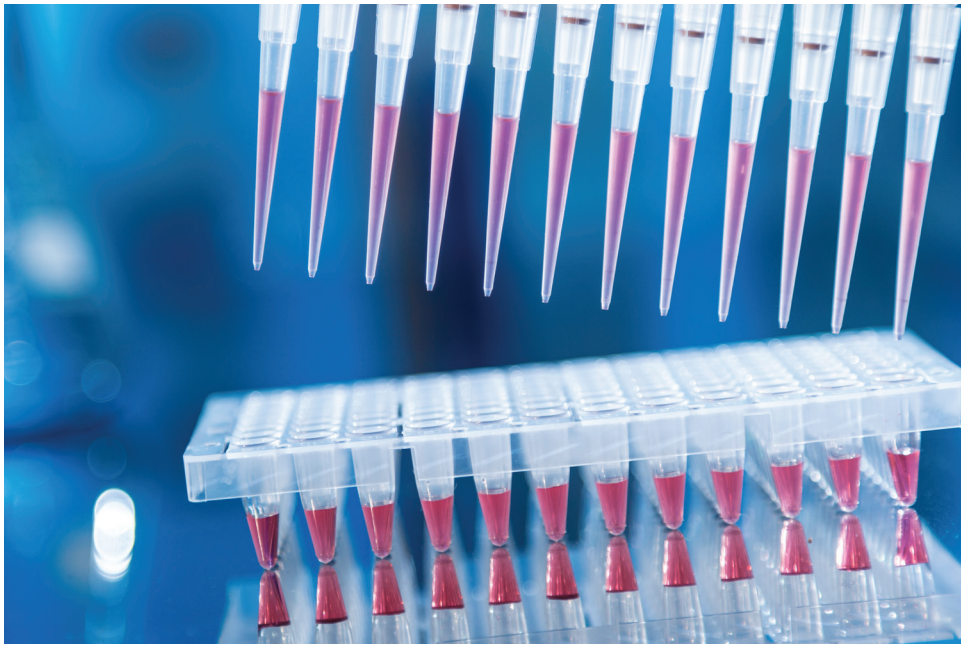

Development Strategies for Cell and Gene Therapy Methods

The increased number of regulatory filings for cell and gene therapies requires reevaluation of bioassay methods because of their complexity.

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As cell-based methods continue to evolve in support of gene therapies to assess product potency through gene or protein expression, the increased complexity and variation of assay readouts can bring questions on best-practice approaches for method development and analysis. According to FDA, the number of investigational new drug (IND) filings for cell and gene therapies (CGT) has undergone rapid growth in the past several years. There were approximately 50 related filings per year from 1995 to 2015, but that number increased to more than 300 filings between 2016 and 2020, indicating an industrywide trend toward the development and implementation of CGT products. In fact, FDA has approved multiple types of viral therapies for commercial use since 2015, including herpes simplex virus, adeno-associated virus (AAV), lentivirus, and retrovirus (1).

As the biotech industry moves further into gene therapies, we have to acknowledge the changes in production and clinical processes. Gene therapy development time is cut in half with potency and titer method development, further overlapping with process development. Autologous and allogenic cell therapies have different production processes resulting in varied cell-based testing panels that continue to evolve as we learn from the data. Considering that process changes commonly correlate with shifts in potency and titer methods, continuous data collection and review of trending data to confirm impact of process changes on these results are important to maintain acceptable method performance.

ASSAY TYPES

Mechanism of action of viral-mediated gene therapies is measured by multiple approaches, with a fundamental aspect being viral infectivity, which is core to its ability to deliver

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a therapy. Several assay types may be used to measure infectivity, such as plaque assays, focus-forming assays, and tissue culture infectious dose 50 ($TCID_{50}$). For these assays to be used in a good manufacturing practice (GMP) environment, they must be objective in their measurements and statistically robust.

The $TCID_{50}$ method meets these considerations and is frequently performed in a 96-well-plate format, which lends itself to higher throughput. It measures viral infectivity by using a dilution scheme to back calculate the starting titer based on the dilution where the virus infects cells at a statistical frequency of 50%, often using approaches such as quantitative polymerase chain reaction (qPCR) or droplet digital polymerase chain reaction (ddPCR) to detect viral nucleic acids. Given the variety of $TCID_{50}$ approaches, multiple choices must be made in assay development to create an assay that best suits the virus being titered and the end use.

First, a cell line must be chosen that is permissive for viral replication, and unique cell lines must be engineered in the case of replication-incompetent viruses. For example, titering a replication-incompetent AAV requires a cell line that expresses the Rep and Cap genes of AAV to induce productive infection. Second, a dilution scheme that suits the state of the drug product must be designed. The broader the dilution range, the greater the titer range of the assay, though this is at the cost of accuracy. The use of 10-fold dilutions in $TCID_{50}$ is common and can produce assays with wide ranges, even up to 10% to 1000% of an expected value. However, due to the wide jumps between levels, the statistical data is limited and produces less accurate results. The $TCID_{50}$ method works across dilution schemes with narrow dilution steps generating more accurate results. For drug products undergoing manufacturing optimization, the range should be kept wide while late-phase products could benefit from narrow dilution steps and more accurate results.

Perhaps the most common debate when developing $TCID_{50}$ methods is what readout should be used. Any approach that can differentiate infected wells from uninfected wells could be chosen, pending several considerations. First, the detection technique must be able to identify wells with de novo viral replication. Defective viral particles may infect cells and even express protein. Therefore, the approach should rely on the generation of progeny virus. Second, the signal being measured should be as close to viral replication as possible. Relying on secondary effects of viral replication such as cell death may be misleading. Third, the measurement should be objective. Many traditional $TCID_{50}$ approaches rely on microscopy and human identification of viral foci. This type of approach may produce different results depending on which analyst reads the assay, leading to uncertainty in the data.

Finally, the detection method need not be overly sensitive and care should be taken to avoid an overly restrictive system suitability to prevent false positives. Ideal methods of detection give repeatable thresholding for determining if a well was infected without detecting the inoculum or nonproductive infections. Exact genome copy number across replicates has no impact since the statistical modeling used to calculate the $TCID_{50}$ value only relies on whether the well was positive or negative for infectious virus.

ASSAY REQUIREMENTS FOR CELL AND GENE THERAPIES

CGT products are intended to elicit a cellular response that is directly responsible for the therapeutic effect. This response is often in the form of protein expression or modification in the target cells and tissues. While an understanding of how the virus behaves is an important part of the process, how that behavior translates into the therapeutic effect is key to determining drug efficacy, dosing, and, ultimately, patient response.

Potency assays are a direct means of measuring the targeted result of the viral infection. Although potency assays have been used in drug development for decades, application in CGT products is a more recent development. CGT products present specific challenges due to complex mechanisms of action and manufacturing processes. Often CGT products have higher variability due to cell lines, donors, and errors in replicating virus. These products also may have limited stability and potential interference from multiple expressed genes.

FDA has issued recommendations for the development of potency assays to support the drug manufacturing process for cell and gene therapies. Because the potency assays are specific for each product, it is the responsibility of the drug developer or contract research organization (CRO) to ensure the assays are a true representation of the drug's mechanism of action and that the best statistical and laboratory practices are followed.

Potency assays measure the strength or therapeutic activity of a product and are part of product conformance testing, comparability studies, and stability testing. Together, these tests measure product quality and manufacturing controls throughout all phases of the clinical study and determine if any manufacturing changes have a detrimental effect. While assays that are designed to characterize the virus are typically focused on nucleic acid analysis, such as qPCR, sequencing, or ddPCR, potency testing focuses on the outcomes of the viral transductions or infections. This often takes the form of gene expression by protein modification, activity, or production. For example, the virus may modify immune cells in such a way that they are now expressing novel receptors that can recognize cancer cells, resulting in customized immune response to a particular disorder. Potency assays

designed to measure the outcomes of such modifications are recommended to be cell-based in later stages to better demonstrate biological activity.

Common potency readouts include the enzyme linked immunosorbent assay (ELISA) platform where antibodies recognizing specific proteins or protein modifications can be used to detect an analyte in cell lysates, fixed intact cells, or fixed permeabilized cells. ELISAs produce valuable quantifiable information about the production of a specific analyte but do not measure protein's functionality and so are often combined with functional cell-based assays to tackle quantity and function.

Western blot is considered less powerful than ELISA due to its qualitative limitations but can still be used to determine protein production from cell lysates after viral transduction or infection. Semi-quantitative measurements can be made if a known amount of protein is loaded and its intensity derived using software to detect pixels. However, as with ELISA, protein functionality cannot be established from this test.

Ligand and receptor binding assays are used to evaluate the drug's mechanism of action and interaction with cell receptors. CGT products evoke a response in signal transduction and undergo optimization comparable to antibodies with aspects regarding the reference standard preparation and layout, cell surface receptor, cell line, and specificity. Ligand and receptor assays and their subsequent readout can lead to a quantifiable, functional output. Receptors can elicit a response without binding and, alternatively, signal transduction may not occur upon binding, thus, rigorous examination at these levels is imperative.

Enzyme activity assays provide functional readouts after cell infection or transduction and highlight the intended biological activity from CGT products aimed at replacing misfolded enzymes or an overall replenishment of these proteins. Dose-response relationship needs to be demonstrated between the enzymatic capabilities of a CGT vector and its readout.

TESTING FOR POTENCY

The potency test is required by FDA before releasing any lot of product from the manufacturer. Although mandated, FDA allows for considerable flexibility in determining the measurements for each product. However, all potency assays must meet minimum current good manufacturing practice (CGMP) regulations, such as indicating biological activity specific to the product, providing quantitative data, meeting pre-defined acceptance criteria, and including appropriate reference materials and controls.

FDA also includes requirements, such as establishing the accuracy, sensitivity, specificity, and reproducibility through method validations. Because of the complexity of CGT products, FDA recommends an incremental approach to implementing potency assays and recognizes that potency measurements may evolve as the product is developed.

The biological properties of CGT products must be well understood as part of the approval process. FDA recommends that a wide array of attributes be examined in addition to the routine tests for lot release. These attributes may be used to help develop robust potency assays that demonstrate a specific mechanism of action and which components of the product are contributing to any observed effect.

FDA also has recommendations on how refence material and controls should be used. A product-specific in-house reference material should be used when possible, such as well-characterized clinical lots. When applicable, commercially available material may be used, such as adenovirus type 5 retrovirus vectors and AAV type 2 vectors. All standards used for potency testing must be assigned data-driven expirations with appropriate retest dates.

In the past decade, the focus of pharmaceutical development has shifted from monoclonal antibody (mAb) therapy to gene therapy. While potency assays for cell and gene therapies have additional challenges compared to traditional products such as mAbs, there are many lessons to be learned from previous experiences. Due to the similarity in traditional potency assays between mAb therapy and gene therapy, the *United States Pharmacopeia* (*USP*) bioassay chapters, specifically <1032> *Design and Development of Biological Assays*, provide guidance that remains valuable in method development for evaluating gene therapy pharmaceuticals (2).

Similar bias and variation sources exist across CGT methods, and so should be similarly considered during optimization. New assay designs bring different ways to assess robustness and inherent variation, which may make it harder to see shifts in potency or infectious titer. Infectivity methods still have room to establish best practices for striking the appropriate balance between range and sensitivity. Since process and method development now overlap more than they had previously, closer attention also must be paid to which is driving method performance changes in order to optimize the method

Proper design of experiment should be performed when developing method qualifications and validations. Cell lines and the type of potency assays should be selected to demonstrate mechanism of action and biological activity. Assays should be linear, precise, and accurate, but assay criteria should be established based on sound statistical analysis while remaining phase appropriate for the study with more stringent criteria for later phase products.

REFERENCES

1. R. Vatsan, *Regulatory Expectations for Cell and Gene Therapies*, 4th PQRI/FDA Conference on Advancing Product Quality: Patient-Centric Product Design, Drug Development, and Manufacturing (Rockville, Md., April 9, 2019).
2. USP, General Chapter <1032>, "Design and Development of Bioassays," *USP* (Rockville, Md., 2020). ◆