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Risk Assessment and Comparability Analysis of Adherent Versus Suspension Cultures for Gene Therapy Manufacturing

The importance of de-risking process
improvements for large scale AAV
gene therapy production

Authors: Aígee Duenas, Caroline Smith-Moore



tkdsolns.com



INTRODUCTION:

With the most FDA approvals of any viral vectors, adeno-associated virus (AAV) has become a popular gene therapy delivery platform. AAVs provide a safe and efficacious platform for *in vivo* gene delivery as AAVs are non-pathogenic and do not integrate into host genomes yet enable sustained gene delivery to target tissue^[1].

As such, AAVs are a preferred gene therapy vector for *in vivo* applications. Despite the popularity of AAV gene therapies, large-scale production of AAVs remains a challenge. Many early phase AAV production utilizes adherent cell cultures for efficient, small-scale transfections. However, to meet clinical demand, late phase AAV programs often are transitioned to suspension cultures for improved scalability and yield. Transferring AAV production from adherent to suspension cultures requires a thorough risk and comparability assessment.

The present study highlights TKD Solutions' approach to de-risking the transition of an AAV gene therapy from an adherent production method to

suspension culture. TKD Solutions developed this approach to identify potential process attributes that could be at risk in a suspension culture when compared to the original adherent culture process. The process attributes were evaluated in a side-by-side comparability assessment using analytical tools to de-risk the process changes and validate the suspension culture method. TKD Solutions' risk assessment and comparability analysis resulted in a validated suspension method that enabled the program to scale its AAV production to meet clinical demand.

Market Need

There are more than 250 AAV gene therapies in clinical trials^[2]. The growing number of patients dosed with AAV gene therapies underscores the necessity for robust and reproducible large-scale production of AAVs. To meet the dosing demands of clinical trials, it is often required to move adherent cell cultures to suspension cultures as the adherent production logistics become infeasible with patient population increases. Notably, a Phase 1/2 trial for an AAV gene therapy required more than 400 ten-layer adherent cell stacks to generate enough doses for six patients in the clinical trial^[3]. To circumvent the inefficiency of adherent culture cell stacks, late phase AAV processes are modified to utilize suspension cultures and bioreactors.

A risk assessment and comparability analysis can be utilized to de-risk implementation of AAV suspension cultures. Risk assessments involve a comprehensive scoring metric to evaluate (1) severity (2) occurrence and (3) detection of failure for each process step and impact to CQAs. Process attributes evaluated as high risk can be evaluated in a comparability assessment. The risk assessment and comparability analysis are a powerful set of tools to de-risk and implement a process change to an AAV gene therapy program.

Methods

TKD Solutions developed a method to evaluate the implementation of a suspension culture for a client's AAV gene therapy program. The program required a change from adherent to suspension culture to improve yield and meet clinical demand. To ensure the change from adherent to suspension cultures did not adversely impact the process or product, TKD Solutions performed the following steps:

1. A risk assessment was developed with side-by-side comparison of adherent and suspension process details
2. Process attributes were identified based on a scoring system that accounted for severity, occurrence, and detection of process step failures and impact to CQAs
3. Analytical comparability between adherent & suspension cultures was performed using historical GMP and development data to evaluate process attributes

The results from the risk assessment and comparability analysis were used to determine the validity of the suspension culture in the AAV gene therapy production process.

Risk Assessment

TKD Solutions' risk assessment is a comprehensive evaluation of the entire manufacturing process. Each process attribute is evaluated for criticality by assessing the failure mode's potential impact on safety, identity, strength, purity, and quality (SISPQ) when a process attribute fails. The process attributes are scored and ranked by looking at severity of the failure, failure occurrence, and failure detection. This was done in a side-by-side matrix for both the adherent and suspension cultures to evaluate deviations in the process attributes between the two methods.

The risk assessment was performed to identify any potential risks of the following process attributes for the

Manufacturing Location	Polishing Chromatography Ion Exchange
Cell Bank	Transient Transfection
Inoculum Development	Transfection Reagent
Cell Expansion I	Media Exchange or Media Quench
Cell Expansion II + Transfection	Total Transfection Incubation Time
Cell Culture Media	Endonuclear Treatment + Media Release
Plasmids	Cell Lysis
Fill + Finish	Clarification
Sterile Filtration	Volume Reduction: Large Scale Tangential Flow Filtration
Harvest	Capture Chromatography: Affinity Chromatography
Final Formulation	Empty / Full Capsid Separation: Gradient Ultracentrifugation

Table 1: Process Attributes

current AAV9 adherent process (Table 1).

A risk-based approach has been adopted for identification and assignment of criticality. AAV9 adherent process was compared side-by-side to the suspension process in order to assign a risk ranking score for each process attribute identified in Table 1. Each process attribute is evaluated for criticality by assessing its potential impact as it relates to the quality and safety of the product.

Risk Assessment Scope

TKD Solutions identified and documented the process steps or details that are critical during the production of AAV9 using the adherent process. Such process steps or details were then compared side-by-side to that of the suspension process. The process attributes evaluated have been summarized in [Table 2](#).

Process Attributes	Adherent Process	Suspension Process
Manufacturing Location	USA	EU
Cell Bank	HEK 293 Adherent Cell Line	HEK293 Suspension Derived Line
Inoculum Development	T-225cm ² Flasks	Shaker Flask
Cell Expansion I	CellSTACKs	WAVE Bioreactor
Cell Expansion II + Transfection	HYPERStacks	Thermo Scientific™ HyPerforma™ Single-Use Bioreactor (Thermo SUB, 250 – 500 L)
Cell Culture Media	Dulbecco's Modified Eagle Medium (DMEM) with Calf Serum (BCS)	Cell Culture Media (Animal Component-Free Expression Medium)
Plasmids	Transgene Plasmid (Vector Plasmid with Ampicillin Resistance Marker), RepCap + AdHelper	Transgene Plasmid (Vector Plasmid with Kanamycin Resistance Marker), RepCap + AdHelper
Transient Transfection	Triple Plasmid Transfection	Triple Plasmid Transfection
Transfection Reagent	Calcium Phosphate Ca ₃ (PO ₄) ₂	Polyethyleneimine (PEI) transfection, PEIMAX®
Media Exchange / Media Quench	Media Exchange	Media Quench
Total Transfection Incubation Time	Up to 1.5 days	Up to 3 days
Endonuclease Treatment + Media Release	Endonuclease Treatment	No Nuclease Treatment
Harvest	Media from the HYPERStacks are harvested pooled into a single volume	Bioreactor batch harvest; no centrifugation
Cell Lysis	No cell lysis involved	Chemical lysis + flocculation
Clarification	Capsule pre-filter, followed by a sterilizing grade capsule filter chased with buffer only	Depth filtration followed by a sterilizing grade filter chased with buffer and NaCl
Volume Reduction Process	Tangential Flow Filtration	No Volume Reduction Post Clarification
Chromatography (Capture)	No Capture Chromatography	Affinity Chromatography
Empty / Full Separation: Gradient Ultracentrifugation	2-Step Iodixanol Gradient Process	1-Step Iodixanol Gradient Process
Chromatography (Polishing)	Cation Exchange Chromatography	Anion Exchange Chromatography
Final Formulation (TFF)	Final Formulation TFF; excipient added post formulation	Final Formulation TFF; the excipient is added to the formulation buffer and used during Final Formulation TFF
Sterile Filtration	Single 0.2 µm Sterile Filtration	Double 0.2 µm Sterile Filtration
Fill + Finish	Aseptic Fill in PP Microtube	Aseptic Vial Fill in Crystal Zenith® Vials

Table 2: Process Attributes Evaluated for AAV9 (Adherent vs. Suspension)

Each process attribute was evaluated for criticality by assessing the failure modes potential impact on quality and safety when a process attribute fails. The process attributes were scored and ranked by looking at severity of the failure, failure occurrence, and failure detection. The risk ranking definitions for failure severity are summarized in **Table 3**, ranging from a score of 1, no impact, with product expected to meet specifications, to a score of 10, high, with high impact to quality, with product expected to fail specifications and high impact to product safety and quality.

Risk Ranking Score	Impact	Description
1	None	No impact to quality attributes; no/low impact to performance indicators; product expected to meet specifications
4	Low	No, or acceptable impact to quality attributes; low/moderate impact to performance indicators; product expected to meet specifications
7	Moderate	Moderate impact to quality attributes; Moderate/high impact to performance indicators; Product expected to meet specifications
10	High	High impact to quality attributes; Product expected to fail specifications; Specifically high impact to product safety

Table 3: Severity (S) of Failure

Similarly, the risk ranking definitions for failure occurrence are summarized in **Table 4** ranging from a score of 1, probability of failure is remote (no reported excursions) to a score of 10, high probability of failure based on repeated excursions.

Risk Ranking Score	Impact	Description
1	Remote	Probability of failure is remote, based on no excursions to date as well as automated control
4	Low	Probability of failure is low, based on no excursions to date, but manual control
7	Moderate	Probability of failure is moderate, based on at least one observed excursion regardless of type of control (automated or manual)
10	High	Probability of failure is very high, based on repeated excursions to date, regardless of type of control (automated or manual)

Table 4: Occurance (O) of Failure

The ability to detect a process step failing is scored separately to inform the impact of the critical process steps. The risk ranking definitions for failure detection are summarized in **Table 5**, ranging from a score of 1, high detection of failure to a score of 10, no detection of failure and effect of failure may not be detected until release and can impact product quality. An increase in detection can help mitigate overall risk of a process attribute and its failure mode.

Risk Ranking Score	Impact	Description
1	High	Defined automation and process outputs ensure almost certain detection of both failure itself (e.g. via alarm) and the effect of failure at this step (e.g. by suitable IPS, such as protein content, ISS concentration, etc.)
4	Moderate	Either automation or defined process outputs are in place to detect the failure itself or the effect of the failure at this step by suitable IPC/IPS
7	Low	There are no alarms or systems to detect the failure itself and the effect of failure may not be detected by the defined process outputs at this step
10	None	There are no alarms or systems to detect the failure itself and the effect of failure may not be detected until release

Table 5: Direction (D) of Failure

Critical Process Attribute determination is as follows (**Table 6**): severity and occurrence scores are used together to inform process attributes of high risk to the product quality in the event a failure occurs. The combined score is the multiplicative product of the severity and occurrence scores. Risk scores less than or equal to 10 are considered to be low to minimal risk. Scores between 16 and 40 are moderate risk and are considered to be process attributes that could have potential impact to product quality. Scores between 49 and 100 were considered to be process attributes with high criticality or of high risk.

Severity Risk Score	Occurrence Risk Score			
	1	4	7	10
1	1	4	7	10
4	4	16	28	40
7	7	28	49	70
10	10	40	70	100

Table 6: Overall Attribute Risk Scores

Critical Process Attribute Identification

Critical Process Attributes with scores between 49 – 100 were compared between processes to determine if the risk was culture-independent or if the suspension culture added an additional risk to a process step. The attribute was further evaluated for its ability to be detected early in the process using the detection scoring matrix.

Analytical Comparability

Critical quality attributes (CQAs) of the AAV gene therapy product were evaluated in analytical methods in a side-by-side analysis of an adherent GMP lot and three suspension lots (one development lot and two GMP lots). The comparison was made using release assays with testing conducted at the same time for all lots to eliminate inter-assay variability. Results were evaluated for meeting acceptance criteria and performing relative to each other.

Results


The risk assessment identified high risk attributes as lysis, empty/full separation (gradient ultracentrifugation) and dilution of material to target titer (DS). However, these were consistent between both the adherent and

suspension AAV gene therapy processes, independent of platform. Thus, no additional risk was introduced as a result of the suspension culture conditions.

The comparability report evaluated the full panel of release assays for the AAV gene therapy product across one adherent GMP lot, two suspension GMP lots, and one suspension development lot. The comparability report evaluated CQAs across six categories: strength, purity, identity, safety, general quality and characterization. In all instances, the assays passed acceptance criteria with some moderate improvements in suspension cultures compared to adherent cultures. Results of the comparability report showed no deviations between culture conditions.

Appendix A captures the process criticality assessment scoring and justifications in detail.

Appendix B outlines our detection rating scores when adapting a suspension platform vs. an adherent platform.



The risk assessment identified high risk attributes as lysis, empty/full separation and dilution of material to target titer.

Results: Analytical Comparability Assessment

Here we summarize the approach of assessing analytical data derived from the testing conducted at an EU CDMO comparing an AAV9 adherent lot to three suspension lots produced. Many of the assays were conducted using side-by-side testing to ensure equivalency of the methods. However, some of the compendial methods were assessed by comparison of the respective CoAs of the lots. The data is analyzed to assess the comparability of the suspension material to the adherent material previously used in the clinic. Attributes that were not part of the comparability are discussed for information purposes only to provide context.

Analytical Comparability Purpose

The purpose of analytical comparability is to assess the data provided in the comparability report using the comparability study acceptance criteria. The analytical comparability study was conducted to assess the impact of the manufacturing change from an adherent process at a US CDMO to a suspension process at an EU CDMO. A risk assessment of the manufacturing process changes was performed in a separate assessment and is not discussed in this document.

Lot Selection

The batches included in the analytical comparability study are listed in [Table 7](#). In accordance with the agency feedback received, as there is only one lot available for the adherent material, statistical evaluation of the variability of that process is not possible. For this reason, a direct comparison is made between the adherent lot and the suspension lots, and the associated acceptance criteria are reported.

Mfg. Year	Mfg. Site	Description
2018	US CDMO	AAV lot produced by triple transfection of plasmids in adherent cell culture
2020	EU CDMO	AAV lot produced by triple transfection of plasmids in suspension cell culture
2021	EU CDMO	AAV lot produced by triple transfection of plasmids in suspension cell culture
2021	EU CDMO	AAV lot produced by triple transfection of plasmids in suspension cell culture

Table 7: AAV9 Lots Incl. in the Analytical Comparability Study

Analytical Comparability Study Assays

The assays included in the analytical comparability study are listed below. Comparability was evaluated using either side-by-side testing or a Certificate of Analysis comparison, as reflected in [Table 8](#). For side-by-side testing, actual analytical data cannot be provided due to confidentiality reasons, but methods and acceptance criteria are outlined for each respective assay.

Analytical Method	Side-by-Side Testing	CoA Comparison
General Characteristics		
Appearance		•
pH	•	
Osmolality	•	
Particle Size Distribution	•	
Identification		
Genomic ID	•	
Capsid ID	•	
Strength		
Vector Genome Titer (vg/mL)	•	
TCID ₅₀	•	
Potency	•	
Purity		
Vector Purity	•	
Full / Empty Particle Ratio	•	
Aggregation	•	
Residual Process Chemicals (those that apply to both processes)	•	
Residual Host Protein		•
Residual Host Cell DNA + EIA		•
Residual Plasmid DNA		•
Safety		
Mycoplasma		•
Adventitious Agents		•
Endotoxin		•
Replication Competent AAV	•	
Sterility		•

Table 8: AAV9 Lots Incl. in the Analytical Comparability Study

Strength Assays

Vector Genome Titer: Transgene-Specific ddPCR

The vector genome titer by transgene specific ddPCR quantifies the total number of DNase I resistant genomes. This titer is transgene and product-specific and will be used as the dosing titer. The lots adherent and two suspension lots were all titered in parallel using this method, while one suspension lot was titered as part of batch release testing. This assay was included as the dosing titer and is a critical measure of the AAV, and also to demonstrate bridging of the method with the titer of the adherent batch. The acceptance criteria set for this attribute is that the batches need to meet the specification that was set for titer when the batches were manufactured. This specification is derived from the clinical need for dosing. The appropriate patient dose is calculated in the pharmacy using the patient's weight in kilograms, and the titer value for the specific lot being dosed.

Vector Genome Titer: ITR qPCR

The vector genome titer by ITR-qPCR quantifies the number of DNase I resistant genomes using the conserved AAV ITR sequence. This titer is not product specific but is a platform assay used by the EU CDMO. This qPCR assay is not used for a dosing titer as its precision and accuracy are inferior to the Transgene specific-ddPCR assay. It is primarily included here as it serves as the inoculation titer

for the calculation of the infectious titer. Considering the titer values reported for the transgene specific-ddPCR, where the adherent batch has a lower titer than the suspension material, and the method variability for the ITR qPCR, the values reported are as expected.

Infectious Titer (TCID₅₀)

The infectious titer TCID₅₀ assay measures the ability of the AAV to infect a cell. In this method HeLa RC32 cells expressing rep/cap are infected with serially diluted AAV drug product in the presence of Adenovirus, to allow for viral replication. The infectious AAV titer is measured using ITR qPCR, with the Spearman-Kärber method for the TCID₅₀. This assay is included in the comparability as it is an indicator of the function of the AAV. The acceptance criteria for this attribute is that the lots will be comparable where the difference between the adherent lot and suspension lots is within method variability, approximately 2-fold. The fold change is calculated by dividing each of the suspension batches by the adherent batch to get the fold difference. All fold differences are within 2-fold. Lots are deemed comparable and meet the acceptance criteria.

Identity Assays

Genomic Identity

The genomic identity of the AAV9 drug product is confirmed utilizing Sanger sequencing of the AAV genome transgene compared to the reference sequence of the plasmid used for production. To ensure the genomic identity of the AAV9 drug product is identical between batches, this assay is included in the comparability study with an acceptance criteria stating that the genome sequence would be identical between the adherent lot and the suspension lots.

Protein Identity

The identity of the capsid is confirmed using SDS-PAGE with a western blot with an antibody specific for VP1, VP2, and VP3 of the rAAV capsid. Confirming the identity of the viral proteins between the adherent and suspension lots is critical for the comparability of the batches and is thus included in the study. The acceptance criteria for this attribute is that the materials are comparable if VP1, VP2, and VP3 are detected in the adherent and suspension lots.

Purity Assays

Full to Empty Particles Ratio

The SDS-PAGE/Silver Stain assay is a general purity method used to evaluate the presence of contaminating proteins in addition to confirming the presence of the AAV capsid proteins, VP1, VP2 and VP3. This assay is included in the comparability study as it provides support for the purity of the AAV9 drug product produced using the new suspension process compared with the previous clinical adherent batch. The acceptance criteria for comparability is that for all samples VP1, VP2, and VP3 would be the predominant bands, which is the specification for batch release.

Residual Chemicals (Iodixanol)

Iodixanol is used during the separation of full and empty particles by ultracentrifugation. The assay used to measure residual iodixanol is an RP-HPLC method using a limit test. If the area of the iodixanol peak in the sample is lower than the peak area in LOD standard (0.1 ppm), the content of iodixanol in the sample is reported as lower than LOD (0.1 ppm). If the area is higher, additional standard solutions (0.2, 1, and 10 ppm) are prepared and injected. In this case, result is reported as an interval whose limits correspond to the concentrations of the standards between which the sample falls. The safety of iodixanol is supported by its use as a clinical radiographic contrast agent, where it is used in significantly higher concentrations (FDA Visipaque (iodixanol) injection) (See Reference 5) than reported in the adherent and suspension lots. As there are limited lots for comparison and this attribute was not conducted for early-stage development lots, the assay is primarily a limit test and was not validated for precision; therefore, setting a meaningful acceptance criteria for this attribute was not possible. However, given the very low concentrations of iodixanol across all lots, it is believed the lots are comparable and the manufacturing change does not negatively impact product safety based on this attribute. The residual iodixanol will continue to be monitored as further lots are produced.

Full to Empty Particles Ratio

The full to empty particle ratio for AAV provides a measurement of the number of AAV particles that contain a genome compared to the number of empty capsids. It is hypothesized that the presence of empty particles can have an effect on the efficacy and potentially the safety of the AAV product, though this has not been definitively proven to date. However, a measure of this attribute is still important for product quality and is thus included in the comparability study. All lots were measured by cryo-TEM to obtain their full to empty particle ratio. This method provides a percentage of full particles, empty particles, and particles which have undetermined packaging by this method. All lots met the acceptance criteria of $\geq 50\%$ full capsids, which was the specification for the GMP suspension lots. From this assessment all lots are comparable; however it is also noted that the suspension lots show a higher overall percentage of full capsids. While this method is not validated, precision data is not available to assess the method variability, this trend is seen for all suspension lots.

Vector Aggregation

The method used for measuring vector aggregation is negative stain TEM (nsTEM). In this analysis, individual particles are reported as percentage of particles < 40 nm, while aggregates and clusters are reported as a percentage of particles > 40 nm. All lots were analyzed on the same assay during the same time. As this method is not validated, a measure of assay variability is not available to set acceptance criteria. The acceptance criteria are set using the previous data for the development batches. All lots showed a high percentage of individual particles, which agrees with the particle size distribution data, which is an orthogonal method to evaluate aggregation.

Safety Assays

All of the safety assays, aside from the replication competent AAV, were compared based on their CoA values instead of tested side-by-side. As these are compendial methods with specific regulatory expectations, this comparison was deemed appropriate. The replication competent AAV assay was performed side-by-side for all lots utilizing the same assay and titering method.

Endotoxin

Endotoxin is a critical safety assay measuring the endotoxin units per mL of product using a kinetic chromogenic assay as defined in USP <85> and EP 2.6.14. The lots were compared based on the CoA values.

Replication Competent AAV

The purpose of this assay is for the detection of replication competent AAV, which could be formed as part of the production process. For this assay, all lots were tested side-by-side, and the titer of the AAV9 Drug product used to infect the cells was measured using the Transgene specific ddPCR. As this is an important safety assay and a parameter that can vary based on production processes, it was included in the comparability study with the acceptance criteria that all lots will have no detection of rcAAV above the LOD of the assay. The LOD of the assay was determined to be 10 infectious particles rcAAV in 10^{10} viral genomes.

Appearance

Visual inspection of the lots was conducted as defined in USP <790> and EP 2.9.2. The lots are compared based on their CoA values. Development batch (suspension) was not assessed for appearance due to the low filling volume of the batch (0.120 mL).

Mycoplasma

Mycoplasma testing was conducted either by agar isolation and indicator cell culture assay (USP <63>) for the adherent lot, or by a PCR-based method (EP 2.6.7) for the suspension lots. All lots were found to be negative for mycoplasma and are compared based on their CoA values.

Adventitious Virus

Adventitious virus detection was conducted for the GMP suspension lots using the transfection pool (harvest before lysis), and the adherent lot analysis was conducted using the bulk harvest. In both cases the assay used to detect adventitious virus involved inoculation of three permissive cell lines (MCR-5, Vero, and HEK293/A549) with the sample, followed by extended culture and observation for cytopathic effects including hemadsorption and hemagglutination. The suspension lot samples were tested at a different vendor compared to the adherent lot. The developmental suspension batch was not tested as this was a development batch. All tested lots were found to be negative for mycoplasma and are compared based on their CoA values.

Sterility

Sterility was assessed in the adherent lot using direct inoculation, and in the GMP suspension lots using membrane filtration in accordance with USP <71> and EP 2.6.1. The developmental suspension lot was also assessed using direct inoculation; however, as the fill volume was very small (0.12 mL), and it was a development batch, the compendial method was not followed. All lots were found to meet the acceptance criteria of no growth and passed sterility.

General Quality Assays

Osmolality

Osmolality of all lots was assessed on the same day by freezing point depression using an instrument that was calibrated and had its performance verified with standard solutions in compliance with EP 2.2.35 and USP <785>. All lots were found to meet the specification for the suspension lots 388-426 mOsm/kg, and thus pass the acceptance criteria.

pH

The pH of all lots was assessed on the same day using potentiometry on an instrument that had been calibrated and had its performance verified with standard solutions in compliance with EP 2.2.3 and USP <791>. All lots were found to meet the specification 7.5-8.5 and thus the acceptance criteria for comparability.

Particle Size Distribution

Particle size distribution is an assessment of the population of particle sizes that exist in a given sample. The particle size distribution assessment was conducted side-by-side using dynamic light scattering (DLS) for all lots. Particle size distribution was included in the comparability study as it can provide information of sample aggregation if larger particle sizes are detected in a sample. For AAV, the predicted particle size by DLS is approximately 25 nm. The acceptance criteria was set based on previous development lots.

Characterization Assays

Due to sample, assay, and other technical constraints as described below, certain attributes were not included as part of the comparability study. Data contained in this section is provided for information purposes only.

Strength / Potency

A specific potency assay was originally not included in the comparability study, as a suitable method was not fully developed at the time. A method is validated for the detection of enzyme activity but was not ready for use for this study.

Purity / Residual Host Cell Protein

Residual host cell protein was not included as part of comparability as it was predicted that the lots may not be comparable for this attribute due to the inherent differences in the adherent and suspension processes. Suspension processes have a higher cell density leading to an increased amount of HCP entering into the downstream manufacturing process, leading to increased amounts of this residual.

While HCP was not included in the comparability study, this attribute was quantified for all of the lots, though not side-by-side using the same assay. This assay data is provided for informational purposes only.

Residual Host Cell DNA and EIA

Residual 18s host cell DNA and residual EIA were not included in the comparability study as it is predicted that due to the higher cell density, the lots would not be comparable for this attribute. Residual 18s host cell DNA was measured for all of the lots, but for the suspension lots quantification of residual EIA was added to be in line with regulatory guidance and to ensure product safety.

The 18s residual host cell DNA assay for the adherent lot involved qPCR amplification and quantification of the 18s gene with three amplicon sizes (102bp, 401bp, and 765bp), while the assay used for the suspension lots had two amplicon sizes (123bp and 254bp). As only the approximately 100bp amplicons are similar in size, those values are shown, with this assay data provided for informational purposes only.

Residual Plasmid DNA

Residual plasmid DNA was not included for side-by-side comparison in the comparability study as these materials

cannot be compared on the same residual plasmid assay. The material produced in adherent cell culture utilized plasmids carrying the ampicillin resistance gene, whereas the suspension carry the kanamycin resistance gene on the plasmids. This change was made to be more compliant with regulatory guidance and for patient safety concerning the use of β -lactam antibiotics. The residual plasmid assay involves quantification of the antibiotic resistance gene from the plasmids by qPCR, as the same primers and probes could not detect both the kanamycin resistance gene and the ampicillin resistance gene, these lots could not be compared side-by-side on the same assay. Additionally, as an increased amount of residual plasmid may be predicted for suspension produced during production, it was predicted that the lots from the adherent process and from the suspension process may not be comparable for this residual.

Residual plasmid is quantified for all lots, and an appropriate specification will be set when sufficient batches have been produced. Given the caveat that the values were derived using different qPCR assays with different targets (Amp vs. Kan) at different locations, the data represents the amount of residual plasmid per vg for the 4 lots. This data is provided for informational purposes only.

Residual Chemicals and Process-Related Residuals

Many of the process residual chemicals that are tested in the suspension batches do not overlap with the adherent process. For that reason, other than overlapping residual chemicals and process residuals were not included as part of the comparability. These residuals are tested as part of lot release.

Safety: Bioburden

Bioburden was not included in the comparability side-by-side testing as the only samples available for testing were drug product, and the samples volumes required for bioburden testing were too high. However, bioburden testing was conducted for all lots.

Safety: Subvisible Particles

Subvisible particles testing was not included as part of the comparability study due to sample volume limitations. This attribute was not assessed for the adherent lot but is assessed for the suspension lots.

Conclusion

The analytical comparability results demonstrate that the adherent lot and the suspension lots were comparable based on the acceptance criteria set in this study. Due to the limited number of lots and materials available, a true statistical comparison was not possible, however from the data it was seen that the manufacturing change did not have an impact on the safety of the product, which was the primary concern for this Phase I study. As further process and product knowledge is gained with more lots produced, these attributes will continue to be assessed and further assay and process controls implemented.

Summary

In this study we present the combined approach of a risk assessment and comparability analysis to support a process change in gene therapy manufacturing. Risk assessments are a comprehensive tool to score and rank individual process steps in a biomanufacturing workflow. TKD Solutions leveraged the risk assessment in a side-by-side method to compare an adherent culture to a suspension culture to produce an AAV gene therapy. The side-by-side risk assessment identified high risk process steps as being significant to both adherent and

suspension cultures with no new risks being introduced as a result of the suspension culture. Following the risk assessment, TKD Solutions demonstrated process comparability between adherent and suspension cultures by evaluating analytical results across four different lots of material. This method for risk assessment and comparability analysis enabled the client to confidently implement a suspension culture in a process that historically was limited by adherent culture conditions.

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Need to de-risk your AAV-based gene therapy process? Reach out to learn how we can help.

TKD Solutions LLC
Science-driven Consulting for Advanced Therapies

+1 617-803-6631
info@tkdsolns.com



tkdsolns.com

Appendices

Appendix A: Process Risk Assessment when adapting a suspension platform vs. an adherent platform

Example of risk assessment failure mode severity and occurrence likelihood with justification for one process detail:

Process Detail	Process (Adherent)	Process (Suspension)	Failure Mode	S	Justification	O	Justification	Overall Risk
Mfg. Location	USA	EU	None GMP production at new manufacturing location	7	GMP lots are required for clinical trial material	1	EU CDMO is a GMP certified manufacturing facility that has produced clinical stage lots of viral vectors	7

The complete risk assessment appendix is shown below with individual scores and justifications redacted for confidentiality purposes:

Process Detail	Process (Adherent)	Process (Suspension)	Overall Risk
Manufacturing Location	USA	EU	7
Cell Bank	HEK 293 Adherent Cell Line	HEK293 Suspension Derived Line	16
Plasmids	Transgene plasmid (vector plasmid with ampicillin resistance marker)	Transgene plasmid (vector plasmid with Kanamycin resistance marker)	28
	AAV9 RepCap–Helper Plasmid	AAV9 RepCap–Helper Plasmid	28
	Ad Helper Plasmid	Ad Helper Plasmid	28
Cell Culture Media	Dulbecco's Modified Eagle Medium (DMEM) with calf serum (BCS)	Cell culture media (animal component-free expression medium)	7
Vessels for Cell Expansion (Inoculum Development, Cell Expansion I, Cell Expansion II & Transfection)	T-225cm ² Flasks / CellSTACKs HYPERStacks	Shake Flasks: WAVE Bioreactor 2 x 250L Thermo SUB	40
Transient Transfection	Triple Plasmid Transfection	Triple Plasmid Transfection	28
Transfection Reagent	Calcium Phosphate Ca ₃ (PO ₄) ₂	Polyethyleneimine (PEI) transfection, PEIMAX [®]	28
Media Exchange	Post transfection, the medium is changed	Post transfection: Media quench	16
Total Transfection Incubation Time	Up to 1.5 days	Up to 3 days	28
Endonuclease Treatment + Media Release	Endonuclease Treatment	No Nuclease Treatment/ NA	16
Harvest	Media from the HYPERStacks are harvested pooled into a single volume	Bioreactor batch harvest; no centrifugation	28
Cell Lysis	Cell lysis is not applicable in the adherent platform	Chemical lysis + flocculation	49
Clarification	Capsule pre-filter, followed by a sterilizing grade capsule filter chased with buffer only	Depth filtration followed by a sterilizing grade filter chased with buffer and NaCl	16
Volume Reduction	Tangential Flow Filtration	No Volume Reduction Post Clarification	16
Chromatography (Capture)	No Capture Chromatography	Affinity Chromatography	49
Empty / Full Separation: Gradient Ultracentrifugation	2-Step Iodixanol Gradient Process	1-Step Iodixanol Gradient Process	49

Appendix A: Cont.

Process Detail	Process (Adherent)	Process (Suspension)	Overall Risk
Chromatography (Polishing)	Cation Exchange Chromatography	Anion Exchange Chromatography	28
Final Formulation (TFF)	Final Formulation TFF; excipient added post formulation	Final Formulation TFF; the excipient is added to the formulation buffer and used during Final Formulation TFF	28
	Diavolumes: Less	Diavolumes: More	28
	Hollow fiber cartridge; smaller	Hollow fiber cartridge; larger	1
Dilution of Material to Target Titer (DS)	During TFF (DS)	Post TFF Eluate (Stored for a maximum of 7 days then diluted to DS)	70
Sterile Filtration	Single 0.2 µm Sterile Filtration	Double 0.2 µm Sterile Filtration	28
Fill + Finish	Aseptic Fill in PP Microtube	Aseptic Vial Fill in Crystal Zenith® Vials	7

Appendix B: Detection (D) Rating when adapting a suspension platform vs. an adherent platform.

Process Detail	Process (Adherent)	Process (Suspension)	(D)	Justification
Manufacturing Location	USA	EU	1	EU CDMO is a GMP certified manufacturing facility that has produced clinical stage lots of viral vectors. New lots are produced at EU CDMO.
Cell Bank	HEK 293 Adherent Cell Line	HEK293 Suspension Cell Line	4	Cell bank must meet specification upon release and during the duration of the stability study before introduction into the process.
Plasmids	Transgene plasmid (vector plasmid with ampicillin resistance marker)	Transgene plasmid (vector plasmid with Kanamycin resistance marker)	4	The required volumes are calculated and recorded in the batch records at time of addition. Step is verified by second operator. Detection during release testing for CQA impact. Residual Plasmid DNA is also an IPT.
	AAV9 RepCap–Helper Plasmid	AAV9 RepCap–Helper Plasmid	4	
	Ad Helper Plasmid	Ad Helper Plasmid	4	
Cell Culture Media	Dulbecco's Modified Eagle Medium (DMEM) with calf serum (BCS)	Cell culture media (animal component-free expression medium)	1	There is a specific cell culture media for the suspension process that is serum-free and animal component-free. This is an approved raw material and listed in the batch record for material recording.
Vessels for Cell Expansion (Inoculum Development, Cell Expansion I, Cell Expansion II & Transfection)	T-225cm ² Flasks / CellSTACKs HYPERStacks	Shake Flasks: WAVE Bioreactor 2 x 250L Thermo SUB	4	Cell expansion is conducted in sterile conditions. The overall process is a closed system process once outside ISO 5/Grade A areas. Daily monitoring during each cell growth phase.
Transient Transfection	Triple Plasmid Transfection	Triple Plasmid Transfection	7	The required plasmid ratios are calculated and recorded in the batch records prior to and at time of cocktail preparation. Step is verified by second operator. Detection during measurement of components. Detection is also during release testing for CQA impact
Transfection Reagent	Calcium Phosphate Ca ₃ (PO ₄) ₂	Polyethyleneimine (PEI) transfection, PEIMAX [®]	4	The required volumes are calculated and recorded in the batch records at time of addition. The step is verified by a second operator. Detection during weigh out of components.
Media Exchange	Post transfection, the medium is changed	Post transfection: Media quench	4	The time dictated by batch record and calculated by an operator and verified by a second.
Total Transfection Incubation Time	Up to 1.5 days	Up to 3 days	4	The time dictated by batch record and calculated by an operator and verified by a second.
Endonuclease Treatment + Media Release	Endonuclease Treatment	No Nuclease Treatment/ NA	4	The suspension process has an affinity chromatography step that deals with DNA and HCP clearance.
Harvest	Media from the HYPERStacks are harvested pooled into a single volume	Bioreactor batch harvest; no centrifugation	4	Target weight calculated. Weight at end of collection is recorded and verified in batch record.
Cell Lysis	Cell lysis is not applicable in the adherent platform	Chemical lysis + flocculation	7	Quantity of reagent is calculated and addition amount recorded in batch record. Detection during release testing for CQA impact. Residual HCP and HCDNA also an IPT.

Appendix B: Cont.

Process Detail	Process (Adherent)	Process (Suspension)	(D)	Justification
Clarification	Capsule pre-filter, followed by a sterilizing grade capsule filter chased with buffer only	Depth filtration followed by a sterilizing grade filter chased with buffer and NaCl	4	Pressure is monitored during the buffer addition rate and pump flowrate may be adjusted. Pump speed recorded in the batch record; Flush volume is specified and recorded in the batch record.
Volume Reduction	Tangential Flow Filtration	No Volume Reduction Post Clarification	4	All clarified material for suspension process is loaded onto the affinity chromatography step.
Chromatography (Capture)	No Capture Chromatography	Affinity Chromatography	7	Fractionated elution and QC testing of eluates for titer. Pooling criteria required to be met; detection during release testing for CQA impact.
Empty / Full Separation: Gradient Ultracentrifugation	2-Step Iodixanol Gradient Process	1-Step Iodixanol Gradient Process	7	Completed by operator under aseptic conditions in Biosafety Cabinet (BSC). Manually controlled per SOP and MBR. Detection during release testing for CQA impact.
Chromatography (Polishing)	Cation Exchange Chromatography	Anion Exchange Chromatography	7	Detection during release testing for impurity impact.
Final Formulation (TFF)	Final Formulation TFF; excipient added post formulation	Final Formulation TFF; the excipient is added to the formulation buffer and used during Final Formulation TFF	4	Buffer is formulated manually and released prior to use. Poloxamer is being tested pre- and post-TFF as a characterization assay to determine loss across TFF.
	Diavolumes: Less	Diavolumes: More	7	Permeate volume is monitored and recorded throughout diafiltration; impurity levels would be detected at release. TFF is ran using a fully automated system.
	Hollow fiber cartridge; smaller	Hollow fiber cartridge; larger	4	PN dictated and recorded in batch record. Operator verifies correct PN during use and verified by second operator.
Dilution of Material to Target Titer (DS)	During TFF (DS)	Post TFF Eluate (Stored for a maximum of 7 days then diluted to DS)	7	TFF eluate processing and Fill finish is scheduled no more than 7 days. Detection during release testing for CQA impact.
Sterile Filtration	Single 0.2 µm Sterile Filtration	Double 0.2 µm Sterile Filtration	1	Flow rates are recorded during filtration.
Fill + Finish	Aseptic Fill in PP Microtube	Aseptic Vial Fill in Crystal Zenith® Vials	1	Weight checks are performed periodically throughout filling.