ABSORPTION SYSTEMS®
A Pharmaron Company

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Immunogenicity
Best Practices

Immunogenicity Best Practices

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Best Practices

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1. Promises of Cellular and Gene Therapy

1.1 Cell and gene therapy (CGT)

Only 8% of rare diseases have an approved therapy and two-thirds of these rare diseases are congenital or genetic conditions. Cell and gene therapies (CGT) aim to treat such genetic diseases by either introducing, replacing, or inactivating genes in cells.

As it is a relatively new treatment strategy that usually targets smaller patient cohorts, there is limited clinical experience with CGT products in a large population. This can lead to uncertainty about the safety and safety assessments required. Additionally, some of these products may persist long-term or have a permanent effect after treatment.

1.2 CGT statistics

Recent years have seen a surge in CGT clinical trials. The majority of clinical trials performed worldwide are primarily focused on cancer (60%), cardiovascular diseases (6%), and central nervous system conditions (5%). CGT clinical trials are mainly conducted in the US (63.3%), followed by Europe (23.3%), China (7%), Japan (2%), and Australia (1%) (Ref: The Journal of Gene Medicine, Volume: 20, Issue: 5, First published: 25 March 2018).

In 2018, the Alliance for Regenerative Medicine presented significant increases in global spending on CGT, with a total of $13.3 billion raised in 2018, a 73% increase from the previous year. The increased funding generated a rise in 64% for gene and gene-modified cell therapy ($9.7 billion), 64% for cell therapy ($7.6 billion), and 258% for tissue engineering ($936.9 million) (Ref: Alliance for Regenerative Medicine annual report 2018).

> **The US Food and Drug Administration (FDA) also acknowledges the major surge of cell and gene therapy products in early development and predicts to be approving 10-20 cell and gene therapy products per year by 2025.**

(Statement from FDA Commissioner Scott Gottlieb and Peter Marks Director of CBER on new policies to advance development of safe and effective cell and gene therapies 2019)

1.3 Examples of successful treatment

Some of the successes in CGT are shown in recent approvals of therapies treating genetic conditions. For example, in 2018, the European Medicines Agency (EMA) approved a novel gene therapy for patients with a rare form of inherited vision loss (Leber congenital amaurosis). Luxturna (voretigene neparvovec) is an AAV-based therapy that delivers a functional copy of the RPE6 gene to treat patients with a biallelic RPE65 mutation-associated retinal dystrophy.

In 2019, the FDA approved Zolgensma (onasemnogene abeparvovec-xioi) for the treatment of patients under the age of 2 with spinal muscle atrophy (SMA). This therapy harnesses an AAV (serotype 9) vector to deliver a functional copy of the SMN1 gene to motor neurons.

2. Adeno-associated virus (AAV) vectors

2.1 Types of vectors and benefits

AAV vectors harness several features that make them ideal delivery tools for gene transfer. AAV can drive transgene expression and function for several years, even though they do not integrate efficiently into the host genome. Since the parental virions are replication-deficient and non-pathogenic, these vectors are ideal for use in CGT.

In recent years, a portfolio of natural AAV isolates (AAV serotypes) has been developed as vectors. There are currently eleven known AAV serotypes. AAV2 is the most commonly used for CGT. Different isotypes are capable of infecting different cell types in humans, allowing for specific targeting of distinct tissues by choosing the right AAV vector.

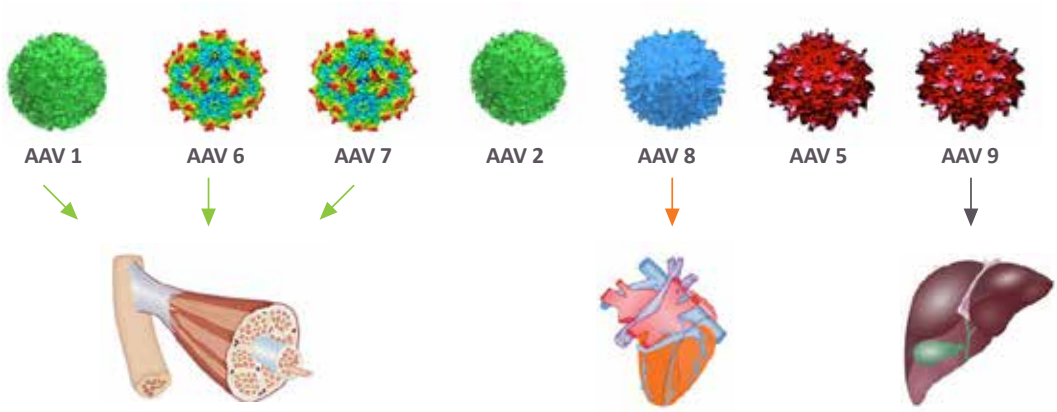

Figure 1: Various AAV serotypes differ in tissue tropism. Adapted from: Smith JK, Agbandje-McKenna M. PLoS Pathog. 2018;14(5); Mingozzi F, High KA. Blood. 2013 Jul 4;122(1):23–36.

Additional specificity can be achieved by adequate control of gene expression of the transgene. Expression that is too high or uncontrolled may result in adverse effects not intended with the therapy. To address this issue, regulatory systems that require the activation from a small molecule can be used. An example of such a system is a rapamycin-inducible system that uses the immunosuppressant drug rapamycin or an analog to activate and regulate transgene expression.

2.2 AAV immunogenicity

AAVs are not known to cause pathogenic responses and only induce mild immune responses. Therefore, on itself, AAVs are not strongly immunogenic but can provoke cellular and humoral immune responses. Additionally, the transgene product may contribute to the overall immunogenic impact, as it may encode a non-self protein.

The magnitude and speed at which an immune response is initiated is dependent on several factors:

- The tissue targeted
- The dose of the viral particles
- The route of administration
- Pre-existing antibodies

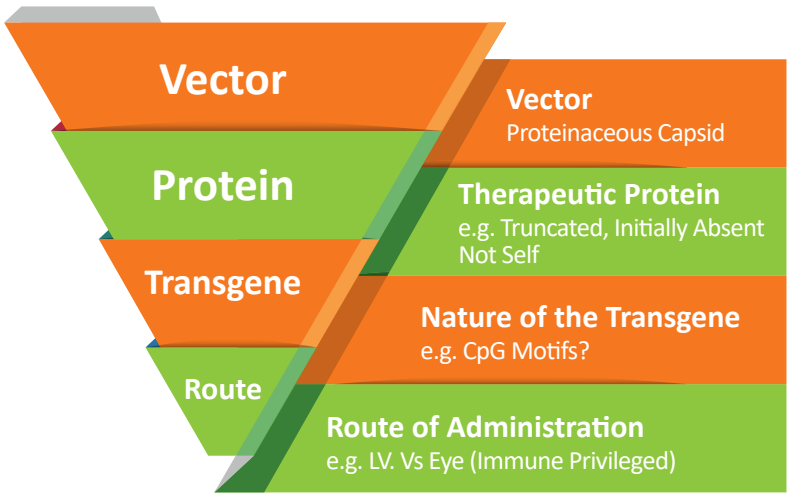

Figure 2: Factors affecting the magnitude and the speed of initiation of an immune response

2.3 Types of immune responses to AAVs

During a natural infection with a wild type AAV capsid, specific immune responses can be triggered with the development of anti-AAV antibodies and the establishment of a pool of long-lasting capsid-reactive memory B and T cells.

Upon *in vivo* administration of recombinant AAV vectors, pre-existing anti-AAV antibodies can neutralize vector particles. At the same time, memory B and T cells can be reactivated and expanded, leading to *de novo* production of anti-AAV antibodies or potential destruction of transduced cells presenting the capsid-derived antigens.

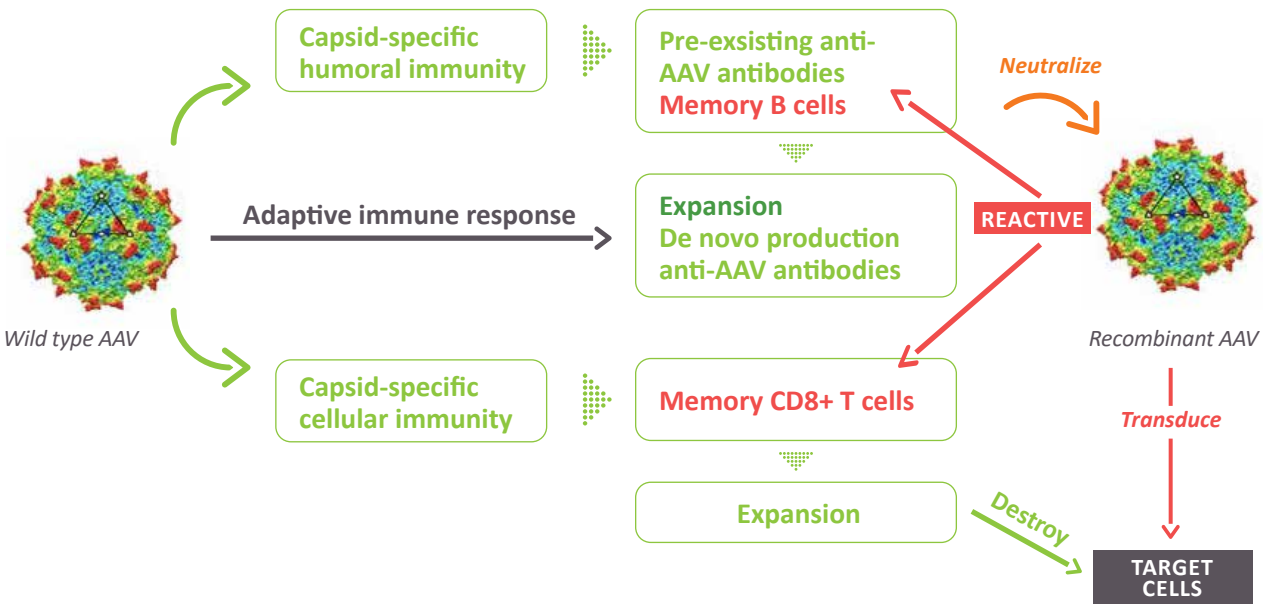

Figure 3: Overview of humoral and cellular immune response directed against AAVs

Given the rapid expansion of the CGT pipeline, regulators and industry are defining best practices for Nab, ADA, potency, and other critical assays. Early experiences with CGT products indicated that some products might pose substantial risks, including the potential for prolonged biological activity after a single administration. Therefore, the EMA and FDA have provided specific guidelines regarding immunogenicity testing of CGT products.

Guidelines provided by the EMA include:

> "The extrapolation of immunogenicity data for therapeutic applications of AAV vectors from animal models to humans is not straightforward, and the route of administration may also have an impact on the immunogenic profile of the product. It is, therefore, recommended that consideration is given to the potential of subjects having pre-existing antibodies to the serotype of AAV under investigation, and that evaluation of the immunogenicity of both the vector and the transgene is assessed in terms of neutralizing and non-neutralizing antibody formation after administration during clinical trials. The relationship (or lack thereof) between safety or efficacy and any response should be evaluated and discussed. This will be of particular importance if the aim is to re-administer the vector."

The FDA's recommendations for developing and validating assays for ADAs:

> "There are additional considerations for selecting the subject population for certain gene therapies. Pre-existing antibodies to either the vector or the transgene may influence the safety or effectiveness of the product; therefore, the study might exclude subjects with such antibodies." From: Immunogenicity Assessment for therapeutic protein products US Food and Drug Administration and Considerations for the design and early-phase clinical trials of cellular and gene therapy products *(FDA guidance for industry 2017 and 2015)*

4. Humoral immune response

4.1 Overview of antibody responses against AAVs

Two main types of AAV-specific anti-drug antibodies (ADAs) can be differentiated depending on their activity. The absence of ADAs, particularly neutralizing antibodies (Nabs), is often a prerequisite for enrollment in gene therapy trials, as the immune response can prevent or reduce transgene expression.

Binding antibodies (total Abs)

Binding antibodies are capable of binding the AAV capsid but may lack AAV neutralizing activity. In most cases, ADA assays should be designed to detect multiple antibody isotypes, including IgG, IgM, and IgA, if the mucosal route of administration is used. These antibodies can typically be detected in an immunoassay, such as the Enzyme-Linked Immunosorbent Assay (ELISA) or the Meso Scale Discovery (MSD) method.

Neutralizing antibodies (NAbs)

NAbs are a subpopulation of the total Abs that inhibit the functional activity of the AAV. NAbs can be detected by *in vitro* cell-based transduction inhibition (TI) assays and *in vivo* TI assays. It is estimated that up to 70% of the population has pre-existing NAbs directed against AAVs. Such NAbs can be induced by a natural AAV infection or can be present due to transmission from mother to child. The presence of NAbs can be detrimental to the efficacy and safety of the treatment.

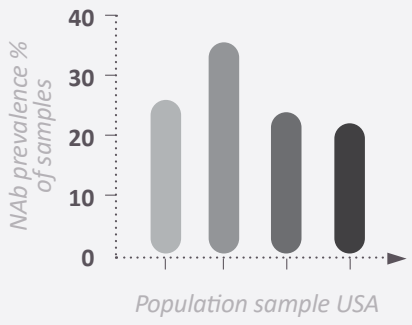

Figure 4: NAb prevalence in US population. Adapted From: Calcedo R et al. J Infect Dis. 2009 1;199(3):381–90.

Evading antibody neutralization

Techniques have been developed to prevent antibody neutralization. By encapsulating the AAV in vesicles, exosomes, or lipid-based 'cloaks', the AAV can resist neutralizing antibodies and reach the site of activity.

4.2 Anti-drug antibodies

A multi-tiered approach is recommended to assess ADAs, though it is currently not required by regulatory agencies in all situations.

4.2.1 Three-tiered approach

1. Screening assay to detect the presence of any ADAs
2. Confirmatory assay to confirm positive samples (for ADA) in the screening assay
3. Titration to determine the antibody titer
4. Additional tests, like the characterization of the ADAs, which includes isotyping, functional neutralizing TI assays (to determine the neutralizing capacity), and binding affinity assessment, may be performed as needed

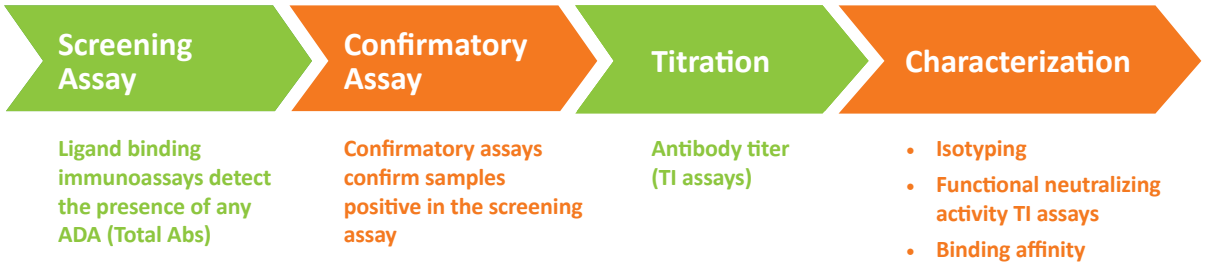

Figure 5: Multi-tiered approach to assess ADAs

These assays can be used as enrollment criteria for clinical trials or post-dose immune response monitoring.

4.2.2. Available ADA assays

Analysis of total Abs can be performed rapidly and is relatively easy to conduct. It has the advantage of higher sensitivity and can detect NAbs with a low potency that are not identified by TI assays. The downside of this approach is that the total amount of anti-AAV antibodies is not always proportional to their neutralizing activity. The total Ab assay might be useful for the determination of patient NAb titers before AAV therapy.

The most commonly used assays to assess ADAs are ELISA and MSD. The ELISA is very widely used and is a well-established technology, which is relatively inexpensive to perform. The downside of this assay is that it has a relatively narrow analytical range (usually 50-100 fold). The MSD method, on the other hand, has a broad analytical range, has a higher sensitivity, and offers the ability to multiplex. However, it does require more expensive equipment and reagents. Both techniques can be developed in an indirect or bridging format.

> **Less commonly used methods are Luminex, which allows for simultaneous detection and quantification of multiple antibody types, and Western blot.**

4.2.3. Requirements for development of a potent ADA assay

For the development of a potent ADA assay, several assay parameters should be evaluated. These include, but are not limited to:

1. **Coating conditions:** the analyte protein concentration, coat buffers, and incubation time should be optimized for each assay
2. **Positive control selection:** One of the challenges for human clinical trials is the availability of human ADA to use as a positive assay control
3. **Blocking buffer and diluent selection**
4. **Secondary antibody selection and titer:** choices include using polyclonal or monoclonal antibodies, and which concentration to use
5. **Matrix effect and background** from individual healthy human serum samples should be determined
6. **Preliminary threshold/ cut point determination:** a robust and sensitive cut point is a critical aspect of every ADA assay. For the screening assay, a cut-off factor should be established, and for the confirmatory test, a % inhibition cut-off is required.
   At least 30 samples should be used to determine the cut-off, and the samples should represent the age and the gender of the target population. Confirmed positive samples and outliers should be excluded for calculation of the cut-off factor. For the screening assay, a plate-specific cut-off is preferred in which the negative control is multiplied by the cut-off factor.
7. **Preliminary repeatability:** the intra-and inter-assay precision is determined

4.2.4. Potential challenges

Costs and time

The time and costs of assay development can be significantly affected by the availability of reagents. Development and validation of an assay typically require 3-5 mg of capsid or protein. Some capsids and proteins are available from commercial sources, but the quantity may be limited or costly. To prevent delays and high costs, we recommend the production of extra AAV capsids during the research-grade or GMP production phases, or to bulk order the transgene.

Developing a proper cut-off

There is a high prevalence of pre-existing anti-AAV antibodies in the healthy, normal population. This makes selections of the samples to develop a proper cut-off more challenging.

Positive control selection

The availability of a human ADA positive control is often a challenge for human clinical trials. To circumvent this issue, three types of positive controls might be used:

- (A) Samples from healthy individuals can be screened to identify pre-existing AAV antibodies. Positive samples can be pooled for use as positive control. The downside of this approach is that the Abs are not specifically raised against the target and are selected based on non-validated tests.
- (B) Non-human primate samples obtained during a pre-clinical study can be pooled for use as positive control. The downside of this approach is the difference in species, which will require different secondary Abs.
- (C) Mouse or rabbit ADA can be used in a bridging assay format. This approach requires additional steps of making biotinylated and/or ruthenylated protein.

4.3 Neutralizing antibodies

4.3.1 *In-vitro* testing of NAbs

In vitro cell-based transduction inhibition (TI) assays

Regulators prefer cell-based TI assays over plate-based ligand binding assays, as these provide mechanistic insight into immunogenicity. The assay can be designed to reflect the *in vivo* biology by selecting appropriate cell types as the cell lines used in the assay.

This type of assay can be performed in three days. On the first day, cells are plated and cultured overnight. On day 2, serial semi-log dilutions of the test samples are made. An AAV luciferase vector is most commonly used for AAV reporter neutralization. After that, *in vitro* transduction takes place by mixing with the target cells and incubating for 16-24 hours. On day 3, the luciferase is detected with a luminometer. The NAb titer is determined as the first reciprocal dilution at which <50% inhibition of transduction is observed though sometimes a different %inhibition can be determined for the specific assay.

Challenges for *in vitro* cell-based TI assays

The major challenge with *in vitro* cell-based TI assays is that it may require extensive time for development and validation. These assays may be less precise or robust than ligand-binding assays due to biological variability. *In vitro* TI assays work well with serotypes such as AAV2, which efficiently transfect cells *in vitro*. However, less permissive serotypes, such as AAV8, require higher MOIs for an appreciable signal, limiting assay sensitivity at low NAb titers. The choice of the right cell line is, therefore, crucial to ensure efficient transfection and should reflect the mechanism of action and viral tropism.

The choice of reporter transgene is also pivotal, as it can directly influence assay sensitivity.For instance, luciferase is sensitive and has a wide dynamic range of detection when under the control of a strong promotor like CMV. Cell-based assays are more susceptible to matrix-effects, and drug inference and cell maintenance can be time consuming and expensive.

4.3.2. *In vivo* testing of NAbs

In vivo transduction inhibition (TI) assays

For this assay type, mice are typically pre-conditioned and receive plasma or serum from patients and control preparations with or without anti-AAV antibodies. After an incubation period of a couple of days, the AAV vector is administered, and the reporter or transgene expression is measured.

Challenges for *in vivo* TI assays

The *in vivo* assay is more cumbersome, expensive, variable, and time-consuming to perform than *in vitro* assays. Furthermore, the extrapolation of immunogenicity data for therapeutic applications from animal models to humans is not straightforward.

5.1 Overview of cellular response

Cell-mediated adaptive immunity is dependent on T cells responding to AAVs. To activate a T cell response, antigen-presenting cells (APCs) display the foreign antigens and present these to T cells. Interleukins secreted by APCs and helper T cells aid in the activation of T cells. Once taken up in cells, proteasomes degrade AAVs. Subsequently, capsid fragments are transported as peptides through the transporter associated with antigen processing (TAP) and attached to MHC class I molecules. MHC class I presentation of the peptides on the cell surface can then be recognized by activated cytotoxic T cells and lead to the destruction of the cell.

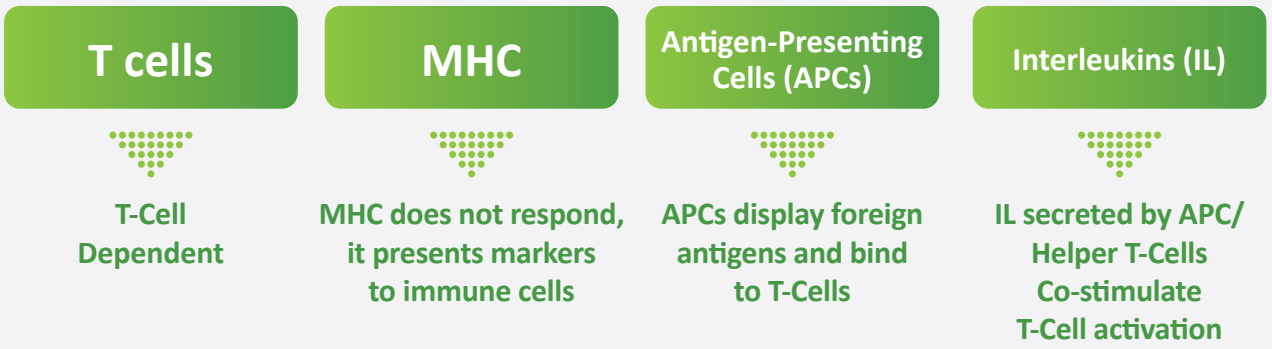

Figure 6: Cellular immune responses

Evading cellular immune responses

If an AAV causes a strong and potent cellular immune response, the transgene expression might get lost fast. If there is any pre-existing immunity in the patient treated, it may significantly hinder the performance of CGT. One way to overcome this challenge is to pre-treat the patient with an immunosuppressive drug, such as rapamycin. This immune suppression will allow the engraftment and the development of long-term T cell tolerance for the transgene.

5.2 ELISpot technology to detect cellular immune responses

Cellular responses to AAVs can be measured using the enzyme-linked immune absorbent spot (ELISpot) assay. This gold standard method quantifies the number of cytokine-secreting cells.

The assay is performed by adding PBMCs (peripheral blood mononuclear cells) to a plate that is coated with a capture antibody. After incubation for 18-20 hours, cells are removed, and a secondary biotinylated antibody is added. This antibody binds the captured analyte produced by PBMCs (often IFN-γ), which is then visualized using a detection reagent and chromogenic substrate.

> **The ELISpot technique is beneficial as a screening tool that is sensitive enough to detect low-frequency cells without requiring *in vitro* cell expansion.**

The challenges of this technique is that PBMCs need to be isolated, stored, and shipped before performing the assay, which may introduce variability.

For these reasons, we recommend the same company perform the isolation and testing.

Development of an assay for the detection of ADA in human serum samples:

- This assay was developed using an ELISA assay format in which an analyte was used to coat a 96-well plate, and human serum samples were added to test. Abs bound to the plate were detected with an HRP-conjugated anti-human IgG Ab and a solution of 3,3', 5,5'-Tetramethylbenzidine (TMB).
- The anti-analyte rabbit antibody was used to create Positive Control (PC) samples.

The validation parameters evaluated for this assay were:

- Repeatability (intra-assay) and intermediate precision (inter-assay)
- Determination of the assay cut point factor
- Sensitivity
- Specificity/drug interference
- Short-term stability
- Freeze-thaw stability
- Long-term stability
- Assay robustness: time and temperature
- Plate positioning effects (beginning to end variation)

To achieve this task, two analysts ran a total of 27 ELISA plates.

7. Why partner with Absorption Systems for advanced therapy studies?

Absorption Systems consists of a seasoned team led by study directors with over 10 years of experience in the immunogenicity field. We support gene therapy products approved by the FDA. Absorption Systems develops cell-based NAb and potency assays for biologics and small molecules.

> **We have over 20 years of experience with approved and emerging therapies. We also have optimized over 12 ADA and NAb assays, including αAAVrh8 and AAV-LK03 vectors.**

Absorption Systems has supported 2 out of 4 FDA-approved CGT products to phase I clinical trials. In 2020, we provided bioanalytical support to >35 CGT programs at various stages of development (Figure 7)

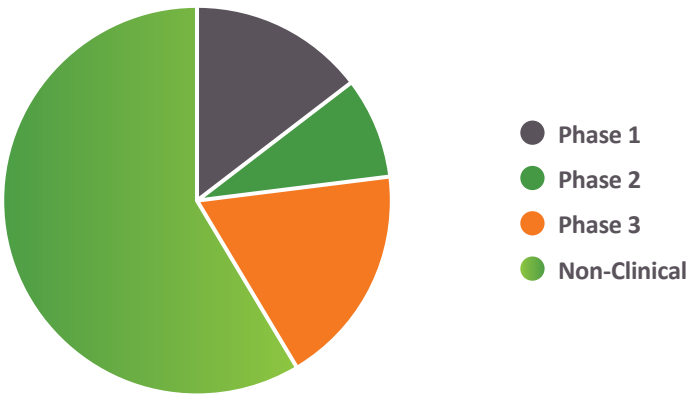

Figure 7: Recent CGT programs supported by Absorption Systems.

Reasons for outsourcing work to an analytical, nonclinical CRO:

- To concentrate internal resources on more critical tasks instead of routine sample testing
- Tests need to be run according to GLP/GMP regulations
- Lack of expertise or infrastructure to develop and validate immunogenicity assays

The goal of both parties is to have a CRO as an extension of your lab, and we are proud to contribute to the development of new therapies that can significantly benefit patients. Good communication and on-time information sharing is vital to the success in any study.

The time and effort you invest into developing a trusting relationship will pay off multiple times over.

What you need to provide for immunogenicity testing:

- Analyte and sample type
- Intended use and interpretation of the data.
  Do you need quantitative, semi-quantitative, or qualitative assays?
- Regulatory requirements: RUO, GLP, GCLP.
  Do you need optimization, qualification, or validation of your assay?
- Timelines, testing schedule, turnaround time
- Any previous development, qualification, validation, or other relevant data, if available
- Desired sensitivity (recommended <100 ng/ml but based on the application
  250 ng/ml or higher can be acceptable), accuracy, and precision
- Sample or reagent stability data and storage conditions used
- Reagents: typically assay development and validation requires 3-5 mg of capsid
  or protein. However, the design of the assay may significantly affect the amount
- Positive controls or samples availability

Why Absorption Systems

- *Full Service Integrated CRO*
- *Small and Large Animal Expertise*
- *AAALAC Accredited*
- *USDA, NIH registration*
- *FDA-Inspected*
- *ISO-Certified*
- *General Surgical Expertise*
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- *Custom Designed Programs*
- *Acute and Chronic Studies*
- *Non-GLP and GLP*
- *In Life and Bioanalysis*

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ADME Profiling

Biochemical
Metabolism
Transporters

Bioequivalance
BCS Biowaivers
Complex Drug Products

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Small & Large Animals
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