid or viral particles,
- percentage of full rAAV capsids,
- particle size,
- assessment of aggregate formation,
- stability,
- replication-competent virus production, and
- capsid charge.

In Cole et al. (2021), the researchers characterized the key attributes and stability profiles of rAAV samples, regardless of serotype, over a wide range of viral concentrations (titers) at different levels of genome load, sample heterogeneity, and sample conditions using biophysical methods. The results support the possibility of using dynamic light scattering (DLS) and multiangle dynamic light scattering (MADLS) to determine the size of full and empty rAAV5 (27 ± 0.3 and 33 ± 0.4 nm, respectively). The vector structural stability and viral load were determined by a combination of methods – DLS, size-exclusion chromatography with multiangle light scattering (SEC-MALS), and differential scanning calorimetry (DSC). One of the advantages of the described biophysical techniques for confirming the identity and purity of rAAV-based products is the rapid speed of the analysis not relying on the use of specific reagents and costly reference standards.

In February 2024, The United States Pharmacopeia (USP) hosted a forum on innovative analytical approaches to cell and gene therapy (USP 2024). One of the widely discussed issues was the control of related impurities in AAV-based gene therapy products – empty and

incorrectly assembled viral particles, aggregates, capsids with additional plasmid DNA, host cells – whose presence in the finished product can trigger an immune response and result in loss of efficacy of the product. The most widely used methods for the analysis of full/empty capsids ratio are analytical ultracentrifugation, gel permeation chromatography-multi-angle light scattering along with PCR and ELISA, as well as electron microscopy. However, analytical ultracentrifugation, which is used for product characterization during development, is less suitable for quality control during batch release according to GMP requirements; SEC-MALS, on the contrary, is optimal for quality control during GMP release testing, but is not capable of separating partially filled capsids. Thus, characterization of impurities in AAV-based products by orthogonal methods recognizes the advantages and limitations of these techniques, often relying on PCR and ELISA for quality control during batch release, and on their combination with, e.g., analytical ultracentrifugation, for product characterization during development (Debauve 2024).

Potency characterization of gene and cell therapy products

Potency testing should reflect the mechanism of action of the product, and, ideally, the obtained results should correlate with the clinical response. GCT products often have multiple mechanisms by which the intended effect is achieved; therefore, potency testing can cover, for example, determination of both gene activity and secreted proteins or factors, including surrogates, in demonstrating their correlation with clinical efficacy (CHMP 2008; CAT 2018; CBER 2024b). Various assay formats can be used, both *in vitro* assays and *in vivo* models; however, the timely release of products for clinical trials or commercial use requires the use of validated quantitative in vitro assays. The FDA requires the use of quantitative potency assays during batch release (FDA 2011), allowing an approach based on comparing the test sample with a reference standard (USP 2024a; European Pharmacopoeia 2025).

Reliable potency assays are also fundamental for comparability studies, process validation, and stability assessment. Given the complex composition of GCT products (nucleic acids, viral vectors, viable cells), demonstration of their potency requires a combination of methods to address multiple functional mechanisms of the product, for example:

- for cell therapy products, cell viability and the target cell phenotype are mandatory attributes, but alone will not be sufficient to establish potency;
- for cells transduced with a viral vector, potency is related to the transgene expression, characteristics of target cells, and transduction efficiency/copy number of the transgene in the cells;
- genome editing together with other cell manipulations can lead to multiple changes in the characteristics and biological activity of the cells, therefore, in addition to cell properties characterization, further assessment is required of the expected subsequent biological modifications resulting from editing (e.g., alterations in cell function);
- for recombinant viral vectors, the demonstration of the transgene presence should be accompanied by the assessment of the expression product activity.

The EMA and FDA guidelines recommend the use and evaluation of multiple potency assays for GCTs early in the development. This is due to the fact that many functional assays may be difficult to validate, while some assays, especially cell-based assays, may have high intrinsic variability that may preclude the use of such assays for release or comparability testing. Analytical methods used for characterization do not require validation according to ICH Q2 (R1) (ICH 1995), but must be qualified to ensure reliability. However, validated methods are expected for release and stability testing and in-process control testing (process consistency) during early clinical development (CBER 2020; CAT 2024).

Orthogonal approach for potency characterization of various types of GCT products

Biological activity of somatic cell-based products can be confirmed by a combination of the following attributes:

- cell viability;
- specific cell surface markers;
- functionality:

1) for chondrocytes, surrogate markers such as glycosaminoglycans (GAGs), aggrecan or collagen type 2 are often used, but their ability to form hyaline cartilage requires *in vivo* or specific *in vitro* methods (Bartz et al. 2016; EMA 2021; Guillén-García et al. 2023);

2) for MSCs, the potency assessment is based on the differentiation status (Trento et al. 2018) and the proposed mechanism of action – assessment of immunomodulatory properties (depending on whether the cells are intended for anti-inflammatory or immunostimulating action (paracrine effects)) during determination of relevant cytokines expression and MSCs interaction with host immune cells/assessment of regenerative properties (Da Silva Meirelles et al. 2009;

CAT 2011; Weiss and Dahlke 2019; Zha et al. 2021). For example, Alofisel mechanism of action is based on MSCs anti-inflammatory effect, that is, on suppression of lymphocyte proliferation and inhibition of pro-inflammatory cytokines release, thereby allowing the tissues around the fistulas to heal. The demonstration of its potency involved the determination of: ability to differentiate, immune regulation, immune-related proteins, and proteins associated with regenerative and reparative functions (CHMP 2017);

3) for dendritic cells (DCs): in the case of DCs presenting specific tumor antigens to T-cells, functional assays would preferably include assessment of stimulation of antigen-specific T-cells, testing of antigen uptake by DCs, DC maturation and tumor growth inhibition (De Wolf et al. 2018); in the case of DCs used to induce tolerance (e.g. peptide-loaded DCs), the testing will include assessment of the ability to generate regulatory T-cells (T_{regs}) (Fucikova et al. 2019);

4) for induced pluripotent stem cells (iPSCs), functionality characteristics are closely related to the differentiation status (appropriate markers and proliferation) and their intended mechanism of action in each indication. For example, when they are used for the treatment of retinal diseases like retinitis pigmentosa (RP) or age-related macular degeneration, it is necessary to control iPSCs differentiation into photoreceptor or retinal pigment epithelium cells; therefore, potency testing should include: determination of differentiation markers, assessment of the target cell structure (morphological analysis and assessment of the tissue engineered construct as a whole) and functionality (phagocytic ability by flow cytometry, secretome assessment by ELISA) (NIHS, DMD 2013; Ahmed et al. 2021).

5) for genetically modified T-cells and NK-cells: cytotoxicity, cytokine secretion, assessment of proliferative potential (CHMP 2016b, 2019, 2020; Wagner et al. 2019). For example, biological activity characteristics of CAR-T cells are presented in Fig. 2 (CHMP 2018c; CAT 2022; Mazinani and Rahbarizadeh 2022; CBER 2024a).

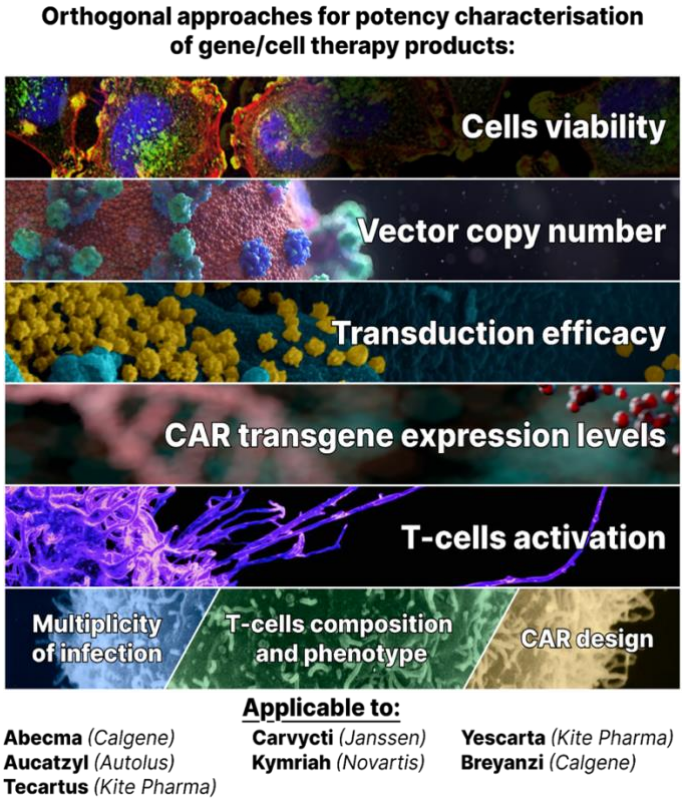


Figure 2. Orthogonal approach for potency characterization of CAR-T therapy products.

The demonstration of clinical functionality of genetically modified CD34+ cells in products based on integrating lenti- or gamma-retroviral vectors is performed after their administration, using clinical samples from the treated patients. The bioanalytical techniques measure engraftment capacity (e.g., colony formation assay), differentiation into various cell lineages (flow cytometry), and expression level/quality of the transgene product (CHMP 2016b, 2020). For products based on genetically modified cells relying on gene editing systems, it is necessary to determine the activity of the components used in production, for example, viral vectors, as well as to control all added/removed/altered cell characteristics.

Examples of a complex approach to the determination of biological activity of rAAV-based gene therapy products and plasmids used for delivery of therapeutic gene(s) are presented in Fig. 3 (Couto et al. 2016; EMA 2016; CAT 2018; Buck and Wijnholds 2020; Li and Samulski 2020; Schofield 2021).

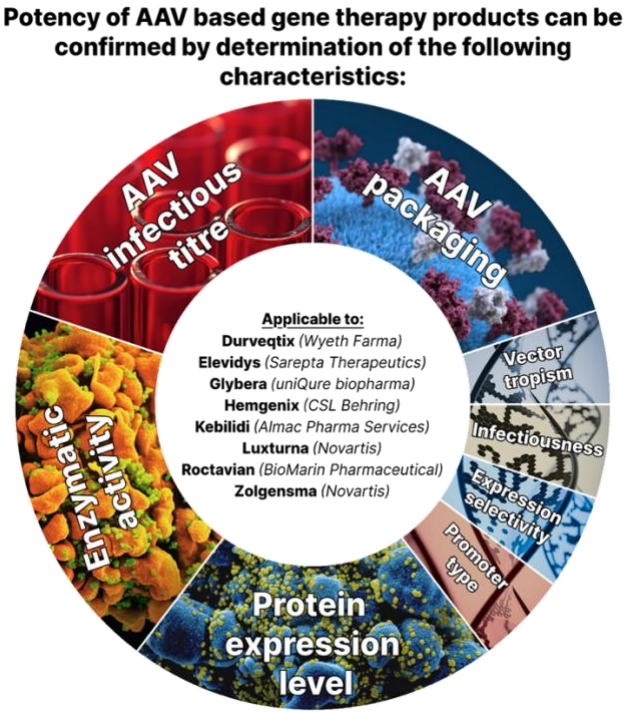


Figure 3. Orthogonal approach for potency characterization of viral vector-based gene therapy products.

Protein expression levels can be assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB) (as used, for example, in quality control of Zolgensma (CHMP, CAT 2020), as well as alternative methods – cell staining or flow cytometry. All of these approaches require a specific antibody against the protein being expressed.

The enzymatic activity measurement is used in the quality control of products intended for replacement therapy in genetic diseases, for example, lipoprotein lipase and retinoid isomerohydrolase expressed by AAVs in Glybera (EMA 2016) and Luxturna (CHMP 2018b; Melnikova et al. 2023), respectively.

The measurement of AAV infectious titer is carried out using cell-based assays in specific cell lines (for example, HEK293T) and requires the use of helper viruses (adenoviruses) (Buck and Wijnholds 2020).

The assessment of the viral capsid correct packaging for AAV is necessary due to the fact that "empty" particles containing no or only part of the genomic sequence within the capsid may impact efficacy due to the competition for receptor binding sites in target cells (Schofield 2021).

To demonstrate the potency of oncolytic viruses, whose general mechanism of action is based on either direct or indirect (via activation of immune cells) killing of tumor cells, the following set of characteristics can be used:

- infectious titer;
- ability to lyse infected tumor cells *in vitro;*
- assessment of the expression of transgenic molecules:

a) having direct (apoptosis, inhibition of angiogenesis) and indirect (stimulation of immune cells and antigen presentation by macrophages and DCs to enhance the immune response) pleiotropic antitumor effect, for example IFNα2b, whose gene is delivered to tumor cells by an adenoviral vector (Adstiladrin) (FDA 2022; Narayan et al. 2024);

b) interfering with tumor signaling (for example, TGF β), and their effects (Cristi et al. 2022), such as antitumor immune responses in the case of Imlygic (HSV expressing human granulocyte-macrophage colony-stimulating factor, GM-CSF) (Ferrucci et al. 2021);

c) mediating cell cycle arrest and DNA repair or causing apoptosis, aging and/or autophagy, depending on cellular stress conditions, for example, p53 when delivered by an adenoviral vector (Gencidine) (Zhang et al. 2018; Melnikova et al. 2021).

The analytical methods required to assess potency are specific to the product type and clinical indication. It is critical that the potency testing strategy considers the mechanism of action, the relevance of non-clinical models, and the ability to establish correlation with clinical efficacy. One of the major challenges in testing the activity of GCT formulations is that biological activity can be mediated by numerous factors, and thus a single marker or assay may not fully reflect the functionality of the product, which explains the use of an orthogonal testing approach. Developing quantitative, relevant, rapid potency assays for GCT formulations can be complex and time-consuming. The potency testing strategy and development of appropriate assays should be considered early in advance of pivotal non-clinical studies.

Conclusion

Gene and cell therapy products, characterized by intrinsic biological complexity, require stringent quality assessment frameworks to address critical attributes such as identity, potency, and purity. The integration of complementary analytical methods – termed the orthogonal approach – provides a robust mechanism to mitigate risks associated with product heterogeneity and variability. For cellular therapies, layered evaluations encompassing genetic stability, phenotypic markers, and functional activity are essential, while viral vector-based products require meticulous analysis of capsid integrity, transgene fidelity, and impurity levels. In case of products containing viable cells (including genetically modified), there is a complex of approaches capable of confirming identity and potency that includes methods like flow cytometry, ELISA, STR, PCR (and its modifications) and karyological chromosome analysis. For products based on viral vectors, capsid identification is carried out with physicochemical methods like DLS, MADLS, and SEC-MALS. Methods like PCR, restriction analysis, insertion sequencing allow for confirming the presence of target genes in recombinant virus. Potency (protein expression) can be determined by ELISA, SDS-PAGE, and WB. In addition, we can use *in vitro* tests to determine the infection titer, mechanism of action (cytotoxicity, induction of production of cytokines that can stimulating effect on immune cells or is responsible for regenerative effect and others). Regulatory mandates from agencies like the FDA and EMA underscore the necessity of validated, reproducible assays to ensure batch consistency and clinical safety. Case studies, including AAV vector characterization and CAR-T cell potency testing, exemplify the application of these methodologies in real-world settings. Challenges such as assay sensitivity limitations and the need for clinically relevant endpoints further emphasize the importance of adaptive testing strategies tailored to product-specific mechanisms. Collectively, these approaches not only align with global regulatory expectations but also reinforce the reliability of GCT products, fostering their transition from development to therapeutic use while maintaining rigorous safety and efficacy standards.

Additional Information

Conflict of interest

The authors have declared that no competing interests exist.

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Data availability

All of the data that support the findings of this study are available in the main text.

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Author Contribution

Ekaterina V. Melnikova, PhD, Head of the Laboratory of Biomedical Cell Products, Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation, Moscow, Russia; e-mail: melnikovaev@expmed.ru; **ORCID ID:** http://orcid.org/0000-0002-9585-3545. The concept and design of the study, writing the text.

Marina A. Vodyakova, PhD, Lead Expert of the Laboratory of Biomedical Cell Products, Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation, Moscow, Russia. e-mail: vodyakova@expmed.ru; **ORCID ID:** http://orcid.org/0000-0002-6008-0554. Writing the text, collection and processing of material.

Nikita S. Pokrovsky, 1st category expert of the Laboratory of Biomedical Cell Products, Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation, Moscow, Russia. e-mail: pokrovsky.ns@gmail.com; **ORCID ID:** http://orcid.org/0000-0002-2355-0879. Writing the text, collection and processing of material, creating tables and images.

Vadim A. Merkulov, Dr. of Medical Sciences, Prof., Deputy General Director for the Expertise of Medicines, Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation, Moscow, Russia. e-mail: merkulov@expmed.ru; **ORCID ID:** http://orcid.org/0000-0003-4891-973X. Editing.