

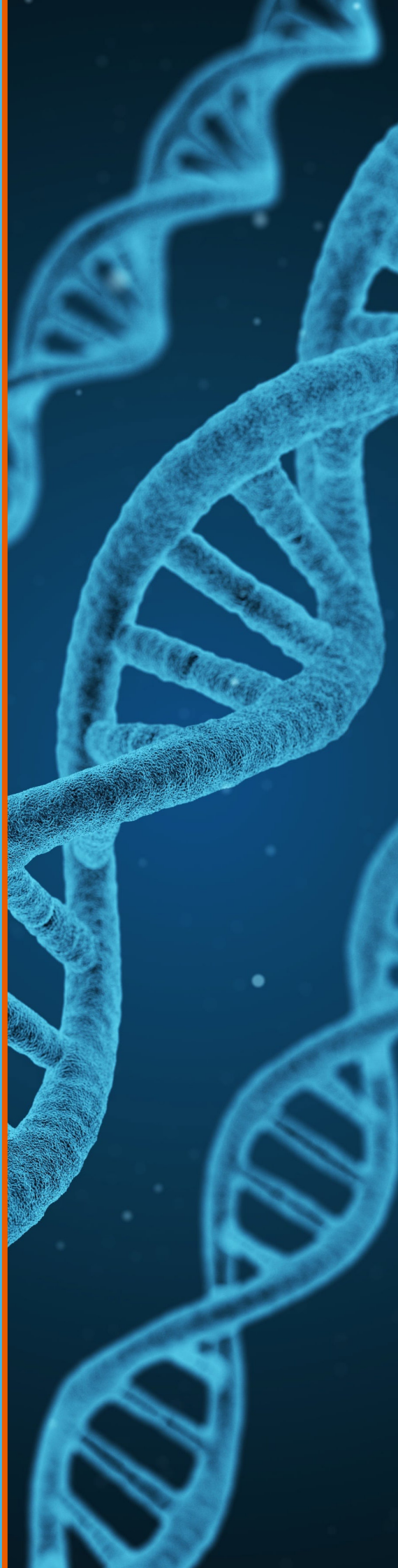
Long-Read NGS as a Highly Sensitive and Specific Advanced Platform for GMP-Compliant Adventitious Agent Detection

VirusSure GmbH
Hans-Peter Fasching, Natascha Hodosi



VIRUSURE

Quality is no coincidence



Executive Summary

Adventitious agent testing (AAT) is undergoing a significant regulatory and technological transformation. Recent updates to ICH Q5A(R2) and European Pharmacopoeia Chapter 2.6.41 recognize next-generation sequencing (NGS) as a valuable tool to complement, and in certain cases replace, conventional testing approaches. This shift enables biopharmaceutical developers and manufacturers to move beyond fragmented testing strategies toward more comprehensive, risk-based biosafety assessments.

As product modalities, manufacturing platforms, and regulatory expectations continue to evolve, organizations require testing solutions capable of detecting a broad range of known and unknown contaminants while maintaining efficiency, scientific rigor, and regulatory compliance. NGS addresses this need through agnostic detection of viral nucleic acids within a single analytical workflow, reducing reliance on multiple orthogonal assays and providing deeper biological insight throughout biosafety programs.

At the vanguard of this transition, ViruSure has developed the first GMP-compliant long-read NGS platform for adventitious agent detection, leveraging Oxford Nanopore long-read sequencing technology to combine unbiased detection with advanced viral characterization. Unlike conventional short-read approaches, long-read sequencing generates extended viral sequence information that enables more confident taxonomic classification, reduced ambiguity in database searches, simplified bioinformatics, and improved characterization of detected viral signals. As a result, the platform supports not only sensitive detection of adventitious agents, but also more informed investigation and risk assessment of positive findings.

Validation studies using the WHO International Reference Panel for Adventitious Virus Detection, together with representative cell bank and virus seed matrices, demonstrated the platform's sensitivity, specificity, robustness, and suitability for routine GMP-compliant testing through the reliable detection of diverse DNA and RNA viruses across biologically relevant matrices.

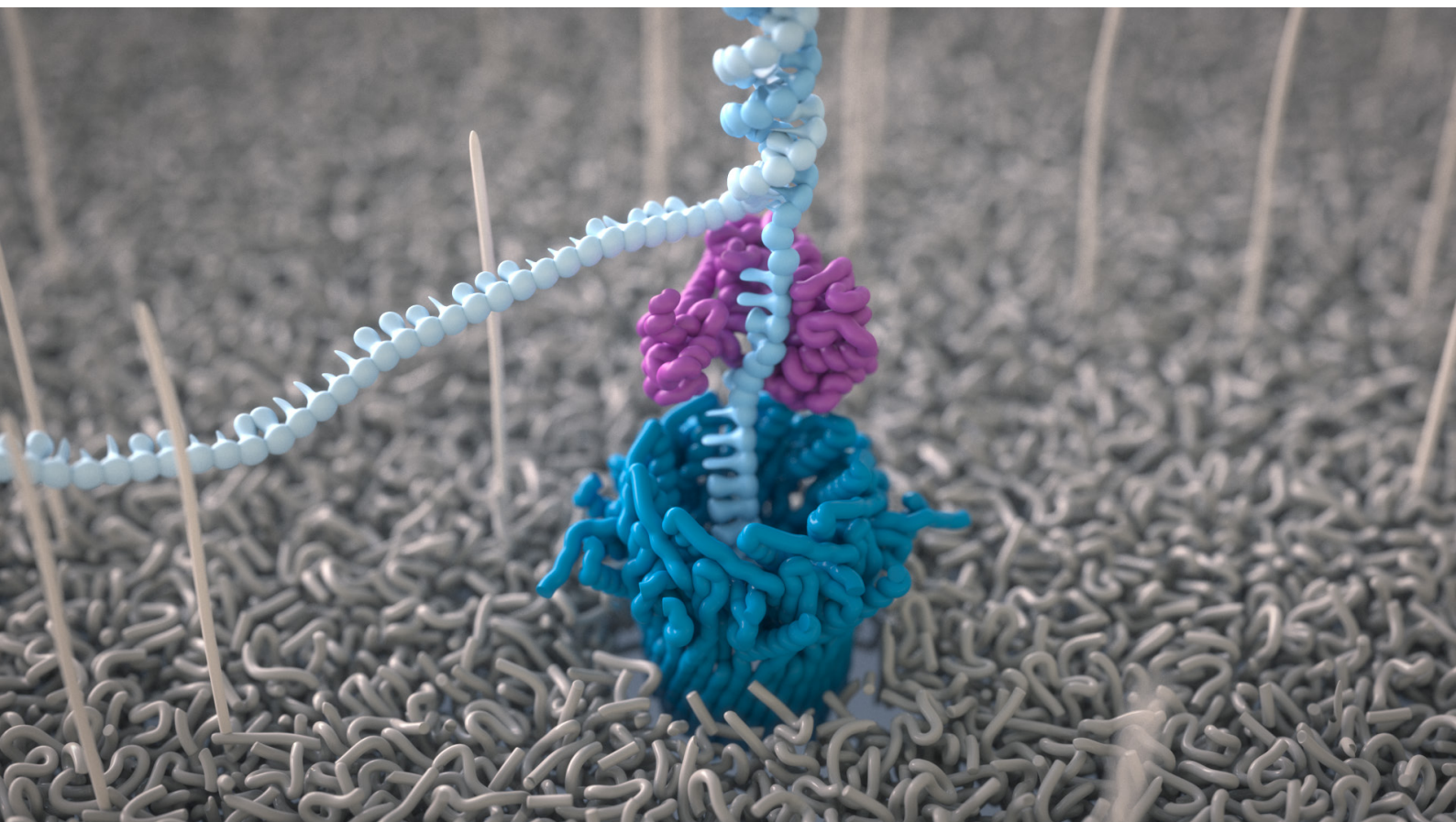
This white paper presents the scientific rationale, technological design, validation strategy, and performance of ViruSure's long-read NGS platform. More importantly, it demonstrates how long-read NGS can move beyond proof-of-concept applications and be successfully implemented as a practical, GMP-compliant solution for routine adventitious agent testing, helping biopharmaceutical organizations navigate an evolving biosafety landscape.

Background

Ensuring the absence of adventitious viruses is a fundamental requirement in the manufacturing of biological products. Cell substrates, raw materials of biological origin and complex production processes can introduce unintended viral contaminants at various stages of development and manufacturing. Such contaminants may originate from cell banks, culture media, animal-derived reagents or cross-contamination between production lines. Because many viruses can replicate silently, remain noncytopathic or persist at low levels without producing visible effects in cell culture, their presence often cannot be excluded through routine process monitoring or testing alone.

Undetected viral contaminants pose significant safety and regulatory risks. They can compromise product quality, impact patient safety and lead to costly batch failures, process shutdowns or product recalls. Historical contamination events across the biopharmaceutical industry underscore the importance of thorough viral safety strategies and have shaped current regulatory expectations^{[4][5][6][7][8]}. As a result, adventitious agent testing (AAT) has become a central component of viral safety programs, complementing risk assessments, raw material controls and upstream and downstream process clearance studies.

AAT serves two primary purposes: verification of the viral safety of production cell lines and raw materials and release testing of intermediate and final products. To fulfil this role, testing strategies must be sensitive, specific, broad in scope and capable of detecting both known and unknown viruses. Traditionally, this has been achieved through a combination of in vivo assays, cell-based in vitro tests and targeted molecular methods. However, the increasing complexity of modern biologics and the limitations of classical assays have driven the need for broader, more sensitive detection technologies—setting the stage for next-generation sequencing (NGS).



Images courtesy of Oxford Nanopore Technologies Plc.

Why NGS?

Recent regulatory developments have accelerated interest in NGS as a viable approach for adventitious agent testing. To understand why, it is important to examine the limitations of conventional methods and the unique advantages that sequencing-based technologies can provide.

PARAMETER	<i>In vivo</i> AAT	MAP/HAP	<i>In vitro</i> AAT	9 CFR	Retroviruses (PG-4)	TEM	RT activity	qPCR	NGS
Detection of unknown viruses	✓	✗	✓	✗	✓	✓	✓	✗	✓
Detection of replicating viruses	✓	✓	✓	✓	✓	✗	✗	✗	✓ / -
Identification of viruses	✗	✓	✗	✓	✗	✗	✗	✓	✓
Detection of non-cytopathic viruses?	✗	✗	✗	✗ / ✓	✗	✗	✗	✗	✓
Specificity of Detection	UNSPECIFIC	SPECIFIC	UNSPECIFIC	SPECIFIC	UNSPECIFIC	UNSPECIFIC	UNSPECIFIC	SPECIFIC	UNSPECIFIC
Sensitivity	+	+	+++ / ???	+	+	-	+++	+++	+++

Table 1 Summary of strengths and weaknesses of the legacy testing panel. Comparison with the benefits of NGS

Update of regulations – ICH Q5A, EP 2.6.41

The publication of Revision 2 of ICH Q5A (2023)^[1] marked a turning point for the use of next-generation sequencing (NGS) in adventitious agent detection. NGS is not only formally acknowledged in the guideline; its use is actively encouraged, both as a replacement for *in vivo* testing and as a replacement or supplement to *in vitro* AAT. This applies particularly in cases where the nature of the sample limits the sensitivity of legacy assays. A prominent example is viral vaccines either lacking neutralising antibodies or where antibody neutralisation is ineffective and predilution steps are required and sensitivity is consequently reduced.

Since the release of ICH Q5A(R2)^[1], NGS has rapidly gained acceptance, and regulatory authorities increasingly accept datasets generated by NGS as part of submissions. The method has demonstrated high sensitivity, broad applicability and reduced susceptibility to assay interference.

Recently, the European Pharmacopoeia published chapter 2.6.41^[2], which provides explicit guidance on validation requirements for NGS-based assays for adventitious virus detection. This additional regulatory clarity has further facilitated the routine implementation of NGS technologies in testing strategies and is discussed later in this white paper.

Comparison with traditional methods / Benefits over traditional methods

Before NGS became part of regulatory guidance, viral safety strategies relied on a combination of classical assays to mitigate the risk of adventitious viral contamination. While this multi-assay approach has historically been effective, each legacy method carries inherent scientific and practical limitations that restrict overall assay sensitivity, specificity and breadth of detection (see Table 1 for a structured comparison of advantages and limitations).

Limitations of classical assays

Untargeted assays like *in vivo* and *in vitro* adventitious agent tests (AAT) are designed to be broad but depend entirely on the susceptibility of the animal model or cell substrate. Viruses that do not induce cytopathic effects (CPE) or replicate inefficiently in the chosen assay can remain undetected. Moreover, the readouts of these assays, such as clinical signs or CPE, are easily influenced by interfering factors and often require highly experienced personnel to detect reliably.

Targeted methods, like MAP/HAP, 9 CFR and qPCR, are highly specific but require prior knowledge of the virus of interest.

They will only detect contaminants for which assays have been explicitly designed. Any unexpected, novel or distantly related virus can remain entirely outside the scope of detection.

Missed contamination events

The limitations of classical assays have contributed to several well-documented contamination events. For example, Minute virus of mice (MMV) has repeatedly caused noncytopathic, silent contamination incidents and Moody et al.^[3] demonstrated that such events can easily bypass in vitro AAT due to the absence of observable CPE.

A similar phenomenon occurred with porcine circovirus (PCV) in the Rotarix vaccine ^{[4][5]}, which was only identified after market release using NGS, as PCV replicates without causing CPE in Vero cells, the substrate used for routine testing. Comparable challenges have been seen with the persistently infecting Sf9 rhabdovirus, which is entirely noncytopathic and was detected only through NGS-based analysis ^[6] by Arifa Khan's group at CBER, FDA. Furthermore, Vesivirus 2117 induces only very weak CPE, resulting in a substantial risk of being overlooked in classical AAT workflows ^[8].

In addition, Barone et al. conducted a comprehensive analysis of historical contamination events, identifying recurrent viral contaminants, their sources, affected cell lines and associated corrective actions ^[7]. Their work illustrates that many of these incidents share a common feature: the contaminating virus produced little to no detectable cytopathic effect and therefore evaded traditional in vivo and in vitro assays.

This body of evidence collectively underscores the limitations of classical AAT and reinforces the need for broader, agnostic viral detection strategies.

Practical challenges: time, cost, and sample volume

Legacy testing presents not only scientific limitations but also several practical challenges in routine application. It is cost-intensive, as multiple orthogonal assays must be conducted in parallel to achieve sufficient coverage. These workflows additionally require substantial sample volumes, often in the range of 30–50 ml for a complete QC panel. These volumes can be difficult to obtain, particularly for small-scale or early-stage manufacturing processes. Turnaround times are typically long, which can delay batch release and increase storage burden.

Furthermore, results from the various assays must be interpreted collectively, adding logistical and analytical complexity for QC teams.

How NGS overcomes these limitations

NGS overcomes many of these limitations by combining the strengths of legacy testing into a single, highly informative assay. Because the method is sequence-agnostic, it can detect any viral nucleic acid present in a sample, covering known, unexpected and novel viruses alike. Deep sequencing provides high analytical sensitivity and specificity, enabling the detection of contaminants even at very low abundance. The approach is broadly applicable across DNA and RNA viruses, enveloped and non-enveloped viruses, as well as lytic and noncytopathic agents. Moreover, NGS readouts are not dependent on cell susceptibility or virus-induced phenotype, reducing the risk of interference that often impacts in vivo and cell-based assays.

In some workflows, the sequencing profile can further offer indications of whether viral signatures stem from active, replication-competent virus, for example through genome coverage patterns or the detection of viral RNA intermediates. Importantly, these advantages can be achieved at lower overall cost and with significantly shorter turnaround times compared to executing the full legacy testing panel.

Relevance for advanced modalities

NGS offers additional benefits for advanced modalities such as AAV-based gene therapies, CAR-T cell products and other ATMPs. These products are often manufactured in very limited batch sizes, have short shelf lives and provide only small sample quantities for QC. For such cases, NGS is particularly well suited, requiring as little as approximately 1 ml of material. This makes NGS a practical and robust option for viral safety testing in modern biomanufacturing environments.

NGS at ViruSure

While NGS offers significant advantages for adventitious agent detection, its successful implementation in a GMP environment depends on carefully designed workflows, validated analytical processes, and robust bioinformatics capabilities. This section describes ViruSure's long-read NGS approach and the technological principles that form the foundation of its adventitious agent testing platform.

The Viromics Approach

For adventitious agent testing using NGS, three principal analytical strategies can be applied:

- 1. Genomics** – The complete nucleic acid content of a sample is sequenced without prior enrichment or depletion steps, providing an unbiased view of all genetic material present.
- 2. Transcriptomics** – RNA molecules are selectively analysed to characterize viral transcripts, providing insight into active viral replication and transcriptional activity.
- 3. Viromics** – Background host DNA and RNA are reduced through an upfront nuclease treatment, enriching viral genomes and improving sensitivity for viral detection.

At ViruSure, the viromics approach is the primary strategy, as it offers several advantages over the other two. In contrast to transcriptomics, the viromics workflow can be applied to virtually any sample type and enables the detection of all viral contaminants present, regardless of whether they are capable of replication in that matrix. Transcriptomics, by comparison, is limited to situations where viruses can actively replicate in the sample; viruses that cannot replicate in the underlying system, as well as latent infections, may go undetected.

Compared to the genomics approach, viromics provides significantly higher sensitivity due to the initial reduction of host-cell background through nuclease treatment. This enrichment step improves sensitivity for detecting low-level contaminants in high-background samples such as cell banks, bioreactor harvests or viral stocks, making it a robust and broadly applicable workflow for routine adventitious agent testing.

Long-reads vs short-read NGS

At ViruSure, Oxford Nanopore–based long-read sequencing was established as the core technology for adventitious agent testing, in contrast to the more commonly used short-read sequencing platforms. Short-read sequencing technologies rely on sequencing-by-synthesis. In this method, the DNA is fragmented into ~150–300 bp fragments, followed by PCR amplification.

Sensitivity with short-read sequencing is strongly influenced by background DNA, and high background levels can result in overloading of the flow cells, resulting in excessively high cluster density, poor cluster separation and therefore inefficient sequencing performance and reduced sensitivity. In contrast, with Nanopore sequencing, DNA molecules are passed through a protein pore embedded in a membrane. As the molecule moves through the pore, characteristic disruptions in ionic current occur, which are measured and directly translated into sequence information. The technology is therefore less impacted by background DNA and continues to generate data as long as DNA is available at the pore.

This fundamental difference in sequencing chemistry results in several key advantages for adventitious virus detection:

- 1.** Short-read sequencing requires PCR amplification during library preparation, which can introduce biases because not all genomic regions amplify with equal efficiency (e.g. GC-rich or structured regions). These PCR-related effects can cause some viral regions to be underrepresented or even missed completely. Because Nanopore sequencing does not rely on PCR amplification, it avoids these sequence-dependent issues and produces readouts that more accurately reflect the true composition of the sample.

2. Short-read workflows require fragmentation of DNA into small pieces (~150–300 bp). Reconstructing viral genomes from these fragments requires computationally intensive assembly processes and complex bioinformatics, which present a barrier when explaining the output in regulatory submissions. This can be challenging, particularly for samples containing mixtures of unrelated viral sequences or high levels of host-cell DNA. Nanopore sequencing, however, preserves long fragments, eliminating the need for complex de novo assembly in many cases and significantly simplifying downstream bioinformatics and analysis.

Taken together, the absence of amplification bias and the availability of long, contiguous sequence information make Nanopore workflows highly suited for AAT, particularly in terms of sensitivity, specificity (i.e. low false-positive rate) and interpretability.

Benefits of long reads in database searches

Long reads provide clear advantages when searching against viral reference databases. Simply through their length, the probability of a random match is substantially reduced, which in turn lowers the background noise in taxonomic classification. As a result, false-positive hits are significantly reduced, which is a key benefit when analysing complex biological matrices that contain a variety of host-cell sequences.

Because long reads already cover substantial stretches of the viral genome, bioinformatic assemblies are often unnecessary, eliminating an entire computational step that is both resource-intensive and error-prone in short-read pipelines. The bioinformatics workflow becomes simpler, more deterministic and more transparent. This significantly simplifies the presentation of data in regulatory submissions. Furthermore, long reads that align across extended regions of a viral reference genome provide stronger evidence for a true viral hit, as they reduce the likelihood that the alignment represents artefacts or accidental matches to conserved motifs. Overall, long reads increase specificity, reduce noise and enable much clearer differentiation between true viral signals and background.

Simpler bioinformatics and easy-to-interpret results

At ViruSure, bioinformatics pipelines are designed to require minimal operator intervention, are easy to understand even by non-bioinformaticians and run in a largely automated fashion. Because Nanopore sequencing produces long reads and thereby inherently limits the total number of hits returned, the resulting output is compact, interpretable and manageable.

Instead of thousands of ambiguous short-read hits that require extensive filtering, clustering and computational decontamination, the long-read workflow returns a manageable set of high-confidence alignments. This low number of candidate hits, which is orders of magnitude lower than the number of potential hits using short-read technologies, allows analysts to review each result individually, without the need for complex filtering strategies. As a result, the pipeline remains accessible to trained analysts who can directly evaluate and interpret the data without the need for advanced bioinformatics expertise.

This transparent analysis structure provides several advantages. Datasets are easier to understand and communicate. Regulatory review benefits from straightforward, traceable evidence, since each long read contains enough sequence information to support a viral identification. By limiting reliance on highly complex computational steps, the workflow becomes more transparent and easier to verify, leading to greater confidence and reproducibility.

In summary, the combination of long reads and streamlined bioinformatics results in an approach that is both scientifically robust and operationally practical, making long-read NGS a highly suitable method for routine adventitious virus testing at ViruSure.

Virusure Workflow

In the following sections, each step of the Virusure pipeline is outlined, giving a rationale for the implementation of the step. The overall goal was to design an assay that is robust and broadly applicable, ensuring consistent assay performance across multiple sample matrix types while minimizing the risk of potential matrix-derived interference.

Nuclease treatment

As a first step, a nuclease treatment is performed on the input material (typically 10^6 cells or 1 mL of sample). This step reduces background DNA and RNA originating from the sample itself (e.g. host-cell DNA) and it also removes free viral nucleic acids released from damaged or non-intact viral particles. In contrast, viral genomes enclosed by intact viral particles remain protected, thereby significantly reducing the risk of generating false-positive viral signals.

The reduction of background nucleic acid improves the overall signal-to-noise ratio and increases the sensitivity of the assay. An additional advantage of this step is that it normalizes background nucleic acid levels across different sample matrices, ensuring that the method can be applied robustly to a wide range of sample types with a reduced risk of matrix-derived interference.

Extraction

Following nuclease treatment, total nucleic acid extraction is performed. Efficient recovery of both DNA and RNA is essential, as it directly influences the assay's ability to detect viral contaminants with high sensitivity. Because the nuclease step reduces and harmonizes background nucleic acid levels across matrices, extraction can be carried out with high consistency across a wide variety of sample types. As a result, a single extraction method can be applied across many different sample types and matrices, including cell banks, virus seeds, bulk harvests, and process intermediates. To ensure efficient and reproducible nucleic acid recovery, internal assay controls are included and monitored throughout the workflow.

Reverse transcription (RT)

Reverse transcription is performed to convert RNA molecules present in the sample into complementary DNA (cDNA). The first step generates a single cDNA strand from RNA templates, followed by second-strand synthesis to produce double-stranded DNA. This second-strand synthesis step also enables conversion of any single-stranded DNA viruses into a double-stranded form, ensuring uniform compatibility with subsequent library preparation.

As RNA viruses possess various genomes, efficient and unbiased reverse transcription is crucial for the implementation of a broad-range and high-sensitivity assay. The reaction conditions are therefore designed to maximize inclusivity across a broad range of RNA virus families (ss(+) RNA, ss(-)RNA, dsRNA), including those with long, structured, or segmented genomes.

Library preparation

After double-stranded DNA is generated, library preparation is performed to convert the purified nucleic acids into sequencing-ready molecules. For Nanopore sequencing, this process typically includes end-repair and adapter ligation. Besides QC checks during library preparation at various stages, including DNA quantification and fragment size determination, sequencing controls are spiked into the sample. These controls help monitor the robustness and reproducibility of the library preparation process, which is essential for ensuring consistent assay performance.

NGS run (Oxford Nanopore)

Once the libraries are prepared, samples are loaded onto the Nanopore flow cell for sequencing. During this process, DNA strands pass through nanopores embedded in a membrane, causing characteristic disruptions in an applied ionic current. These current changes are measured and are subsequently translated into nucleotide sequence information during the basecalling process.

Another benefit of Nanopore technology is the capability for real-time data analysis. Sequencing data become available immediately as they are generated, enabling rapid assessment of results. This makes the platform particularly well suited for time-critical applications, such as the rapid identification of a known contaminant in a sample.

Data analysis

After base calling, reads are quality-filtered according to QV score and minimum read lengths. The quality data set is analysed using automated, in-house-developed bioinformatics pipelines. Each pipeline is fully GMP-validated and complies with regulatory requirements such as 21 CFR Part 11 and EU GMP Annex 11. In-house development enables complete control over the workflow. Furthermore, the pipelines are designed so that they can be executed by any trained operator without requiring in-depth bioinformatics expertise.

The long reads generated by Nanopore sequencing simplify downstream bioinformatics and result in outputs that are straightforward to interpret. Only a low number of background hits is typically obtained, significantly reducing the risk of false-positive viral signals. Furthermore, this allows for the evaluation of each obtained hit by a trained virologist, without the need to apply any complex bioinformatics filtering steps. The simplicity and transparency of the analysis process also facilitate regulatory understanding and support broader acceptance of the approach.



Figure 1 Modules of the NGS workflow

Validation of NGS Workflows

The value of any testing platform ultimately depends on its demonstrated performance. As regulatory acceptance of NGS continues to grow, rigorous validation is essential to ensure that sequencing-based approaches deliver reliable, reproducible, and scientifically defensible results. This section outlines the validation strategy used to establish the suitability of ViruSure's long-read NGS platform for GMP-compliant adventitious agent testing.

Modular approach

Due to the complexity of NGS workflows, Ph. Eur. Chapter 2.6.41 on validation of NGS technologies for adventitious agent detection describes a modular validation approach. This principle allows for the subdivision of the workflow into its functional components (sample prep, library prep, sequencing, bioinformatics). Each module is validated individually to demonstrate that the respective process step is suitable for its intended purpose.

A key advantage of this validation strategy is the increased flexibility for the implementation of improvements. When a change to a single module is made, normally only the affected module needs to be revalidated. A single subsequent confirmation run of the complete workflow is then performed to demonstrate that the modification does not negatively impact the overall method performance.

In contrast, with a non-modular approach, any change would require complete revalidation of the full workflow.

For module validation, appropriate reference materials were used to assess sensitivity, specificity, and robustness. After the modular validation was completed, a full end-to-end validation was conducted in accordance with the HTS validation requirements described in Ph. Eur. Chapter 2.6.41. This two-step approach (modular validation followed by generic end-to-end validation) ensures regulatory compliance while maintaining flexibility for future method updates under GMP change-control requirements.

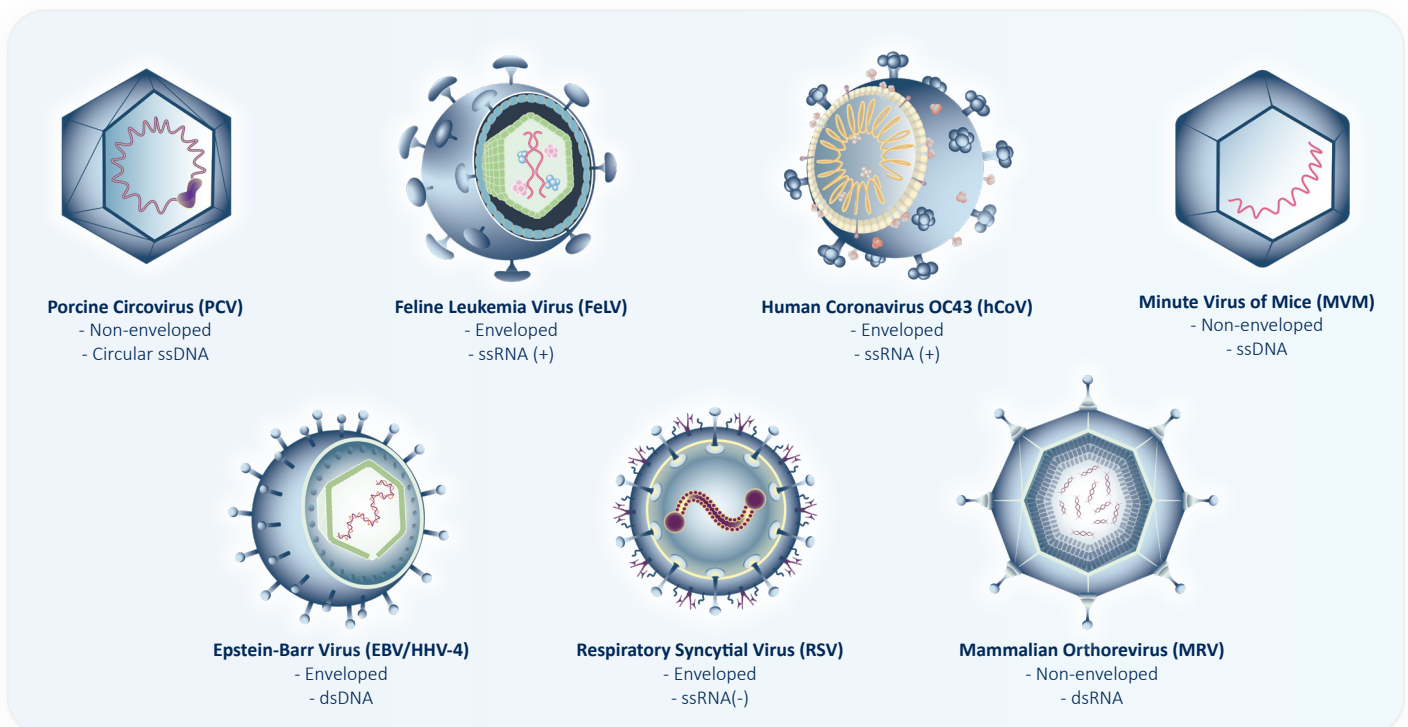


Figure 2 1st WHO International Reference Panel for Adventitious Virus Detection in Biological Products by High-Throughput Sequencing

Generic validation

NGS for adventitious virus detection is considered a qualitative limit test. According to ICH Q2(R2), this requires the validation of the method's sensitivity (LOD) and specificity. To do so, spiking studies need to be performed, where representative sample matrices (e.g. cell banks or virus seeds) are spiked at defined concentrations with the 1st WHO International Reference Panel for Adventitious Virus Detection in Biological Products by High-Throughput Sequencing.

This panel was prepared by CBER, FDA for method validations and consists of 7 viruses covering the known genomic and physicochemical attributes of the viral kingdom (Figure 2). To confirm the suitability of the panel for validation studies, it underwent extensive characterisation and was evaluated across multiple laboratories, demonstrating consistent viral recovery and acceptable DNA/RNA background profiles^[8]. The ability to recover the complete panel in an NGS workflow demonstrates the capability to detect any viral contamination within a given sample.

During the validation, spiked samples at defined concentrations are tested in three independent end-to-end runs. Upon completion of the spiking studies, the LOD is determined as the lowest concentration at which the complete virus panel could be recovered. Specificity is demonstrated by the correct identification of the spiking panel, as well as the absence of any inexplicable (false-positive) results.

Matrix-specific validation (interference testing)

Once the generic validation using representative matrices is completed, the European Pharmacopoeia requests the performance of matrix-specific validations to ensure the absence of any interfering effects in a given sample matrix that might negatively impact the sensitivity of the NGS assay. For this purpose, the respective sample is spiked with the previously described virus panel at the LOD concentration defined in the generic validation. The recovery of the complete virus panel at the same concentrations demonstrates the absence of interfering effects. Should it not be possible to recover the complete virus panel, interfering effects are anticipated and a predilution of the sample or a higher spike concentration is recommended.

Segregation and Controls

Due to the complexity of the workflow and the nature of high-throughput sequencing technologies, comprehensive contamination-control measures, similar to PCR, are essential. Because NGS can process any nucleic acid present in a sample, there is a substantial risk of introducing unwanted sequences through reagents, consumables, or the laboratory environment. To mitigate these risks, strict cleaning, segregation and control procedures are implemented throughout the entire workflow.

Cleaning and segregation measures are applied before and after each sample-processing step to minimize the potential for carryover or operator-derived contamination. All critical workflow stages – extraction, reverse transcription, library preparation and sequencing – are performed in physically separated laboratory areas to ensure unidirectional sample handling and reduce the chance of cross-contamination.

In addition to these procedural safeguards, multiple controls are incorporated across the workflow to verify performance and detect potential contamination events. Negative controls (blank samples) are processed alongside test samples to monitor for contamination originating from reagents or handling steps. Positive controls are introduced at defined workflow stages, such as extraction, reverse transcription and library preparation, to confirm that material is efficiently recovered and that each module functions as intended. Together, these measures ensure that results generated by the NGS pipeline are reliable, traceable and free from process-related artefacts.

Validation data of AAT pipeline in different matrix types

Taking all these points into consideration, ViruSure's AAT pipeline was validated using CHO cells and undiluted BVDV virus harvest as representative matrices. The resulting limits of detection (LODs) were 10^4 viral genomes per 10^6 cells for CHO cell banks and 10^3 viral genomes per mL for virus seeds. A summary of the validation data is presented in Figures 3 to 4. These figures show the number of reads obtained for each spike as well as the corresponding percentage of genome coverage achieved at the validated LOD.

Figure 3 and Figure 4 illustrate the recovery of 10^4 viral genomes spiked into 10^6 cells, which represents the LOD for cellular matrices. For viral seed matrices, however, the LOD is 10^3 viral genomes/mL, reflecting the increased sensitivity in these matrices due to their substantially lower background DNA/RNA levels compared with cell banks.

Specificity was demonstrated by the correct identification of all seven viruses from the WHO reference panel, with no false-positive hits detected in any sequencing run. Together, these results confirm that the AAT pipeline delivers high sensitivity, high specificity, and consistent performance across multiple matrix types.

Reads numbers - 1×10^4 viral genomes / 10^6 cells

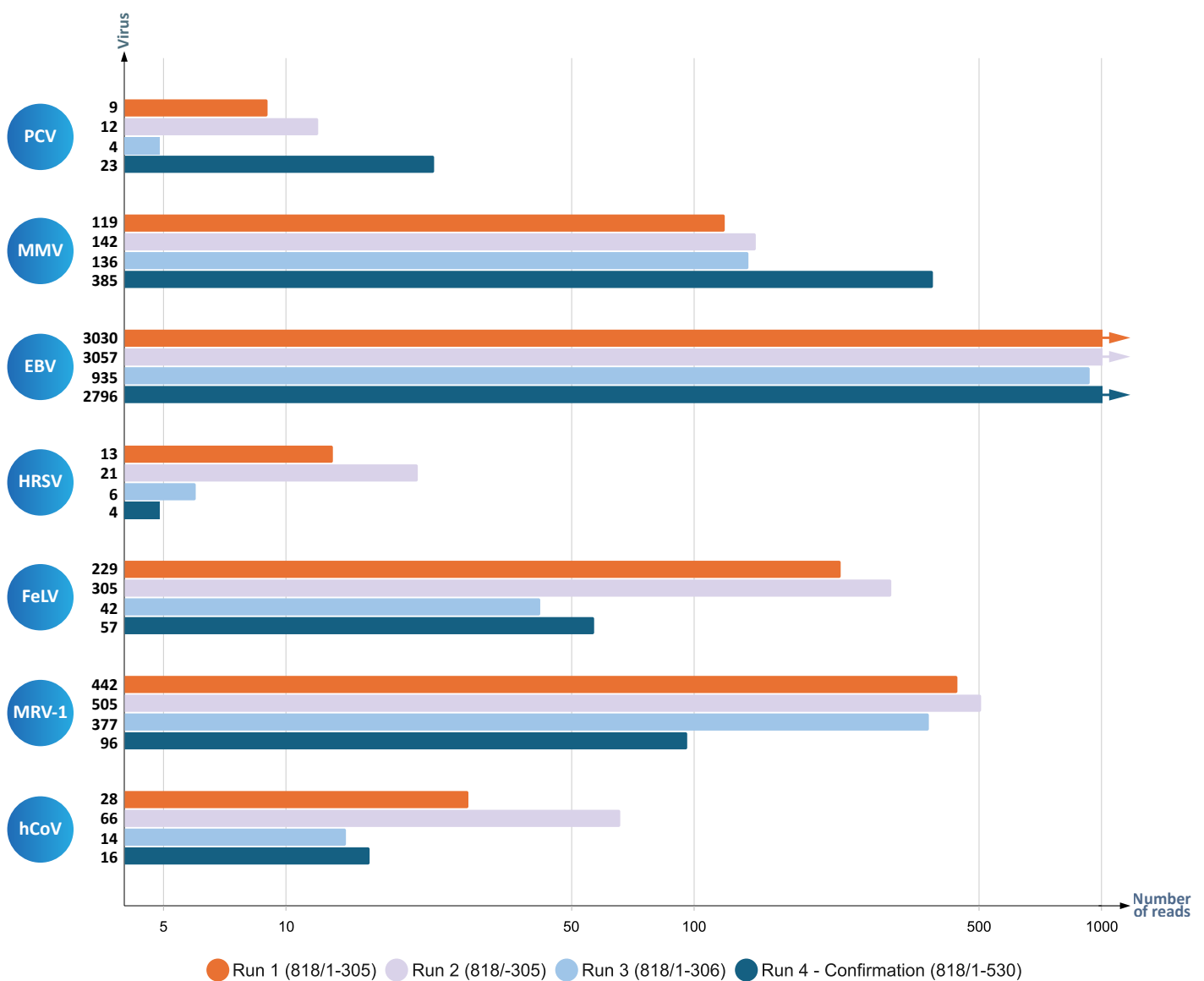


Figure 3 Number of reads obtained in the 10^6 background cells using 10^4 viral genomes
Read counts are displayed on a logarithmic scale

% Coverage - 1×10^4 viral genomes/ 10^6 cells

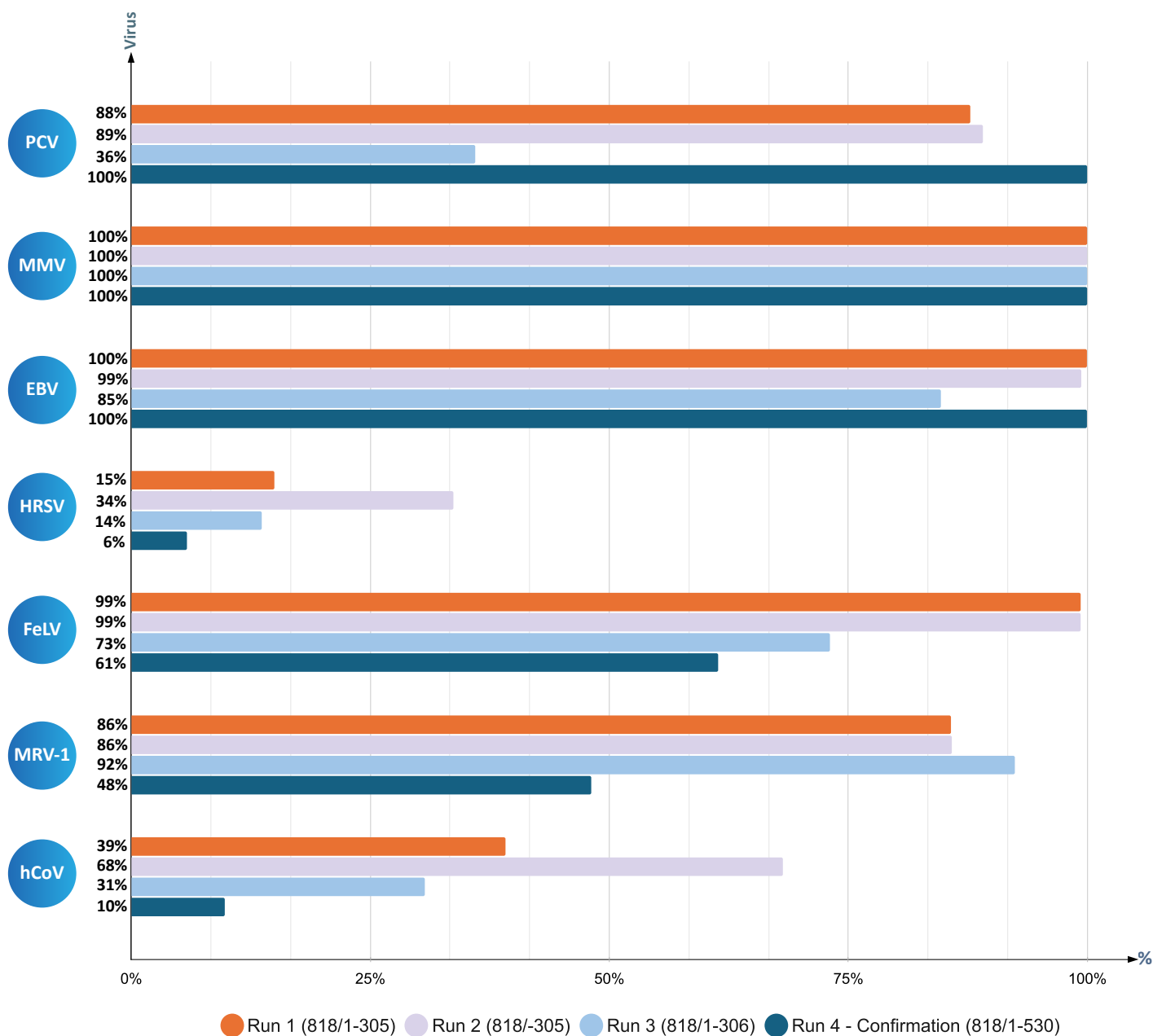


Figure 4 Percentage of viral genomes recovered in 10^6 background cell using 10^4 viral genomes

Conclusion

Next-generation sequencing (NGS) has become an increasingly important tool for adventitious virus detection following the publication of ICH Q5A(R2) and European Pharmacopoeia Chapter 2.6.41. These regulatory updates explicitly support the use of NGS as a replacement for in vivo testing and as a supplement or alternative to traditional in vitro adventitious agent tests.

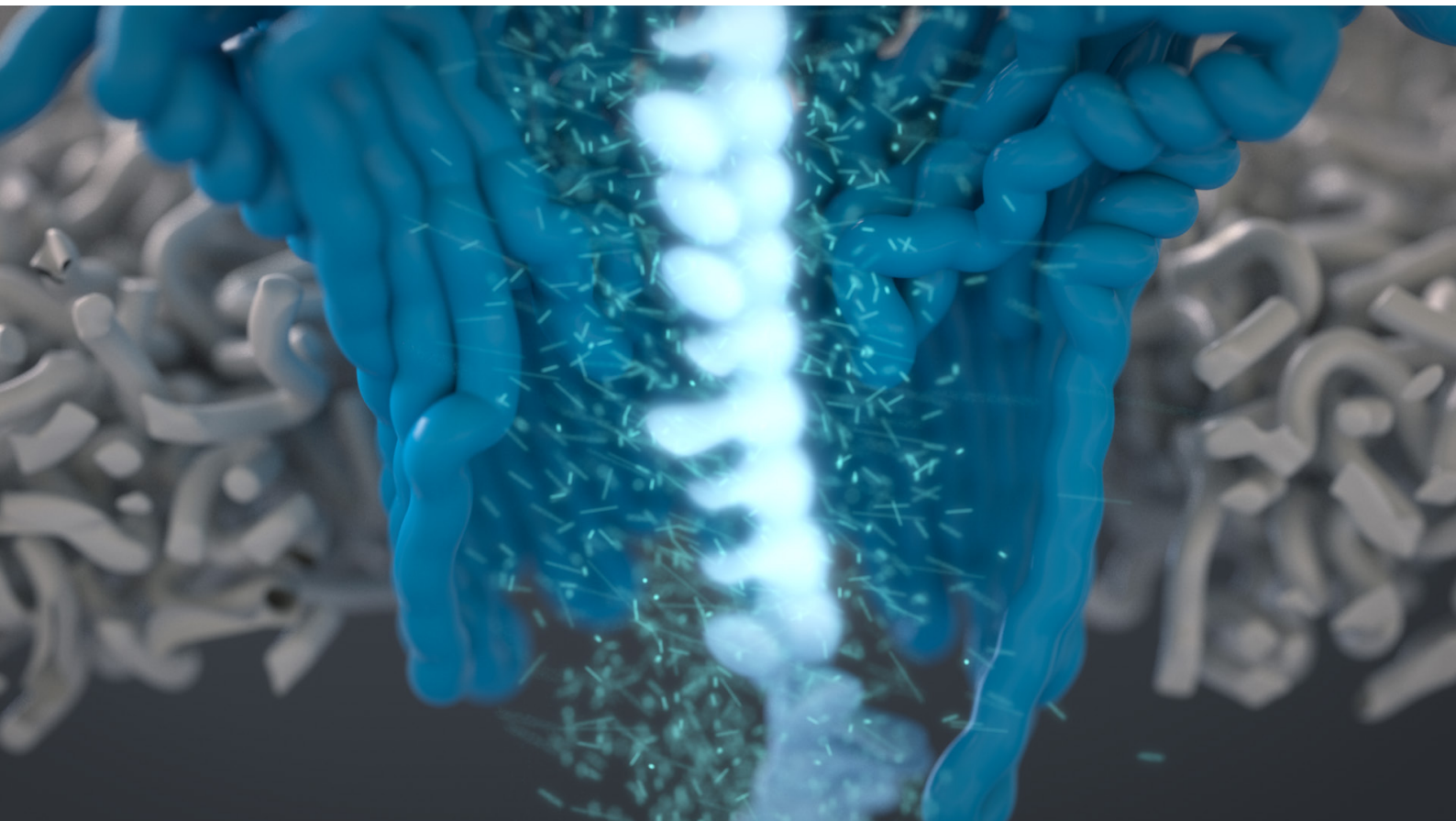
Classical assays, although historically effective, have well-known scientific and practical limitations, including restricted detection scope, dependence on host-cell susceptibility, and long turnaround times. Several contamination events in recent decades illustrate these shortcomings.

NGS addresses many of the limitations of legacy methods by enabling sequence-agnostic detection of known and unknown viruses with high sensitivity and broad applicability. Long-read sequencing, particularly Oxford Nanopore technology, provides additional advantages by eliminating PCR-related biases, reducing the need for de novo assembly and generating long, easily interpretable reads that greatly reduce false-positive rates.

Virusure has implemented a robust NGS workflow for adventitious virus detection based on a viromics approach, which employs an upfront nuclease treatment to enrich for intact viral particles. This strategy ensures high sensitivity across diverse sample types, including cell banks, virus seeds, bulk harvests, and advanced modalities such as AAV gene therapies and CAR-T products. The pipeline incorporates validated extraction, reverse transcription, library preparation, sequencing, and automated bioinformatics modules, each developed and qualified under GMP.

Validation was performed using the WHO reference panel for adventitious virus detection and representative matrices, demonstrating high sensitivity, specificity, and robustness. The modular validation concept outlined in Ph. Eur. 2.6.41 supports method flexibility while ensuring regulatory compliance.

In summary, long-read NGS provides a comprehensive, sensitive, and operationally efficient solution for adventitious virus detection and is well positioned to become an integral component of modern viral safety testing strategies.



Images courtesy of Oxford Nanopore Technologies Plc.

References

1. ICH Q5A (R2) *Guideline on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin*
2. Ph Eur 2.6.41 *High-Throughput Sequencing for the Detection of Viral Extraneous Agents*
3. M. Moody, W. Alves, J. Varghese, F. Khan. *Mouse Minute Virus (MMV) Contamination--A Case Study: Detection, Root Cause Determination, and Corrective Actions*, PDA J Pharm Sci Technol. 2011 Nov-Dec;65(6):580-8.
4. JG Victoria, C Wang, MS Jones, C Jaing, K McLoughlin, S Gardner, et al. *Viral Nucleic Acids in Live-Attenuated Vaccines: Detection of Minority Variants and an Adventitious Virus*, J Virol 2010;84:6033e40
5. G. Dubin, JF. Toussaint, JP. Cassart, B. Howe, D. Boyce, L. Friedland, R. Abu-Elyazeed, S. Poncelet, HH. Han, S. Debrus. *Investigation of a regulatory agency enquiry into potential porcine circovirus type 1 contamination of the human rotavirus vaccine, Rotarix™*, Hum Vaccin Immunother. 2013 Aug 28;9(11):2398–2408.
6. H. Ma, TA Galvin, DR Glasner, S Shaheduzzaman, AS Khan. *Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines*, J Virol. 2014 Jun;88(12):6576-85.
7. PW. Barone, ME. Wiebe, JC. Leung, ITM. Hussein, FJ. Keumurian, J. Bouressa, A. Brussel, D. Chen, M. Chong, H. Dehgani, L. Gerentes, J. Gilbert, D. Gold, R. Kiss, TR. Kreil, R. Labatut, Y. Li, J. Müllberg, L. Mallet, C. Menzel, M. Moody, S. Monpoeho, M. Murphy, M. Plavsic, SL. Springs. *Viral contamination in biologic manufacture and implications for emerging therapies*, Nature Biotechnology 38, pages563–572 (2020)
8. A. Oehmig, M. Buettner, F. Weiland, W. Werz, K. Bergemann, E. Pfaff. *Identification of a calicivirus isolate of unknown origin*, J Gen Virol. 2003 Oct; 84(Pt 10):2837-2845.
9. AS. Khan, PJ. Chin, SM. Fuentes, JH. Tsou, et al. *A Collaborative Study to Evaluate the Proposed First WHO International Reference Panel for Adventitious Virus Detection in Biological Products by High-throughput Sequencing (HTS) Technologies*, Expert Committee on Biological Standardization, Geneva, 11 to 14 March 2024



VIRUSURE

Quality is no coincidence

Virusure GmbH
Donau-City-Straße 1, A-1220
Vienna, Austria

Tel: +43-(0)1-269-9120
virus_safety@virusure.com

AsahiKASEI
BIOPROCESS