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Validation of a Next Generation Sequencing Method for Adventitious Virus Detection in Biologically Derived Products

With Case Study on implementation in a live attenuated vaccine product

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Agenda

1 **Virus Detection Method Description and Validation Approach**

2 **Case study demonstrating implementation for a vaccine product**

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Regulatory Guidance

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Ph. Eur. 2.6.41 High-Throughput Sequencing for the Detection of Viral Extraneous Agents (DRAFT)

Final Document – October 2025

Effective – April 2026

1. Describes the NGS methods that can be used for virus detection in various sample types.

2. Outlines the general steps of the NGS workflow and makes recommendations for method design, analysis strategies and controls.

3. Provides guidance on method validation including the critical parameters to be evaluated as well as the selection of spike material to demonstrate limit of detection.

4. Ph. Eur. 2.6.41 has been adopted by the European Pharmacopeia, will be published in October 2025 and will become effective April 1, 2026

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NGS Method for Virus Detection

Broad Detection Coverage

NGS allows for simultaneous detection of a wide range of viruses without the need of prior knowledge of specific agents.

Viromic Assessment

- Sequences all nucleic acid in the test sample to detect both RNA and DNA viruses
- Nuclease sample treatment included to increase virus detection
- Suitable for complex matrices such as virus seeds or virus harvests.
- Illumina based platform

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Validation Approach

- Demonstrate that the method is suitable for its intended purpose considering the sample type and testing approach.
- Virus detection methods are qualitative limit tests where specificity and sensitivity of the method are assessed.
- End to end validation of all steps in the process is required but can be subdivided into the individual steps or modules to establish criteria to demonstrate performance consistency at each step of the process.
- A modular validation strategy also provides flexibility of updating the validated parameters of individual steps without the need to revalidate the entire process.

 *Nucleic Acid Extraction*	Minimum quantity and quality for nucleic acid yields
 *DNA Library Preparation*	Acceptable library yield and fragment size range
 *Sequencing*	Quality score for sequencing data and cluster density (if applicable)
 *Data Analysis*	Confirmation of complete analysis run with no errors

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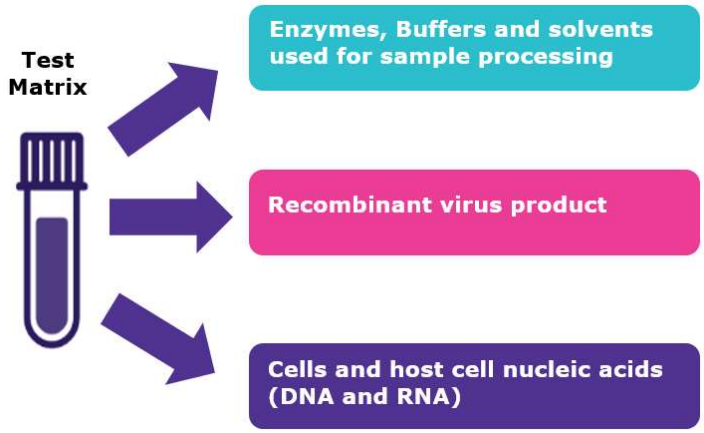
Specificity and Sensitivity

Assay specificity evaluated by:

- Recovery of positive controls
- Recovery of reference virus spikes from spiked samples
- No recovery of virus spikes from unspiked samples

Assay sensitivity – Limit of Detection (LOD)

- LOD depends on the test article matrix
- Assessments of LOD are accomplished through **spiking studies**
  - A spiking study is performed in an end-to-end analysis in the test matrix to demonstrate the limit of detection



Spiking Material

- Ph. Eur. 2.6.41 Recommends the use of model virus spikes or relevant infected cell models
- Spike viruses should represent viral diversity
  - Structure (enveloped/non-enveloped)
  - Nucleic acid content (DNA, RNA, single or double stranded)
  - Size
  - Morphology
  - Chemical resistance
- Virus stocks should be well characterized
- WHO International Reference Panel for Adventitious Virus detection is recommended as the minimum model virus panel.
  - Additional viruses can be added based on a risk assessment

Virus name	Genome	ss/ds	Genome Size (kb)	Particle size (nm)	Env.	Chemical Resistance
Epstein Barr Virus (**EBV**)	DNA	ds	172	120-180	Yes	Low/Medium
Mammalian orthoreovirus type 1 (**REO**)	RNA	ds	23.6	80	No	Medium/High
Human respiratory syncitial virus (**RSV**)	RNA	ss	15	150-200	Yes	Low/Medium
Feline Leukemia Virus (**FeLV**)	RNA	ss	8.5	80-100	Yes	Low
Porcine Circovirus (**PCV**)	DNA	ss	1.8	16-18	No	High
Minute Virus of Mice (**MVM**)	DNA	ss	5.1	26	No	Medium/High
Human betacoronavirus OC43 (**hCoV**)	RNA	ss	30.7	80-120	Yes	Low

Limit of Detection (LOD) Determination

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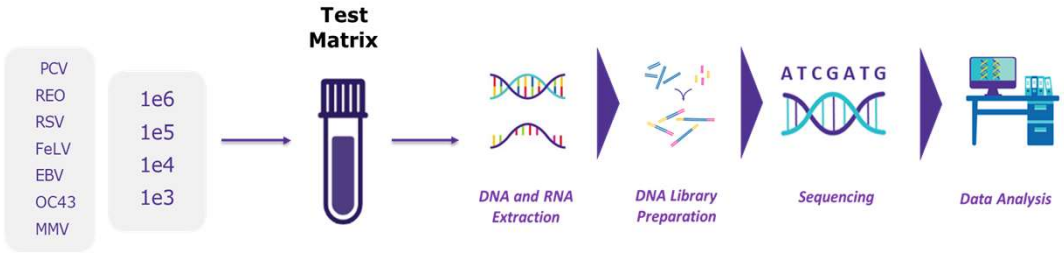
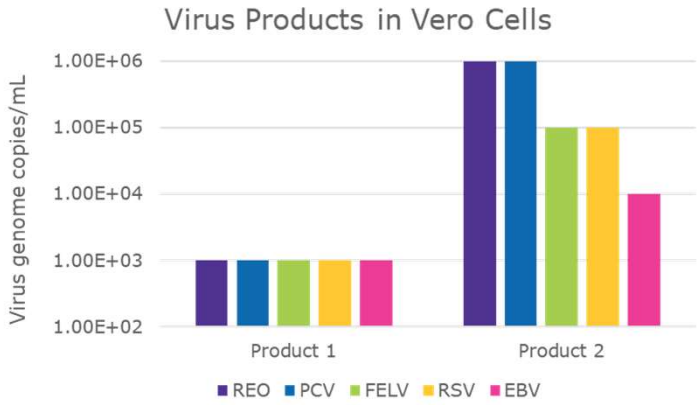
Sensitivity

- LOD is established for each matrix tested through a spiking study
- Spiking study should include at minimum:
  - Viruses from the WHO International Reference Panel for Adventitious Virus spiked at appropriate concentrations to establish a limit of detection.
  - Additional viruses can be included based on risk assessment



Example of LOD variability in products with similar background



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Case Study:

Validation of an NGS virus detection method in a live vaccine matrix

Biologicals
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Validation of a Next Generation Sequencing Method for adventitious agents detection in a live vaccine matrix

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Case Study: *in vivo* replacement

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- Goal: Replacement of *in vivo* animal model testing for Adventitious viruses with NGS
- Test Sample: Bulk Harvest Material for Quadrivalent Influenza Vaccine
- Matrix: Allantoic Fluid - 4 separate influenza strains
  - H1N1
  - H3N2
  - B Yamagata
  - B Victoria
- Influenza vaccine products are manufactured using SPF eggs
  - Testing to assess virus safety is conducted in compliance with:
    - Ph. Eur. 2.6.16 Test for Extraneous Agents in Viral Vaccines for Human Use
    - FDA Guidance to Industry Characterization of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications
  - Ph. Eur. 2.6.16 specifies testing in mice and embryonated eggs for virus harvest propagated in avian tissues.

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NGS supplies an appropriate replacement for the *in vivo* adventitious virus test

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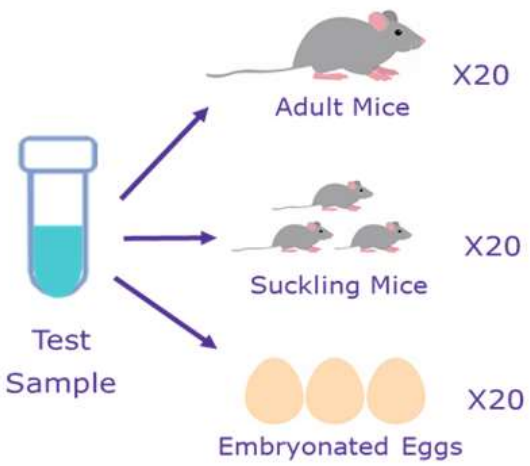
Traditional *in vivo* method

- Well-established method utilizing animals to detect adventitious virus in a biological material
- Viral contaminant indicated by the impact of infection on the host animal
- No direct identification of a contaminant without further testing
- Subject to high invalid test rates due to inherent variability of the animal model



NGS Alternative

- ✓ Broad spectrum virus detection
- ✓ Ability to demonstrate comparable or better virus detection
- ✓ Reliable methodology with objective results
- ✓ Reduction in use of animals for virus detection methods

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Study Design

Each individual vaccine component tested separately = 4 Test Samples

Viruses used for Spiking:

- WHO Virus Reference Panel
  - Epstein Barr Virus (EBV)
  - Respiratory Syncytial Virus (RSV)
  - Feline Leukemia Virus (FeLV)
  - Porcine Circovirus (PCV)
  - Reovirus (Reo)
- Additional Virus included based on risk assessment
  - Bovine Diarrhea Virus (BVDV)

Spike Concentrations used for each virus matrix:

- 1e6 Viral Genome Copies/mL
- 1e4 Viral Genome Copies/mL
- 1e3 Viral Genome Copies/mL
- 1e2 Viral Genome Copies/mL
- Unspiked Sample

Replicates included at each level

Demonstration of method suitability
Spiking Study in an Influenza background

Method

- Fully validated methodology used for spiking study
- Performed to GMP compliance to fulfill regulatory expectation
- Utilized WHO-CBER standards for virus spiking + BVDV
- Instrument used: NextSeq2000™ System

Results	
Data Sets Analyzed	32
Reads per data set	190 million to 640 million
Average Read Size	139 to 150 base pairs
Q30 levels	88.7% to 90.4%
Data normalized to "Hits per Million"	
All runs VALID	
Cluster Density in line with patterned flow cell used	

Criteria for detectability

1 hit = detection

7/8 replicates required

Results of Method Suitability

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Table 2
Spike detectability for 1e6 VGC/mL.

Sample Tested	EBV	RSV	FLV	PCV	REO	BVDV
A/Darwin/6/2018	+	+	+	+	+	+
A/Sydney/22/2018	+	+	+	+	+	+
B/Victoria/2113/2019	+	+	+	+	+	+
B/Guyane/005/2018	+	+	+	+	+	+
Total Positive/Total	**4/4**	**4/4**	**4/4**	**4/4**	**4/4**	**4/4**

→ Positive Control: All passed with detectability

Table 3
Spike detectability for 1e4 VGC/mL.

Sample Tested	EBV	RSV	FLV	PCV	REO	BVDV
A/Darwin/6/2018	+ +	+ +	+ +	+ +	+ +	+ +
A/Sydney/22/2018	+ +	+ +	+ +	+ +	+ +	+ +
B/Victoria/2113/2019	+ +	+ +	+ +	+ +	- +	+ +
B/Guyane/005/2018	+ +	+ +	+ +	+ +	+ +	+ +
Total Positive/Total	**8/8**	**8/8**	**8/8**	**8/8**	**7/8**	**8/8**

→ All spikes detected in 7/8 replicates

Table 4
Spike detectability for 1e3 VGC/mL.

Sample Tested	EBV	RSV	FLV	PCV	REO	BVDV
A/Darwin/6/2018	+ +	+ +	+ +	- -	- -	+ +
A/Sydney/22/2018	+ +	+ +	+ +	+ +	- -	+ +
B/Victoria/2113/2019	+ +	+ +	+ +	- -	- -	- +
B/Guyane/005/2018	+ +	+ +	+ +	- -	- -	+ +
Total Positive/Total	**8/8**	**8/8**	**8/8**	**2/8**	**0/8**	**7/8**

→ EBV, RSV, FeLV, BVDV detected in 7/8 replicates
PCV and REO not detected in 7/8 replicates

Table 5
Spike detectability for 1e2 VGC/mL.

Sample Tested	EBV	RSV	FLV	PCV	REO	BVDV
A/Darwin/6/2018	- -	+ -	- -	- -	- -	- -
A/Sydney/22/2018	+ +	+ +	- -	- -	- -	- -
B/Victoria/2113/2019	+ -	- -	- +	+ -	- -	- +
B/Guyane/005/2018	+ -	+ +	- +	- -	- -	+ -
Total Positive/Total	**4/8**	**5/8**	**2/8**	**1/8**	**0/8**	**2/8**

→ No spikes detected in 7/8 replicates

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Results of Method Suitability

Combined limit of detection[a].

Sample Tested	EBV	RSV	FLV	PCV	REO	BVDV
Sample + 1e6 Spike	+	+	+	+	+	+
Sample + 1e4 Spike	+	+	+	+	+	+
Sample + 1e3 Spike	+	+	+	–	–	+
Sample + 1e2 Spike	–	–	–	–	–	–
Sample + No Spike	–	–	–	–	–	–

[a] Note Combined limit of detection is based on detection of a minimum of 7/8 replicates.

VGC/mL and TCID50 limits of detection[a].

Virus Spike	Genomic Copy/mL	TCID50 Equivalent/mL
EBV	1000	29.73
RSV	1000	1.10
FLV	1000	0.43
PCV	10,000	0.44
REO	10,000	12,727.27
BVDV	1000	8.86

[a] Note: Values presented represent limits of detection for the representative virus stocks tested in the representative samples provided by the client. Different stocks may have different GC/TCID50 ratios.

LOD Results

- Final results showed Detectability of EBV, RSV, FeLV and BVDV at 1,000 GC/mL.
- Final results showed Detectability of PCV and REO at 10,000 GC/mL

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Conclusions from Spiking Study

1. NGS was able to detect representative virus spikes at a suitable limit of detection, in-line with previous matrices published through worldwide studies (e.g. AVDTWG).
2. Results are IN-LINE with expectations regarding limit of detection between 1e2 and 1e4 Viral Genome copies per mL.
3. Higher Limit of Detection in REO and PCV is not unexpected given the nature of those 2 viruses
   - REO- Double-stranded RNA virus
   - PCV- Very small single-stranded DNA virus
4. Read numbers ≥100 Million reads per sample are ideal for achieving detection limit based on "Hits per million" assessment.

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From Qualification to Implementation

- ✓ WHO-CBER standards for virus spiking
- ✓ All Assays and controls passed validity criteria with expected LOD between 1e3 and 1e4 viral genome copies per mL.
- ✓ No "hits" in unspiked samples

- ✓ Validation package and product specific qualification report
- ✓ Client contamination control assessment of manufacturing and QC labs
- ✓ Client risk assessment for the introduction of NGS as an alternate to *in vivo* AAT

- ✓ Validation details of the NGS AAT method
- ✓ Interpretation of data generated

- ✓ Market approval in specific regulatory jurisdictions

- ✓ Implementation approach based on approval timelines and agency requirements (may be phased approach).

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Takeaways

NGS for Adventitious Virus Detection

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1 NGS is a well-supported alternative to traditional testing for Adventitious Viruses

2 Guidance documents provide information on method design and validation

3 NGS testing has been successfully implemented in vaccine products

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