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European Federation of Pharmaceutical Industries and Associations

Considerations for Validation and Implementation of Next Generation Sequencing for Adventitious Virus Detection for Biological Medicinal Products

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Date: June 2024 **Version:** **final**

Executive Summary

Viral safety of biological medicinal products relies on extensive testing of the materials used in manufacturing, including cell banks of animal origin as a critical starting material. The ICH Q5A guideline, used worldwide as a reference for viral safety, has been recently revised to integrate the most up-to-date scientific knowledge and approaches developed over the last decades. Among them, Next Generation Sequencing (NGS) has emerged as a promising technology to detect a broad spectrum of viruses. Also known as high-throughput sequencing, NGS allows massive parallel generation of nucleic acid sequence data without prior sequence information, offering the potential to detect unknown or unexpected viruses.

This article aims at complementing the recent revision of ICH Q5A by providing the position of the EFPIA Supportive Group "Clonality, Characterisation and Viral Safety of Cell Lines" on NGS implementation for adventitious virus detection. Envisaged as a practical guide, the paper addresses questions related to analytical method validation and corresponding pre-requisites originating from method development. It discusses the benefits and limitations of analytical comparability studies between NGS and conventional virus detection assays . Finally, it provides a regulatory perspective to aid manufacturers implementation of NGS for viral safety testing of development and authorised products.

The development and manufacture of biological medicinal products remains technically challenging and significant pressure exists to make these processes more efficient. Emerging technological advances and capabilities represent a clear opportunity to meet these challenges. Among recent advancements are those associated with molecular analytical technologies for genetic characterisation with the most prominent examples being collectively referred to as **Next Generation Sequencing (NGS)** (also named High Throughput Sequencing) platforms. NGS has transformed nucleotide sequencing capabilities with improved speed and throughput while drastically reducing the per-base sequencing costs.

NGS methodologies are being evaluated for a wide-range of applications across the biological therapeutic landscape from traditional protein-based therapeutics to gene and cell therapies. They are being implemented by manufacturers in areas such as genetic characterisation and adventitious agent testing.

The scope of this position paper is focused on the **use of NGS for adventitious virus detection**. The first section provides some background on NGS technologies and a comparison to conventional methods for virus detection. In the second section, approaches to conduct the validation of NGS-based methods for the detection of adventitious viruses are described, together with technical considerations. In the third section, the replacement of conventional virus detection tests by NGS is addressed and the topic of analytical comparability (head-to-head comparison) is discussed in detail. Finally, the last part presents the current regulatory framework and possible regulatory strategies to implement NGS for adventitious virus detection in development or commercial biological products. In general, the recommendations included in this position paper have been made by considering recombinant proteins and vaccines application. Advanced Therapy Medicinal Products are out of scope.

1 Analytical Methods for Virus Detection in Biological Medicines

Demonstrating the absence of viruses in biological medicinal products is a key safety concern, illustrated by the occurrence of a small number of cases of contaminated products or processes[1]. In addition to appropriate environmental measures to limit contamination during manufacturing, regulatory requirements related to virus safety of biologicals manufactured in animal cells prescribe a mitigation strategy based on three complementary approaches: a comprehensive selection and testing of incoming materials, an assessment of the capacity, if any, of the manufacturing process to clear potentially present infectious viruses, and routine testing of the product at appropriate stages for the absence of viruses — see for example[2,3,4]. Testing of cell banks, seed lots, materials from human or animal origin, or manufacturing intermediates is requested based on the risks inherent to the production system, and the corresponding assays are described in Pharmacopoeias or regulatory guidelines[3,5,6]. In addition to tests for adventitious virus contaminants, tests for endogenous viruses (such as retroviruses) should be performed as described in ICH Q5A[2].

1.1 Conventional Assays

The assays commonly used for adventitious virus detection are based on different analytical principles and are used in combination due to their orthogonal and complementary nature, as highlighted in Table 1.

Table 1: Comparison of Adventitious Virus Detection Assays used to Demonstrate Viral Safety of Biological Products

Characteristics	*In vivo* assays	Cell-based infectivity assays	PCR-based methods	NGS (HTS)-based methods
Main tests	*In vivo* viral screening assays[a]; Antibody production tests (MAP, HAP, RAP tests)	*In vitro* infectivity assays using at least three detector cell lines; other detector cell lines may be added as needed (e.g. use of raw materials of animal origin (bovine, porcine …))	PCR assays for specific viruses (e.g. MVM, bovine polyomavirus, vesivirus) ; Degenerate PCR assays for multiple target virus detection	NGS assays for specific or broad virus range: Genomics, transcriptomics or viromics approach; Non-targeted or targeted approaches
Test responses	*Viral screening assays*: signs of viral pathogenicity in animals and embryonated hen eggs *Antibody production tests:* antibody response to specified rodent viruses	Detectable phenotype upon infection of permissive indicator cell lines: cytopathic effect, hemadsorption or hemagglutination	Specific amplification of virus genomic sequences	Sequencing of all nucleic acid in a sample (or a subset), and mapping against a virus database
Application	Cell banks, viral seeds	Cell banks, viral seeds, manufacturing intermediates, animal-derived raw materials	Cell banks, viral seeds, manufacturing intermediates	Cell banks, viral seeds, cell therapy drug substance Investigational tool Potential replacement/ supplementary assay for *in vivo* tests and *in vitro* tests
Breadth of virus detection	*Viral screening assays:* Broad detection of viruses for viral screening assays, including potential detection of new or emerging viruses; *Antibody production tests*: specific detection[2]. Limitations: susceptibility of animals to viral infection	Broad detection of viruses, depending on the indicator cell line panel, including detection of novel viruses. Limitations: susceptibility of indicator cells to viral infection and detectability of infection phenotype	Aimed at detecting rapidly specific known viruses Limitations: may detect viral nucleic acid not related to infectious particles; may not detect some variants depending on primer design.	Broad detection (for non-targeted approaches), including possibility to detect variants and novel viruses Limitations: may detect viral nucleic acids not related to infectious particles, except in case of transcriptome sequencing

[a] The terminology used in USP <1237> was chosen to designate *in vivo* assays in adult mice, suckling mice, guinea pigs or embryonated hens' eggs, to distinguish them from antibody production tests.

In vivo assays and *in vitro* cell-based infectivity tests can detect viruses based on their infectivity in permissive biological systems. Animal models and detector cell lines have different susceptibility patterns and are selected based on the viruses they promote growth of[6,7]. Those tests have proven so far to ensure a suitable level of safety for biological products, in part thanks to their complementary coverage. But they also have some limitations, as discussed below. In addition, follow-up confirmations are usually needed as well in case of positive results, to identify the virus causing the response. Molecular methods such as NGS are powerful investigational tools in that respect.

In vivo viral screening assays can only detect pathogenic viruses, and the long duration of the assays limits their use in routine testing. They are inherently prone to high variability, and while they can detect some viruses with a high sensitivity[7], their sensitivity to other viruses is low[7,8]. The current initiatives to reduce, as much as possible, the use of animals for testing (3R: replacement, reduction, refinement[9]) is a strong incentive to find alternatives for *in vivo* virus detection assays.

Originally developed for vaccines, cell-based infectivity assays are widely used as broad range virus detection tests or as virus-specific tests. These tests can detect many viruses with a good sensitivity and are used to demonstrate the absence of infectious viruses for release of biological products[5,10,11,12]. Nevertheless, some viruses of concern to biologicals production may not be detected as reported[1], either because of absence of propagation in culture, or because of absence of a visible phenotype[13]. The use of multiple indicator cell lines helps minimise this limitation.

Complementing those assays, tests based on polymerase chain reaction (PCR) (see also Table 2) are routinely used to detect specific viruses of high concern which might not be detected by other tests[2,13]. Those tests have a high sensitivity and are not dependent on virus infectivity. Because they are based on the use of specific primers for virus detection, PCR tests usually have a narrow breadth of detection and will detect a limited number of viruses, with the risk to miss detection of variants presenting mutations in the regions targeted by primers. Despite this limitation, PCR assays have strong advantages, including easy implementation and short assay duration. They have already been shown to be effective in rapidly detecting viral contamination, as reported for example[1]. In addition, the use of degenerated or consensus sequence primers can widen the breadth of detection of those types of assays beyond virus species[14].

1.2 Introduction to NGS Technologies

In addition to conventional assays, novel molecular techniques with broad virus detection scope are being developed and provide interesting alternatives to current *in vivo* viral screening assays and cell-based infectivity assays, as stated in some Pharmacopoeias[8] and described[14]. Among them, NGS is a molecular biology technique generating huge amount of sequence information in a massive parallel manner, without the need for prior sequence information. This makes NGS technologies able to generate sequences for any nucleic acid present in a tested sample, including those of virus origin. NGS further makes possible to assign those sequencing reads to any virus for which sequences are known, and possibly novel viruses based on sequence homology with other viruses. NGS may achieve comparable sensitivity as PCR, but this requires significant sequencing capacity and intensive bioinformatics data analysis, leading to longer analysis durations and potentially significantly higher costs.

NGS is a rapidly evolving field with a diversity of coexisting technological platforms, which can be divided in two main families: short-read (< 500 nt) and long-read sequencers (corresponding to the average length of the sequences generated, also called reads) (see Table 2). The main characteristics of the various platforms have already been described extensively in other publications[15,16,17]. In addition, NGS can be applied using targeted (i.e. scope of detection limited to a subset of viruses of

interest) or non-targeted approaches. Targeting can be achieved at the bioinformatics level, when only one or few reference sequences are taken into consideration for the analysis, which contrasts with the non-targeted approach searching the raw data against a broad viral sequence database. Targeting can also be achieved at the sample preparation level, using centrifugation, ribosomal RNA gene depletion, amplicon enrichment or hybridization-based enrichment[18] to enrich for specific genetic sequences prior to bioinformatic analysis. While enrichment focuses the analysis on the viral sequences of interest, leading to increased sensitivity, simpler analysis and lower overall costs, it may also lead to exclude some viral sequences and decrease breadth of detection. Finally, NGS methods can be differentiated based on the targeted nucleic acid population (based on sample preparation): genomics (all genomic viral nucleic acids, DNA and RNA, obtained from cells), transcriptomics (RNA from cells including viral mRNAs), or viromics (encapsidated viral genomes, obtained from supernatant).

Table 2: Diversity of PCR and NGS Technologies for the Purpose of Virus Detection

Technology	Platforms	Main features	Expected performance
PCR	Quantitative PCR Digital PCR	Detects specific regions of virus genomes Needs prior sequence information (targeted technology) Easy to implement	Narrow breadth of detection (generally at the species or even type level) (the use of degenerate primers can increase breadth of detection) Not capable of detecting novel or distant viruses High sensitivity
NGS: short-read technologies	Ion semiconductor Sequencing by synthesis Sequencing by ligation	Non-targeted methods: do not need prior sequence information (adventitious virus detection) Targeted methods: NGS analysis against specific viruses with high sequence coverage Needs sophisticated bioinformatics tools and dedicated expertise Breadth of detection and specificity is dependent on chosen methods	Non-targeted: Wide range of virus detection possible, including unknown viruses Targeted: detection of low frequency mutants High sensitivity in case of high read depth Low sequencing error rate (<1%)
NGS: long-read technologies	Single molecule real-time sequencing Nanopore sequencing		Wide breadth of detection possible Sensitivity in some cases limited by data capacity Potentially higher sequencing error rate

NGS-based method for virus detection are complex multi-step methods, and the diversity of approaches described above leads to multiple possible workflows (a detailed description of the main options is provided in the draft Ph.Eur. chapter 2.6.41[19]). The performance of NGS-based methods can be impacted by several steps in the experimental procedure, and careful method development is therefore needed. For example (and non-exhaustively), the protocol used for nucleic acids extraction, reverse transcription and library preparation will impact sensitivity and specificity[20]; the content of the database used to align reads will impact specificity and breadth of detection; the overall sequencing capacity of the technology will impact sequencing depth and therefore the detection limit; the bioinformatics pipeline used to assemble the reads and/or align them against the database will impact the rate of false positives and false negatives[21].

While the capabilities of molecular biology methods (NGS and PCR) make them very valuable tools for virus detection, they differ from conventional *in vivo* and cell-based assays in a fundamental aspect, i.e. the very different nature of the analytical responses (Table 1). *In vivo* and cell-based assays detect infectious viruses through the observation of pathogenic or cytopathic effects, whereas molecular

biology methods detect nucleic acid sequences that may not be related to infectious particles, triggering the need to potentially conduct additional follow-up confirmation in order to confirm infectivity. Only NGS methods using a transcriptomics approach in cell samples would rather detect the potential presence of active viruses, but specific care should be given to potential high background of cellular RNAs being sequenced together with viral ones, as well as genomic viral signals in case of RNA viruses.

The development of NGS-based methods for adventitious virus detection is encouraged by multiple Health Authorities as a promising route to further secure viral safety of biological products. The potential of this technology to detect viruses missed by conventional *in vivo* and *in vitro* assays has already been demonstrated[22,23]. WHO stated in its 2013 guideline[3] that "it is probable that application of methods of this type will be expected or required by regulatory agencies in future". With regards to vaccines, the European Pharmacopoeia has introduced the possible use of NGS and other new sensitive molecular techniques with broad detection capabilities as an "alternative to *in vivo* or specific nucleic acid tests, or as a supplement/alternative to cell-based infectivity assays, based on a risk assessment and in agreement with the competent authority"[5,8], and is working on the elaboration of a general chapter describing methods and validations requirements[19]. More recently, the revision of ICH Q5A has included recommendations for the use of NGS for adventitious virus detection, either as supplement or as replacement of conventional tests[2]. However, in order to fulfill the potential of NGS-based methods for virus safety testing of biologicals, several challenges need to be addressed related to assay design, assay validation, comparison with conventional assays and regulatory acceptance. The next sections of this paper discuss those challenges in detail.

2 Validation of NGS-based Analytical Procedures for Adventitious Virus Detection

The objective of validating an analytical method is to demonstrate that the method is suitable for the intended purpose. While the validation of methods based on NGS has to follow international regulatory guidelines on method validation[24], NGS is still an emerging technology when it comes to its application for broad virus detection in a regulated environment. The purpose of this section is therefore to give a perspective on how to interpret and adapt international guidelines to a NGS-based method aimed at detecting the presence of viruses. The following considerations assume that the purpose of the NGS-based method is the detection and species identification of a broad range of potential adventitious viral contaminants in either cell banks, viral seeds, or manufacturing harvests, but not the quantification of these contaminants. Given the capability of NGS methods to detect unknown viral species or strains, the species identification is only intended as the preliminary assignment to a defined taxonomic group, such as viral family or better according to the claims of the method. Of note, in case of a positive result (*i.e.* viral sequence detected in the sample), it is assumed that the conclusive species identification of the virus would be determined in the context of a follow-up investigation.

Before discussing the details of validation parameters, a couple of terms need to be defined:

1. Analyte: viral nucleic acid whose sequence has significant similarity with a known sequence present in the viral database.
2. Matrix: type of sample in which the potential virus contamination can be found (e.g. cells, viral seeds, or manufacturing harvests, intermediates).

3. Impurities: any component that is not expected in the product (intermediate or final) composition. For the purpose of this section, the term refers in particular to viral contaminants.
4. Unknown viral sequences: viral sequences absent from the viral database, but with sequence similarity to viruses present in the database.
5. Background signals: nucleic acids sequences detected in the matrix that do not originate from viral contaminants.

With these premises, NGS virus detection methods can be considered "limit tests for the control of impurities" (*i.e.* viral contaminants) according to ICH Q2 (R2)[24]. Therefore, the validation of NGS analytical procedures must evaluate at least specificity and detection limit (DL), in addition to robustness.

2.1 Specificity

In agreement with ICH Q2(R2) definition, *specificity/selectivity* can be addressed demonstrating that the identification of the analyte is not impacted by the presence of other substances, matrices, or impurities. For NGS, this means demonstrating the method's ability to detect a viral contaminant in a complex matrix, discriminating viral nucleic acids from the background.

It should be kept in mind that possible false-positive results of the test (*i.e.* nucleic acid wrongly identified as viral) are mostly a quality and business risk, without a negative impact on the safety of the product. More importantly, NGS-based methods should ideally be able to detect viruses from biological raw materials, drug product (intermediates or final) or the environment, even if they are only distantly related with the species or strains included in the viral database. Results of the specificity assessment should therefore be evaluated considering that a low level of non-specific signals might be acceptable as long as a follow-up strategy is in place to confirm or infirm the result.

According to the intended use, the following two aspects should be verified:

1. The method should report positive results only in the presence of actual contamination. In this case, the specificity can be demonstrated using *negative* samples, represented by the matrix without viral contaminants. The analysis of negative samples is required to verify that false-positive background signals are properly identified and filtered out by the method through appropriate bioinformatic analysis.
2. In the presence of actual contamination, the method identifies exclusively the viral sequences of the contaminant virus or closely related viral species, belonging to the same taxonomic group. This assessment can be conducted by testing *positive* samples artificially contaminated with known viruses or cells infected with known viruses representing different virus categories (see section 2.6). This approach allows for evaluation of the potential effect caused by the matrix in the context of method validation, and it is required to verify that the method can report the correct taxonomic group, species or strain of the viral contaminant.

An optimal level of specificity can be achieved with a properly designed laboratory workflow and bioinformatic analysis pipeline (see section 2.8 for additional details). The discrimination of real viral signals from the background can be achieved with a high stringency of the bioinformatic analysis (*e.g.* assembling and/or alignment methods, threshold definition, and taxonomic assignment approach). At the same time, high sensitivity requires a more relaxed stringency of the analysis, particularly for the detection of unknown viruses, with the possible downside of increasing background noise. Several factors can lead to uncertainties or inaccuracies regarding the origin of a sequence, including highly

conserved regions among different viruses, endogenous viral sequences like retroviruses, homologous phages, vector sequences inserted into the host cells' genome, viral sequences derived from laboratory reagents or materials, and poor quality of the sequences in the database. Moreover, since part of these background signals originates from the sample matrix, the specificity is highly dependent on the matrix itself.

Another complexity in assessing the specificity of an NGS-based method is that the bioinformatic pipeline might not always be sufficient to automatically discriminate genuine viral sequences from background noise. Ultimately, like any broad range test, NGS usually requires a final level of manual curation from a field expert in order to validate the pipeline results and exclude background noise coming from multiple sources (*e.g.* sequencing error, environment, conserved regions).

2.2 Detection Limit

The *detection limit (DL)* of an analytical method is the lowest amount of analyte in a sample that can be detected, but not necessarily quantified as an exact value[24]. During validation, it is therefore required to demonstrate the performance (analytical sensitivity) of the NGS-based method to detect low levels of contaminant viruses present in the sample. To assess the DL, samples can be artificially spiked with different quantities of model contaminants to mimic different levels of contamination. Of note, the sample should be spiked prior to the initial steps of the method (*i.e.* sample preparation and nucleic acids extraction), then the standard method workflow is applied to verify at which analyte concentrations the method can detect the viral contamination.

The exact approach to be followed for DL assessment should be decided based on the sample type (analyte and matrix) and method workflow, but at least two approaches have been proposed, in which the analyte is either viral particles or infected cells[20]. The first approach interprets the DL as the lowest number of viral particles (preferably expressed in viral genome copies) that the method is able to detect. This can be achieved by spiking samples with known quantities of the virus in a defined matrix background (*i.e.* fixed number of cells, or manufacturing harvest, or viral seed). In the second approach, the DL is defined as the minimal number of infected cells that can be detected in non-infected cells. Various infected cell models can be used, such as naïve infections, latent infections, or chronically infected cells. Table 3 summarizes the proposed approaches for DL determination, highlighting the type of sample, application, advantages, and limitations. Other approaches can also be used if scientifically justified.

Table 3: Possible Approaches for DL Determination of NGS-Based Methods

	Viral Particles Approach			Infected Cells Approach
DL Unit	Lowest number of viral genome copies that can be detected			Lowest number of infected cells into non-infected cells that can be detected
Analyte for DL determination	Viral nucleic acid			
Sample type examples	Viral Seed	Cell Line	Manufacturing harvest	Cell Line
DL experiments	Fixed amount of sample as background, spiked with different known quantities of model viruses			Non-infected cells as background spiked with different known quantities of model virus-infected cells
Advantages	Control on the number of genome copies at the time of DNA/RNA extraction Easy to test several model viruses			Mimic a sample where cells are infected and the virus is actively replicating Sensitivity is similar to a real-life contamination
Limitations	Does not mimic a test sample containing infected cells (possible underestimation of method sensitivity)			Cell type and growing conditions impact viral replication, thus influencing the DL estimation Demanding to test several model viruses for the cell model during validation

While NGS can detect a broad range of viral species, it is not possible to determine the DL for all the detectable viral contaminants. Instead, viral and infected cell models representing the different viral categories should be selected and used to prepare the spiked samples, covering different classes of viral genomes (DNA/RNA, single-stranded/double-stranded), as well as enveloped and non-enveloped, and the different morphologies. A risk assessment might be put in place to select the viruses used for the validation. Viral and cellular models should be thoroughly characterized as described below (see section 2.6). Depending on the experimental conditions and workflow, multiple viruses can be used to spike one single sample, before the extraction step, thus allowing to reduce the total number of samples that should be analyzed in the frame of the validation[25].

Giving the diverse morphological and biochemical nature of viral particles, and also giving the different interactions between viruses and cells, the sensitivity of any NGS protocol is different for the different virus types (*e.g.* DNA vs RNA viral genomes). As a result, the detection limit of a NGS-based method depends on the actual contaminant species. Since different DL values are usually obtained for the different viral and cellular models tested, the DL of the NGS-based method can ultimately be expressed in three possible ways: i) multiple DL values, each one specific to a single model virus tested; ii) a range of concentrations (*i.e.* lower and upper values, corresponding to the DL of the viruses for which the method has the best and worst sensitivity); iii) the DL of the virus for which the method has the worst sensitivity. The third option is an underestimation of the real performance for most viruses, but it is the only way of summarising the minimal sensitivity expected for all the viral families in a single value. A risk assessment can be put in place to assess the risk of missing the detection of some viruses based on their DL.

The detection limit can be impacted by the applied NGS experimental workflow[26] and by the matrix. The different sample types can indeed influence the performance of the laboratory workflow and therefore inhibit the capability of NGS to generate strong positive signals in the presence of actual contaminations. Moreover, the sensitivity of the method, similarly to its specificity, depends also on the approach of the bioinformatic pipeline[21], as well as on the coverage metrics used to discriminate

genuine signals from the background and the other information integrated during data analysis (see section 2.8).

An important aspect to be considered during the assessment of the detection limit is the variability introduced by the sequencing depth, because the achievable sensitivity in the detection of viral sequences is directly correlated with the number of sequencing reads generated for any particular sample. However, in most sequencing experiments the generated sequencing throughput per sample cannot be exactly defined upfront. While method design should include validity criteria for the sequencing depth to be reached during the sequencing run, sequencing depth variability within the accepted range would still affect the measured DL. To control this effect, different strategies could be adopted. One way is to conduct DL measurements at the lowest accepted value of sequencing depth (*e.g.* applying random down-sampling on the generated reads). A second possibility is to conservatively set a single acceptance criterion after the validation, using the lowest measured sequencing depth where DL could be confirmed. Finally, a third approach is to model the relationship between the measured signal and the sequencing throughput in order to statistically infer the DL at the lowest accepted value of sequencing depth.

In summary, the detection limit demonstrated for a NGS-based method may depend on many parameters, including the specific workflow, the model viruses, or the sample matrix. The observed detection limit(s) needs to be considered in the context of the overall virus control strategy (as further developed in the next section 3).

2.3 Robustness

In addition to specificity and detection limit, *robustness* should be evaluated. Robustness can be either included as part of the validation or evaluated during the development phase as described in ICH Q2(R2)[24] and ICH Q14[27]. Robustness should show the reliability of the analysis with respect to deliberate variations in method parameters. NGS-based methods are made up of different analytical steps, therefore a risk assessment could be applied to identify critical steps and consequently critical parameters. This risk assessment can be based on generic knowledge about the technology used, on method-specific knowledge acquired during method development and/or on historical knowledge of similar methods. Then, the critical parameters identified should be deliberately variated to demonstrate the robustness of the analytical method. Examples of steps of the workflow in which critical parameters can be identified and tested are the sample extraction, library preparation, and sequencing. Robustness can be evaluated using artificially contaminated samples, such as the ones used for the DL determination. In addition, whenever the method requires the analysis of different sample types with substantial matrix differences, the reliability of the analysis with respect to matrix variations should be assessed. If all the different matrix types cannot be tested, an approach could be to use a matrix with a high load of interfering substances like host cell nucleic acids or vaccine virus load as a worst-case scenario to be tested.

2.4 Structure of the Method Validation

When evaluating the parameters mentioned above, all steps of the NGS-based method for adventitious virus detection, from nucleic acid extraction to bioinformatics analysis, should be included and validated. There are essentially two possible approaches to address validation. In the first approach, a single validation protocol is prepared in which the end-to-end performance of the test (from nucleic acid extraction to bioinformatics) is evaluated. In the case of future method modifications, a risk assessment approach can be applied to determine if the modification introduced

has an impact on intermediate steps or on the final result of the test. In case the modification is critical (for example for a change of matrix or in the workflow, both potentially impacting detection limit), an addendum validation protocol can be executed in which only the parameters impacted by the modification are re-evaluated. If the modifications only impact the bioinformatic part, sequence data generated during the previous validation studies can be reanalysed with the modified bioinformatic workflow, without the need to replicate the experimental part of the validation.

In the second approach, a dedicated protocol is edited for the single steps of the workflow (nucleic acid extraction, library preparation, NGS sequencing platform, bioinformatics), as well as for optional steps (*e.g.* amplification, depletion…). The complete NGS-based method validation is therefore obtained by a combination of protocols. In case of modifications, protocols must be re-evaluated independently through a risk assessment, according to the modifications applied to the respective step (*e.g.* kit, equipment, materials, etc.). The impact on the final test result should also be evaluated. For instance, if the sequencing platform used in the method is replaced by a new generation of equipment and from the risk assessment there is no impact on the test result, the entire workflow should not be revalidated, but only the protocol related to the sequencing step.

2.5 Pre-requisites for Method Validation

In order to validate a NGS-based test method, several necessary elements are needed, including trained personnel, as well as established Standard Operating Procedures (SOP) and Record Forms for the method under validation. Moreover, all the equipment must be qualified and the bioinformatic pipeline validated before being used for the method validation. Among other things, suitable controls should be implemented to ensure data integrity for all the input and output data of the electronic and software systems, including the implementation of measures to protect data against accidental loss or damage (*i.e.* backup system), and tampering or unauthorized manipulation (*i.e.* security controls to limit access to computerized systems only to authorized persons)[28]. Furthermore, procedures should be in place to guarantee data completeness, availability, and readability throughout the retention period. With respect to the bioinformatic pipeline, the validation should be conducted according to relevant guidelines[29,30,31], and this step will require documenting several aspects, including the pipeline version, code, user requirements, and system suitability criteria. The performance qualification (PQ) of the bioinformatic pipeline can be assessed using datasets containing known viral signals to verify the defined operating performance parameters. However, PQ does not need to qualify the capability of the software to identify viral signals because this aspect is assessed in the context of the overall method validation.

In addition, the method should be well-developed, understood, and documented. Among other things, for those cases in which only an aliquot of the tested material is analyzed with NGS, the effect of the sampling procedure on the overall probability of virus detection should be assessed during method development, ensuring that the volume of the sample and its preparation for testing are fit for the intended use[2]. In addition, because several experimental steps of the NGS workflow can impact the overall method performance, method development should include the evaluation of the relevant alternative technological approaches, in order to assess their impact on the final performance and guide the definition of an adequate experimental workflow. This consideration is true for all the steps of the analytical procedure but is particularly critical for nucleic acid extraction, a step known to significantly impact the overall method performance. The final method workflow defined during method development and its performance should be fit for the intended method application. This includes, in order to ensure appropriate specificity, that nucleic acid background signals originating from the matrix are characterised.

In order to guarantee the expected performance during routine method execution, the workflow of the method should include suitability tests to check for unexpected behavior that could affect the method performance. Since a NGS workflow is made of multiple steps, each part of the method flow should include tests having pre-defined system suitability criteria. Whenever the system suitability criteria of a specific step are not satisfied, the performance of the method might be affected, therefore the test would be invalid. This approach should cover the entire laboratory workflow, from nucleic acid extraction (*e.g.* testing extracted material quality and quantity), to library preparation and sequencing, for which system suitability criteria will depend on the library and sequencing technologies of choice (*e.g.* assessing library quantity and size distribution or verifying sequencing yield and average quality). Moreover, a similar approach should be used for the bioinformatic analysis, assessing all the critical parameters in the raw sequencing data (*e.g.* minimum number of reads per sample) and in the relevant intermediate steps of the pipeline. These criteria depend largely on the bioinformatic approach, but possible tests include assessing the performance of the read trimming, host cell subtraction, mapping (*e.g.* mapping frequencies), assembly (*e.g.* contig count or length statistics), and others. A possible additional approach to ensure the overall method performance is to add in the test sample a known quantity of a virus or control nucleic acid as an internal control. The signal on this internal control can then be used during bioinformatic analysis to verify that the method performed as expected. This type of control can be especially important to exclude false-negative results whenever the test indicates the absence of viral contamination. A similar approach could be used by spiking *in silico* reads in generated data to confirm that the bioinformatic pipelines are performing as expected.

2.6 Standards for Method Validation

Well-characterized reference standard materials should be used during method validation. Viral stocks can be either made in-house or purchased. Before the use in method validation, several aspects of the model viral stocks should be determined, including the closest sequence ID to their viral genome, as well as the TCID50 (infectivity) and/or the genome copy number and/or the number of virus particles and the presence of contaminant sequences (of potential interest during specificity evaluation). Efforts are currently ongoing at NIST (National Institute of Standards and Technology), U.S. FDA (Food and Drug Administration), and NIBSC (National Institute for Biological Standards and Control) to provide well-characterized reference virus stocks to support the implementation of innovative virus detection methods. In 2019, a NIST-FDA workshop on standards used for NGS detection of viral adventitious agents in biologics and biomanufacturing was organized and the outcome was published[32]. The workshop aimed to identify what viral standards are publicly available and in use for NGS virus detection and to identify gaps in terms of the availability of viral standards. In the last years, FDA prepared the reference viral stocks for NGS platform evaluation and standardization. Details were presented at the 2020 NIST-FDA workshop[33]. Recently, these stocks were included in the WHO reference standard material panel[34]. Finally, the NIBSC is developing reference materials that could be used in bridging and validation studies[33]. Those reference materials have been recommended as the minimum panel of model viruses for validation in the draft EDQM general chapter on NGS for viral extraneous agent detection[19].

As well as for viral stocks, chronically or latently infected cells used in the frame of the validation (infected cells approach) should be characterised. At least the closest sequence ID to the viral sequence, the quantity of viral nucleic acid generated by the cells (number of copies of viral RNA per million cells), and the presence of contamination should be determined before method validation. To our knowledge, there is currently no publicly available reference standard material of this kind.

2.7 Requirements of the Viral Database

The capability of NGS-based methods to detect viral contaminants relies heavily on the completeness of the viral database used for the analysis. If specific viruses should be detected by this method, it is important that sequences of those viruses are included in the viral database, or that at least a distantly related sequence from the same family is included. The database might annotate information on single sequences (*e.g.* taxonomical information) and its structure should be compatible with the bioinformatic pipeline workflow. An example of such database is the Reference Viral Database (RVDB), a publicly available and routinely updated database that was generated using semantic selection criteria to include all viral, viral-like, and viral-related sequences, but excluding bacteriophages[35]. While RVDB is a very comprehensive viral database, the content of the database to be employed should be defined based on the needs of the testing laboratory in terms of the viral families that must be detected. Of course, the broader the database, the more viral contaminants could be detectable by the NGS method. However, larger databases will require higher efforts for results interpretation and more computational resources and time during data analysis[35]. Depending on the test sample, it is possible to restrict the type of viral sequences in the database to those viral species that could infect the test sample (*e.g.* in the case of mammalian cells, only vertebrate-infecting viruses). This strategy can simplify the result interpretation and reduce computational time/resources, but the choice of viral families/species included should be justified through a risk assessment approach.

Proper versioning of the database and its content should be in place in order to track the viral sequences covered by the method. Depending on the application, the database should be updated regularly. Defining a standard update frequency is however not always possible *a priori*, because the need to update the database depends largely on the general knowledge of the field. For example, it would be recommendable that a database is updated if a previously unknown viral contaminant capable of infecting the test sample has been recently reported in the literature, especially if identified in a relevant context (*e.g.* large-scale contamination of a manufacturing facility). Moreover, an updated database could be used during follow-up investigations of positive results to include in the analysis the most up-to-date sequence information.

An update of the database may potentially affect the specificity and sensitivity of the method; therefore, it might have an impact on a previously validated method. Assuming that the format and structure of the database are not modified (in which case the pipeline might have to be adapted and revalidated), it is necessary to verify that the pipeline still operates properly. Regression testing can be performed by re-running tests developed during pipeline validation to ensure that the previously developed and tested pipeline still performs as expected. The employed data can be the controlled datasets used during pipeline validation. Moreover, prior to using a new database version for production, it should be verified that the new version of the database has no impact on the method validation. This assessment can be performed by re-analyzing previously generated datasets with the new database version, to exclude a significant effect on method sensitivity or specificity. The employed data could be a subset of controlled datasets built on purpose for validation.

2.8 Requirements of the Bioinformatic Analysis

Among other aspects, the performance of the NGS-based method depends greatly on bioinformatic analysis[21]. For this reason, as previously discussed, it is important that the bioinformatic pipeline is validated prior to method validation. The validation of the pipeline can increase the software usability and reliability and ensures that the analytical approach applied during routine method execution is fixed and well defined.

The bioinformatic pipeline should provide the optimal level of sensitivity and specificity by automatically evaluating different coverage parameters on the reference viral sequence (*e.g.* number of mapped reads, horizontal coverage, distribution along the sequence, sequence similarity to the reference), as well as by integrating other information, such as on conserved regions, endogenous retroviruses, known background sequences, or sequencing data from previous reference samples. Bioinformatic analysis should use this information to discriminate real viral signals from the background. Notably, bioinformatic parameter cutoffs for identification of positive signals can be adjusted during method development and possibly in the context of the method validation, but they should be fixed during routine method execution. Moreover, since they are part of the method definition, the same cut-off values should be applied during the entire method validation, including the assessment of specificity and detection limit.

For a non-targeted NGS test, it should be considered that the breadth of virus detection of the method is highly related to the capability of the bioinformatic pipeline to detect contaminant sequences, even those with some distance to the reference viral sequences included in the database. Among other factors, the method performance for any particular virus will depend on its similarity to the sequences present in the database and the stringency of the bioinformatic pipeline. During method development, it is, therefore, advisable to assess the capability of the pipeline to detect unknown viral contaminants based on sequence similarity. A way to systematically assess the effect of sequence similarity on the method performance is the analysis of datasets that have been "contaminated" *in silico*, spiking the data with a series of sequences having a variable degree of similarity to known viral species included in the database. Therefore, with this approach it is possible to verify whether synthetic novel viruses (*i.e.* unknown and therefore not present in the viral database) can reliably be detected by the method, thus assessing not only *in silico* sensitivity/specificity but also the breadth of detection. Notably, very good sensitivities would be without any practical interest if they are obtained at the price of decreasing the breadth of detection. In addition, if the bioinformatic pipeline is not too stringent, it should be possible to detect new strains or viral species even in regulated environments. Despite that, detection of the novel or distantly related viruses (*e.g.* members of a new genus or family) might be challenging during routine method execution, because the full characterization of a new virus usually requires manual data interpretation and further analyses defined case-by-case. This type of analysis can however be conducted in the context of an investigation, or for research purposes in non-regulated environments.

3 Comparability of NGS-based Methods with Existing Virus Safety Tests

Validation studies evaluate and confirm the performance of a novel method for its intended purpose. When a new method is implemented as a supplementary or complementary test in the virus safety testing panel, validation studies are sufficient to support implementation, along with appropriate regulatory activities (see section 4). However, when a new method is implemented to replace a conventional virus detection assay, method validation may be expected to be supplemented with comparability studies between the new method and the test to be replaced, demonstrating that the new method provides equal or greater assurance of viral safety for the biological product.

The comparability evaluation of two analytical methods should not necessarily be restricted to the strict statistical equivalence of experimental results. This is not the only valid approach, and it may have limitations when the two methods do not measure the same attribute or use different analytical principles, as it is the case for NGS-based methods compared to conventional virus tests. One may also prefer the broader term of non-inferiority, which makes clear that a method with a better performance

is acceptable as replacement. In this section, we will use the term comparability as encompassing non-inferiority. This is aligned with the interpretation proposed by US FDA of the concept of analytical comparability[36]: an analytical comparability study should demonstrate that "the new method coupled with any additional control measures is equivalent or superior to the original method for the intended purpose".

Considering the diversity of existing virus detection tests that could be replaced by NGS (as presented in the section 1), a general methodology for analytical comparability is proposed. It is applied to several possible cases, highlighting the type of data or evidence that can support the intended replacement, as well as some limitations and challenges.

The proposed general strategy to address comparability between two analytical procedures with the perspective of replacing one with the other includes three main steps:

1. A pre-assessment to compare the intended purpose and targeted quality attributes of each analytical method to verify the extent to which it is scientifically justified to compare the analytical results or decisions provided by each of them, and to guide the design of a potential comparability study.
2. When justified, an experimental comparability study to compare the performance characteristics and analytical results of the two procedures, to determine their ability to reach the same pass/fail decision for a given attribute, and to provide an equivalent level of control of product quality.
3. In case the experimental comparability study is not scientifically justified or provides partial justification for the replacement, additional elements identified through a risk/gap analysis should be provided to Health Authorities to justify that the proposed replacement is acceptable.

The pre-assessment includes three questions that can guide the user to determine to what extent an experimental comparability study is scientifically justified. The pre-assessment is theoretical and scientifically driven:

- **Q1:** Is the **intended purpose** of the alternative method achieved by measuring the same type of **Product Quality Attribute**[b,37] as the reference method, or minimally is there a relationship between them?

  In some cases, the same intended purpose can be achieved by focusing on different Product Quality Attributes. For adventitious virus detection, demonstrating the absence of contaminants is considered the same intended purpose (see Table 4). The extent of the correlation between the Product Quality Attributes helps to identify if a comparability study is scientifically justified and if it provides meaningful information.

- **Q2:** Do the two methods use the same **signals or read-outs**, or is there at least scientific evidence of a relationship or correlation between them?

  The design of the experimental comparability study depends on the possibility of establishing a strong correspondence between the responses obtained with the two compared methods.

[b] A Product Quality Attribute is a molecular or product characteristics selected for its ability to help indicate the quality of the product. Collectively, the quality attributes define identity, purity, potency and stability of the product, and safety with respect to adventitious agents (ICH Q5E (2004) Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Processes).

The level of required correlation relies on the analytical measurement type (e.g. qualitative, semi-quantitative or quantitative).

* **Q3:** Is the **test category** of the alternative method the same as the reference method?

  There are three main test categories: qualitative (e.g., identification, characterisation), semi-quantitative (e.g., limit test), and quantitative (e.g., purity, assay). Usually, methods with the same test category are compared together, however different test categories may be compared, for example if a quantitative method can appropriately replace a semi-quantitative or qualitative test.

The answers to these three questions are used to define whether an experimental comparability is scientifically justified and sufficient to support the replacement of the reference method with a new one. It clarifies the type of information that will be provided, and if additional elements / rationale are necessary to justify method replacement.

3.1 Pre-assessment of a Broad Range NGS-based Method as Potential Replacement for Conventional Virus Safety Assays

Table 4 presents the pre-assessment considerations for the replacement of various conventional virus detection assays with NGS. The assessment focuses on a broad range NGS-based method aimed at detecting viral genomes in a non-targeted manner (i.e. no prior sequence information considered). A similar pre-assessment can also be performed for other formats of NGS-based methods.

The Table 4 illustrates that the comparability of NGS-based methods with conventional assays is not straightforward due to differences at the Product Quality Attributes level: most conventional assays (*in vivo*, cell-based, antibody production tests) detect signals produced by infectious viruses (mortality, cytotoxicity, antibodies…), while nucleic-based methods (NGS, PCR) detect viral nucleic acids. In such cases, only a partial correlation can be made between the results of NGS and the conventional method. For example, a sample containing viral genomes without infectious capacity would be ranked as positive by NGS or PCR and negative by *in vivo* or cell-based infectivity tests. Also, NGS is capable of detecting live viruses that failed to be identified by infectivity assays. In order to avoid the unnecessary rejection of batches, it is recommended that the typology of the potential virus sequences identified by NGS is examined by qualified experts who will inspect the available information on the reference viral sequence (*e.g.* source, biological sample type, sequence completeness) and combine it with information on the testing environment, test controls and the history of the test sample. This assessment will allow to i) reject the results as false positive or as inert sequences upon justification or, on the contrary, ii) identify the result as suggestive of a contamination that can be confirmed, if necessary and possible, by other methods, such as infectivity assays or expression analyses.

Additional complexity arises from the fact that virus detection assays detect a plurality of viruses rather than a single analyte. Each analytical method exhibits a different breadth of detection, depending on its analytical principle and design (see Table 1). For this reason, the breadth of detection of the proposed NGS-based method should be compared to the current method and shown to be appropriate for the intended purpose considering the overall virus control strategy.

Table 4: Pre-Assessment of a Broad Range NGS-based Method as Potential Replacement for Conventional Adventitious Virus Detection Assays

	Non-targeted NGS	***In vivo* viral screening assays**	**Cell-based infectivity assays**	**Antibody production tests (HAP, MAP, RAP)**	**PCR**
Q1: Is the intended purpose of the alternative method achieved by measuring the same type of Product Quality Attribute as the reference method, or is there at least a relationship between them?					
Intended purpose of the analytical procedure	Detection and **identification** of the viral nucleic acid sequences	Detection of live viruses	Detection of live viruses	Detection of live viruses	Detection and identification of specific viral nucleic acids
Quality Attribute tested	Absence of viral nucleic acid sequences	Absence of live viruses with pathologic effect in permissive animals	Absence of live viruses with visible phenotype in permissive cells	Absence of viruses with immunological response in rodents	Absence of specific viral nucleic acids
Answer to Q1	-	PARTIALLY: Any live virus generates virus nucleic acids. However, naked nucleic acids, inactivated and non-replicative virus particles, and virus-like genomic sequences will not always trigger a reaction in animals or indicator cells.			YES: Product Quality Attributes are similar.
Q2: Do the two methods use the same signals or read-outs, or is there at least scientific evidence of a relationship or correlation between them?					
Read-out / signal measured	Generation of sequences attributable to specific viral nucleic acids	Animal mortality and testing of tissue or fluids for the presence of viral proteins	Observation of cells productively infected by a virus: cytopathic effect (CPE), hemagglutination (HA), or hemadsorption (HAD)	Animal antibodies against specific viral components	Amplification of a specific virus nucleic acid fragment
Answer to Q2	-	NO: No direct quantitative relationship exists between the indicators.			
Q3: Is the test category of the alternative method the same as the reference method?					
Test category	Limit test	Limit test	Limit test	Limit test	Limit test
Answer to Q3	-	YES Because the tests are limit tests, it would be sufficient to demonstrate equivalent or superior capability of detection for the alternative method compared to the reference one to be able to reach the same decision.			
Conclusion of theoretical pre-assessment	-	Comparability can be partially evaluated experimentally using model viruses. Similarity/ superiority of breath of detection needs to be justified in addition.			Comparability can be fully evaluated experimentally for viruses in scope.

Finally, since all these tests are considered as limit tests, the ability to obtain the same pass/fail decision for viral detection can be evaluated by comparing their detection limits. As analytical sensitivity depends on viral type and characteristics, such a comparison should be performed using a reference virus panel representative of the virus population (see section 2.6). However, quantitative comparison of detection limits is dependent on establishing a correlation between the read-outs measured by the two analytical methods.

These general observations translate differently considering the specific conventional virus assay that is intended to be replaced with NGS. The next sections cover each of the conventional assays potentially replaced by NGS.

3.2 *In Vivo* Viral Screening Assays

The replacement of *in vivo* viral screening assays by NGS-based methods focuses a lot of interest. While NGS is recognized for providing very broad virus detection (which is the primary purpose of *in vivo* viral screening assays), it brings multiple advantages such as: a decreasing animals use (3R initiative), improving virus detection, and information on the identity of the viral contaminant at the time of detection. It has been widely recognized that the experimental comparability of NGS-based methods and *in vivo* assays provides limited added value. The conventional *in vivo* assay is not validated according to the current regulatory requirements[24], "making a formal one-to-one comparison challenging or even impossible in some cases", according to chapter 5.2.14 from the European Pharmacopoeia[8]. Consequently, the execution of animal tests as part of a validation or comparability study is strongly discouraged in some countries. In this context, it is noteworthy that several Health Authorities have agreed, as part of the recently approved ICH Q5A(R2)[2], on the possibility to use NGS-based methods as replacement for conventional *in vivo* assays without comparison with animal data. As ICH Q5A(R2) indicates, "A head-to-head comparison is not recommended due to the different end points of the assay systems and limitations of the breath of virus detection by the […] *in vivo* method compared to the enhanced capability of NGS for broad virus detection".

In the absence of an experimental analytical comparability study, a justification that the alternative NGS-based method has appropriate specificity, detection limit and breadth of detection is required to support the implementation of the method for the intended purpose and proposed replacement of the reference method (see section 2). For the replacement of *in vivo* viral screening assays, the demonstration of an extensive breadth of virus detection for the new assay is important, indicating the ability to detect unexpected viruses. The experimental demonstration that the method is capable of detecting all classes of viruses (addressed during validation) can reasonably be complemented by the theoretical evaluation of the scope of virus families included in the viral genome database. In addition, as previously detailed in the validation section, spiking of sample and/or in silico datasets with synthetic nucleic acids mimicking distant strains and even novel viruses can provide further characterisation of the ability of the NGS-based method to detect viruses not yet included in viral genome databases.

Defining what can be considered an appropriate detection limit is challenging without reference data and may be subject to interpretation. There were few published studies comparing the detection limit of *in vivo* viral screening assays with NGS-based methods. Charlebois et al. used the virus stocks developed by Gombold et al.[7] to determine the detection limit of their viromic NGS-based method and compare it with the sensitivity reported for *in vivo* assays and cell-based infectivity assays[25]. The study demonstrated that the NGS-based assay has better sensitivity (10 to 10^6-fold) than *in vivo* assays, except for two viruses detected at extremely low levels in the animal models[25]. Another comparative study published by Beurdeley-Fehlbaum et al. in 2023 demonstrated as well broad detection of diverse

viruses using the transcriptomic NGS approach[38]. The study demonstrated a robust performance of the NGS method with the ability to successfully detect one infected cell in a background of 10^3 to 10^7 non-infected cells. This analytical sensitivity was at par or superior to the sensitivity of *in vivo* assays for 7/9 model viruses[38]. The results from these two studies using different NGS formats (viromic and transcriptomic) further support the benefit of replacing *in vivo* assays by NGS-based methods.

However, it must be highlighted that utilising the output of few available studies on detection limit comparison of *in vivo* assays versus NGS-based methods should be interpreted with caution, since the detection limit (as determined in a validation study) results from a trade-off between the ability of the method to detect very low levels of viruses and the cut-off criteria set to minimize the risk to retain false positive signals coming from other nucleic acids. Ultimately, the acceptance of NGS-based method as replacement for *in vivo* animal assays should be decided in the context of overall viral control strategy, including the other performed tests and the capability of the process to clear or inactivate potential contaminants (usually more than 4 logs of clearance are demonstrated).

3.3 Antibody Production Tests (MAP/HAP/RAP)

In addition to *in vivo* viral screening assays, replacement of other animal-based virus safety tests with NGS can also be considered in the mid-long term. Antibody production tests (MAP/HAP/RAP) target a much narrower scope of viruses and are usually considered as highly sensitive tests. Therefore, they can be appropriately replaced by PCR-based assays targeting the subset of viruses mentioned in ICH Q5A[2]. As an example, multiplex degenerate PCR methods have already been developed, validated, and successfully implemented as part of viral control strategy of development products. NGS-based methods can also replace the antibody production tests if they are developed to ensure sufficient sensibility (with the corresponding effort in terms of cost and time due to the need to reach a sufficient sequencing depth). ICH Q5A(R2) indicates that "NGS (targeted or non-targeted) can replace […] rodent antibody production tests […] without a head-to-head comparison"[2].

3.4 Cell-based Infectivity Assays

ICH Q5A(R2) indicates: "Non-targeted NGS may also be used without a head-to-head comparison to supplement or replace the *in vitro* cell culture assays for detection of known and unknown or unexpected viruses. This could address general limitations of the in vitro cell culture infectivity assay (e.g., susceptibility of cell lines to infection) and specific limitations of the production system (e.g., test article-mediated interference or toxicity)."[2], opening the door to cell-based infectivity assay replacement without the need for an analytical comparability study.

It needs to be highlighted that cell-based infectivity assays play a more critical role than *in vivo* assays for viral safety strategies, as they have been able to detect several virus contamination events in actual manufacturing conditions[1,39]. Therefore, when considering replacing cell-based infectivity tests by NGS-based methods, it is important to thoroughly characterise the performance of the new method and ensure that contamination events possibly detected by cell-based infectivity assays would be detected by the alternative test as well, if replaced. In this respect, the generation of analytical comparability data is seen as valuable, even if it may be envisaged to replace cell-based infectivity assays with NGS without a comparability study when NGS validation results demonstrate extensive and sensitive virus detection in line with the needs outlined by the virus risk assessment.

NGS-based methods may also be proposed to supplement the cell-based infectivity assays to overcome specific limitations of these assays. Those limitations include absence of virus detection, for example

when neutralization is limiting, or when the viruses are not replicating in the indicator cell lines, but also incompatibility of the test with some cytotoxic samples. These applications lead to an improvement of the adventitious virus control strategy and do not require analytical comparability for implementation.

The comparability between NGS-based methods and *in vitro* cell-based assays may be more easily studied experimentally than for *in vivo* assays, due to the absence of ethical concerns, and the possibility to generate bridging data. But intrinsic variability related to the use of cell-based assays may significantly limit the power of the comparability study, in addition to the impossibility to exhaustively compare the performance of the two tests for all the viruses in scope. Experimental comparison may be envisaged using a panel of viruses carefully selected to represent a worst-case scenario, either because they are detected at low levels by one of the methods, or because they represent a significant risk in terms of viral safety and/or contamination. Considering that the two methods do not detect the same Product Quality Attributes nor the same signals, the detection limits would need to be compared using virus stocks which are characterised both in terms of genome copy numbers and infectivity units. To that purpose, the well-characterised virus reference stocks already referenced in section 2.6 constitute useful resources; although the method developer can also generate and characterise its own virus stocks.

Some differences in terms of performance between cell-based infectivity assays and NGS-based assays may be expected for the following (non-exhaustive) reasons: direct NGS-based methods may present a lower sensitivity for some viruses, considering that the conventional cell-based assays include a 28-days cellular amplification step. On the other hand, as already mentioned, NGS-based methods are expected to detect viruses failed to be picked up by cell-culture infectivity assays. In that respect, it is important to re-emphasise that even if comparability results are useful to characterise the performance of the new method and its fitness for purpose, equivalent specificity, detection limit or breadth of detection is not necessary. Differences in analytical sensitivity (e.g. the capability to detect low amounts of substance) and breadth of detection should be discussed with consideration of the diagnostic sensitivity (e.g. the probability to detect a contaminated sample) of the overall virus safety control strategy, specific risks arising from the manufacturing process and corresponding mitigations in place (see for example[38]).

3.5 PCR

Replacement of a PCR test with an NGS-based method can be relevant. An NGS-based broad virus test can cover the purpose of a pre-existing PCR virus-specific test, and in some cases increase virus detectability of viruses having many variants (which can impact PCR performance). In this situation, ICH Q5A(R2) indicates that the replacement can be envisaged "without head-to-head comparison"[2]. Analytical comparability evaluation between PCR and NGS is straightforward due to the similarity of the Product Quality Attributes tested by the two methods. Because the breadth of detection is also limited, the comparison would focus mostly on detection limits. However, some biases may limit the significance of the results generated. For example, in PCR assay, the detection limit is often validated with a model virus whose sequence perfectly fits the primers (best case situation), leading to possible overrating of the method sensibility. In contrast, NGS-based methods may be more robust to small sequence differences, not relying on prior sequence information.

In conclusion in this chapter, a general methodology to approach comparability study between NGS-based methods and conventional virus safety tests was proposed and illustrated with several examples. Ethical, scientific and operational limitations of experimental comparability studies were

highlighted, prompting consideration of other elements to support the replacement of some conventional tests, especially *in vivo* virus safety assays. It was indicated that the performance of the NGS-based analytical methods is the primary driver for acceptance, and as described in revision 2 of ICH Q5A, it does not necessarily require a head-to-head comparison. The generation of comparability results can provide strong evidence of appropriate method performance, especially in case of replacement of cell-based infectivity assays or PCR, but the demonstration of equivalent performance is not necessary. The results should rather be discussed based on a risk analysis considering the overall virus safety control strategy. Finally, the comparability study or the justification for absence thereof is one of the elements that will have to be provided to Health Authorities during a regulatory submission to implement an NGS-based virus safety test as a replacement for a conventional one.

4 Regulatory Strategy for Registration of NGS for the Control of Biological Products

This last section is intended to serve as a guide to elaborate a suitable regulatory strategy with the objective of receiving Health Authorities' approval for the implementation of NGS for adventitious virus safety testing.

The section first clarifies the regulatory framework and provides an overview of the latest Trade Association discussions and positions on the topic. It also provides guidance on the regulatory pathways and national requirements to be followed within a defined scope of key countries (WHO, EU and United Kingdom, USA, Brazil, China, Japan, Canada). Finally, potential regulatory barriers and recommendations to facilitate adoption of NGS are highlighted. Some case-studies presenting recent experience of industry on NGS registration or interactions with Health Authorities are also presented.

It should be noted that the information extracted from regulatory guidelines is effective at the time of writing of this document, and up-to-date guidelines should always be consulted.

4.1 Regulatory Framework

Table 5 summarizes the analysis of regulatory guidelines relevant for the viral safety control for biological medicinal products and vaccines, discussing how they may impact the introduction of NGS-based assays for adventitious virus detection.

Table 5: Key Messages from Relevant Regulatory Texts in Relation with NGS Introduction (non-exhaustive list)

Organisation (Country)	Guideline / Standard Reference	Regulatory environment and considerations impacting implementation of NGS
ICH	ICH Q5A (R2)[2] Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (2023)	The introduction of new methodologies such as NGS for detecting a broad range of adventitious viruses is encouraged. Non-targeted NGS is specifically encouraged as a replacement for *in vivo* assays, especially to identify unknown or unexpected virus species, without requiring a head-to-head comparison. Additionally, NGS may supplement or replace cell culture infectivity assays without a head-to-head comparison. The use of NGS is considered for the characterisation of the cell line or the testing of cell banks, virus seeds or unprocessed bulk harvests. Suitable reference materials should be used for method qualification and validation to evaluate performance of the different steps involved in the workflow and to demonstrate sensitivity, specificity, and breadth of virus detection. For any NGS method applied, validation/qualification should be provided to support its intended use for the application. This includes method validation and matrix-specific verification when used as a replacement method. Additionally, when used as a supplementary method, this includes method qualification and matrix-specific verification.
WHO	TRS 978 Annex 3 "Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks" (2013)[3]	The guideline recognizes the emergence of new and sensitive molecular methods with broad detection capabilities, highlighting their growing importance in the evaluation of cell substrates. It also recommends that the sensitivity of these methods, as well as their breadth of detection, should be considered when evaluating their applicability.
WHO	TRS 993 Annex 2 "Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine" (2015)[40]	The guideline suggests the use of NGS as a powerful investigational tool that can be explored further with conventional techniques.
ANVISA (Brazil)	ANVISA_RDC 55/2010: RESOLUTION OF THE COLLECTIVE BOARD – RDC No. 55, OF DECEMBER 16, 2010[41]	The regulation provides recommendations for the registration of biological products, notably production and quality control, but it doesn't include specific requirements related to viral safety testing in the control strategy.
ChP (China)	Chinese Pharmacopoeia "Viral Safety Control for Biological Products" 3302 (2020 edition)[42]	The text does not recommend specific analytical methods but rather encourages the use of advanced techniques and methods, such as NGS.
ChP (China)	Chinese Pharmacopoeia "Preparation and Quality Control of Animal Cell Substrates Used for Manufacturing of Biological Products" (current 2020 edition and draft for comments intended for 2025 edition)[43]	The text does recommend specific analytical methods as essential requirements, including traditional tests such as *in vivo* assays (MCB, End-of-Production Cells) and cell-based infectivity assays (MCB, WCB, End-of-Production Cells). For species-specific adventitious viruses, a door is open to implementation of NGS. This guideline is under revision inviting implementation of NGS to replace or complement *in vivo* and *in vitro* assays.
EMA (EU)	EMA/CHMP/CVMP/JEG-3Rs/450091/2012 Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches[9]	This guideline represents EU engagement to reducing or eliminating the use of animals in the development (safety, non-clinical studies) as well as quality control (in-process and/or final product batch testing) of pharmaceutical products. It aims to encourage stakeholders and authorities to initiate, support and accept the development and use of 3Rs testing approaches. Regulatory acceptance is considered as follows:

Organisation (Country)	Guideline / Standard Reference	Regulatory environment and considerations impacting implementation of NGS
		• incorporation into a regulatory testing guideline and/or the Ph.Eur. • acceptance by regulatory authorities of new approaches not (yet) incorporated in testing guidelines but used for regulatory decision making (through a submission of a MA application or variation or qualification of novel methodologies for human medicines) Regulatory acceptance of NGS is progressing through the elaboration of a dedicated Ph.Eur. general chapter (see below, Ph.Eur. 2.6.41) and its qualification as novel methodology. The last part of this section includes case studies illustrating interactions with Health Authorities and NGS-related submissions.
EMA (EU)	EMEA/CHMP/BWP/398498/2005 GUIDELINE ON VIRUS SAFETY EVALUATION OF BIOTECHNOLOGICAL INVESTIGATIONAL MEDICINAL PRODUCTS (2008)[44]	The guideline references to ICH Q5A for the testing of the cell banks and unprocessed bulk harvest, with some flexibility regarding to the extent of the testing.
EDQM (EU)	Ph.Eur. 5.2.3 Cell substrates for the production of vaccines for human use[12] Ph.Eur. 2.6.16 Tests for extraneous agents in viral vaccines[5] Ph.Eur. 5.2.14 Substitution of in vivo method(s) by in vitro method(s) for the quality control of vaccines[8]	Ph.Eur. 5.2.3: This chapter encourages the usage of sensitive molecular techniques, which can serve as alternative to *in vivo* or specific Nucleic Acid Tests (NAT) or as a supplement/alternative to cell culture infectivity assays, in agreement with the competent authority. Ph.Eur. 2.6.16: This chapters allows, with the agreement of the competent authority, the use of broad molecular methods such as NGS either as an alternative to *in vivo* tests, or as a supplement/alternative to cell culture infectivity assays based on the risk assessment. NGS can be carried out with or without prior amplification in suitable permissive cells. In cases of positive results, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health. Ph.Eur. 5.2.14: This chapter encourages the use of *in vitro* methods (including immunological, molecular and physicochemical tests) to replace the animal tests. The implementation of new molecular methods such as NGS as substitutes for *in vivo* methods requires a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods. Considerations developed around these comparisons are considered in the corresponding section of this paper (see section 3).
EDQM (EU)	Ph.Eur. 2.6.41 High Throughput Sequencing for the detection of extraneous agents in biological products (draft published in 2024)[19]	This general chapter on NGS used for testing of viral adventitious agents reflects the consensus emerging from multiple discussions between industry and regulatory agencies. It describes NGS methodologies, providing general considerations on the workflow depending on type of material to be tested, and details on each step of the workflow. The chapter also provides guidance for the validation of HTS methods: general consideration on validation parameters and approach, selection of spiking material, generic and product specific validation aspects. A specific chapter is dedicated to targeted NGS.
JP (Japan)	Japanese Pharmacopoeia “Basic Requirements for Viral Safety of Biotechnological / Biological Products” <G3-13-141> (2021 edition)[45]	The text acknowledges that virus detection methods are improving as science and technology progress. It encourages the application of the most advanced technologies to enhance the assurance level of virus detection. While the recommendation is made for testing cell banks, specific tests are not mentioned. The document provides a list of infectious viruses known to be common between human and animal. Japan refers to Notice Iyakushin No. 329 entitled ``Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin'', reflecting ICH Q5A guideline.

Organisation (Country)	Guideline / Standard Reference	Regulatory environment and considerations impacting implementation of NGS
FDA (USA)	Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993)[46]	The guideline recommends the use of traditional *in vitro* and *in vivo* tests for quality control of cell lines. However, this text dated 1993 and it is expected that US FDA will accept alternative control strategies established according to ICH Q5A(R2), which it adopted in 2024.
FDA (USA)	FDA Guidance for Industry "Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications" (2010)[4]	The guideline recommends specific virus safety tests, but it allows some flexibility to modify or replace some of these tests with advanced technology where scientifically justified (except if the replacement is prohibited by Regulation, i.e. tests included in CFR). It also encourages manufacturers to consider replacing, refining, or reducing the use of in vivo tests.
FDA (USA)	9 CFR 113.53 « Requirements for ingredients of animal origin used for production of biologics"[47] 9 CFR 113.47 Detection of extraneous viruses by the fluorescent antibody technique[48]	These texts applies to raw materials, including those used for cell bank establishment. NGS is not mentioned in the text, but selected viruses detection could be covered by adding the corresponding sequences to the NGS database.
FDA (USA)	21 CFR 610.9 Equivalent methods and processes[49]	This text requires demonstration that the modification of any particular test method provides equal or greater assurance of the biological product quality, safety and efficacy. Considerations and discussions around comparability of test methods are discussed in the corresponding section of this paper (see section 3).
FDA (USA)	USP <1042> CELL BANKING PRACTICES FOR RECOMBINANT BIOLOGICS[50] USP <1050> VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN[51] USP <1239> VACCINES FOR HUMAN USE —VIRAL VACCINES[52]	<1042> Conventional tests (incl. *in vivo* assays) are listed as examples, but cross reference is made to the version in force of ICH Q5A as appropriate reference to establish the testing panel. <1050> Conventional tests (incl. *in vivo* assays) are listed as examples, but the list is not all-inclusive or definitive, and considered as valid at time of chapter publication. Proposals for alternative techniques resulting from scientific progress, when accompanied by adequate supporting data, may be acceptable. <1239> Traditional tests (incl *in vivo* assays) are listed as examples. The option to use new sensitive molecular methods with broad detection capabilities, such as NGS, is included, either as an alternative to *in vivo* tests and specific nucleic acid amplification techniques (NAT), or as a supplement/alternative to *in vitro* cell culture tests based on the risk assessment and with the agreement of the competent authority.
FDA (USA)	FDA Guidance for Stakeholders and Food and Drug Administration Staff "Considerations for Design, Development, and Analytical Validation of Next Generation Sequencing (NGS) – Based In Vitro Diagnostics (IVDs) Intended to Aid in the Diagnosis of Suspected Germline Diseases" (2018)[53]	Although the scope of the guideline is for NGS in vitro diagnostic (IVD) tests, considerations regarding bioinformatic pipeline as described in chapter 6 (Test Elements and Methods, A (iii) Bioinformatics) are applicable to NGS used for adventitious virus detection.

In addition to the regulatory documents mentioned in Table 5, three international conferences on "Next Generation Sequencing for adventitious virus detection in biologics for humans and animals" were organized by IABS (International Alliance for Biological Standardization) since 2017, regrouping members from industries but also regulatory agencies such as FDA, WHO or EMA. The goal of the meetings was to share the current state of the scientific knowledge, as well as the readiness in regards of replacement of adventitious agent testing by NGS. In 2017, 2019 and 2022, the presentations included efforts for standardisation, validation and bioinformatics steps, as well as case studies on applications in biologics, comparison with routine virus detection assays, and current regulatory thinking[54,32,55]. It was concluded in 2019 that "NGS can be used for the detection of a broad range of viruses, including novel viruses, and therefore can complement, supplement or even replace some of the conventional adventitious virus detection assays"[32]. In 2022, regulatory Authorities further stressed the need for a testing strategy based on risk assessment, followed by appropriate methodology including NGS[55].

In conclusion, the regulatory framework for adventitious agent safety testing has recently evolved to integrate new molecular technologies, specifically NGS, as powerful method for viral testing of cell banks/viral seeds, unprocessed bulk harvest/intermediates or drug substance. ICH encourages the replacement of *in vivo* assays, and potentially *in vitro* assays with NGS, using a risk-based approach for NGS implementation in the overall viral control strategy.

4.2 Implementation Strategy Based on Intended Use for NGS, and Corresponding Regulatory Pathways / CMC Variations

As presented in the introduction, this position paper covers the implementation of NGS for adventitious virus detection, and more precisely the following possible applications that should derive from a viral risk assessment:

- Viral testing of cell banks and viral seeds. In that case, the time of implementation of the NGS-based method is linked to the characterisation of the new cell bank or seed system.
- Viral control of raw materials from animal origin, used in the manufacture of vaccines or recombinant proteins. In practice, there are some limitations to implementation of NGS in testing raw materials, since being able to detect non-infectious viruses. However, it could be used for the readout once subcultures are conducted on indicator cell lines.
- Safety Quality Control (QC): viral safety controls can be performed during the manufacturing process, on unprocessed bulk harvest or on another intermediate, or on the drug substance. Depending on the company strategy and viral risk assessment of the product, tests are reported in the drug substance specification, or only as in-process controls. Therefore, any change in the control strategy would need to be reported according to the corresponding category — change to in-process control or change to drug substance specification — as described in the following tables.

The following paragraphs present the regulatory impact and required documentation for registration of NGS depending on product types — *i.e.* development and established products. The focus is mainly on the implementation of NGS as replacement of a conventional test. Indeed, addition of NGS as a new test to the existing testing panel does generally not represent a regulatory challenge, when it is not consequential to a quality issue or safety concerns. Therefore, demonstration of comparability with other methods would not be relevant. Although the corresponding submission categories (for test addition) are not discussed in detail, deliverable requirements and particularly dossier content recommendations are explained.

4.2.1 Development Products

Table 6: Regulatory impact and deliverables for registration of NGS for development products

Development stage	Testing of cell banks / viral seeds	Testing of raw materials including cell culture media components	In-Process Control (IPC) testing	Release Quality Control testing (for vaccines)
Early phase (Clinical Trial Phase I, II)	When incorporating NGS in the first Clinical trial application (CTA), there is an opportunity to consult with the Health Authority via pre-IND or pre-IMPD meetings. This is particularly relevant when aiming to replace conventional tests with NGS, especially for cell banks (MCB, EOPC) / viral seeds, IPC and Release Quality Control. The intention to implement NGS must be declared in the CTA application. Method validation (see section 2) is expected from initial CTA application specifically for cell banks / viral seeds and quality control of drug substance and intermediates. The use of NGS should demonstrate its suitability for its intended purpose. The appropriateness of the overall viral control strategy, whether integrating NGS in lieu of or in conjugation with traditional tests, should be discussed comprehensively and will be a subject of review. In case Health Authorities do not accept the approach, the possibility of parallel testing or retesting with conventional tests can be considered as mitigation plan.			
Late phase (Clinical trial Phase III)	Introduction of NGS as a change in viral safety testing is covered by introducing a new cell bank/viral seed such as the switch from MCB/MS to WCB/WS. This change requires submission as a substantial modification (Major change - Prior Approval). This is typically included in CTA or Information amendment for entry into Phase III.	Applicable to raw materials of animal origin: No regulatory action is required for replacement of a current viral test by NGS for raw materials with a CEP and when they comply with pharmacopeias requirements. Otherwise, there might be the need for regulatory action depending on the risk introduced in the overall control of adventitious agents, as described in Module 3 section 3.2.A.2 Adventitious Agents and Safety Evaluation.	Replacement of an existing method by NGS, whether applied as in-process control (e.g. unprocessed bulk harvests) or registered in the specification for quality control of the drug substance/product, is submitted as substantial modification (Major change - Prior Approval). This is typically included in CTA or Information amendment for entry into Phase III.	

The recommended deliverables for submission of a substantial modification (Major change - Prior Approval) are: the Rationale for the proposed change, the demonstration of analytical comparability when relevant, or an alternative justification for the replacement (see section Comparability), revised IMPD/IND dossier sections including supportive NGS data (see section 4.2.2).

The recommended deliverables for submission of a non-substantial modification (Minor change – Implement and Notify) are : Rationale for the change, revised IMPD/IND dossier sections (see section 4.2.2).

4.2.2 Established Products

Table 7 below aims to compile the reporting categories and deliverables required for the registration of NGS for the previously listed applications during life cycle management of Established Products.

For cell bank / viral seed testing, three possible scenarios can be considered, depending on the pre-existing registration of a Qualification / Comparability Protocol for future working cell banks/seeds:

1. No protocol registered: the new working cell bank/seed needs to be reported and implementation of NGS testing would be covered under this single variation,
2. No protocol registered: a protocol including NGS testing can be submitted as a variation. A second variation may be needed in some cases to notify the new cell bank/seed established according to the new protocol. This strategy allows to save several months compared to scenario 1 and is particularly beneficial when the stock of current WCB/WS is very low and a new one needs to be rapidly implemented.
3. Protocol registered: the protocol needs to be amended to integrate NGS. In order to benefit from its advantages, amendment to the existing protocol should be submitted before the qualification of new cell bank/seed, otherwise the registered protocol would be considered null and void (see scenario 1).

Table 7: Regulatory Classification and Deliverables for Registration of NGS Testing for Authorised (Established) Products

Application	WHO*	EU / UK	US	China	Canada	Japan	Deliverables
Change in Cell bank/seed testing when no protocol is registered would be covered by the change of cell bank/seed itself	If WCB is issued from the approved MCB: 2.c. for biopharmaceutical products & 9.b/10.b for vaccines - Moderate by default with condition 4 not met (prior approval). For MCB: 2.b. for biopharmaceutical products & 9.a/10.a for vaccines Moderate (prior approval) if issued, for example, from the same clone. New marketing authorization may be needed in some cases.	B.I.b.2.d) Type II – Major (prior approval)	Prior Approval Supplement - Major	Moderate change (prior approval) for WCB/WS issued from the same MCB/MS, Major Change (prior approval) for new MCB/MS	Notifiable change (prior approval)	Partial Change Application – Major (prior approval)	In addition to the requirements expected for a change in cell bank/seed: -Justification for the changes and risk assessment -Inclusion of NGS test results in the comparability/qualification of the new cell bank/seed -Amendment of the relevant dossier sections including Control of materials for cell banks/seeds and Adventitious agents Safety Evaluation-Compliance to relevant national pharmacopeias chapters
Introduction of a qualification protocol for new	Supplement (prior approval)	B.I.a.2.c. Type II – Major (prior approval)	Prior Approval Supplement - Major	No dedicated category, ref to ICH. ICH Q12: Prior Approval	No dedicated category. Prior Approval following ICH Q12 and	Partial Change Application – Major (prior approval).	-Working cell bank / seed qualification protocol - Justification for the change in cell banks/seed testing strategy, when relevant

Application	WHO*	EU / UK	US	China	Canada	Japan	Deliverables
working cell banks/viral seeds					extrapolation from category 16, which requires a Notifiable change for a change to an approved protocol (see below)		
Change to an approved cell bank/seed qualification protocol	Replacement of a test by NGS: 5. for biopharmaceutical products & 12. for vaccines - Moderate (prior approval)	Type IB - B.I.a.2.a, or Type II - B.I.a.2.c, as relevant depending on the complexity of the change, ex which test is aimed to be replaced and can equivalence be demonstrated	Prior Approval Supplement - Major	Concept of post-approval change protocol in China is under implementation	Notifiable change (prior approval), 16	Partial Change Application – Major (prior approval). Protocol is usually included in Registered Form appendix.	-Justification of the change to the cell bank/seed qualification protocol and risk assessment. -Updated cell bank/seed qualification protocol -Brief description of the method
Change to raw material/reagents specification	Not described in the guideline for biotherapeutic products, so considered not reportable. For vaccines: closest category would be 14.a. Moderate change (prior approval), but could also be considered not reportable if detected virus and acceptance criteria are the same (as reported in the dossier)	B.I.b.2.d) Type II – Major (prior approval), however information on analytical procedures used to test raw materials are not often included in the registered dossier, but rather limited to the list of viral strains which are controlled > this could become not reportable if unchanged	CBE-30 – Moderate; or annual report if the raw material specifications are registered in a way that does not mention the method used but only the parameter (viruses tested) and acceptance criteria. It is assumed that one could demonstrate overall same or increased level of assurance of the viral control on the raw material.	Not reportable (no change to test item) to Minor change (addition of test item)	Notifiable change (prior approval), 10a. Condition 8 not met. Based on scientific evaluation (no/positive quality impact) and absence of dossier impact, this could however be considered not reportable.	Not reportable if not described in the Registered Form	-Justification for the change and risk assessment -Amendment of the relevant section(s) of the dossier. -Information on revised quality control and specification of the raw material, including a description of the analytical methodology and a summary of validation data. -Comparative validation results as relevant, or if justified comparative analysis results (IPC and QC) showing that the current test and the proposed one are equivalent. -Certificate of analysis (CoA) of the raw material including NGS testing, provide batch analysis data/CoA of DS manufactured with the raw material pre- and post-change if needed for some markets. -TSE certificate for the concerned raw mat as relevant
Change to in-process tests or limits applied during the manufacture of the active substance	15.f for biopharmaceutical products and vaccines - Moderate change (prior approval). closest category being related to quality/safety issue	B.I.a.4.b)&e) Type II – Major (prior approval) OR B.I.a.4.f) Type IB – Minor (Tell, wait&do)	Prior Approval Supplement - Major	Major change (Guideline informs Moderate change but since related to viral safety – condition 1 -, advise to file under Major change or consult	Notifiable change (prior approval), 18.b condition 3 not met (NGS is a novel non-standard test procedure)	Partial Change Application – Major	-Comparative table of current and proposed in-process tests. -Amendment of the relevant section(s) of the dossier. -Details of NGS method as non-pharmacopoeial analytical method and validation data, where relevant. -IPC and Batch analysis data on three consequential commercial production batches

Application	WHO*	EU / UK	US	China	Canada	Japan	Deliverables
				CDE for moderate change.			of the active substance for all specification parameters. -Justification of the proposed change for the new in-process test and limits, and associated risk assessment.
Change in test procedure for antigen (for vaccines)	18.c. Moderate change (prior approval)	B.I.b.2.d) Type II – Major (prior approval)	Prior Approval Supplement - Major	Moderate change if equivalence can be demonstrated, or Major change	Notifiable change (prior approval), 25.c condition 10 not met	Partial Change Application – Major	- Amendment of the relevant section(s) of the dossier including a description of the revised specifications, analytical methodology, and method validation data. - Comparative validation results as relevant, or if justified comparative analysis results showing that the current test and the proposed one are equivalent. -Justification of specifications and risk assessment associated to the proposed change

*For vaccines, WHO could be consulted ad-hoc to confirm the submission category, allowing to potentially use a lower reporting category if certain conditions are met including a proposed control strategy that is considered “more stringent” than the current one.

General recommendations regarding information provided in the dossier include explaining that NGS is a new technology. A minimum of information is required in the submission for acceptance, particularly method validation and bioinformatic pipeline robustness, but not all information may necessarily need to be captured in the Module 3 of Common Technical Document (CTD) sections. The following paragraphs summarise the general recommended information for application of NGS in different viral testing steps.

- If NGS is used for cell banks / viral seeds testing: brief description of method principle, summary of validation results and test results for cell bank / viral seed safety testing can be presented in "Control of materials" and/or "Adventitious agents safety evaluation" section.
- If NGS is used as in-process control (IPC) or for intermediate testing (vaccines), and not part of the drug substance specification: details about NGS test, acceptance criteria, a brief description of method principle, and its validation status should be provided in section of "Control of Critical Steps and Intermediates". Generally, information on IPC methods validation is not specifically requested in the CTD Module 3. However, an executive summary can be submitted in the variation application to facilitate proper evaluation of the proposed change. The specific location for this information should be defined based on company practices.
- If NGS is used for drug substance Quality Control (QC) or for intermediate testing (vaccines) and is part of the release specification: updated sections should be provided for "Specification", "Analytical procedures" (description of the analytical procedure for experimental part and bioinformatic part) including established conditions if relevant, "Validation of analytical procedures", "Batch analyses" and "Justification of Specification". Information related to intermediate testing can be alternatively provided in section "Control of Critical Steps and Intermediates".

For the previously mentioned applications, considering the complexity and innovative nature of NGS methodology, some Health Authorities may request additional information and validation details. Furthermore, providing details about the tools and parameters used in the bioinformatics pipeline may be necessary for an informed review of the application, in addition to a discussion around analytical comparability, if relevant. However, these elements can be provided outside of Module 3 when needed (location defined based on company practices).

- If NGS is used for raw material testing: information on analytical procedures used to test raw materials are not often required in the registered dossier. Instead, it is usually limited to specifications including a list of controlled viral strains and their associated acceptance criteria (absence of contamination). The introduction of NGS as a broad-range detection technique for raw material testing may impact the expression of raw materials specifications. In the rare occasions that there is a concern about viral safety affecting any raw material(s), an amendment to section "Adventitious agents and safety evaluation" should be considered.

4.3 Regulatory Strategy, Tools and Recommendations

As a general consideration, to increase the likelihood of adoption of a new technology, the reasons why innovative technology is perceived to be advantageous in comparison to the current methods should be explained. These explanations should be relatively easy to understand without having in-depth knowledge of the innovative technology, with a certain degree of consistency and compatibility with the existing regulation, standards and norms. In addition, an early discussion/dialogue with Health Authorities to align on the expectations and identify technical and regulatory barriers for adoption is desirable.

Some Health authorities may be currently more familiar than others with the technology. One way to get an early discussion with the Health Authority and to receive scientific advice to establish a strategy for data generation using the new technology is through a product or a non-product specific Health Authority meeting. Some Health Authorities have dedicated procedures and teams responsible for Chemistry, Manufacturing and Controls (CMC) innovation matters as listed below:

- EMA (EU): Innovation Task Force (ITF), Process Analytical Technology (PAT) Team, and Quality Innovation Group (QIG).
- FDA (USA): non-regulatory advice (FDA CBER office – committee of experts dedicated to NGS that could address scientific and technical questions under unformal exchange to guide industry, contact CBER office for request), CDER Emerging Technology Team (ETT), CBER Advanced Technologies Team (CATT).
- PMDA (Japan): Innovative Manufacturing Technology Working Group.

Health Authorities can also be contacted in the context of product-specific scientific advice following the national procedures. During product development, the sponsors can take the opportunity of the regular Health Authorities interactions to address scientific questions and concerns, and implementation of NGS in the control strategy could be a topic for discussion besides clinical aspects as relevant.

For established products, once the implementation strategy is defined with the key required study elements, Post-Approval Change Management Protocols (PACMP) may be used. These protocols, also known as Qualification/Comparability Protocols allow to seek Health Authority approval on the strategy (planned studies, controls and acceptance criteria) before execution, ensuring a more streamlined process.

This approach is particularly valuable for the qualification of working cell banks and working viral seeds. Cell bank qualification protocols are widely accepted globally, and in many countries, the implementation of the new working cell bank/seed can be done without regulatory action after approval of the strategy described in the qualification protocol. In some countries, regulatory action is still required, but with a downgraded submission category at time of execution. It is important to note that not all countries accept the PACMP/Qualification/Comparability Protocol approach. Additionally, registering a protocol limits flexibility in execution and is therefore most beneficial when there is enough experience to ensure that the proposed approach will be followed without modification.

Another potential strategy for the registration of NGS involves giving priority to replacing animal tests. This aligns with Health Authorities current trends, particularly in Europe, which encourages minimizing or discontinuing the use of animals or animal derived products for ethical reasons[9].

With this changing mindset, advancements in science and technology, and increased experience and understanding of the innovative technologies, there is a higher likelihood of adoption in countries used to risk-based approach evaluations. The challenge currently remains for worldwide adoption (including non-ICH countries), but some strategies can be established:

- Ensure adoption in reference member states/countries by prioritising submissions in countries of (regional) influence.
- Use reliance procedures that give significant weight to assessments by other National Regulatory Authorities or trusted institutions as references. The receiving authority can incorporate this shared regulatory work, aligning with its scientific knowledge and regulatory procedures while retaining its regulatory responsibilities. This approach fosters regulatory convergence of Health Authorities decisions and is perceived as a key regulatory mechanism to promote worldwide adoption of innovative technologies.

Staggered approaches for implementation of NGS to replace conventional virus detection tests can also be considered. For example, existing available conventional tests could be used on Master Cell Banks/Master Viral Seeds for Established Products in addition to NGS, while Working Cell Banks/Seeds and End of Production Cell Banks/Seeds would be tested by NGS only if applicable.

4.4 Case Studies and Experience Sharing

This final sub-section gathers Health Authorities' interaction experiences related to NGS and presents practical cases of NGS applications for registration.

One company has benefited from the possibility to meet USA FDA through scientific non-regulatory advice (FDA CBER office – Committee of Experts dedicated to NGS) early in the development of its NGS-based method. Throughout these meetings, the company shared its strategy and details on its NGS workflow and detailed its approach to substitute *in vivo* viral screening assays with NGS at a time when no scientific data was yet available to support this objective. The Company performed sensitivity comparison of the analytical procedures based on Ph.Eur. 5.2.14. Details on the workflow, from sample preparation to bioinformatic assessment were also discussed, as well as the validation approach with the parameters to be assessed, the nature and type of spiking materials. Similarly, the developed strategy was also shared ahead of submissions with other Health Authorities, for example with EMA Innovation Task Force. Those early discussions facilitated subsequent submissions, as they allowed the company to meet Health Authorities expectations, and to anticipate on and to address potential concerns prior to any registration.

One company requested a technical consultation with the French ANSM GIO (Guichet Innovation and Orientation) toward the Comité Scientifique Permanent "Sécurité et Qualité des Médicaments, Formation Restreinte Sécurité Virale". During a face-to-face meeting in 2022, scientific data were presented comparing results from *in vivo* viral screening assays and *in vitro* cell culture infectivity tests with NGS detection limit results, highlighting the good sensitivity of the developed NGS-based test. The ANSM agreed that *in vivo* assays could be substituted by NGS for the testing of MCB, WCB and End-of-Production cells (EOPC) for vaccine production. The ANSM also agreed that *in vitro* cell-culture assays could be substituted by NGS for the testing of cell banks and for in-process control of unprocessed bulk for production of recombinant proteins for clinical trial applications. Some elements on dossier content were also discussed. The conclusions are publicly available[56]. The Company had another technical consultation with EMA ITF (Innovation Task Force) in 2024. In addition, a type V Biological Master File was filed in 2023 to US FDA CBER to present the validation package for NGS, and a technical consultation was initiated with the CATT (interface "CBER Advanced Technologies Team").

One company held several type C meetings with US FDA ETT, where the intended use and design of the NGS-based analytical method were presented. The bioinformatic approach was deeply scrutinized, with a focus on the design and content of the virus genome database, and the capability of the bioinformatic pipeline to detect novel viruses not included in the database. Robustness and validation results were presented and found appropriate. Additional points of discussion included: the robustness of the extraction step, the characterisation of the virus stocks, and the justification of the limit of detection of the NGS-based method.

One company conducted a Type C meeting with US FDA ETT to discuss the application of NGS as a replacement method to the current tests in adventitious viral detection. The proposed approach, along with supporting data, was presented. The Agency agreed that *in vitro* and *in vivo* assays could be replaced by NGS to control cell banks (MCB, WCB, EOPC) and in-process unprocessed bulk harvest in clinical biologic production. The ETT agreed on the provided data on NGS assay sensitivity, robustness

and detection limit. In addition, the ETT provided comments on the characterisation of the virus stocks, the titer of detection limit, tested volume and validation approach of bioinformatics pipeline.

A last company opted for offline discussions with different Health Authority agencies during a conference on Next Generation Sequencing, where technical and regulatory experts were present, to outline their position for NGS testing as a replacement of the *in vivo* viral screening assays. No formal regulatory meetings were held with the Agencies after these initial discussions. The approach to implement NGS for a drug substance matrix was to partner with a Contract Research Organisation (CRO). The CRO already had a modular validated analytical procedure in place, and a formal Product Specific Qualification (PSQ) was completed on the drug substance matrix. In line with ICH Q5(A)R2 guidance, no direct head-to-head comparison of *in vivo* tests vs NGS was completed. The PSQ was performed using the WHO reference viruses developed by USA FDA CBER (see section 2) and an additional virus from a different virus family to strengthen the demonstration of the breadth of detection. After determination of the detection limit had been determined, the Company submitted the following package to Health Authorities:

- Validation summary of the modular validated method.
- Data package containing the spiking studies for the PSQ (including all raw data).
- Risk Assessment for replacing the *in vivo* viral screening assays with the NGS assay (including the suite of registered safety assays to continue for lot release purposes, potential risks identified of switching to NGS, requirements of investigation approach if a positive hit is determined, analytical life cycle management and a discussion on known genetic sequences within the drug substance which was investigated and accepted as known to be within the drug substance).
- Contamination control for manufacturing drug substance (including raw materials, different testing stages).

Questions were received from the Health Authorities regarding the submission package, mainly focused on the data analysis and the viral database used for analysis and interpretation. These were responded to in a timely manner, and all Health Authorities approved the NGS-based method as a replacement for *in vivo* assays.

In conclusion, the above shared companies' experiences allowed to clarify Agencies expectations with regards to the followings:

- Comparability when NGS is aimed to replace in vitro assays: provision of comparability data to support the substitution, and particularly for what concerns method performance. Such comparability is not expected for replacement of in vivo assays.
- Limit of Detection: agencies expect to get insights on how validation was conducted and lead to the claimed LOD, also verifying if it is aligned with the publicly available knowledge for NGS such as the one generated by AVDTIG collaborative studies.

5 Conclusion

In this article, the EFPIA Supportive Group on "Clonality, Characterisation and Viral Safety of Cell Lines" has provided its extended position on topics of interest related to the implementation of Next Generation Sequencing for viral safety testing. The detailed discussion and recommendations on method validation, analytical comparability and regulatory strategies are shared with the purpose to facilitate NGS use, stimulate discussion on challenging technical issues, and foster global alignment on

expectations related to this innovative technology. With this initiative, the EFPIA Supportive Group on "Clonality, Characterisation and Viral Safety of Cell Lines" is contributing to the ongoing efforts of the active scientific community and of Health Authorities to move forward the use of Next Generation Sequencing for the detection of adventitious viruses in biological products.

7 Acknowledgement

This paper represents the view of EFPIA. The contribution of the following EFPIA MQEG Biomanufacturing Clonality, Characterisation and Viral Safety of Cell Lines team members is acknowledged:

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The other members of the Clonality, Characterisation and Viral Safety of Cell Lines team, as well as additional colleagues from contributing companies are acknowledged:

Stephanie Bartels, Kristi Daris, Jean-François Dierick, Luigi Grassi, Diane Hatton, Matthew Hinchliffe, Martina Kopp, Christophe Lambert, Fides Lay, Helen Newton, Sebastien Renouf, Kevin Smith, Jennitte Stevens, Anne Stokes, Olivier Vandeputte

Marie Murphy (Lilly) is thanked for her helpful interaction with the ICH Q5A(R2) EWG and EDQM.

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