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Application Note

Viral Clearance Considerations for Adeno-Associated Virus (AAV) Viral Vectors

Introduction

Viral clearance studies for biotherapeutics are a critical step in viral risk mitigation and one of the three key elements to viral safety noted in International Council for Harmonization (ICH) Q5A **(Figure 1)** and commonly known as the safety triangle [1]. First, careful selection and testing of raw materials and product materials is performed. The assessment of these materials is to prevent virus from entering the production process. Second, the detection of potential viral contaminants during processing is assessed by testing of relevant process intermediates. The testing of cell lines, raw materials and in-process intermediates is an important step, because human cell lines are often used to produce gene therapy vectors. Third, the removal or inactivation of potential viral contaminants by unit operations of the downstream manufacturing process is assessed in a customized viral clearance study. These three elements should form the basis of viral safety from the very beginning of development, through Investigational New Drug (IND) application to Biologics License Application (BLA) and after commercial licensure of a biopharmaceutical drug product.

The upcoming revision of ICH Q5A will address the viral safety of novel therapeutics, such as Adeno-associated virus (AAV) vector-based gene therapy [2]. Viral safety for enveloped viral vectors used in cell therapies will need to focus solely on “prevention” and “detection” of potential viral contaminants because downstream inactivation or removal steps are not compatible with these products. However, for non-enveloped viral vectors, such as AAV, viral clearance needs to be demonstrated. Typically, these viral vectors are resistant to inactivation by detergent or low pH, and large pore (35-70nm) virus reduction filters will allow AAV to pass through while retaining larger viruses.

The design of viral clearance studies for monoclonal antibodies and recombinant proteins derived from Chinese hamster ovary (CHO) cell lines is well established within the industry. Similar purification steps are often evaluated, and clearance expectations are well understood. Rodent cell lines produce retroviral-like particles, and the clearance of these particles, or a surrogate virus, must exceed the number of particles in the bulk harvest. Viral clearance studies for non-enveloped viral vectors have been performed less frequently. Therefore, the virus inactivation steps and the model virus panel to be used for clearance studies may not be as clear. We address some of the questions with respect to viral clearance studies for AAV vectors below.

Figure 1. Safety Triangle

Adeno-associated virus

Adeno-associated virus (AAV) is a small virus that belongs to the *Parvoviridae* family. AAV belongs to the *Dependoparvovirus* genus, is only 20-25nm in diameter, has a 4.7 kb linear, single-stranded DNA genome and is replication defective.

To replicate, the virus requires factors from helper viruses, such as adenovirus or herpes simplex virus type 1 (HSV-1)[3]. AAV has become increasingly popular as a gene therapy approach for a number of reasons, including it is not known to cause any disease, can be grown to very high titers, and can support transgene expression without integration into the host genome.

Manufacturers can produce recombinant AAV vectors in several ways **(Figure 2)**. A plasmid DNA vector containing critical genes and portions of the AAV genome, the therapeutic transgene and helper virus genes can be transfected into producer cells. These genes can also be supplied by more than one plasmid or by helper virus.

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Another way to produce these vectors is to transfect Sf9 insect cells with recombinant baculovirus vectors that contain the transgene and key AAV genes required for AAV replication. These different strategies require different cell lines for plasmid transfection and helper virus infection. Usually a human cell line, like HEK 293 is used, but baculovirus transfection requires an insect cell line, like Sf9. The impact on the virus model used for viral clearance will be based on the AAV production strategy discussed below.

The purification strategy of AAV products can vary from product to product. Often an enveloped virus inactivation step, like solvent/detergent or low pH, is included in the downstream manufacturing process. When adenovirus is used as a helper virus, a heating step at approximately 50°C is usually included to ensure inactivation of any residual helper virus [4].

A large pore virus reduction filter, such as ones with 35nm, 50nm or 70nm nominal pores, is typically added to the process, which will retain larger, enveloped viruses, but allow the small, approximately 20nm AAV vector to pass through. AAV affinity or anion exchange chromatography columns may be included in the purification process, and some manufacturers also evaluate these columns for virus reduction. It is important to understand that other parvoviruses, like murine minute virus (MMV) or porcine parvovirus (PPV) may co-elute with AAV and therefore not provide clearance of these related viruses. The structural similarities between AAV and other parvovirus may complicate clearance of parvovirus in an AAV downstream manufacturing process.

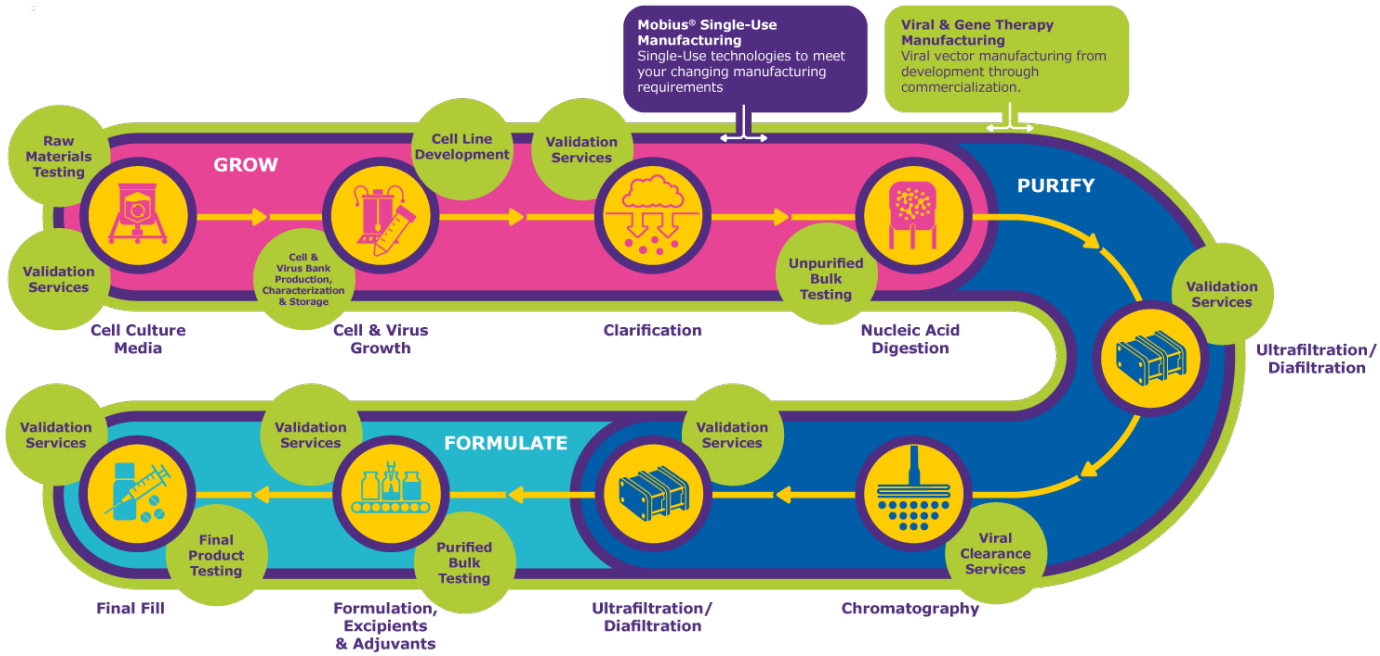

Figure 2. AAV Manufacturing Process

Virus Panel Selection

ICH Q5A states "Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general"[1]. Each manufacturer should evaluate their AAV manufacturing process and identify potential viral contaminants, based on a risk assessment. Furthermore, if the cells have been exposed to bovine serum, porcine trypsin or any other animal-derived material, the risk of potential contaminants from these materials must also be considered. These potential contaminants or viruses that model them should be used in the viral clearance study. Because AAV can be produced in more than a single cell line with or without a helper virus and with a plasmid or a baculovirus vector, the potential viral contaminants will vary with each manufacturing process.

ICH Q5A (1998) guidance discusses three types of model viruses that can be used in a virus panel for a viral clearance study. Relevant, specific model and non-specific model viruses **(Table 1. Examples of Model Viruses).** A relevant virus model is a virus that may be a potential contaminant of the manufacturing process. An example is Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV), a member of the *Baculoviridae* family. For AAV produced in a baculovirus system, it is important that the viral clearance study demonstrates that the purification process has the capacity to inactivate or remove any residual baculovirus that might be present.

Table 1. Examples of Model Viruses

Virus	Family	Structure/Genome	Sizes	Physico-Chemical Resistance	Model/Host
(Ad-2) Human Adenovirus 2	*Adenoviridae*	Non-enveloped, Double Stranded DNA	70-90 nm	High	Specific model for adenovirus used as a helper virus. Non-specific model for non-enveloped viruses with a DNA genome.
(AcMNPV) Autographa californica multicapsid nucleopolyhedrovirus	*Baculoviridae*	Enveloped, Double Stranded DNA	30-60 x 250-300 nm	Low	A model insect virus and considered a host agent for baculovirus expression systems (BESV).
(BVDV) Bovine viral diarrhea virus	*Flaviviridae*	Enveloped, Single Stranded RNA	40-60 nm	Low to medium	Model for potential togavirus or flavivirus contaminants. Non-specific enveloped model with RNA genome.
(EMCV) Encephalomyo-carditis virus	*Picornaviridae*	Non-enveloped, single stranded RNA	25-30 nm	Medium to high	Model for hepatitis A virus or rhinovirus, who may infect human cells. Also models other picornavirus contaminants.
(PRV) Pseudorabies virus	*Herpesviridae*	Enveloped, Double Stranded DNA	120-200 nm	Low to medium	Model for human herpes virus type 1, which may be used as a helper virus for AAV. Surrogate for other herpes viruses that can infect human cells.
(Reo 3) Reovirus type 3	*Reoviridae*	Non-enveloped, Double Stranded RNA	60-80 nm	Medium to high	Infects human and animal cells. Non-specific model virus.
(VSV) Vesicular Stomatitis Virus (Indiana strain)	*Rhabdovirida*	Enveloped, Single Stranded RNA	50-200nm	Low to medium	Model for rhabdoviruses, including the insect rhabdovirus.
(XMuLV) Xenotropic Murine Leukemia Virus	*Retroviridae*	Enveloped, Single Stranded RNA	80-100nm	Low	Surrogate for human and bovine retroviruses. Represents retrovirus-like retrotransposons that may be found in insects and baculovirus.

Similarly, HSV-1, a member of *Herpesviridae* family, may be used as a helper virus, and in that case would be a relevant virus that should be used in the clearance study to verify that the downstream process can remove any residual HSV-1.

An adenovirus, which belongs to the *Adenoviridae* family, can also be used as a helper virus and could be used in a viral clearance study as a relevant virus.

Specific model viruses are viruses that are closely related to known or suspected contaminants of a manufacturing process. Specific model viruses are used when the relevant virus cannot be readily propagated for use in a clearance study. Vesicular stomatitis virus (VSV), a mammalian virus and a member of the *Rhabdoviridae* family, is a model for an insect rhabdovirus when insect cell lines are used[5].

Non-specific virus models are used to demonstrate that the clearance process is robust and can show clearance of classes of viruses. Xenotropic murine leukemia virus (XMuLV), a member of the *Retroviridae* family, is an enveloped, RNA virus. This virus can be used to demonstrate clearance of enveloped viruses, enveloped viruses with an RNA genome and viruses that are approximately 80-100nm in diameter. A relevant or specific model virus can also demonstrate clearance of viruses with various characteristics. For example, HSV-1, which may be a relevant virus for some manufacturing processes, can also be a surrogate for enveloped viruses with a DNA genome and viruses that are approximately 180-200nm in diameter.

Human Cell Line-derived AAV

When AAV is produced in well-characterized human HEK 293 cells using a plasmid transfection system and using a medium that is free of animal-derived components, a virus panel that provides various viral characteristics should be considered.

XMuLV is a surrogate virus for human and animal lentiviruses, as well as other retroviruses. XMuLV is an average sized, enveloped virus (80-100nm) with an RNA genome and is susceptible to physio-chemical inactivation methods.

Pseudorabies virus (PRV), a member of the *Herpesviridae* family, models human or animal herpesviruses, including HSV-1, which can be used as a helper virus for AAV infections. PRV is 180-200nm in diameter, has a lipid envelope, a double-stranded DNA genome and is susceptible to physio-chemical inactivation methods.

Bovine viral diarrhea virus (BVDV), a member of the *Flaviviridae* family, is a 50-70nm enveloped virus with an RNA genome. Of the enveloped viruses, it is more resistant to inactivation than are XMuLV or pseudorabies virus (PRV). While certainly a relevant virus for any cell line that has been exposed to bovine

serum, it can also be used as a general, non-specific model for enveloped viruses with an RNA genome.

Encephalomyocarditis virus (EMC), a member of the *Picornavirdae* family is a non-enveloped RNA virus that has an icosahedral structure. EMC is 25-30nm in diameter and relatively thermostable, is resistant to organic solvents, non-ionic detergents and is stable at acidic pH. In addition to a model for a small, non-enveloped virus with an RNA genome, it can be used as a surrogate for hepatitis A virus or for rhinovirus, which can grow in HEK 293 cells. It must be noted, however, this virus is likely to pass through a large pore virus reduction filter (i.e., 35-70nm).

Reovirus type 3 (Reo 3) is a member of the *Reoviridae* family. Reoviruses have been detected in a wide range of species and can infect animal and human cells. Reo 3 is 60-80nm in diameter, is non-enveloped and contains an RNA genome.

Adenovirus type 2 (Ad-2) is a member of the *Adenoviridae* family and is a non-enveloped virus that is 65-80nm in diameter containing a double-stranded DNA genome. It is resistant to organic solvents and extremes of pH and is relatively thermostable.

Ad-2 could be a specific model virus for AAV produced using an adenovirus helper virus, but for AAV processes that only use plasmid transfection, it can represent non-enveloped viruses with a DNA genome. It will likely be retained by a 35nm or 50nm virus reduction filter.

Unless your risk assessment suggests it is needed, it is not necessary to use all these viruses to evaluate viral clearance for AAV produced in HEK 293 cells without helper viruses. Representative enveloped viruses containing RNA and DNA genomes as well as non-enveloped viruses with RNA and DNA genomes is suggested. We recommend that you discuss biologically relevant selection of your virus panel with a member of our Technical and Scientific Solutions team.

Helper Virus AAV

The production of AAV gene therapy products can also be done using helper viruses. HSV-1 or adenovirus are often used as helper viruses. If one of these viruses are used in an AAV manufacturing process, the process usually contains a step that would inactivate the helper virus. Incubation at a low pH or exposure to detergent or solvent/detergent are often used to inactivate HSV-1 and heat treatment at approximately 50°C can be used to inactivate adenovirus. Other steps of the downstream process may also be evaluated to ensure that no residual helper virus remains in the final drug product.

PRV, a herpes virus can be used as a specific model virus for HSV-1 and for helper virus for AAV. For manufacturing processes that use a helper virus, a model for the helper virus must be included in the virus panel along with other relevant and/or model viruses.

Baculovirus-derived AAV

AAV gene therapy products using baculovirus-derived expression system in an insect cell line have a different viral contamination risk when compared to AAV produced in human-derived HEK 293 cell lines. For these viral clearance studies, a virus panel of relevant, specific, and non-specific should be considered. Recombinant baculoviral vectors derived from the insect baculovirus (such as AcMNPV) should be used in viral clearance studies supporting vectors produced using this expression system.

AcMNPV is an enveloped virus with a DNA genome and is 30-60 x 250-300 nm. The manufacturing process must demonstrate the capacity to clear all baculovirus.

We recommend that VSV be included in the virus panel for clearance studies for all baculovirus-expressed AAV products. This mammalian virus is a model for the insect rhabdovirus that was found in Sf9 cells[5].

While baculovirus and VSV must be included in viral clearance studies for all products derived from baculovirus expression systems, other viruses may also be included in the panel. Bovine viral diarrhea virus (BVDV) can be a surrogate for West Nile virus (WNV), which is known to be transmitted by mosquitoes. Both BVDV and WNV are members of the *Flaviviridae* family. Retrovirus-like transposons may be found in insect cells infected with baculoviruses, and XMuLV may be a model for these elements. As noted above, non-enveloped viruses may also be included in the virus panel for a baculovirus-expressed AAV product. Ad-2, the non-enveloped virus with a DNA genome and Reo-3, the non-enveloped virus with an RNA genome may also be used.

Other Considerations for the Virus Panel

During the risk assessment, the cell line and the process should be evaluated to determine if animal-derived raw materials, such as fetal bovine serum or porcine-derived trypsin were ever used.

These animal-derived raw materials carry a risk of being contaminated with animal viruses. Your Technical and Scientific Solutions team can help identify any relevant model viruses that should be included in the clearance virus panel as surrogates for animal-derived potential contaminants

Viral Clearance Study Design for Non-Enveloped Viral Vectors

The design of a viral clearance study for a non-enveloped viral vector or a virus-like particle (VLP) is very similar to studies designed for monoclonal antibodies and recombinant proteins, with respect to how specific process steps are evaluated. Inactivation steps must include an evaluation of the kinetics of inactivation. Chromatography steps using resins that will be re-used should include an evaluation of resin sanitization process and the efficacy of virus removal by repeatedly used/sanitized resins.

As always, when known, worst-case parameter settings should be used. There are a number of regulatory documents that can provide guidance on designing viral clearance studies [1, 6, 7].

Summary

There is no one-size-fits-all model for AAV gene therapy products when it comes to viral clearance studies. Several key factors need to be considered when selecting the virus panel that will be used in these studies based on the raw materials going into the process, the cell line used to make the product, and the product itself. The goal is to show a broad range of virus removal or inactivation capability in the manufacturing process in the event of a virus ingress into the production stream. Design and execution of a robust viral clearance strategy is a critical piece of the safety triangle. It complements the biosafety testing, which is designed to detect any potential viral contaminant. These strategies ensure that AAV gene therapy products are free of contaminants and are safe for patients.

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