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NGS-Based Viral Safety Testing for ATMPs: Mandatory or Just an Option?

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Abstract

The heterogenous group of advanced therapy medicinal products (ATMPs) are biologics with frequently limited viral safety profiles. As compared to well-established biologics such as monoclonal antibody products, the risk of virus contamination is significantly higher for some ATMPs. The standard approaches and tools used to mitigate the viral risk have limitations, leaving open the chances of missing virus contamination in an ATMP manufacturing process in both upstream and downstream. Next-generation sequencing (NGS) technology can overcome the residual risk by having the potential to detect any kind of virus contamination based on its inherent capability to detect any kind of nucleic acid in a sample. It perfectly combines the benefits and compensates for the downsides of the existing testing tools. It will replace a bunch of different established testing methods at improved turnaround times and, in the end, reduced overall costs. The combination of these characteristics is making NGS-based virus testing an in-demand and preferred approach to mitigating the virus contamination risk across all kinds of biologics mid- and long-term.

Introduction

Advanced therapy medicinal products (ATMPs) describe a heterogenous group of biologics (animal or human-derived biopharmaceuticals). There are typically three categories:

- Gene therapy medicinal products (GTMP)
- Somatic cell therapy medicinal products (sCTMP)
- Tissue-engineered products (TEP)

They are frequently derived from living organisms like viruses and cells, and/or deliver and alter genetic material through different kinds of shuttle systems. Examples include viral vectors, oncolytic viruses, processed and genetically modified cells derived from human tissues, and also naked DNA and different types of RNA delivered directly or through different kinds of biological vesicles.

Oncolytic viruses, viral vectors, and virus-like particles are typically produced on continuous mammalian cell lines like HEK 293 or Vero cells similar to non-ATMP recombinant products, which are frequently produced on Chinese hamster ovary (CHO) cell lines. Cellular products are derived from primary human tissues like chondrocytes, keratinocytes, stem cells, or cells from the hematopoietic system.

The Risks of ATMPs

What all ATMPs have in common are the starting materials, the product itself, and animal/human-derived critical raw materials that can potentially be contaminated with viral contaminants and/or are highly susceptible to virus contamination. Many of these materials are of human origin, and viral contaminations have significant relevance for humans treated with ATMPs. This is different from biologics produced in cells from different species. For instance, most therapeutic recombinants and monoclonal antibodies (mAbs) are produced on a rodent cell line like CHO cells. These cells are also susceptible to virus contamination, but not many of them can typically infect humans. Most cell therapy products (genetically unmodified and modified) are isolated from human donors, and human cells are frequently used for ATMP production. They contain endogenous viruses which are either fragmented (human endogenous retroviruses) or in a latency stage like some herpesviruses.[1,2] These viruses can be activated or recombined during the manufacturing process or in the patient after administration.[3] Raw materials of different biological origins are frequently involved in the manufacturing process of ATMPs. Recombinant cytokines and growth factors are examples of materials with a well-controlled risk. But much less risk-controlled materials like animal/human serum, platelet extracts, feeder cells or extracts of feeder cells—and even viruses (viral vectors or helper viruses) are also required in the manufacturing process. Finally, manufacturing processes based on human cells or cell therapy products still require a lot of manual handling

from material collection to patient administration. The high susceptibility of these cells to human viruses increases the contamination risk through the process environment or human operators. Cell substrates can be a perfect amplification machine if susceptible to a virus. Low viral particle numbers can be sufficient to initiate active virus infection and replication. The amplification in an *in vitro* or *in vivo* system can be much more effective than any PCR system.

Overall, the virus contamination risks for a broad range of ATMPs is significantly higher versus conventional biologics like recombinants and mAbs.

The Risk Mitigation Principles

The ICH Q5A guidelines issued in 1997 and comprehensively revised very recently[4] provide a risk mitigation strategy to minimize viral contamination. This strategy is frequently called the "three-pillar concept" but can be expanded further (see **Figure 1**).

The guidelines outline the importance of careful sourcing of materials with low or well-controlled viral risk, applying a comprehensive testing program adapted to the overall risk and, if possible, analyzing and demonstrating the capacity of the manufacturing process to clear potential viral contaminants through inactivation or removal. Hygiene measures, a fourth pillar, are equally important to avoid the introduction of viruses through the environment or operators during manufacturing. This is even more important when working with human cells, as outlined above. Finally, the base of all these principles is "knowledge." The combination of manufacturing, test methodology, and virology expertise/experience ensures the proper and correctly balanced application of all four principles. These principles should not only be applied to the manufacturing process of the final product but also to the critical raw materials of biological origin frequently required in the production of ATMPs.

The Risk Mitigation Principles for ATMPs

The different pillar models in **Figure 1** illustrate the virus risk mitigation potential for different types of products.

CHO-derived recombinants do have a low risk profile as all principles can be applied effectively based on the industry's experiences collected for more than 25 years. Taking this as a standard, one can well illustrate the reduced risk mitigation options for different types of ATMPs. Importantly, the risk mitigation through viral clearance is generally much lower for many ATMPs. While some virus removal or inactivation can still be demonstrated for adeno-associated viral (AAV) vectors, this capacity does not exist for other ATMPs like retroviral vectors, oncolytic viruses, and any kind of cell therapy products. This has many disadvantages as viral clearance is the most effective virus risk mitigation tool. Virus inactivation and removal is based on the biophysical features

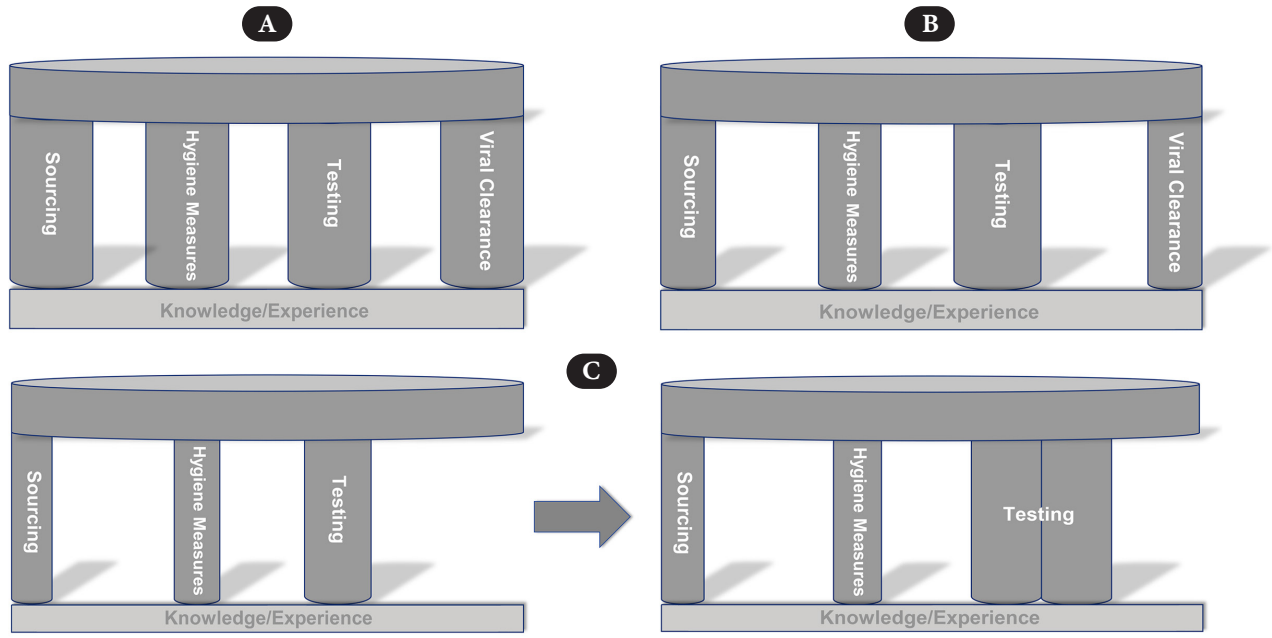

FIGURE 1. Safety pillar concept for different product types. The size of the pillars and the bottom platform illustrate the strength of the mitigation tool. A thick pillar/bottom stands for high strength and a thinner pillar for a reduced strength. **(A)** Highly mitigated risk. Recombinants like mAbs derived from well-established cell lines such as CHO, NS0, SP2/0; **(B)** Medium mitigated risk. Viral vectors like AAV produced on HEK293, PER.C6, Sf9; and **(C)** Significantly reduced mitigation capabilities. Viral vectors like retro and adeno, allogeneic cell therapy products derived from human donor cells (peripheral blood mononuclear cells [PBMCs], stem cells, induced pluripotent stem cells [iPSCs]), and oncolytic viruses. The extended testing requested for the products illustrated here are intended to compensate for the reduced or missed risk mitigation options.

of viruses and less on the virus biology. Many biophysical characteristics like lipid enveloped or non-enveloped, the size of the viruses, and the genome type of the viruses are common across a broad range of known and even unknown viruses of much different biologies. This makes viral clearance a robust and broad virus mitigation tool.

The sourcing of low-risk starting materials (or raw materials) has some limitations either because of the potential high human virus susceptibility of production cells (HEK 293 and Vero), the known and unavoidable presence of endogenous viruses in human donor cells (leukocytes, stem cells, iPSCs), and the animal (or even human) source of raw materials like serum, viruses, or feeder cells. Some ATMPs like viral vectors used *in vivo* (AAV, adeno) or as critical raw material *ex vivo* (retro/lentiviral vectors) present an inherent risk of the development of replication-competent viruses during the manufacturing process—although this risk has been significantly reduced through vector engineering over the last couple of years.

Hygiene measures for the production of biologics are typically well-established, but many ATMPs still require a lot of manual processing, which bears a higher risk of virus contamination through the operator or the environment. These factors weaken the strength of the "sourcing" and the "hygiene measures" pillars in the viral risk mitigation strategy of many ATMPs. Finally, the knowledge and experience in the manufacture of ATMPs, with respect to virus safety, is much more limited compared to the accumulated knowledge/experience of therapeutics like mAbs over the last 25 years.

No viral clearance capability, restriction in sourcing of safe material, reduced hygiene measures, and reduced experiences stress the importance of comprehensive safety characterization of the starting material and critical raw materials through both routine in-process testing, and testing on the final product. It demands the application of different testing methodologies and most advanced technologies to detect a broad range of viruses including unknown viruses.

Testing Tools

There are well-established virus testing methods summarized in **Table 1**. They can be subdivided into general

TABLE 1. Qualitative strength and weakness evaluations of the established virus safety testing methods with respect to the subjects of testing. Dark green stands for high strength going down gradually to high weakness (light green to light orange and dark orange).

Method			Specificity	Breadth of Detection	Detection of Unkown Viruses	Virus Identification	Discriminate Infectious Viruses	Sensitivity
General Screening Methods	TEM	Microscopy	All Viruses	High	Yes	Limited	No	low
	In vivo	Animal-Based (mouse, egg, guinea pig)	Unkown	Limited	Yes	No	Yes	Unkown
	In vitro	Cell-Based (3-4 indicator cells)	Unkown	Limited	Yes	No	Yes	Unkown
Retrovirus Screening	Retro Cell	Cell-Based (S+L-/XC-plaque)	Retroviruses	Restricted	Limited	No	Yes	Unkown
	Retro RT (PERT)	PCR (reverse transcriptase activity)	Retroviruses	Restricted	Limited	No	No	High
Specific Viruses	Antibody Production Test	Animal-Based (MAP/HAP/RAP)	Determined	Restricted	No	Yes	Yes	Unkown
	Replication Competent Viruses	Cell-Based Assay/PCR	Determined	Restricted	No	Yes	Yes	High
	9 CFR §113	Cell-Based Assay	Bovine/Porcine Viruses	Restricted	No	Yes	Yes	High
	Specific Viruses	PCR (single/multiplex)	Determined	Restricted	No	Yes	No	High

(TEM) transmission electron microscopy; (PERT) product-enhanced reverse transcriptase; (PCR) polymerase chain reaction; and (MAP/HAP/RAP) mouse/hamster/rat antibody production

screening assays (*in vivo, in vitro*, TEM), assay testing for a specific group of viruses like retroviruses, and more specific tests targeting predefined viruses. The "9 CFR §113" test targets specific porcine and bovine viruses. The viruses that are supposed to be detected by the antibody production test MAP/HAP/RAP are outlined in the ICH Q5A guideline.[4] PCR-based methods target specific viruses either individually or with a group of predefined viruses by multiplex approaches. The methodologies comprise particle screening (TEM), animal assays (*in vivo*, MAP/HAP/RAP), cell culture-based assays (*in vivo*, retroviruses, 9 CFR §113, replication-competent virus testing) with different readout methods (phenotype changes, adsorption of erythrocytes, immunostaining, PCR), and molecular biology methods (PERT). The table defines the pros and cons of all different assays with respect to the subject of testing:

- Specificity
- Breadth of virus detection
- Capability to detect unknown viruses
- Virus identification
- Differentiating infectious from non-infectious viruses
- Sensitivity

For instance, TEM can detect all viruses, in principle, but the sensitivity is very low. PCR approaches do have a high sensitivity typically but require upfront information on the targeted viruses. There is a risk that variants of the targeted viruses are missed. The unspecific general *in vivo* and *in vitro* virus screening assays have the potential to detect even unknown viruses, but the specificity and the sensitivity are not known. Both assays cannot be validated following ICH Q2 principles.

For starting materials like production cell lines and some critical raw materials, a combination of these assays is requested[4] for virus safety characterization while single assays are selected for in-process—or release testing, depending on determined risk. In fact, the combination of different assays compensates for some of the cons of the individual assays.

All assays open windows to detect known or unknown viruses, but even their combination can miss certain contaminants for different reasons:

- The virus will not grow in the animal, on the indicator cell lines, or cannot be detected by the readout method applied (*in vivo*, *in vitro*)*
- The sensitivity is too low (TEM)
- The virus-specific assays might miss variants (MAP/HAP/RAP, PCR, 9 CFR §113)

In fact, even though the tools have been applied successfully in the past 25 years, there were cases where all of the methods failed to detect a viral contaminant. The insect production cell line Sf9 was thought to be virus-free until a contamination was detected using a more sensitive technology—next-generation sequencing (NGS).[5] More seriously, a viral contamination was detected by the same advanced technology in a commercial human vaccine.[6] Fortunately, the porcine viral contaminant (porcine circovirus) did not infect humans and did not cause any diseases.

Next-Generation Sequencing

These findings put much attention on the NGS technology. It was not a new technology at that time, but it was well-recognized as an advanced technology for viral safety testing of biologics in general. Without going into too much details, the essential characteristics of the technology are:

- It targets any kind of nucleic acid (RNA/DNA) in a sample.
- It sequences millions of fragments simultaneously in one run.
- Next to detection, it sequences the nucleic acids and provides information about the kind of nucleic acid.
- It's a hypothesis-free approach that does not require prior knowledge of sequence information like PCR-based methods (agnostic approach).
- The technology was optimized from first to third or fourth generation.
- Different technologies with different features are available, and the appropriateness depends on the target and the application.

There are many review articles describing the technology in different details. A great overview is provided in a newly drafted Ph. Eur. general chapter 2.6.41 currently made available for comments.[9] There are additional good review articles and technology providers that also provide reviews either as publications, white papers, or videos (see relevant web pages of technology providers).

There are multiple publications demonstrating the power of the technology for virus safety testing with respect to the:

- High breadth of detection in agnostic approaches
- Sensitivity being determined to be comparable to PCR
- Capability to differentiate infectious from non-infectious viruses through transcriptome-based approaches
- Capability to detect different variants and even unknown viruses[10–14]

*One also needs to consider a special characteristic of viral contaminants in products derived from primary human tissue (allogeneic chimeric antigen receptor [CAR] T, iPSCs). Frequently, primary viral isolates from human tissue do not grow well in continuous cell lines and might not be detected in the cell-based *in vitro* assay, which uses immortalized, continuous cell lines.[7,8]

TABLE 2. Qualitative strength and weakness evaluation of NGS for virus safety testing with respect to the testing subjects (in comparison to **Table 1**). Dark green stands for high strength going down gradually to low weakness (light green).

Method			Specificity	Breadth of Detection	Detection of Unkown Viruses	Virus Identification	Discriminate Infectious Viruses	Sensitivity
General Screening	NGS	Sequencing	All viruses	High	Yes	Yes	Yes	High

Overall, the technology overcomes the cons of the traditional assays and has the potential not only to supplement the conventional methods but also replace those methods (see **Table 2**). The two contamination cases reported[5,6] demonstrated clearly the enhanced capability for virus detection when established methods fail.

These finding[5,6] have triggered activities by regulatory bodies and the industry. A general chapter in the Ph. Eur. EP 5.2.14 recommends the use of NGS for replacing *in vivo* methods.[15] Other EP chapters for vaccines, like EP 5.2.3 and EP 2.6.16, indicate the technology for alternative testing strategies[16,17], and both chapters are referenced in the EP chapter 5.14 related to ATMPs.[18] Most recently, EDQM published a draft general chapter 2.6.41 for comments, which describes the technology with respect to virus testing in much detail.[9] Importantly, the meaning of NGS, for virus safety testing, is thoroughly stressed in the recently released revision two of the most important guidelines for virus safety testing, the ICH Q5A(R2).[4] NGS is mentioned more than 40 times and contains a full chapter on NGS. The technology is recommended to replace the animal-based assays specifically. This is of high importance considering the different governmental initiatives in the EU and US to reduce, refine, and replace animal-based testing (the 3R principles) methods whenever possible.[19,20] The ICH Q5A guidelines also offer the option to supplement or even replace other assays like the *in vitro* cell-based assays and virus specific-assays like the MAP/HAP/RAP or PCR-based assays explicitly. The industry is supporting and pushing NGS for virus safety testing through different initiatives like the Advanced Virus Detection Technologies Interest Group (AVDTIG) together with members of the regulatory bodies[21], or the Consortium on Adventitious Agent Contamination in Biomanufacturing (CAACB).[22] These regulatory, academic, and industry activities are complemented by bi-yearly conferences on NGS for adventitious virus detection where all stakeholders meet and discuss further progress.[23]

The challenges coming with this technology should not be obscured—it's a complex technology.[9] Next to the wet chemistry, including sample and library preparation and sequencing, huge data packages must be processed, and evaluation requires deep bioinformatics knowledge combined with virology expertise. This is a major reason why many companies still hesitate to use NGS for supplementing and/or replacing traditional testing. It goes along with limited experiences by regulatory bodies to accept such data. Qualification of equipment and validation of methods require huge efforts and new approaches. How to qualify databases, how to justify data processing (filtering and/or removal of data), and how to deal with false positives are just a few challenges. It is strongly advised to place the bioinformatics portion into the hands of partners who have gained immeasurable experience by executing many projects, being involved in regulatory body meetings and discussions, and offering combined expertise in bioinformatics, virology, and quality assurance.

Despite all the challenges, considerable progress has been made in the past four to five years. The AVDTIG has published a number of papers and is running different projects to address many of the challenges, including the preparation of viral reference materials to validate NGS approaches.[14,24–27] The new general chapter draft EP 2.6.41 addresses critical aspects of the technology and provides directions to overcome the challenges (*e.g.*, by recommending validation strategies).[9] In addition, a number of organizations have successfully used the technology in IND and BLA submissions, and the number of such submissions is increasing.[23]

Risk Mitigation Strategy

As outlined earlier, testing is an important virus risk mitigation tool for ATMPs due to the higher risk of starting and/or raw materials in general, and no viral clearance capabilities in particular.

Testing can be applied at two different stages:

1. On starting materials like cell banks or donor cells and on raw materials before usage in the manufacturing process (offline)
2. During manufacturing either on process intermediates or on the drug substance/drug products (online)

The requirements for these two testing approaches are significantly different. The characteristics are outlined in **Table 3** (on the following page).

Starting materials and critical raw materials can be comprehensively analyzed offline without delaying the manufacturing process. The more intense viral safety testing

TABLE 3. A description of testing characteristics for critical materials like starting and raw materials (offline) versus testing during manufacturing and final release testing (online).

Testing Characteristics	Offline	Online
Turnaround Time (TAT)	Less critical	Highly critical
Complexity	Can be high	Should be low
Data/Program	Comprehensive	Simple
Costs	Can be high	Should be low
Material/Volume	Less critical	Minimal

is applied on these critical materials, the safer the routine manufacturing. This justifies a more comprehensive testing program using more complex technologies like NGS despite the fact that the cost might be higher, the data evaluation might take longer, more material might be needed and, most importantly, the completion might take longer (TAT). However, this testing program is applied offline on materials typically used for the manufacture of multiple batches like master cell banks or serum batches used in media. This won't delay the manufacturing process itself.

The online testing during manufacturing, or at the final product release, needs to meet other characteristics. The TAT should be as short as possible, the sample needed should be small (not wasting too much product), and the method as simple as possible to create clear data—ideally, a simple yes or no answer with no need for interpretation. In addition, the costs should be low as it will be part of routine testing and contribute to overall manufacturing costs.

In fact, the more comprehensive the safety analytics in preparation of manufacturing, the less testing or more simplified testing can be applied during manufacturing and release testing. This can significantly reduce the manufacturing TAT, the costs, and increase the reliability of safety. The online testing program would just address residual viral risk identified in the risk assessment based on the results of the comprehensive testing of starting materials, and critical raw materials, among others. The logic should be applied in an overall testing program, although it might not always be applicable for all kinds of ATMPs.

So far, NGS-based virus safety testing solutions typically fall into the offline category. Costs are still high (in a GMP setting), TATs are better than any *in vivo* testing but still subject of improvement, the technology is complex and results might require interpretation or even follow-up testing either to invalidate false positive or to verify real positive findings. However, the extended safety determined through NGS makes it the right tool for deep safety characterization of critical starting and raw materials. The exceeding capability of NGS-based testing, as compared to the conventional testing programs, opens the option to replace some, if not all, standard testing methods. In return, this will reduce overall costs and TAT significantly for the offline testing programs. This benefit can also be specifically important for ATMPs where comprehensive virus safety testing is still needed on the final product, for example, allogeneic cell therapy products produced from primary human resources like PBMCs, stem cells, and iPSCs.

Fortunately, NGS technologies are developing further and can make comprehensive testing of critical materials even more efficient and can finally meet online testing requirements. Amplicon-based sequencing or capture-based targeted NGS solutions reduce the amount of data processing and evaluation dramatically. Results can be made available much quicker. Oxford Nanopore (Oxford, United Kingdom) or PacBio (Menlo Park, California USA) technologies use direct sequencing without prior amplification and offer long-read testing. This also can dramatically improve the TAT and reduce the complexity of data analysis.

Conclusion

Overall, different NGS solutions adapted to specific needs will make it a comprehensively applicable safety testing technology with the potential to replace most, if not all, conventional testing methods for both offline and online testing requirements. It will significantly contribute to a higher virus safety profile for ATMPs despite their generally higher risk for virus contaminations.

Importantly, the technology can be expanded to analyze other pathogens like mycoplasma. This can be achieved in one experimental approach by just adding additional data banks specific for the evaluated pathogens in the data processing pipeline. Finally, in certain cases, the sequencing data of a virus screening test can also be used to address other quality attributes. For instance, agnostic sequencing data derived from a bulk harvest virus testing of a viral vector product will include sufficient product data suitable to determine the vector genome sequences and control the product identity and purity, in parallel.

In summary, NGS technology will dominate pathogen safety testing programs in general for the production of biologics and ATMPs specifically mid- to long-term. The enhanced virus detection capability and the benefits, compared to established testing methodologies, will make NGS the widely preferred, and by regulatory bodies, the mandatory pathogen testing solution. Further developments, including more automation in data processing, will reduce costs and TAT so significantly that NGS becomes more the standard than an option.

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