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Quality by Design (QbD) for Adeno-Associated Virus (AAV)

A Framework for a QbD Assessment for AAV Products Within the Chemistry Manufacturing and Controls (CMC) Documentation

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1 Abstract

Chemistry Manufacturing and Controls (CMC) for gene therapies is one of the biggest obstacles when moving towards regulatory approval and presents a significant risk to the success of new gene therapy drug candidates today.

A key aspect to the CMC documentation of such complex biological products is the application of the Quality by Design (QbD) principle: A rationale of quality being achieved by process design rather than relying on final quality testing alone. The work presented here provides a framework to illustrate the concept and initial thoughts on the use of the QbD concept for gene therapy, specifically AAV manufacture.

For the four most prominent AAV upstream manufacturing platforms, process and product related impurities as well as adventitious agents are identified. A preliminary hazard analysis of the impurities allows the identification of the critical quality attributes in AAV manufacture for each step, from media filtration in upstream processing (USP) to final sterile filtration of the AAV drug substance. With combined knowledge taken from literature, industry and in-house experience, critical material attributes and critical process parameters are assessed. This work shows that a wide process understanding is already created within the industry despite AAV manufacture being a new field. This knowledge may provide a template for future development of AAV-based biotherapeutics.

2 Introduction

After several successful approvals of gene therapy products in the past years, 2020 saw some clinical trials face regulatory setbacks. The reason for these rejections primarily seems to be the lack of sufficient data in the chemistry, manufacturing and controls (CMC) documentation [1]. The interpretation and implementation of the various frameworks released for advanced therapy medicinal products (ATMPs) seems to be unclear to the industry.

A key aspect to the CMC documentation of biological products is the application of the quality by design (QbD) principle. The QbD approach is heavily based on prior knowledge with a detailed understanding of both product and process variables. In gene therapy, experience today is limited to only a small number of approved drugs for a relatively small patient population. As a result, manufacturers cannot rely on the depth of accumulated information as for other, broader used biological products like recombinant proteins and monoclonal antibodies. With only limited information at hand, the QbD approach comes with hurdles that need to be understood and overcome for successful Investigational New Drug (IND) applications.

This framework shows how QbD can be applied to adeno-associated virus (AAV) gene therapy products and collects initial thoughts on where data gaps or interpretation uncertainties of the regulatory framework impede the chances of regulatory approval.

3 Quality by Design

For well over a decade, the FDA has advocated for QbD in pharmaceutical processes. For new approvals, QbD approaches are requested by regulatory authorities [2]. QbD follows the rationale of quality being achieved by built-in design rather than confirmation of quality through final testing alone. QbD combines product knowledge and process knowledge to define the target quality attributes, parameters in the process that impact the quality, and their operating ranges and control options, as summarized in Figure 1.

The QbD approach is implemented by defining a quality target product profile (QTPP) which includes specifications of safety, purity and efficacy of the drug product. From these specifications, critical quality attributes (CQAs) are derived which characterize the product and describe the desired product quality. To assure that the CQAs are met, a set of critical process parameters (CPPs) and critical material attributes (CMAs) are defined with a specific design space established based on process knowledge. The level of monitoring and controls that are required to maintain each CPP and CMA within its design or control space is defined in the control and test strategy. In its entirety, the QbD process assures that the target product quality criteria defined in the QTPP is achieved throughout the process by maintaining each CPP and CMA within their design space. The definition of the parameters as used in this framework are provided below.

Figure 1

Methodology overview using process and product knowledge to develop the QbD approach to serve safety, purity and efficacy of AAV drug products. QTPP: Quality Target Product Profile, CQA: Critical Quality Attributes, CPP: Critical Process Parameters

Quality Target Product Profile (QTPP)

A prospective summary of the quality characteristics of a drug product to ensure desired quality, safety and efficacy.

Critical Quality Attribute (CQA)

A physical, chemical, biological or microbiological property that should be within an appropriate limit, range or distribution to ensure the desired product quality.

Critical Process Parameter (CPP)

A parameter whose variability has an impact on a critical quality attribute and should therefore be monitored or controlled to ensure the desired product quality. Process parameters are defined as variables that are controlled in the production suite.

Critical Material Attribute (CMA)

A material characteristic whose variability has an impact on a critical quality attribute and should therefore be monitored or controlled to ensure the desired product quality. Within this framework, materials are defined as goods that are purchased or prepared outside of the production suite.

Design Space

The multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality.

Control Strategy

Summary of control and monitoring strategies derived from process and product understanding to ensure process performance and product quality.

4 AAV Reference Process

In the last decade, adeno-associated virus (AAV) has emerged as one of the most widely applied vectors for gene therapy and vaccines. For the framework presented here, the focus is therefore laid on AAV manufacture where the four most used upstream production methods are evaluated:

- Transfection of adherent HEK293
- Transfection of suspension HEK293
- Baculovirus infection of Sf9 suspension cells
- Adenovirus infection of HeLa producer cells

An overview of the four AAV processes is given in Table 1 and shows how each platform uses specific vehicles to convey the genetic information to the cell line. The goal of this work is to evaluate the product- and process-related impurities related to each of these upstream manufacturing methods as well as their downstream platform. For this purpose, the reference processes as used in this framework are shown in Figure 2.

Table 1

Overview of the four AAV production methods reviewed in this work.

	Transfection of Adherent HEK	Transfection of Suspension HEK	Baculovirus Infection of Insect Cells	Adenovirus Infection of Producer HeLa
Host Cell Line	HEK293 adherent	HEK293 suspension	Sf9 suspension	HeLa prod. cells
Production Method	Triple plasmid transfection	Triple plasmid transfection	Triple baculovirus infection method	Adenovirus AdV5 infection
REP/CAP Gene	Plasmid	Plasmid	BacRep, BacCap	Stable cell line
GOI/ITR Gene	Plasmid	Plasmid	BacITR-GOI	Stable cell line
Helper Genes	Plasmid	Plasmid	with BacITR-GOI	AdV5
Productivity [3, 4]	10^3-10^5 Vg/cell	10^4-10^5 Vg/cell	10^2-10^5 Vg/cell	10^5 Vg/cell [5]

In the transfection-based upstream process (USP) using either adherent or suspension HEK293 cells (human embryonic kidney), the transfection is performed by three plasmids and the transfection agent. In infection-based manufacture, the adenovirus or several baculoviruses are added at a defined concentration per cell (multiplicity of infection, MOI). Details of typical upstream manufacturing processes have been described elsewhere [4].

With this variability in the upstream processing however, the product- and process-related impurities change. The concentrations of the most prominent product-related impurities can vary greatly depending on the manufacturing protocol. These product-related impurities include noninfectious, deamidated, glycosylated or aggregated AAV, as well as the concentration of AAV with either no genetic insert (empty capsids) or faulty genetic information (encapsidated host cell DNA and encapsidated helper DNA). Examples are given in Table 2 where it can be seen that the biggest impact is expected on the concentration of empty capsids: 50-95% of the capsids can be empty when generating AAV through transfection, whereas only 30-60% are expected to be empty in infection-based procedures [6, 7]. Looking at the process-related impurities, the choice of an adherent system over a suspension system adds the risk of residual detachment enzymes or animal-derived cell culture medium components. When selecting transfection, residual plasmid DNA needs to be considered whereas any infection-based manufacture will introduce residual helper viruses. Impurities from the cell line such as residual host cell protein or residual host cell DNA are present in all of the upstream methods.

For the downstream process (DSP), a platform approach has been established in the industry which is used independent from the chosen upstream method. At point of harvest, multiple days after infection or transfection, cell lysis is initiated either through high salt and/or a detergent, and endonuclease is added for host cell and

unpackaged virus DNA digestion. Even though some AAV serotypes, especially type 8 and 9 are partially secreted from the cell, the cell lysis still represents the most common harvest step [8, 9, 10]. A following clarification step using depth and membrane filters aims to remove the bulk of the cellular debris before further purification, such as ultrafiltration/diafiltration (UFDF) or chromatography is performed.

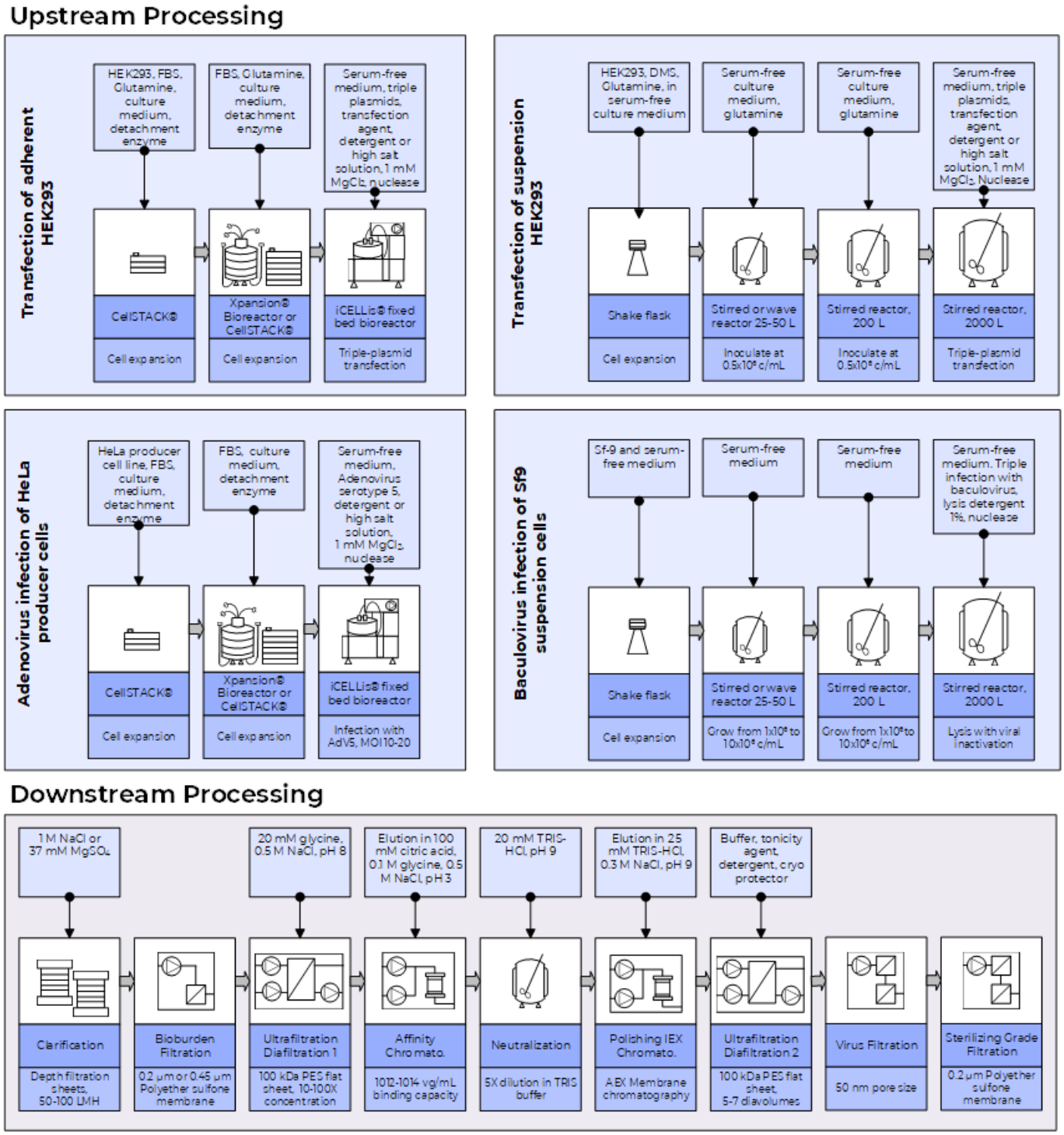


Figure 2

AAV model process for a transfection-based manufacture in adherent HEK 293.

The downstream process typically continues with an immunoaffinity step to capture AAV and adds a subsequent neutralization to raise the low elution pH. Polishing chromatography, typically an anion exchange step, removes the remaining host cell proteins, DNA or leached ligands. Additionally, this step is relied upon to enrich for capsids that contain the therapeutic DNA and reduce the number of empty capsids, devoid of the therapeutic DNA. AEX chromatography is the only step in this template process that can enrich for full capsids. A second UFDF step brings the AAV to the desired concentration and buffer formulation before the final sterile filtration. Some processes include a virus filtration step capable of removing viruses 50 nm or larger, either before or after that second UFDF step. **Introducing a virus filtration step brings the challenge that also AAV product titer can be reduced as the adventitious viruses and residual helper viruses are cleared.**

In DSP, the main impact on the platform design comes from the AAV serotype, as this may influence the choice of chromatography sorbent/membrane and the buffer conditions. These process nuances can impact the removal efficiency for product- and process-related impurities.

The 21 impurities that have been identified based on the reference processes are an important aspect of drug purity and drug safety. They are therefore classified as quality attributes for AAV products within this framework and considered as the basis for assessing critical quality attributes (CQAs). The impurities for the four AAV processes are shown in Table 2.

Table 2

Process-related impurities found in AAV vector manufacturing related to process and product for four different manufacturing processes: transfection of adherent or suspension HEK293, infection of Sf-9 insect cells through baculovirus and infection of HeLa producer cells through Adenovirus Adv5. (x= impurity present in process)

	Impurity	Adherent HEK 293	Suspension HEK 293	Sf9-Baculovirus	HeLa-Adv5
Product-Related Impurity	Noninfectious AAV [11]	1-30%		7-30%	8-30%
	Deamidated AAV	no data			
	Glycosylated AAV	x	x	x	x
	Aggregated AAV	x	x	x	x
	Empty capsids [7] [6]	50-95%	50-95%	30-60%	30-50%
	Encapsidated host cell DNA [11]	0.04%	x	0.02%	x
	Encapsidated helper DNA [11]	0.4-1%	x	0.6-1%	x
	Replication competent rcAAV	x	x	x	x
Process-Related Impurity	Residual host cell DNA/RNA	x	x	x	x
	Residual host cell protein	x	x	x	x
	Residual plasmid DNA	x	x		
	Residual helper viruses			x	x
	Residual animal-derived cell culture medium components	x			x
	Detachment enzyme	x			x
	Detergents	x	x	x	x
	Leachables	x	x	x	x
	Nuclease	x	x	x	x
Adventitious Agents	Endotoxin	x	x	x	x
	Bioburden	x	x	x	x
	Mycoplasma / spiroplasma	x	x	x	x
	Adventitious viruses	x	x	x	x

5 Critical Quality Attributes

Within the QbD framework, quality attributes are typically derived from the QTPP, which describes the desired quality, safety and efficacy of the product. This work does not characterize a QTPP, as it is highly specific to the individual drug product. Instead, this framework evaluates quality attributes based on the purity and potency of an AAV drug product. This includes the functional AAV titer and all impurities that have been identified in the reference processes: process-related impurities, product-related impurities and adventitious agents. Based on these quality attributes, the criticality is evaluated to identify the CQAs of an AAV process.

5.1 Identification of CQAs

For the identification of CQAs, the basic principles of assessing the criticality and risk of quality attributes is well established and described in ICH Q9 [12]. The specific tools used for the risk assessment can however vary depending on the quality attribute and the factors (such as severity, likelihood or uncertainty) that are considered for the assessment. Primary tools used in quality risk management and described in Annex I of ICH Q9 are, among others, the preliminary hazards analysis (PHA) or a risk ranking and filtering [13, 12]. PHA is identified as the method of choice within this framework because the analysis tool is based on applying prior knowledge of hazards, e.g. data from literature and *in vitro*, animal and clinical studies, to identify future hazards and estimate their probability of occurrence. The risk of a quality attribute is evaluated based on the following criteria:

1. The likelihood of a risk event to happen (score 1-7)
2. The severity of the impact on human health if an event is to happen and the level of uncertainty linked to the impact (score 1-9)

The severity score considers what adverse effects on human safety have been reported for an impurity and the severity of that effect as well as the source of evidence.

The combination of likelihood and severity score ranks the most critical quality attributes and thus, identifies the CQAs. As can be seen in Table 3, 13 of all process- and product- related impurities and adventitious agents have been identified as critical quality attributes. The assessment follows the evaluation of Tanaka *et al* (2020) in large parts [14].While the rating and rationale for high-risk CQAs generally finds consensus in the industry, the reasons for excluding attributes from the list of CQAs can be debated. Therefore, the rationale for rating the 8 low-criticality quality attributes that were not considered as CQAs in AAV processes are given below.

Table 3

Preliminary hazards analysis for the identification of critical quality attributes. Risk score 15-63, medium to high (CQA), risk score 6-14, low (QA), risk score 0-5, very low (QA)

	Quality Attribute	Safety	Likelihood	Risk		
Product-Related Impurity	Noninfectious AAV	3	5	15		**CQA**
	Deamidated AAV	5	3	15		**CQA**
	Glycosylated AAV	1	3	3		QA
	Aggregated AAV	5	3	15		**CQA**
	Empty capsids	3	7	21		**CQA**
	Encapsidated host cell DNA	7	3	21		**CQA**
	Encapsidated helper DNA	5	7	35		**CQA**
	Replication competent rcAAV	3	3	9		QA
Process-Related Impurity	Residual host cell DNA/RNA	5	3	15		**CQA**
	Residual host cell protein	3	5	15		**CQA**
	Residual plasmid DNA	3	3	9		QA
	Residual helper viruses	9	3	27		**CQA**
	Residual animal-derived CC medium components	3	3	9		QA
	Detachment enzyme	7	1	7		QA
	Detergents	9	1	9		QA
	Leachables	7	1	7		QA
	Nuclease	1	3	3		QA
Adventitious Agents	Endotoxin	Effect on safety extremely high				**CQA**
	Bioburden	Effect on safety extremely high				**CQA**
	Myoplasma/spiroplasma	Effect on safety extremely high				**CQA**
	Adventitious viruses	Effect on safety extremely high				**CQA**
Potency	Functional AAV titer	Titer impacts potency				**CQA**

Risk Score 15-63
Risk Score 6-14
Risk Score 0-5

5.1.1 Glycosylated AAV

Glycosylation is very rarely observed in AAV and has only recently been described for AAV8 [15]. The severity is rated low (score 1) because current knowledge does not indicate any impact of AAV glycosylation on immunogenicity or transduction efficiency. Also, the likelihood scores low (score 3) since the levels or glycosylation patterns that may have an effect on safety of the product are not known [14].

5.1.2 Replication Competent AAV

Replication competent AAV species (rcAAV) are AAV capsid particles containing AAV rep, cap and the gene of interest (GOI) which can replicate in the presence of a helper virus. RcAAV may arise from nonhomologous recombination between vector and helper AAV plasmids [16]. Since the vector producer cells carry all viral genes coding for the components of the vector particle, unintended recombination can result in replication-competent infectious parental virus [17]. The potential patient risk comes from the adventitious exposure to helper viruses as process-related impurities, its infectious toxicity and, in second stage, immunotoxic proteins expressed from the product-related rcAAV impurity in presence of the helper virus. However, the risk is scored as a 3 because no study indicates that rcAAV can propagate in cells with a helper virus and has not shown a direct effect on safety in *in vitro* or *in vivo* studies [14]. Also, the wild-type AAV being widely common in humans at 38-72% infection rate

depending on the serotype [18], does not have any known pathology. The likelihood also scores at 3 because rcAAV concentration of < 6.2 rcAAV x 10^7 vg/dose did not result in a safety issue [19] and current standard assays have a sensitivity of up to 1x 10^6 rcAAV/vg.

5.1.3 Residual Plasmid DNA

Plasmid DNA added in the transfection-based manufacturing processes is digested by the nuclease during AAV harvest and partially removed during downstream processing. The levels of residual plasmid DNA in AAV formulations have been reported at ranges of 14-164 pg/10^9 vg depending on the AAV design (severity score 3) and an oncogenic effect is seen as unlikely as plasmids do not contain transforming sequences (likelihood score 3).

5.1.4 Residual Animal-Derived Cell Culture Medium Components

The risk associated with animal-derived cell culture medium components, mainly bovine serum albumin (BSA) from fetal bovine serum, is associated with allergic reactions [14]. An immune response is possible even though not directly shown (severity score 3) and limited analytical sensitivity and unknown BSA levels possibly affecting patient safety indicate a likelihood score of 3.

5.1.5 Detachment Enzyme

A detachment enzyme such as trypsin is used in the seed stages of adherent cell cultures and has shown to cause severe blood coagulations at concentrations of 5 mg/kg in animal studies [20] justifying a high severity score of 7. The likelihood is considered very low (score 1) as the trypsin level is several magnitudes lower (expecting < 0.003% trypsin) already at point of cell harvest.

5.1.6 Detergents

Biocompatible surfactants such as Tween® are used in formulations of pharmaceutical products and come with the risk of severe health incidents and allergic reactions. A warning therefore needs to be issued for formulations containing more than 35 mg/kg/day of Tween (EMA/CHMP/190743/2016) and justify a safety rating of 9. This framework discusses the use of Tween as detergent in the harvest step of cell cultures where 1% is typically added to the cell culture which is expected to be reduced in the subsequent downstream steps. The likelihood scores at 1 since any residual detergent from USP is at minimal concentration in the drug product. It needs to be considered that reducing the likelihood of an adverse effect based on the ability of a process to clear said impurity may not be fully in line with the QbD approach but represents a pragmatic approach to classify the risk.

5.1.7 Leachables

Leachables originating from single-use, membrane and sorbent material can hold a toxicity risk to patients (score 7) and contribute to a rise in impurity levels. The FDA, European Medicines Agency (EMA) and The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) have clear guidelines and regulations regarding extractables and leachables. Removal and prevention of leachables is well understood from recombinant protein manufacture and the likelihood of residual leachables with a negative effect on patient safety can therefore be minimized (score 1). The AAV process contains steps where leachables are more critical because no further removal is possible, for example in the final filtration step. In these steps, the industry uses filters specifically designed for low leachables and a validation is common practice which assesses and mitigates the risk of leachables.

5.1.8 Nuclease

The endonuclease added at point of cell harvest for DNA digestion can come with the risk of catalytic activity and immunogenic reactions in patients. Nucleases such as rhDNase are available as approved drugs to treat cystic fibrosis and require a minimum concentration of > 260 µg per human to achieve a detectable catalytic activity and side effects are rarely reported (safety score 1) [14]. The residual level of endonuclease (< 0.5 ng/mL) is much lower and therefore scores a likelihood of 3.

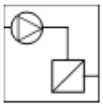
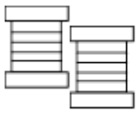
5.2 CQAs in AAV Manufacture

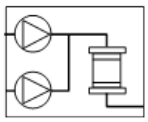
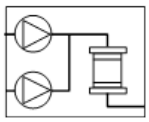
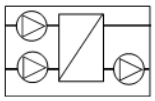
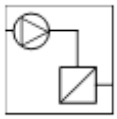
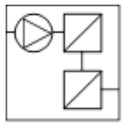
With the CQAs identified it is important to understand at which step of the AAV production process each of the CQAs is impacted. While in USP the product-and process-related impurities are typically at high risk of being generated or introduced, the DSP can reduce the impurity level. A CQA can therefore be impacted either positively or negatively throughout the manufacture process.

In this assessment, experience from AAV processes generated at Pall Corporation or at customers as well as literature knowledge was used to justify the impact of every unit operation on the CQAs. It needs to be considered that AAV manufacture is still in its early stages and that the prior knowledge in the industry is limited. This was included in the assessment by indicating the level of knowledge and assurance when indicating if a CQA is affected by a unit operation:

- Profound data basis proves that the unit operation impacts the CQA
- Few data and solid expert opinion indicate that the unit operation impacts the CQA
- Expert knowledge without data support suggest that the unit operation impacts the CQA

N/A Not applicable: the unit operation does not impact the CQA

		Virus Filtration Media/Buffer	Production: Infection	Production: Transfection	Clarification
					
Product- and Process-Related Impurities	Noninfectious AAV	N/A			N/A
	Deamidated AAV	N/A	N/A		N/A
	Aggregated AAV	N/A			
	Empty capsids	N/A			N/A
	Encapsidated host DNA	N/A	N/A		
	Encapsidated helper DNA	N/A	N/A		
	Residual host cell DNA	N/A			
	Residual host cell protein	N/A			
	Residual helper viruses	N/A		N/A	N/A
Adventitious Agents	Endotoxin	N/A			
	Bioburden	N/A			
	Myoplasma / spiroplasma	N/A			N/A
	Adventitious viruses				N/A
	Functional AAV titer				

		Affinity Chromatogr.	Polishing Chromatogr.	UFDF	Virus Filtration	Sterile Filtration
						
Product- and Process-Related Impurities	Noninfectious AAV			N/A	N/A	N/A
	Deamidated AAV			N/A	N/A	N/A
	Aggregated AAV					
	Empty capsids			N/A	N/A	N/A
	Encapsidated host DNA			N/A	N/A	N/A
	Encapsidated helper DNA			N/A	N/A	N/A
	Residual host cell DNA				N/A	N/A
	Residual host cell protein				N/A	N/A
	Residual helper viruses	N/A		N/A	N/A	N/A
Adventitious Agents	Endotoxin	N/A	N/A	N/A		N/A
	Bioburden	N/A	N/A	N/A		
	Myoplasma / spiroplasma	N/A	N/A	N/A		N/A
	Adventitious viruses	N/A		N/A		N/A
	Functional AAV titer					

6 Critical Process Parameters

There are multiple ways to assess and identify CPPs according to the data available for each specific unit operation. Primarily, CPPs should be identified using a combination of risk assessments, such as failure modes and effects analysis (FMEA) and data generated across the development cycle of a process. In the absence of long-term data trending, a thought-based rationale can be applied to determine preliminary CPPs, which can later be re-enforced once enough data has been collected. Whilst data and rationale can both be used to determine the design space for evaluating a CPP, it is also possible to consider CMAs (e.g., buffer composition acceptance criteria) to determine the design space, typically for chromatography steps, where processing buffers are made. Once the CPPs have been identified, the Normal Operating Ranges (NORs) for these parameters need to be established (if they have not been already) to set up the design space. The final step would be to define the control strategies to ensure the process operates within the design space.

For the purpose of this framework, CPPs were defined as anything that would be controlled in the manufacturing suite, e.g., flow rate, pressure or mixing speed, whilst CMAs were defined as anything that would be brought into the suite, e.g., processing buffers, see definitions of CPPs and CMAs in section Quality by Design.

6.1 Identification and Assessment Based on Data Trending

Ideally, the identification of CPPs (and subsequently, the setting of acceptable limits for these CPPs) should come from long-term data trending, an example of which is shown in Figure 4, as this will be the most accurate way of demonstrating process repeatability using specific parameters. In addition, long-term trending demonstrates the robustness of the step and how susceptible specific parameters may change within the process. Standard deviation is the most commonly used precision metric and setting acceptance criteria for process parameters using standard deviations of the mean is common practice for process characterization [21, 22].

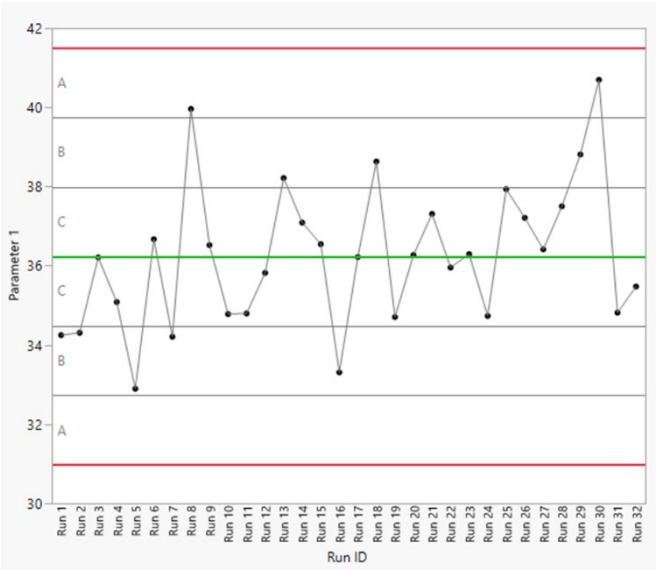


Figure 3

Example control chart with random data points, showing the monitoring of "Parameter 1".
A central green line shows the average of the values, red lines show upper and lower control limits, calculated by ± three standard deviations.

Setting a narrow acceptance criterion may be suitable for a process parameter, if data trending shows that this parameter is unlikely to drift too far from the mean from batch to batch. However, setting acceptance criteria for process parameters in this way needs to be taken into consideration with the control strategy associated with each parameter. The disadvantage in setting acceptance criteria based on a tight data spread is that it may not be possible to determine the true outer limits of the process – a parameter could have a very wide operating limit,

despite the data having a tight spread. If the acceptance criterion is too narrow, then it may not be possible to control the parameter accurately throughout the process and there may not be enough of a safety factor to account for process variance. Therefore, it can also be sensible to determine upper and lower limits for CPPs based on a combination of data trending and thought-based rational.

6.2 Identification and Assessment Based on Rationale

Where long-term data trending is not available, it may be acceptable to determine CPPs and their acceptance criteria based on experience with operating similar processes. When working this way, it is important to consider the likelihood of drift within the process, and how easy each of these parameters are to control at lab, pilot and manufacturing scales.

Table 4 shows an example of identifying suitable operating ranges for a TFF step, using knowledge-based rationale. For modern viral vector processes (which are not yet fully understood), it may not be possible to accurately identify each CPP and their associated operating range in the absence of data trending. This is important to consider for process parameters that are likely to vary from process to process due to variance within the vector itself, for example, the separation of empty/full capsids based on the insert size of a particular plasmid within the vector.

Table 4

Example of selected process parameters for a tangential flow filtration unit operation

Process Parameters	Units	Set Point	Upper Limit	Lower Limit
Feed pressure	barg	0.7	0.75	0.65
Retentate pressure	barg	0.3	0.35	0.25
Transmembrane pressure	barg	0.5	0.55	0.45
Crossflow flux	LMM	5	6	4

6.3 Identification and Assessment Based on Critical Material Attributes

Beyond data collection and knowledge-based assessments, certain CPPs may be identified depending on specific CMAs. In the case of chromatography, it can be acceptable to base the preliminary load, wash and elution conditions on the associated buffer acceptance criteria. Assessing parameters in this way acknowledges the variance in buffer composition from batch to batch due to temperature or the acceptance criteria for each specific buffer reagent. For example, an elution buffer with a pH specification of 7.0 is likely to have its own specification based on this variance (e.g., 6.8 – 7.2, or similar). Therefore, it must be demonstrated that using an elution buffer at pH 6.8 or 7.2 does not result in a critical change to the product quality or impurity profile.

Using design of experiment (DoE) tools to construct a study plan, it is possible to identify specific combinations of buffer properties (e.g., pH, conductivity, etc.) that could result in a significantly different output than expected (e.g., lower functional titer). Should this occur, it would indicate that the current acceptance criteria of the buffer is not suitable for this process, resulting in re-defining the buffer acceptance criteria or re-development of the elution step.

6.4 Identification and Assessment in This Work

The CPPs were identified in this work using a combination of literature, industry data, in-house data and knowledge from process scientists within the Pall Corporation. Each CPP was identified in response to the specific CQAs determined for each unit operation, using the rationale mentioned above to determine why a specific parameter had an effect in controlling a CQA and how a change in this parameter could affect a specific CQA. Where data was available, it was used to define the knowledge space for a specific CPP and where possible, its upper and lower limits.

7 Upstream Processing

7.1 Virus Reduction Filtration of Buffer and Cell Culture Media

Nanofiltration is a key step in assuring virus safety of an AAV product. When implementing nanofiltration in the downstream process, possible vector loss in the closed-meshed membrane and a tight patent landscape is complicating the step. It can also be considered to qualify nanofiltration of raw materials such as buffers and cell culture media used in the manufacture process.

Virus filters for cell culture medium and buffer can consist of a tight pore size (20 nm nominal rating) and have shown log reduction values (LRV) of more than 6. Critical process parameters for a high removal of potential viruses as shown in Table 5 are the typical operating parameters of virus filters, namely the differential pressure, the flux decay and the duration of the process. At extreme values of flux and pressure differential, retention of contaminants can be impacted. The same is true for temperature extremes and pressure decay in start/stop process interruptions. Also, a high throughput with potential premature filter plugging and extended process duration can reduce the retention of contaminants. A critical material attribute is the amount of leachables which is released during filtration and may impact the cell growth and consequently, the functional AAV titer. The filter material quality and integrity are key elements to ensure successful pre- and post-use integrity test results and are summarized under “material performance”.

Table 5

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in virus filtration of buffer and cell

	Adventitious Viruses	Functional AAV Titer
Critical Process Parameters		
Differential pressure	x	N/A
Throughput / flux decay	x	N/A
Duration	x	N/A
Interruptions	x	N/A
Critical Material Attributes		
Membrane leachables	N/A	x
Material performance	x	N/A

Trials performed at Pall Corporation showed high throughputs of more than 1000 L/m^2 when filtering a DMEM-based (Dulbecco’s Modified Eagle Medium) cell culture media using the Pegasus™ Prime nanofilter. Cell culture media, either sterile filtered or nano filtered was used in biological runs at different scales to evaluate the impact of extractables and leachables on the cell culture. No impact of the virus filtration step on cell growth, metabolite concentrations or virus production was observed. In addition, studies have confirmed a consistent virus retention over extended process durations (up to 8 days) and during process interruptions of up to 24 hours. The results suggest that virus filtration of cell culture media is an alternative to nanofiltration of AAV product given that the AAV manufacture process is operated under closed conditions.

This assessment assumes that the nanofiltration is performed directly at the unit operation in the clean room. Therefore, critical process parameters are defined for the filtration process. If media and buffers are filtered offline in a media preparation suite, their quality would be considered a critical material attribute within this framework.

7.2 AAV Production

The four production methods described in the *AAV Reference Process*, were categorized in transfection-based and infection-based methods as the manufacture type significantly impacts the applicable CQAs. Some quality attributes however apply to all four manufacturing types detailed in this report as summarized in Table 6:

- Noninfectious AAV – between 1-30% of AAV particles can be noninfectious or defective in both transfection- and infection- based manufacture. In the framework presented here, noninfectious particles are specified as vectors that do not replicate in the presence of helper sequences and thus do not cause a full infection Even though present in all AAV processes, the formation of noninfectious AAV is mainly researched in transfection processes. Apart from parameters specific to the transfection, noninfectious AAV can be impacted by the temperature and pH of the cell culture.

- Aggregated AAV – several references show that the ionic strength, pH and temperature of the lysis buffer as well as the nuclease addition impact or mediate vector aggregation [23, 24]. Aggregation can be triggered through low temperatures (< 37 °C) or ionic strength and pH outside of the physiological range. A typical design space for minimal aggregated AAV therefore operates at pH 6.5-7.5 but may be increased to pH 8-9.2 during cell lysis for optimal benzonase efficiency. As host cell DNA can mediate aggregation, a nuclease DNA digestion with 15-50 U/mL for 30-120 min during harvest further reduces the risk of aggregation. The conductivity is typically set at 8-12 mS/cm but can increase temporarily, by adding for example up to 0.5 M NaCl or $MgSO_4$ at point of harvest.

- Empty Capsids – in transfection-based processes, 50-95% of AAV can lack the genetic insert [7], with baculovirus-based systems operating in the same range at 30-60% empty capsids. The packaging efficiency is higher for adenovirus-based infection systems with <5% of all AAV capsids lacking the genetic insert [6]. Ionic strength, pH and temperature of the culture media and the length of the gene of interest can impact the encapsidation in both infection and transfection-based processes [7]. The quality of the virus and its phenotype play a role when applying infection-based protocols [25] while the ratio of PEI:DNA and its complexation conditions impact the ratio of full:empty capsids in transfection [26, 7].

- Residual Host Cell Protein (HCP) – significant impact on residual HCP levels comes from the harvest, especially if the cell culture is lysed. Process parameters that impact the level of residual HCP include the time of harvest and the cell viability at point of harvest as well as the conditions of cell lysis (buffer composition and possible shear from agitation or aeration) [27].

- Residual Host Cell DNA – residual host cell DNA (hcDNA) is generated through cell rupture and lysis either during the cell cultivation or at point of harvest and lysis. Both the size of residual hcDNA and the final concentration need to be controlled. A first and significant step in achieving hcDNA reduction is by the addition of nuclease (15-50 UI/mL for up to 120 minutes). Nucleases cut the chain length of DNA which facilitates its removal in subsequent chromatography or tangential flow filtration (TFF) steps and lowers the degree of nucleic acid-induced aggregation of AAV. Furthermore, the risk of carrying full-length oncogenes to the final product is reduced.

- Adventitious Agents – all adventitious agents assessed in this work are well known to be impacted by the AAV upstream processing. Appropriate raw material selection, operator handling, and system design are to be considered as impact factors. As these are universal points for both USP and DSP steps, this topic is further discussed under General CQAs.

- Functional AAV Titer – AAV titer is driven by high cell density and high transfection or infection efficiency. Therefore, CPPs affecting the cultivation conditions such as temperature, cell culture pH or conductivity indirectly affect the functional AAV titer. Same is true for factors impacting complex formation as well as the plasmid or helper virus quality. As virus can attach to the cell membrane through hydrostatic bonds, an increased shear force during harvest can increase the yield by detaching bound AAV. This is to be considered especially if high-pH lysis buffers at pH 8-9 are used that raise the pH above the isoelectric point of AAV (pH 6-7) and thus create negative charges that increases the hydrostatic interactions.

7.2.1 Infection-Based AAV Production

The infection-based processes introduce residual helper viruses such as adenovirus (AdV5) and baculovirus. Excess of these viruses and variants thereof need to be controlled. The virus stock quality, typically at 10-20 PFU/mL, thereby has a significant impact to minimize the virus variants during stock production [11]. Also, keeping the multiplicity of infection (MOI) in the lower range (MOI < 2) of the typical operating range of 0.01-5 MOI can reduce excess of helper virus and thus may reduce the contaminant load. The quality of the virus stock solution with a

minimal modification of the genetic information may furthermore have an impact on the packaging efficiency and by that, the number of empty capsids.

7.2.2 Transfection-Based AAV Production

The transfection process generates AAV with other nucleic acids than the gene of interest encapsidated: both helper component DNA (plasmids) and host cell DNA can be encapsidated. Host cell nucleic acids represent 0.04-3% of the AAV genome containing particles, while 0.4-8% of them can contain helper component DNA [7, 28, 11]. The encapsidation of non-AAV nucleic acids can be influenced by the size of the plasmid backbone and the vector productivity. Data to understand a possible relationship and provide the basis for a design space is yet to be generated.

Functional AAV titer and the amount of noninfectious AAV including empty capsids and deamidated AAV, can mainly be influenced by the ratio of plasmids. A common ratio of the three plasmids for the helper genes (pHelper), the replication and capsid genes (pRepCap) and the gene of interest (pAAV) is 2:1:1 pHelper:pRepCap:pAAV [29, 30, 31], the design space is however very wide. The main focus is typically on assuring a high enough ratio of capsid proteins. The quality of the plasmid is essential since certain DNA modifications can significantly impact the encapsulation efficiency [32]. The efficiency of vector packaging is furthermore dependent on the genome size and has been shown to be most successful if their size is kept <5 kb [32]. The mixing strategy applied to join the plasmids and the transfection reagents can range from manual addition in 2D-biocontainers to more automated mixing in rocking reactors. Adding the mix to the bioreactor can be done through gravity or peristaltic pumps. It is assumed that the method and therefore shear force of preparing and adding the plasmid and PEI impacts the complex formation but it is not fully characterized. Additionally, the complex formation time (typically 5-30 minutes) and the mixing in the bioreactor during complex formation as well as the pH, temperature and the ionic strength of the cell culture medium can impact the complex size and success of complex formation. For an optimal yield of infectious particles an earlier point of harvest typically no more than 2-3 days post transfection can be considered [31], there is however little evidence that longer production times lead to AAV degradation.

Table 6

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in infection- and transfection-based USP of AAV.

	Aggregated AAV	Residual hcDNA	Residual HCP	Noninfectious AAV	Empty Capsids	Deamidated AAV	Encapsidated Host Cell DNA	Encapsidated Helper DNA	Residual Helper Virus	Functional AAV Titer
Critical Process Parameters										
General Process CPPs										
Temperature	x	N/A	x	x	x	x	N/A	N/A	N/A	x
pH of cell culture	x	N/A	N/A	x	x	x	N/A	N/A	N/A	x
Conductivity of cell culture	x	N/A	N/A	N/A	x	N/A	N/A	N/A	N/A	x
Nuclease conc. and activity	x	x	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Time of harvest	N/A	x	x	x	N/A	N/A	N/A	N/A	N/A	N/A
Harvest shear force: agitation	N/A	x	x	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Infection-Specific CPPs										
Multiplicity of infection	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	x	x
Transfection-Specific CPPs										
Plasmid ratio	N/A	N/A	N/A	x	x	x	N/A	N/A	N/A	x
Complexation time	N/A	N/A	N/A	x	x	x	N/A	N/A	N/A	x
Shear force during transfection	N/A	N/A	N/A	x	x	x	N/A	N/A	N/A	x
Critical Material Attributes										
Plasmid or virus quality	x	N/A	N/A	x	x	x	x	x	N/A	x
Helper virus stock titer	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	x	x

Table 7 gives an overview of the CPPs and CMAs that are relevant to control the CQAs in the depth filtration stage of clarification. A filed clarification process is likely to include several more CPPs that cover pre-use flush, filtration and recovery flushes. The framework presented here focuses solely on the filtration stage of the process. The choice of filter media is critical to assure a high filter capacity and removal of host cell proteins and host cell DNA strands. Though removal of these two impurities can be achieved in the clarification step, it is only rarely claimed in a filed process because of the natural variability of depth filter materials. To reduce negatively charged impurities such as endotoxins, a filter material with a positive net charge is required, see Table 7. These filters are likely to contain filter aids, such as diatomaceous earth or charged ligands, such as quaternary amines. It is therefore important to understand the effect of these filter aids on the yield of the viral vector product before implementing these filters into a process.

Endotoxin reduction can be achieved with positively charged filters if the filter capacity is sufficiently large and a suitable filter material is selected. Endotoxin removal in AAV harvest is in its infancy and needs further characterization. The formation of aggregates is a concern in depth filtration which is mitigated by reducing the osmolarity of the used buffers to < 0.3-0.5 M depending on the AAV serotype [7]. This decision however needs to be made already in the harvest stage and conductivity of the feed material is therefore a critical material attribute in filtration.

Typically, a 0.2 µm polyethersulfone (PES) filtration membrane is placed inline downstream of the primary clarification filters. It further reduces the particle load, bioburden load and turbidity of the feed stream and yields in functional AAV titers of up to 100%. Sterile filtration is discussed in more detail in section Sterile Filtration.

Table 7

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in depth filtration for clarification of AAV.

	Aggregated AAV	Bioburden	Residual hcDNA	Residual HCP	Functional AAV Titer	if positive net charge: Endotoxin
Critical Process Parameters						
Pressure	x	x	x	x	x	x
Flux	x	x	x	x	x	x
Critical Material Attributes						
Filter capacity	N/A	x	N/A	N/A	x	x
Conductivity	x	N/A	N/A	N/A	N/A	N/A

8 Downstream Processing

8.1 Affinity Chromatography

The affinity chromatography step typically binds a variety of AAV serotypes at capacities of 10^{12}-10^{14} vg/mL [34, 17]. Immunoaffinity chromatography mainly reduces residual host cell proteins, host cell DNA and other serum protein impurities [35]. Virus-based impurities such as noninfectious, aggregated or AAV with wrong or missing DNA inserts are expected to be bound to the immunoaffinity resin, the variation of the operating parameters can however affect their extent of their binding and co-elution.

Critical process parameters as shown in Table 8 are related to the load flow rate and the load density. The flow rate, typically ranging between 100-450 cm/h, affects the retention time in the chromatography sorbent which influences the binding of AAV and its impurities [36]. Studies have confirmed stable AAV yields of greater than 80% when operating at the higher end of that design space but coelution of impurities also increased with velocities over 150 cm/h [36]. Avoiding an overload of the sorbent is not only critical to maintain high AAV yields but also reduces the risk of aggregate formation. The pH and conductivity of the wash and elution buffers are listed as critical material attributes as they impact not only the binding and release of AAV but also that of several impurities. The pH of the wash buffer (typically at pH 3-5) and elution buffer (pH typically below 3) can affect the nonspecific binding and clearance of product-related impurities [36]. The same goes for the conductivity of wash and elution buffers (10-25 mS/cm) which has shown to impact the binding and elution of noninfectious AAV [36] as well as the nonspecific binding of AAV with encapsidated host cell and helper component DNA, empty capsids, aggregated or deamidated forms and host cell proteins [10].

Table 8

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in affinity chromatography of AAV

	Noninfectious AAV	Deamidated AAV	Aggregated AAV	Empty Capsids	Encapsidated host cell DNA	Encapsidated helper DNA	Residual hcDNA	Residual HCP	Functional AAV Titer
Critical Process Parameters									
Flow rate	x	N/A	x	N/A	N/A	N/A	x	x	x
Load density	N/A	N/A	x	N/A	N/A	N/A	N/A	N/A	x
Critical Material Attributes									
Conductivity of wash buffer	x	x	x	x	x	x	x	x	x
pH of wash buffer	x	x	x	x	x	x	x	x	x
Conductivity of elution buffer	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	x
pH of elution buffer	x	N/A	N/A	N/A	N/A	N/A	N/A	N/A	x

8.2 Polishing Chromatography

An anion exchange chromatography (AEX) with a membrane adsorber or resin is used for AAV polishing. Polishing aims at decreasing the amount of process-related impurities such as HCP, hcDNA, leached affinity ligand and product-related impurities like aggregated or clipped product variants [10, 37]. The ion exchange step can also separate genome-containing, infectious AAV from empty, noninfectious capsids based on the electrical charge between the two particles as has recently been demonstrated [38]. Depending on the aim and design of this process step, the vast majority of all CQAs can be affected or targeted in this step through selective design of the chromatography conditions, see Table 9.

The pH, conductivity and density of the load determine the impurity binding which typically occurs at higher pH and lower conductivity (pH 9, < 5 mS/cm). A capacity of 10^{10}-10^{13} vg/mL represents the expected design space for

the load step, whereby performing at lower capacity is expected to enhance the impurity binding. High salt concentrations increase the risk of aggregates being formed which can co-elute with the AAV product.

The flow rate during the process is set to 3-7 MV/min to assure proper fluid dynamics in the membrane and mixing at optimal residence time.

It must be considered that whilst membrane-based chromatography is more widely used at this stage of the purification process, resin-based chromatography of a similar chemistry can also be employed. This could affect the areas of the design space (e.g., flow rate), but would not be expected to have a major effect on the level of impurity clearance. In addition, the choice of either a strong (e.g., Q) or weak (e.g., DEAE) anion exchanger could potentially influence the level of impurity clearance and yield associated with the step. In any case, this choice should be evaluated on a per-product basis.

Table 9

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in AEX polishing chromatography of AAV.

	Noninfectious AAV	Deamidated AAV	Aggregated AAV	Empty Capsids	Encapsidated Host Cell DNA	Encapsidated Helper DNA	Residual hcDNA	Residual HCP	Residual Helper Virus	Adventitious Viruses	Functional AAV Titer
Critical Process Parameters											
Load density	x	x	x	x	x	x	x	x	x	x	x
Elution volume	x	x	x	x	x	x	x	x	x	x	x
Flow rate	x	x	x	x	x	x	x	x	x	x	x
Critical Material Attributes											
Load pH	x	x	x	x	x	x	x	x	x	x	x
Load conductivity	x	x	x	x	x	x	x	x	x	x	x
Wash condition	x	x	x	x	x	x	x	x	x	x	x
Elution condition	x	x	x	x	x	x	x	x	x	x	x

Since the design space of the AEX process is highly variable and product-dependent, it is essential to carefully define the target CQAs of the polishing chromatography step and perform a DoE of the critical parameters on a per-product basis.

8.3 Ultrafiltration and Diafiltration (UFDF)

A UFDF step with concentration through 100 kDa ultrafiltration membranes followed by a diafiltration with 5-7 diavolumes (DV) serves as a purification step and provides stable conditions for the AAV. UFDF is executed up to two times in every AAV manufacture: prior to the affinity chromatography (UFDF1) and, optionally, prior to the final sterile filtration step (UFDF2).

Depending on the state at which the UFDF is performed, the feed solution characteristics and components vary which impacts the design space of the CPPs that are listed in Table 10. Overall, a limited transmembrane pressure (10-15 psi) leading to a specific volume concentration factor (typically 5-10 X) can keep aggregate formation to a minimum. Also, the permeate flux is a CPP that impacts aggregate formation and is controlled at 50-100 LMH in a typical AAV UFDF step. Aggregates can affect TFF permeability factors and lead to a blockage of the cassette depending on the characteristics of the process solution [39]. Lower molecular weight hcDNA and host cell protein are reduced in UFDF as they penetrate the membrane and are removed with the permeate. Higher molecular weight impurities could however be retained and concentrated with the AAV product. To reduce high molecular weight hcDNA, a nuclease addition step can be implemented during the TFF recirculation. In this case, nuclease clearance may need to be demonstrated prior to the final sterilizing grade filtration. This can be achieved by validating the required diafiltration volume to sufficiently remove residual nuclease from the retentate. HCP reduction can also be validated through diafiltration. Whilst it must be considered that buffer conditions (e.g., pH, conductivity) and excipient levels will play a role in modulating various CPPs, such as aggregation and titer, they are not included within this document since buffer formulations are very process specific.

Table 10

Critical process parameters (CPPs) to meet critical quality attributes (CQAs) in ultrafiltration and diafiltration (UFDF) of AAV

Critical Process Parameters	Aggregated AAV	Residual hcDNA	Residual HCP	Functional AAV Titer
Volume concentration factor	x	N/A	N/A	x
Transmembrane pressure	x	N/A	N/A	x
Permeate flux	x	N/A	N/A	x
Diafiltration volume	N/A	x	x	N/A

8.4 Virus Reduction Filter

Nanofilters with a removal rating for 50 nm viruses can reduce adventitious viruses through depth size exclusion either before or after the UFDF formulation step [40]. More conventional, tighter-mesh virus filters with removal ratings of 20 nm can not be included for virus filtration of viral vectors due to the risk of retaining the viral product on the filter. The process step is not generally applied for all AAV manufactures but can provide patient safety improvements, especially to infection-based processes where reduce residual helper virus needs to be removed. The process of virus reduction is well understood using bacteriophage PR772 under standard test conditions. Details of applying nanofilters for the removal of parvovirus in baculovirus-based AAV processes can be found in patent EP2744895B1 from UniQure and Genzyme [41].

Critical process parameters as shown in Table 11 are the differential pressure, flux decay and the process duration. The impact of potential process interruptions should also be considered in the process design. The filter quality and integrity are key elements to ensure successful pre- and post-use integrity test results and is summarized under "filter performance".

An alternative approach for adventitious virus control consists of nanofiltering cell culture media and buffers used in manufacture and is described in Section 7.1: Virus Reduction Filtration of Buffer and Cell Culture Media.

Table 11

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in virus filtration of AAV.

Critical Process Parameters	Aggregated AAV	Endotoxin	Bioburden	Mycoplasma Spiroplasma	Adventitious Viruses	Functional AAV Titer
Differential pressure	x	x	x	x	x	x
Throughput / flux decay	x	x	x	x	x	x
Duration	x	x	x	x	x	x
Interruptions	x	x	x	x	x	x
Critical Material Attributes						
Filter performance	N/A	N/A	N/A	N/A	x	N/A

8.5 Sterile Filtration

The formulated product is sterile filtered in a redundant double-0.2 µm microfiltration using a polyethersulfone (PES) membrane filter. The reduction of bioburden with its critical process parameters and design space is very well understood as shown in Table 12. As sterile filtration is typically operated and controlled based on flux, a design space characterization should consider the flux, the differential pressure of typically 5-30 psi, the throughput, and specify the filtration time, typically lasting for around 1 hour. The selected filtration membrane should provide adequate material performance to succeed in pre- and post-use integrity testing, a sufficient robustness to withstand potential process interruptions and a well-characterized and minimized leachables profile.

It is standard practice to remove viral aggregates in sterile filtration on viral clearance studies. However, a reduction of viral aggregates in the microfiltration step could indicate product titer loss and may result in earlier filter fouling. Even though sterile filtration can remove aggregated AAV it is therefore not viewed as an aggregate reduction step. It is more important to avoid the formation of new aggregates by controlling the pressure differential and the resulting shear force. In addition, it must be considered that concentration of the vector product may also have an effect on performance and throughput of the filter, particularly if the concentration of the solution is high and/or appears cloudy.

While internal validation projects have shown minimal yield loss in double-0.2 µm microfiltration using the Pall's Supor® EKV membrane, alternative approaches to final filtration are evaluated in the industry. In scenarios where a 0.2 µm microfiltration leads to inacceptable AAV loss, it could be considered to perform the step using filter membranes with a 0.45 µm rating, either in a single or redundant in-series setup. Regulatory authorities open the possibility for such alternative approaches to sterile filtration if 0.2 µm membranes are proven unsuitable. As a consequence, an aseptic manufacturing process might be required which is further discussed in the Section 9: General CQAs.

Table 12

Critical process parameters (CPPs) and critical material attributes (CMAs) to meet critical quality attributes (CQAs) in sterile filtration of AAV.

	Aggregated AAV	Bioburden	Functional AAV Titer
Critical Process Parameters			
Differential pressure	x	x	N/A
Throughput / flux decay	N/A	x	N/A
Flux	N/A	x	N/A
Duration	N/A	x	N/A
Critical Material Attributes			
Filter performance	N/A	x	x
Filter robustness	N/A	x	N/A

9 General CQAs

Certain CQAs around product stability and safety apply to several process steps. This is especially true for AAV aggregate formation in downstream unit operations and introduction of any adventitious agents such as bioburden, endotoxin, mycoplasma or spiroplasma and adventitious viruses.

9.1 General CPPs

Product stability is influenced heavily by the temperature during processing or storage. By controlling the room temperature of the production suite, it can be assured that temperature-induced aggregate formation is minimized. That makes temperature a universally controlled critical process parameter.

Next to temperature, also high osmolarity comes with an increased risk of AAV aggregation. Depending on the AAV serotype, a typically acceptable salt level throughout the different DSP unit operations is < 0.3-0.5 M [7]. The salt concentration can be higher for a limited time like for example during cell harvest or chromatography elution. In this case, the exposure time becomes a critical process parameter.

9.2 General CMAs

A common entry point for adventitious agents is through raw materials, which is addressed by appropriate raw material selection based on quality risk management (QRM) [42]. The risk can be reduced especially in USP by designing processes without fetal bovine serum (FBS) or detachment enzyme as these substances bring a greater risk of contamination with adventitious agents [43]. Further measures as described in the PICS 2231 guide for ATMPs include a sterility assessment of the cell banks and cell cultures and preventive measures to avoid introducing wild type virus.

Adventitious agents can also be introduced from the environment: contaminated air, nonsterile systems or operator handling introduce contamination risks during manual operations when flow paths are connected and disconnected, materials are added, or samples are taken. These risks increase with scale, as larger process volumes become more difficult to handle and manipulate. A QRM can define adequate actions for system handling under GMP including risk mitigation strategies such as sanitization or even sterilization if possible, and aseptic handling. Any material (membrane, resin or single-use component) used in the process needs the robustness (CMA) to withstand an adequate sanitization or sterilization procedure.

If a final sterile filtration of the AAV product is not possible because of an unacceptable product loss in the filter membrane, an aseptic production is required [42]. This means operating in fully closed, sanitized or sterilized systems with a validated pre-use cleaning and testing regime and using only aseptic connectors to join unit operations or add and remove materials. This is to be considered as critical attribute when selecting materials. Small volume purifications can be performed in a microbiological safety cabinet (MSC) but with increasing scale, a fully closed operation becomes more complex. Several unit operations of a standard DSP are not designed and qualified as sterile operations but rather considered “low-bioburden”. Today, most GMP facilities operate in semi-closed- systems that combines fully closed units with open but adequately sanitized steps to maintain a bioburden-controlled state. Fully closed operation is not new to the industry, it is however reserved for processes with highly toxic products (Botox•, antibody drug conjugates) or processes that operate over multiple days (continuous mAb production).

10 Control and Testing Strategy

A control strategy ensures product quality and safety by controlling that the critical process parameters remain within their limits of the design space throughout the manufacture. This work limits the control strategy to only the CPPs discussed in the previous chapters.

A control strategy for an AAV product will be much more extensive and cover all relevant attributes including all CPPs but also key process parameters (KPPs) for process consistency and robustness.

While the traditional drug development approach typically defines the control strategy based on prior experience and end-product testing, the QbD or QRM approach allows to keep the entire process under control based on the rational risk-based assessment and is expected from regulatory authorities [42]. Different categories of control can be implemented:

- Input Material Control: assessment of raw materials and components used in the manufacturing of the product. It includes raw material qualification, raw material specification or supplier quality management. In this assessment any material or component that is prepared outside of the clean-room suite is categorized as input material. The control therefore not only applies to external suppliers but also in-house buffer, media and material supply.
- Procedural Control: assuring reproducible and robust manual operations on the unit operations through operating staff. This includes standard operating procedures (SOPs) and equipment or quality system controls, as well as operator training. Procedural controls are best supported through a QRM system [13].
- Skid Control: assuring reproducible and robust automated operations on the unit operations. This includes parameter programming and feed-back control on automated systems.

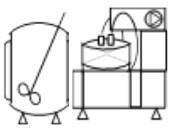
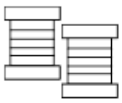
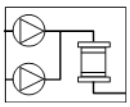
The control strategies are supplemented with a routine quality control testing that verifies that the specifications from the QTPP are met. A range of assays can be applied to evaluate safety, purity, potency and identity of the AAV. Analytical methods are currently being developed at a fast pace and rightly so: the lack of robust and fast in-process quality testing analysis to replace the lengthy lab-based traditional methods are a significant hurdle for the rapidly developed AAV products [44]. In addition, the regulatory expectations towards accuracy, specificity and sensitivity of assays is increasing as the manufacturing processes of gene therapy products gain sophistication, which further directs the focus towards more optimized analytical methods. The faster turnaround times of optimized assays also supports the process analytical technologies (PAT) initiative that allows implementing a highly sensitive and responsive design of experiments (DoE) which is part of the here described QbD framework.

A quality control testing strategy can rely on different elements, of which the following three are included in this assessment in Table 13:

- Process Monitoring: selected attributes or parameters are evaluated to trend product performance or quality to enhance confidence within the design space.
- In-Process Testing: measurements through analytical or functional tests to ensure that the operation is within an acceptable range for the intended product quality.
- Lot Release Testing: test performed at the end of a manufacturing process to confirm that the quality of the drug substance meets the acceptance limits.

Though selecting the appropriate testing methods is within the responsibility of the manufacturer, an example of a routine quality control testing of the quality attributes is defined in this work is given in Table 13.

Before an assay can be implemented in a manufacture process the methods require validation and regulatory acceptance. Using parallel orthogonal methods with suitable data integrity controls is key to meet regulatory demands [44]. This assessment thereby only focuses on currently approved methods and does not include new rapid testing methods currently investigated. The table is non-exhaustive and quality control (QC) testing will include several more assays around AAV identity, potency and safety.

Process Step	CPPs and CMAs	Control
Virus Reduction Filtration of Buffer and Cell Culture Media		
	Differential Pressure	Skid Control
	Throughput/Flux Decay	Skid Control
	Duration	Skid Control
	Interruptions	Skid Control
	Membrane Leachables	Procedural Control
	Material Performance	Input Material Control
General Upstream Processing		
	Temperature	Skid Control
	pH of Cell Culture	Skid Control
	Conductivity of Cell Culture	Skid Control
	Nuclease Conc. and Activity	Input Material Control
	Time of Harvest	Procedural Control
	Harvest Shear Force: Agitation	Skid Control
	Plasmid or Virus Quality	Input Material Control
	Helper Virus Stock Titer	Input Material Control
Infection-Based USP		
	Multiplicity of Infection	Procedural Control
Transfection-Based USP		
	Plasmid Ratio	Procedural Control
	Complexation Time	Procedural Control
	Shear Force during Transfectior	Skid Control
Clarification		
	Pressure	Skid Control
	Flux	Skid Control
	Filter Capacity	Input Material Control
	Conductivity	Input Material Control
Affinity Chromatography		
	Flow Rate	Skid Control
	Conductivity of Wash Vuffer	Input Material Control
	pH of Wash Buffer	Input Material Control
	Conductivity of Elution uffer	Input Material Control
	pH of Elution Buffer	Input Material Control
	Load Density	Skid control with Procedural Control

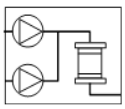
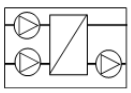
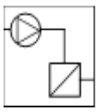
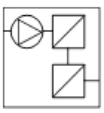
Process Step	CPPs and CMAs	Control
Polishing Chromatography		
	Elution Volume	Skid Control
	Flow Rate	Skid Control
	Load pH	Input Material Control
	Load Conductivity	Input Material Control
	Wash Condition	Input Material Control
	Elution Condition	Input Material Control
	Load Density	Skid Control with Procedural Control
UFDF		
	Volume Concentration Factor	Skid Control
	Transmembrane Pressure	Skid Control
	Permeate Flux	Skid Control
	Diafiltration Volume	Skid Control
Virus Filtration		
	Differential Pressure	Skid Control
	Throughput/ Flux Decay	Skid Control
	Filtration Duration	Skid Control
	Interruptions	Skid Control
	Filter Performance	Input Material Control
Sterile Filtration		
	Differential Pressure	Skid Control
	Throughput/ Flux Decay	Skid Control
	Flux	Skid Control
	Filtration Duration	Skid Control
	Filter Performance	Input Material Control
	Filter Robustness	Input Material Control

Figure 4

Control strategy for the CPPs and CMAs identified in each process step

Table 13

Example of a routine quality control testing for selected CQAs of an AAV process based on [45, 44, 46]. (SEC: Size Exclusion Chromatography, DLS: Dynamic Light Scattering, ELISA: Enzyme-linked immunosorbent assay, TEM: Transmission Electron Microscopy, AUC: Analytical Ultracentrifugation, MS: Mass Spectrometry, qPCR: quantitative Polymerase Chain Reaction, HPLC: High-Pressure Liquid Chromatography, LAL: Limulus Amebocyte Lysate).

	Quality Attribute	Test Strategy	Test Method	Specification
Product-Related Impurity	Noninfectious AAV	Lot-release testing	AD-dependent infectivity in susceptible cells	Product-specific
	Deamidated AAV	Lot-release testing	SEC, DLS	Product-specific
	Glycosylated AAV	Lot-release testing	SEC, DLS	Product-specific
	Aggregated AAV	Lot-release testing	SEC, DLS, light microscopy, TEM, AUC	Product-specific, eg. > 95% monomeric AAV
	Empty capsids	Lot-release testing	ELISA/qPCR, HPLC, MS, TEM, AUC	Product-specific
	Encapsidated host cell DNA	Lot-release testing	qPCR	< 10 ng/dose, ≤ 200 bp
	Encapsidated helper DNA	Lot-release testing	qPCR	Product-specific, eg. < 0.1% VG DNA
	Replication competent rcAAV	Lot-release testing	in vitro assay of cell lines permissive to infection	<1 rcAAV in 10^8 vg
Process-Related Impurity	Residual host cell DNA/RNA	Lot-release testing	qPCR, Picogreen, DNA Threshold assay	< 10 ng/dose , < 200 bp
	Residual host cell protein	Lot-release testing	ELISA, SDS-PAGE, HPLC, TEM	Product-specific, eg. < 1% VP protein
	Residual plasmid DNA	Lot-release testing	qPCR	Product-specific, eg. < 0.1% VG DNA
	Residual helper viruses	Lot-release testing	qPCR, infectious titer or ELISA for virus proteins	Negative
	Residual animal-derived CC medium components (BSA)	Lot-release testing	ELISA	Product-specific, eg. < 1% VP Protein
	Detachment enzyme	Lot-release testing	Various commercially available assays	Product-specific
	Detergents	Lot-release testing	MS, chromatography, TEM	Product-specific
	Leachables	Lot-release testing	LC/MS, GC/MS [47]	Product-specific (eg. 1.5 µg total daily intake for genotoxic impurities)
	Nuclease	Lot-release testing	ELISA	< 0.1% by mass or < 1 pg/ 10^9 VG
Adventitious Agents	Endotoxin	In-process testing	LAL (EP 2.6.14 [48], USP <85> [49], JP 17th Ed. 4.01 [50])	< 2 EU/dose
		Lot-release testing		
	Bioburden	In-process testing	Sterility testing (EP 2.6.1 [48], USP <71> [51], JP 17th Ed. 4.06 [50])	Negative
		Lot-release Testing		
	Myoplasma / spiroplasma	In-process testing	Cell based assay according to 21 CFR and alternative methods, eg. PCR (Ph. Eur. 2.6.7, Ph. Eur 2.6.21 [48], ICH Q2A (R1) [52])	Negative
		Lot-release testing		
	Adventitious viruses	In-process testing	*In vivo* and *in vitro* cellular assay according to 21 CRF (ICH Q5A [53])	Not detected
Potency	Functional AAV titer	In-process testing	ELISA, ddPCR, optical density (UV A260:280)	Product-specific
		Lot-release testing		

11 Conclusions

This document presented a framework for a risk- and science-based QbD assessment by evaluating QTPP, CQAs, CPPs, design space and control strategy based on a model AAV process:

1. Identification of CQAs based on the QTPP through risk assessments. Several risk assessment tools are mentioned by ICH Q9. The PHA represents the method of choice in this example.
2. Identification of the CPPs and CMAs based on risk assessments. The assessment tool used herein combines experimental data and prior knowledge to identify the CPPs and CMAs relevant in each process step.
3. Establishing the proven acceptable ranges (PARs) and normal operating ranges (NORs) for the CPPs and CMAs to establish the design space.
4. Defining the control and testing strategy to ensure consistent operation within the design space so that consistent product quality within the predefined ranges for all CQAs can be guaranteed.

Upstream production of AAV is well understood when it comes to the cell culture operating parameters. As the main source of process-related impurities, AAV purity and quality can be improved especially for transfection-based manufacture. Plasmid design, optimized transfection agents and the complexation conditions have been identified as critical material attributes or parameters that impact the empty-full ratio of AAV. In the clarification stage, the CPPs are characterized and high functional titers are achievable through optimal filter selection. It can be evaluated if a filter with positive net charge can be used as an endotoxin reduction stage.

In downstream processing, the immunoaffinity chromatography is well understood but nonspecific binding of AAV variants could be further reduced through identifying optimal wash conditions. Polishing chromatography has the potential to reduce adventitious viruses and separate empty from full capsids, the design spaces are however highly product-specific and need to be evaluated for every product individually. Challenges are seen in the tight gradient needed for elution and the inline analysis needed to make real-time decisions for the fractionation of empty and full AAV.

To assure viral safety of AAV products, infection-based manufacture includes a nanofiltration step either before or directly after UFDF. The large-pore size nanofilters (50 µm) typically used in this application are yet to be fully characterized for AAV products. If yield loss or patent infringement is a concern when nanofiltering AAV product, it has been discussed to process all raw materials such as buffers and cell culture media through small-pore size nanofilters (20 µm). First results are promising, and data provides a more solid knowledge base for the design space.

Sterility is assured through final sterile filtration of the AAV product with an elaborate design space. The yields achieved in 0.2 µm filtration of AAV are high when choosing a suitable filtration material and alternative approaches discussed for gene therapy products, such as applying a single or redundant in-series 0.45 µm filtration or even a removal of the final sterile filtration step, are not considered for AAV. A remaining challenge is however the very little volumes available for the validation. Here, validating with surrogate materials is a possibility according to PIC/S 2231 but detailed protocols are needed.

AAV manufacture is still a new field of biopharmaceuticals and some of the design spaces are not yet completely understood. However, this work shows that a large amount of process understanding has already been created within the industry. Also, with several approved gene therapies and hundreds of products in the pipeline, industry knowledge is building at a stunning pace. At the same time, the regulatory framework is rapidly developing which is increasing confidence in interpreting guidelines and builds regulatory maturity in both industry and regulators. Data and detailed process understanding thereby supports the prior knowledge needed to implement risk-based QbD strategies. With a strong focus on driving the development of fast and robust analytical tools, near real-time assays for CPPs will enable in-process control and speed up drug development by making DoE a responsive tool in process characterizations. With this work the authors intend to continue advancing gene therapies by combining expert and industry knowledge for the benefit of patients.

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13. Bibliography

[1] S. Viswanathan and M. Puich, "CMC obstacles in cell and gene therapy: four solutions to solve six challenges," *Cell and Gene Therapy Insights,* vol. 7, no. 5, pp. 471-488, 2021.

[2] A. C. Fisher, M.-H. Kamga, C. Agarabi, K. Brorson, S. L. Lee and S. Yoon, "The Current Scientific and Regulatory Landscape in Continuous Biopharmaceutical Manufacturing," *Trends in Biotechnology,* vol. 37, no. 3, pp. 253-267, 2019.

[3] M. Naso, B. P. I. W. Tomkowicz and W. Strohl, "Adeno-Associated Virus as a vector for gene therapy," *BioDrugs,* vol. 31, pp. 317-334, 1 07 2017.

[4] O.-W. Merten, A. A. Kamen and P. Chahal, "Manufacturing of viral vectors for gene therapy: Part I. Upstream processing," *Pharmaceutical Bioprocessing,* 04 2014.

[5] N. Clément and J. C. Grieger, "Manufacturing of recombinant andno-associated viral vectors for clinical trials.," *Molecular Therapy- Methods & Clinical Development,* vol. 3, no. 16002, pp. 1-7, 2016.

[6] O.-W. Merten, "Cell & Gene Therapy insights: challenges and advances in viral vectors," *Bioinsights,* pp. 521-551, 2016.

[7] J. Fraser Wright, "Product-related impurities in clinical-grade recombinant AAV Vectors: Characterization and risk assessment," *Biomedicines,* pp. 80-97, 3 03 2014.

[8] P. S. Chahal, E. Schulze, R. Tran, J. Montes and A. Kamen, "Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery," *Journal of Virological Methods,* vol. 196, no. 2014, pp. 163-173, 29 10 2013.

[9] L. Vandenberghe, R. Xiao, M. Lock, J. Lin, M. Korn and J. Wilson, "Efficient Serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing," *Human Gene Therapy,* 10 2010.

[10] B. Adams, H. Bak and A. Tustian, "Moving from the bench towards a large scale, industrial platform process for adeno-associated viral vector purification.," *Biotechnology and Bioengineering,* pp. 3199-3211, 20 06 2020.

[11] M. Penaud-Budloo, F. Achille and N. A. E. Clément, "Pharmacology of Recombinant Adeno-associated Virus Production," *Molecular Therapy Methods & Clinical Development,* vol. 8, pp. 166-180, 16 3 2018.

[12] ICH Harmonised Tripartite Guideline, "Quality Risk Management Q9," 2005.

[13] CMC Working Group, "A-Mab: A case study in bioprocess development Version 2.1," ISPE, 2009.

[14] T. Tanaka, H. Hanaoka and S. Sakurai, "Optimization of the quality by design approach for gene therapy products: A case study for adeno-associated viral vectors," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 155, pp. 88-102, 2020.

[15] A. Aloor, J. Zhang, E. Gashash, A. Parameswaran, M. M. C. Chrzanowski, Y. Diao, P. Wang and W. Xiao, "Site-specific N-glycosilation on the AAV8 capsid protein," *Viruses 20,* p. 644, 2018.

[16] O.-W. Merten, C. Gény-Fiamma and A. M. Douar, "Current issues in adeno-associated viral vector production," *Gene Therapy,* vol. 12, pp. 51-61, 18 10 2005.

[17] O.-W. Merten, M. Schweizer, P. Chahal and A. Kamen, "Manufacturing of viral vectors: part II. Downstream processing and safety aspects," *Pharmaceutical Bioprocessing,* vol. 2, no. 3, pp. 237-251, 2014.

[18] S. Boutin, V. Montheilhet, P. Veron, C. Leborgne, O. Benveniste, M. Montus and C. Masurier, “Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8 and 9 in the healthy population: implications for gene therapy using AAV vectors,” *Hum. Gene Teher. ,* vol. 21, pp. 704-712, 2010.

[19] A. Nathwani, U. Reiss, E. Tuddenham, C. Rosales, P. Chowdary, J. McIntosh, M. Della Peruta, E. Lheriteau, N. Patel, D. Raj, A. Riddell, J. Pie, S. Rangarajan, D. Bevan, M. Recht, Y. Shen, K. Halka, E. Basner-Tschakarjan, F. Mongozzi, K. High and e. a. Allay, “Long.-term safety and efficacy of factor IX gene therapy in hemophilia B,” *N. Eng J. Med,* vol. 371, pp. 1994-2004, 2014.

[20] A. Taylor and I. Wright, “Intravenous Trypsin,” *Circulation,* vol. 10, no. 3, pp. 331-337, 1954.

[21] X. Wang, A. Germansderfer, J. Harms and S. Rathore, “Using Statistical Analysis for Setting Process Validation Acceptance Criteria for Biotech Products,” *Biotechnology Progress,* pp. 55-60, 2008.

[22] J. Zhang and H. Yang, “Multivariate Analysis for Bioprocess Understanding and Troubleshooting,” in *Statistics for Biotechnology Process Development*, Boca Raton, FL, CRC Press, 2018, pp. 273-298.

[23] D. Blessing, G. Vachey, C. Pythoud, M. REy, V. Padrun, F. M. Wurm, B. Schneider and N. Deglon, “Scalable Production of AAV Vectors in ORbitally Shaken HEK293 Cells,” *Molecular Therapy,* vol. 13, pp. 14-26, 2019.

[24] F. Galdiero, “Adenovirus aggregation and preservation in extracellular environment,” *Arch Virol,* vol. 59, no. 1-2, pp. 99-105, 1979.

[25] D. Grimm, A. Kern, M. Pawlita, F. Ferrari, R. Samulski and J. Kleinschmidt, “Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinatnr AAV-2,” *Gene Ther,* vol. 6, no. 7, pp. 1332-30, 1999.

[26] L. Hajba and A. Guttman, “Recend Advances in tha Analysis Full/Empty Capsid Ratio fnd Genome Integration of AAV Gene Delivery Vectors,” *Current Molecular MEdicine,* vol. 20, no. 10, 2020.

[27] C. E. M. Hogwood, D. G. Bracewell and M. C. Smales, “Measurement and control of host cell proteins (HCPs) in CHO cell bioprocesses,” *Curr Opin Biotechnol,* vol. 30, pp. 153-60, 2014.

[28] J. Fraser Wright, “Manufacturing and characterizing AAV-based vectors for use in clinical studies,” *Gene Therapy,* vol. 15, pp. 840-848, 2008.

[29] F. Achille, M. Bouzelha, E. Lecomte, F. Vroucque, M. Penaud-Budloo, O. Adjali, P. Moullier, V. Blouin and E. Ayuso, “Accurate Tritration of Invectious AAV Particles Requires MEasurement of Biolgically Active Vectore Genomes and Sutiable Controls,” *Mol Ther Methods Clin Dev,* pp. 223-236, 2018.

[30] B. Bosma, F. Du Plessis, E. Ehlert, B. Nijmeijer, M. de Haan, H. Petry and J. Lubelski, “Optimization of viral protein ratios for production of rAAV serotype 5 in the baculovirus system,” *Gene Therapy,* vol. 25, pp. 415-425, 2018.

[31] M. Hildinger, L. Baldi, M. Stettler and F. Wurm, “High-titer, serum-free production of adeno-associated virus vectors by polyethyleneimine-mediated plasmid transfection in mammalian suspension cells,” *Biotechnology Letters,* vol. 29, pp. 1713-1721, 2007.

[32] A. Bennett, M. Mietzsch and M. Agbandje-McKenna, “Understanding capsid assembly and genome packaging for adeno-associated viruses,” *Future Virology,* vol. 12, no. 6, 2017.

[33] B. Raghavan, M. Collins, S. Walls, A. Lambropoulos and S. Bergheim-Pietza, “Optimizing the clarification of industrial scale viral vector culture for gene therapy,” *Cell & Gene Therapy Insights,* vol. 5, no. 9, pp. 1311-1322, 2019.

[34] Q. Wang, M. Lock, A. Prongay, M. Alvira, B. Petkow and J. Wilson, "Identification of an adeno-associated virus binding epitope for AVB sepharose affinity resin," *Molecular Therapy,* vol. 2, 2015.

[35] G. A. Rodrigues, E. Shalaev, T. K. Karami, J. Cunningham, N. K. Slater and H. M. Rivers, "Pharmaceutical Development of AAV-based gene therapy products for the eye," *Pharm Res,* vol. 36, no. 29, 21 08 2018.

[36] M. Toueille, "Development of purification steps for several AAV serotypes using POROS CaptureSelect AAVX affinity chromatography," *Cell and Gene Therapy Insights,* pp. 1-9, 2018.

[37] Pall Corporation, "Scalable Purification of High Yield Adeno-Associated Virus and Lentivirus Gene Therapy Vectors Using Membrane Chromatography," 2019. [Online]. Available: https://www.pall.com/en/biotech/process-workflows/adeno-associated-virus-production.html#dwn-5. [Accessed 01 Jun 2021].

[38] S. Pettit, C. Glover, T. Hitchhock, R. Legman and M. Schofield, "Downstream Manufacturing of Gene Therapy Vectors," Cell Culture Dish, 2020.

[39] J. Fraser Wright, T. Le, J. Prado, J. Bahr-Davidson, P. H. Smith, Z. Zhen, J. M. Sommer, G. F. Pierce and G. Qu, "Identification of Factor that Contribute to Recombinant AAV2 Particle Aggregation and Methods to prevent its occurrence during Vector Pufirication and Formulation," *Mol Ther,* vol. 12, no. 1, 2005.

[40] J. P. Labbrozzi, J. Lubelski, J. F. S. Verbruggen, D. bLankslee, N. Dion, M. Hein and S. McMillan, "rAAV Large-Scale Manufacturing using BEVS Technology," in *ESCGT*, Lausanne, 2018.

[41] W. Hermens, "Removal of contaminating viruses from AAV". Netherlands Patent EP2744895B1, 25 06 2014.

[42] PIC/S, "2231 Annex 2A: Manufacture of Advanced Therapy Medical products for human use".

[43] P. W. Barone and e. al, "Viral contamination in biologic manufacture and," *Nature Biotechnology,* vol. 38, pp. 563-572, 2020.

[44] D. King, E. Botonjic-Sehic, T. Hichcock and M. Krishnan, "Evolution of Viral Vector Analytics for Gene Therapy Manufacturing," *Cell Culture Dish,* pp. 34-46, 2020.

[45] J. Fraser Wright, "Quality Control Testing, Characterization and Critical Quality Attributes of Adeno-Associated Virus Vectors Used for Human Gene Therapy," *Biotechnology Journal,* vol. 16, no. 1, 2020.

[46] World Health Organization (WHO), Requirements for the Use of ANimal Cells as in Vitro Substrates for the Production of Biologics (Requirement for Biological Substrate No. 50), Vols. WHO Technical Report Series, No. 878, Geneva, Switzerland: WHO Expert Committee on Biological Standardization, 1998.

[47] BPSA Bio-Process Systems Alliance, "Extractables/Leachables Considerations For Cell & Gene Therapy Drug Product Development," Arlington, VA, USA, 2020.

[48] Council of Europe, "European Pharmacopeia," Strasbourg, France, 2017.

[49] United States Pharmacopeial Convention, Inc., "U.S. Pharmacopeia <85> Bacterial Endotoxin Test," Rockville, MD, USA, 2018.

[50] The Ministery of Health, Labour and Welfare, "Japanese Pharmacopeia 17th Edition," Tokio, Japan, 2016.

[51] United States Pharmacopeial Convention, Inc., "US. Pharmacopeia <71> Sterility Tests," Rockville, MD, USA, 2009.

[52] INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE, “ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology,” ICH Secretariat, Geneva, Switzerland.

[53] FDA, “Q5A Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin,” 1998.

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