White Paper

A Multifaceted Strategy for Viral Safety in AAV Processes

Viral safety is essential in the manufacturing of biopharmaceuticals and required to ensure patient safety. Assurance of viral clearance can be difficult, however, during production of vectors such as adeno-associated viruses (AAV) used for the purpose of gene delivery. The need to define and deploy an appropriate and robust viral clearance strategy for a process that inherently aims at purifying a virus for therapeutic purposes can be particularly challenging without an overarching strategy in place.

A manufacturing process designed with viral safety as a goal should include specific robust virus inactivation and removal steps, as well as purification unit operations that provide additional viral reduction. Risk mitigation through the careful choice and extensive testing of source materials will help prevent introduction of adventitious viruses while rigorous testing procedures for raw materials, cell banks, virus seed stocks, bulk harvests and drug substances, and assessment of the manufacturing process for inactivation and removal of viruses ensure another layer of safety throughout the process.

This whitepaper describes an approach to adventitious viral risk mitigation which encompasses this multifaceted approach defined as “prevent, detect, remove”. Best practices and advanced technologies to help assure viral safety are also presented.

The Regulatory Landscape

Consideration of a viral safety program should be based on an understanding of the current regulatory landscape. As industry regulations are nascent and still evolving, risk assessments of the production process should be continuously conducted and strategies for adventitious viral clearance as outlined in section A-2 of the Food and Drug Administration’s (FDA) investigational new drug (IND) application or biologics license application (BLA) should be considered prior to filing. The agency encourages open dialogue with gene therapy companies and offers several avenues for interaction including the Initial Targeted Engagement for Regulatory Advice on CBER producTs (INTERACT) meeting, which is an informal non-binding consultation with the Center for Biologics Evaluation and Research (CBER).[1] An INTERACT meeting enables sponsors to obtain preliminary, informal consultation for innovative investigational products at an early stage of development on issues that are not yet at the pre-IND meeting phase.

Similarly, the CBER Advanced Technology Team (CATT) meeting was established to promote dialogue, education, and input among CBER staff and between CBER and prospective innovators and developers of advanced manufacturing technologies.[2] As part of these efforts, CBER provides an interactive mechanism for engagement with their staff related to implementation of new technologies in the development of CBER-regulated products.

The chemistry, manufacturing and control (CMC) guidance document for human gene therapy recently published by the FDA includes a section describing safety evaluation of adventitious agents[1] which should be reviewed carefully while establishing a strategy. The guidelines include risk assessment for adventitious agent contamination and providing information on viral safety studies.

Principles of Virus Mitigation

A viral contamination can have serious clinical and economic implications. Despite rigorous controls applied to all biopharmaceutical products and source materials, adventitious viruses can be inadvertently introduced into the viral vector production process through source materials or during manufacturing. Source materials can become contaminated from infected animals and use of contaminated cell culture components including bovine serum or porcine-derived trypsin. Adventitious viruses can also be introduced into a manufacturing process through handling of cell culture media and contaminated biologics or non-biologic agents.

In addition to multiple entry points for viral contamination, the size of commonly used viruses for gene delivery must be considered when establishing a risk mitigation strategy. AAVs (~20 nanometers in size) and adenoviruses (60 to 90 nanometers) will most likely pass through a 0.2 micron sterilizing grade filter. In contrast, lentiviruses are ~120 nanometers in size and routinely aggregate, causing substantial loss across these filters. Herpes simplex virus (HSV), Sendai and vaccinia viruses are much larger and won't pass through a 0.2 micron filter.

Common techniques for virus mitigation are classified as either inactivation, achieved by pH, detergent or heat, or removal which relies on techniques such as chromatography and/or filtration. Direct exposure of a process intermediate to pH extremes has been extensively used for viral clearance and can provide effective, robust viral reduction. Detergent treatment is also a proven method to inactivate enveloped viruses through solubilization of the lipid membrane structure which prevents the virus from binding to or infecting the cells, thereby rendering it inactive. This process does not inactivate non-enveloped viruses and therefore does not generally affect the potency of the recombinant protein product that is part of the therapeutic.

Wet and dry heat treatment has been used for many human plasma-derived and animal-derived products. This method typically causes three-dimensional changes in the structure of the viral proteins, rendering the viruses non-functional or inactive. While heat treatment can be effective against both enveloped and non-enveloped viruses, some non-enveloped viruses such as parvoviruses require high temperatures to achieve inactivation, and this may not be compatible with certain processes.

Chromatography can also be used to separate enveloped and non-enveloped viruses from a product. In this case, viral clearance depends on the physical, chemical, and biochemical properties of an individual virus; removal of one type of virus, however, does not necessarily mean all other viruses will be removed. Low pH buffers and those containing components that act as detergents in chromatography can inactivate enveloped viruses. For cases in which enveloped viruses are inactivated, quantitative PCR can be used to assess virus removal.

Many purification processes for biopharmaceuticals use virus reduction filtration as an integral part of the overall strategy for virus clearance. Virus reduction filters such as nanofilters can provide robust and effective removal of large, medium and small viruses, including those less than 20 nanometers in size. Additional techniques for reduction of viruses include gamma radiation and ultraviolet treatment.

A Multifaceted Approach

Regulatory guidelines[3] state the need to demonstrate effectiveness of the control, elimination, and risk mitigation of transmissible spongiform encephalopathy (TSE), bacteria, mycoplasma, fungi, viruses and residuals from the biopharmaceutical manufacturing process **(Figure 1)**.

A robust biosafety approach built upon the pillars of "prevent, detect, remove" encompasses use of high-quality raw materials to prevent introduction of viruses, detection using appropriate testing measures and removal of adventitious agents if accidently introduced. Each of these pillars is described in greater detail below.

Figure 1. A comprehensive viral safety strategy relies on a multifaceted approach.

Prevent Contamination

Raw material risk mitigation helps prevent introduction of adventitious viruses both at the point of origin and point of use. Point of origin risk can be prevented with the purchase of raw materials from a low-risk supplier, use of pretreated products such as high-temperature, short-time (HTST) pre-treated glucose or gamma irradiated serum and awareness of the origin of materials with strategic sourcing. At the point of use, virus barrier technologies can be incorporated along with processes to minimize risk of contamination.

An example of a quality raw material is our Sf-9 rhabdovirus-negative cell line and chemically defined media for AAV production. An additional layer of safety is provided by our Emprove® dossier documentation which addresses current and anticipated regulatory requirements to help accelerate internal qualification procedures and preparation of the drug approval process. The program includes more than 400 raw and starting materials, as well as filters and single-use product families. Each product is supported with dossiers containing comprehensive, up-to-date documentation to help navigate through regulatory challenges, manage risks, and improve manufacturing processes.

Remove Adventitious Viruses

Removal of adventitious viruses relies on the use of several technologies, applied at different stages of the manufacturing process **(Figure 2)**.

Figure 2. Techniques for removal of adventitious viruses are applied across the production process.

The Viresolve® barrier filter is specifically designed to process cell culture media and offers robust retention of viruses, mycoplasma, and bacteria. The filters are easy to implement, provide high flux, capacity and speed, and work well in conjunction with chemically defined media as demonstrated by a throughput study in which log reduction values (LRVs) of 6 and 3 were achieved for mycoplasma and parvovirus, respectively **(Figure 3)**. A titer and growth curve with the barrier filtered media and non-media show overlap between the two conditions which should be expected **(Figure 4)**.

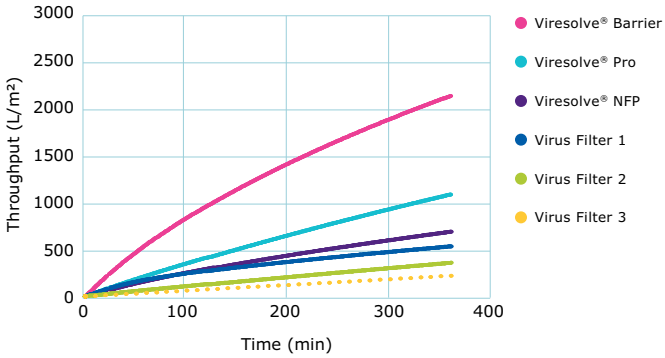


Figure 3. Viresolve® barrier filters offer robust retention of virus, mycoplasma, and bacteria from cell culture media.

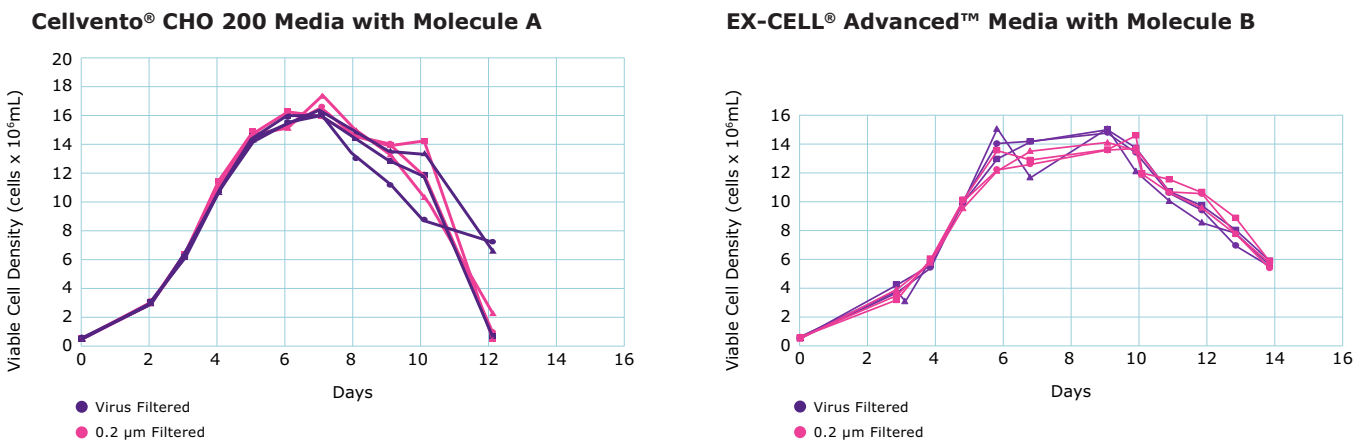


Figure 4. Comparison of titer and growth curve with the barrier filtered and non-filtered media.

Another requirement for viral safety is removal of residual DNA which can be achieved with Benzonase® endonuclease which is highly active against all kinds and forms of nucleic acids including circular, singular and double-stranded. Use of this enzyme has been documented in many clinical trials and commercial products and has long-standing regulatory support in commercial applications. The current regulatory guideline[2] for residual DNA is less than 10 ng DNA/dose, an approximate size of 200 base pairs. One unit of Benzonase® endonuclease is capable of degrading approximately 37 µg of DNA in 30 minutes to a size as small as 3 – 8 base pairs. In addition to nucleic acid degradation, Benzonase® endonuclease also reduces viscosity of process intermediates, prevents fouling of downstream equipment, and minimizes yield loss as a result of virus-nucleic acid complexes.

Two forms of Benzonase® endonuclease are available with similar performance and stability **(Table 1)**. Benzonase® Endonuclease Safety Plus EMPROVE® Expert offers enhanced regulatory compliance, increased process de-risking, and extended quality benefits. The Safety Plus form is completely animal origin free, extending back as far as the upstream raw materials used in fermentation. This form has enhanced lot-release testing for mycoplasma, adventitious viruses, and a drug master file is available. Shipments are accompanied with temperature strips and representative tailgate samples, which offer added convenience in a manufacturing or quality setting.

Specifications	Standard Benzonase® Endonuclease	Benzonase® Endonuclease Safety Plus EMPROVE® Expert
Lot release testing for Mycoplasma	No	**Yes**
Lot release *in-vitro* test for absence of adventitious viruses (3 cell lines) by BioReliance® Services	No	**Yes**
Chemically defined fermentation media to claim non-animal origin	No	**Yes (animal origin-free)**
Microbial testing	< 10 CFU/100,000 U	< 10 CFU/100,000 U
Endotoxins (LAL)	< 0.25 EU/1,000 U	< 0.25 EU/1,000 U
FDA Drug master file (DMF)	Yes	**Yes**
Shipment with temperature strips	No	**Yes**
Tailgate Samples	No	**Yes (with 5M unit size)**
Product Availability	**Both Benzonase® Endonuclease products will stay in portfolio**	

Table 1. Comparison of standard Benzonase® endonuclease and the Safety Plus Emprove® Expert form.

Filtration is also an important strategy for removal of adventitious viruses. Viresolve® normal flow retrovirus (NFR) filters with Retropore® membranes quickly and efficiently remove retroviruses from media and protein feed streams. The pore size is approximately 50 nm, allowing AAV to pass through while retaining larger viruses. High recoveries with more than a six log removal of retroviruses can be achieved **(Table 2)**.

	Average LRV	Fraction 1 27 L/m²	Total Load 114 L/m²	Flush 27 L/m²
XMuLV	≥6.0	≥6.0	≥6.0	≥6.0
PRV	≥5.4	≥5.4	≥5.4	≥5.4

Study Summary

- Capacity > 2e16 vp
- Throughput 115 L/m²
- Recovery > 90%

Data presented at ACS 2019, Orlando FL

Table 2. Log removal values with use of Viresolve® normal flow retrovirus (NFR) filters with Retropore® membranes.

Given that the final volumes of drug substance in gene therapies can be as small as one liter, it is essential to use a sterilizing grade filter with a low hold-up volume. The Millipak® 20 offers a minimum hold up volume and protein binding which is essential to maximize product recovery and cost savings. The unique stacked design minimizes particle shedding while the Durapore® PVDF membranes enable high flow rates and throughputs; various stack sizes are available for optimum flexibility.

In summary, a range of strategies can be applied for removal of different viral contaminants **(Table 3)**. A combination of 0.2 micron sterilizing grade filters and nanofiltration is highly effective. For larger viruses such as HSV, Sendia, and vaccinia, a robust method for prevention of contamination should leveraged in conjunction with 0.45 micron filters.

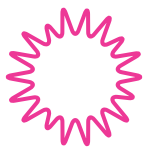
Small to Medium Virus

Adeno-associate virus (AAV)
Adenovirus (AdV)

- Perform normal 0.22µm sterilizing filtration at final filling step

Medium Aggregating Virus

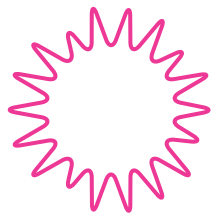
Retrovirus (RV)
Lentivirus (LV)



- Optimize process parameters, in-process buffer and final filling solution to control aggregation
- Perform normal 0.22µm sterilizing filtration at final filling step

Large Virus (> 0.22µm)

Herpes simplex virus (HSV)
Sendai virus
Vaccinia virus



- Use sterilized raw materials to prevent potential contamination
- Implement closed processing
- Adapt appropriate in-process bioburden control method (e.g., 2 x 0.45µm filters)

Table 3. Summary of strategies for removal of adventitious viruses.

Detecting a Possible Contamination

It is imperative that the right testing controls are in place throughout the vector production process to detect the presence of adventitious agents. The main goal is to confirm the identity of the vector, ensure its security and potency, and verify that any process impurities have been removed. For a novel biological product, the approach should be conservative with extensive testing from raw materials and cells to the final product as outlined below and summarized in **Table 4**:

- **Raw Materials:** Manufacturers of traditional biologics such as monoclonal antibodies or recombinant proteins can reduce the potential for viral contamination by eliminating human- or animal-derived materials such as bovine serum or porcine trypsin from their process. For cell therapies, it is very difficult to remove human or animal-derived products from the manufacturing process and it is therefore essential that they be thoroughly tested to ensure the absence of adventitious agents.
- **Cells:** Cell banks used in the manufacture of a biological therapy must be thoroughly characterized according to regulatory expectations and tested to ensure the absence of adventitious agents. This testing may include verification of the cell type, tests for bacteria, fungi, mycoplasma and possibly mycobacteria, broad specificity viral assays, assays for specific viruses and retroviruses.
- **Unpurified Bulks:** Testing of unpurified bulks, which may include transfected producer cells, should include verification of identity and may involve confirmation of the gene of interest. The titer of the vectors should be determined at this stage as well and the absence of replication competent viral vectors should also be confirmed. Tests for microorganisms (e.g., bacteria, fungi, mycoplasma, etc.) are appropriate as are broad specificity assays for adventitious viruses.
- **Process Clearance:** Unlike traditional biopharmaceuticals, enveloped viral vectors and cell therapies cannot claim viral clearance for their manufacturing processes. Given the nature of the product, it is not possible to separate or inactivate potential viral contaminants without adversely impacting the product. Viral inactivation and removal steps can be included in the manufacturing process for non-enveloped viral vectors, and the viral reduction can be claimed for many such processes.
- **Purified Bulks:** Purified bulks should include a verification of identity, such as confirmation of the gene of interest and confirmation of AAV serotype. Quantitation of infectious titer, genome titer and potency is appropriate at this stage as well. The absence of microbial contamination and replication competent vector should be confirmed. Verification of the absence of residual product and process impurities should also be done.
- **Final Product:** The identity of the final vector product should be confirmed, and this testing may include the sequence of the gene of interest, infectivity titer, genomic titer and potency. Sterility and absence of endotoxin should be determined. In addition, characteristics of the final product should be determined. This may include vector aggregation, pH, osmolality, appearance and particulates.

AAV	Lenti/Retrovirus	Adenovirus
Identity • Genomic Region of Interest • AAV Serotype • Vector Sequence	**Identity** • Genomic Region of Interest • Vector Sequence	**Identity** • Genomic Region of Interest • Vector Sequence • Restriction Endonuclease Analysis for ID of Purified Adenovirus Vector
Titer • Infectious Titer • Genomic Titer	**Titer** • Infectious Titer • Genomic Titer	**Titer** • Infectious Titer • Genomic Titer
Potency • r-AAV Expressed Protein	**Potency** • r-LV/RV Expressed Protein	**Potency** • r-Adeno Expressed Protein
Purity • Sterility (**Rapid methods available**) • Mycoplasma/Spiroplasma (**Rapid methods available**) • Endotoxin • *In Vitro* Adventitious Viruses • *In Vivo* Adventitious Viruses • Replication Competent AAV	**Purity** • Sterility (**Rapid methods available**) • Mycoplasma/Spiroplasma (**Rapid methods available**) • Endotoxin • *In Vitro* Adventitious Viruses • *In Vivo* Adventitious Viruses • Replication Competent LV	**Purity** • Sterility (**Rapid methods available**) • Mycoplasma/Spiroplasma (**Rapid methods available**) • Endotoxin • *In Vitro* Adventitious Viruses • *In Vivo* Adventitious Viruses • Replication Competent Adenovirus
Process/Product Residuals • Helper viruses or transfected plasmids • Host Cell DNA • Host Cell Protein • DNA Size Distribution • Empty/Full Capsid • Residual AAV Ligand • Residual Benzonase® nuclease • Residual BSA	**Process/Product Residuals** • Transfected plasmids • Host Cell DNA • Host Cell Protein • DNA Size Distribution • Residual Benzonase® nuclease • Residual BSA	**Process/Product Residuals** • Transfected plasmids • Detection of AAV • Host Cell DNA • Host Cell Protein • DNA Size Distribution • Residual Benzonase® nuclease • Residual BSA
Final Product Characterization • Vector Aggregates • Osmolality • pH • Appearance • Particulates	**Final Product Characterization** • Vector Aggregates • Quantitation of LV Particles • Osmolality • pH • Appearance • Particulates	**Final Product Characterization** • Vector Aggregates • Osmolality • pH • Appearance • Particulates

Table 4. Recommended testing options for different viral vectors.

In addition to the process steps described above, biosafety testing of cell banks can be performed for both master and working cell banks using a variety of assays. While the tests are typically *in vitro*, a number of *in vivo* tests such as tumorigenicity in mice and other infectivity assays can also be considered. There are several drawbacks to these tests, however, including the time to result which can be four to six weeks as viruses in raw materials may need to adapt to growth in cell culture. In addition, components of raw materials (e.g., antibodies, antibody complexes) may inhibit detection of viruses and the components of the test material may be cytotoxic to detector cells used for infectivity assays. Different assay formats may also be needed for virus detection and quantitation of virus contaminants and there remains the challenge of how to detect 'unknown' viruses.

An ongoing development is the move towards new technologies such as next generation sequencing (NGS) which aims to reduce the need for *in vivo* detection assays. While these tools are gaining more visibility and support from regulatory agencies, questions remain related to the validity of the system, contamination effects, and whether it is a true representation of the *in vivo* results.

Conclusion

Developing a viral safety strategy can be facilitated with use of a multifaceted approach defined by “prevent, detect, remove” **(Figure 5)**.

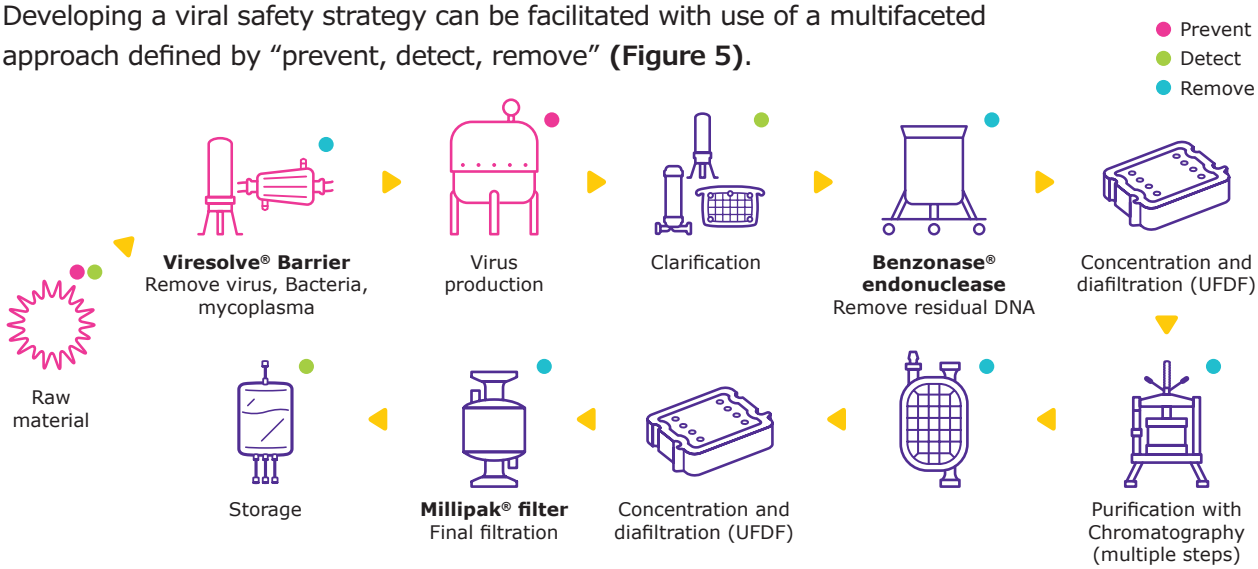


Figure 5. Application of “prevent, detect, remove” strategy for viral safety as applied to the overall vector production workflow.

Using this approach, we evaluated the log removal of bean-associated cytorhabdovirus (BACV) and vesicular stomatitis (VSV) from an AAV production process. Robust log reduction values were observed for all viruses **(Table 5)**. Several factors contributed to the success of this strategy:

- Low pH can achieve inactivation of enveloped viruses, but reduction is dependent on the pH and the susceptibility of individual viruses
- Detergent can achieve effective inactivation of enveloped viruses; in this study we used the eco-friendly detergent Deviron™ C16 detergent (N,N-dimethyltetradecylamine N-oxide) which can be used in place of TRITON™ X-100
- Reduction by chromatography is dependent on the resin, the conditions under which the chromatography step is run, and the virus itself
- Nanofiltration provides another useful assurance to viral vector processes.

Log_{10} Virus Reduction of BACV	Process Step	Log_{10} Virus Reduction of VSV
3.48	**Low pH Inactivation**	1.16
≥5.15	**Detergent Inactivation**	≥4.41
≥6.70	**Chromatography 1**	≥6.08
2.67	**Chromatography 2**	3.93
1.82	**Chromatography 3**	3.84
≥4.41	**Nanofiltration**	≥4.83
24.23		**24.25**

19.82 **19.42**

Table 5. Evidence of viral load reduction in an AAV production process.

With the approval of several AAV-based gene therapies, more than 400 programs in clinical development and evolving regulatory expectations, robust processes for viral clearance will support the ongoing expansion of the field and success of these advanced modalities. While achieving zero risk of viral contamination in the production of vectors for gene and cell therapies is impossible, application of a multifaceted contamination risk mitigation strategy will help ensure viral safety and should encompass:

- Selecting and extensive testing of source materials to prevent entry of contaminants into the process
- Implementing sensitive testing for contaminants in raw materials and process intermediates
- Incorporating virus removal and inactivation steps into downstream purification processes

This “prevent, detect, remove” strategy is foundational for establishing a robust viral safety program, which in turn, leads to improved assurance for patients and increased confidence in a rapidly growing and evolving therapeutic sector.

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