TESTING OF ADVENTITIOUS VIRUS USING NGS PLATFORM- WAY FORWARD FOR VACCINE MANUFACTURERS AND DCVMN MEMBERS

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❑ Vaccine Cell line and Virus Bank Characterization for Adventitious virus

- NGS is most powerful and reliable tools for Adventitious virus genome characterization of cell line Which enables identity
- Regulatory authorities require a confirmation of the characteristics of a cell line to ensure safety, reproducibility, and product efficacy.
- The NGS expertise in microbiology and virology, along with state-of-the-art NGS sequencing, enables us to provide a unique solution for the safety of cell lines and virus stocks to study contamination with Adventitious virus .

❑ Vaccine Cell line Characterization/ Genetic Stability using NGS

- **Verify working and master seed stock testing at genomics level**
  - To demonstrate the stability of the genotype following passages beyond the level used in the production.

    Genotypic characterization of a viral seed includes its sequence, may also include analysis of viral subpopulations and its genetic stability
- Throughout cell passage, mutations may occur which potentially changes the final vaccine product. The vaccine manufacturer to avoid genetic changes should test the identity and integrity of master cell banks, extended cell banks, complementing cell lines or recombinant cell lines expressing transgenes throughout the production process.

❑ NGS Platform- Process Flow For Adventitious Viruses

SAMPLE PROCESSING

- Sample matrix
- Extraction method
- RNA & DNA integrity + quality
- RNA & DNA quantity

LIBRARY PREPARATION

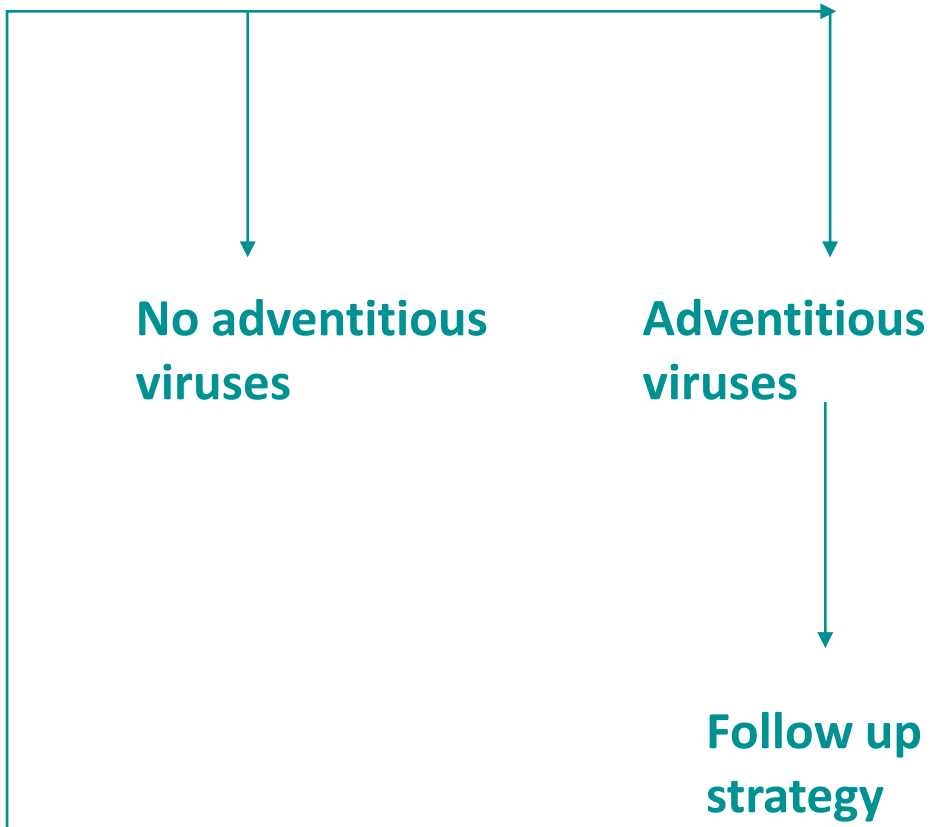
- Manual work
- Fragment size distribution & quantity

SEQUENCING PLATFORMS

- Long reads (SBS)
- Short reads (SBS)
- Long reads (3rd Gen)

BIOINFORMATICS DATA ANALYSIS

- Read quality control
- Reference database quality
- Data analysis strategy



❑ Complexities for NGS

Virus Type

Viruses can have RNA or DNA, each single-stranded, double-stranded, with or without envelope and with different susceptibility to treatment processes and thus the extract procedure has to be robust yet broad enough to encompass all types of virus

Sample matrix

Depending on what within the vaccine manufacturing process has to be tested, such as growth media, suspended whole host cells or cell lysate. The physicochemical properties of the viruses in combination with sample matrix becomes further complicated regarding the extraction method

Computer system validation

Computerized system *includes hardware, software, peripheral devices, personnel, and documentation*. Pharmaceutical manufacturing companies are required to implement quality system as computer server and bioinformatics applications involved in AVT have to demonstrate their GxP-compliance. Including two facts: (1) who has started the application and when (accountability), (2) each feature of the application can be traced to its user requirement through the intermediate phases testing and designspecification (traceability).

❑ Case Study- Example- Viral Seed Characterization as per WHO/ICH

S. NO.	CATEGORY	LIST OF ADVENTITIOUS VIRUS
1	Human Viruses	Immunodeficiency Virus Types 1 And 2 (HIV-1 HIV-2)
2		Hepatitis A Virus (HAV)
3		Hepatitis C Virus (HCV)
4		Human T-Lymphotropic Virus Types 1 And 2 (HTLV-1 And HTLV-2)
5		Epstein Barr Virus (EBV)
6		Hepatitis B Virus (HBV)
7		Human Cytomegalovirus (hCMV)
8		Human Herpes Virus 6 (HHV6)
9		Human Herpes Virus 7 (HHV7)
10		Human Herpes Virus 8 (HHV8)
11		Human Papilloma Virus 16 (HPV-16)
12		Human Papilloma Virus 18 (HPV-18)
13		Human Parvovirus B-19
14	Animal Viruses	Bovine Viral Diarrhea Virus (BVDV)
15		Bovine Adenovirus Type 5 (BAV5)
16		Bovine Parvovirus
17		Bluetongue Virus (BTV) BT-2 Strain
18		Bovine Respiratory Syncytial Virus (BRSV)TN Strain
19		Reovirus Type 3 (REO-3)
20		Rabies virus
21	Porcine Viruses	Porcine parvovirus
22		Porcine transmissible gastroenteritis coronavirus (TGEV)
23		Porcine hemagglutinating encephalomyelitis virus (PHEV)
24		Porcine adenovirus

❑ Workflow for Vaccine Characterization- Viral bank or Cell line

(A) DNA-Sequencing Analysis

Sequencing
- DNA extraction
- Library Preparation
- Sequencing using NGS

Read processing
- Raw Reads
- High quality Reads

Assembly and downstream
- *denovo* assembly of the reads
- Adventitious analysis for identification of Viral Adventitious agents using homology based approach against multiple databases (NCBI ntDB, KaijuDB and RVDB)

(B) RNA-Sequencing Analysis

Sequencing
- RNA extraction
- Library Preparation
- Sequencing using NGS

Read processing
- Raw Reads
- High quality Reads

Preassembly processing
- Mapping on the host cell-line genome to remove any host contamination
- Collecting the viral reads

Assembly and downstream
- *denovo* assembly of the viral reads
- Adventitious analysis for identification of Viral Adventitious agents using homology based approach against multiple databases (NCBI ntDB, KaijuDB and RVDB)

Current EP and IP

EP- 2022

EUROPEAN PHARMACOPOEIA 11.3

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2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

INTRODUCTION

A strategy for testing extraneous agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk detailed in general chapter *5.1.7. Viral safety*. This strategy includes a panel of suitable tests that are able to detect different families of extraneous agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. It also takes into account the capacity of the manufacturing process to remove or inactivate viruses. The list of tests summarised in Table 2.6.16.-1 must be adapted depending on the extraneous agents that have the potential to contaminate the product: for *in vitro* tests, the risk assessment may allow, with the agreement of the competent authority, the use of other permissive cell lines or molecular biology methods depending on the manufacturing process and the incubation temperature for the growth of particular viruses. If *in vivo* tests are more relevant than *in vitro* tests for the detection of some adventitious viruses (e.g. suckling mice for the vesicular stomatitis virus and fertilised SPF eggs for the influenza virus) the decision to maintain or to introduce such *in vivo* assays in a testing strategy must be justified by the risk assessment.

Sensitive molecular methods with broad detection capabilities are available. These approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to *in vivo* tests and specific NAT or in addition/as an alternative to *in vitro* culture tests based on the risk assessment and with the agreement of the competent authority.

Table 2.6.16.-1. – *Relevant tests for extraneous agents at various production stages*

Tests	Virus seed lots	Virus harvests	Production substrates	
			Control cells	Control eggs
Bacterial and fungal contamination	+	+	-	-
Mycoplasmas	+	+	-	-
Spiroplasmas[1]	+	-	-	-
Mycobacteria	+	+[9]	-	-
Test in suckling mice[2]	+	-	-	-
Avian viruses[3]	+	+	-	-
Test for extraneous agents in cell cultures[4]	+	+[9]	+	+
Insect viruses[5]	+	+	-	-
Examination of control cells	-	-	+	-
Haemadsorbing viruses	+	+	+	-
Test for haemagglutinating agents on control eggs	-	-	-	+
Avian leucosis viruses[6]	+	-	+	+
Test for specific viruses by NAT[7]	+	+	-	-
Test for viruses using broad molecular methods[8]	+	+	-	-

(1) If insect cells or raw materials of plant origin are used.
(2) If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.
(3) If the virus is propagated in avian or primary avian tissues. If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.
(4) Test performed in suitable permissive cell cultures based on a risk assessment.
(5) If the virus is propagated in insect cells.
(6) If the virus is propagated in primary avian tissues (including eggs).
(7) Based on a risk assessment.
(8) These methods may be used either as an alternative to *in vivo* tests and specific NAT or in addition/as an alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority.
(9) Not applicable for inactivated viral vaccines.

IP-2022

2.7.2. Cell Substrates for the Production of Vaccines for Human Use

This chapter deals with various cell lines such as diploid and continuous cell lines used for the production of vaccines for human use. Testing to be carried out at various stages (cell seed, master cell bank, working cell bank, cells at or beyond the maximum population doubling level used for production).

Diploid cell line. Diploid cell line has a high but finite capacity for multiplication *in vitro*.

Continuous cell line. A continuous cell line has a capacity to multiply indefinitely *in vitro*. It may be obtained from healthy or tumoral tissue.

For injectable vaccines produced in continuous cell lines the purification process is validated to demonstrate removal of substrate-cell DNA to a level equivalent to not more than 10 ng per single human dose, unless otherwise prescribed.

Cell bank system. Production of vaccines in diploid and continuous cell lines is based on a cell bank system. The *in vitro* age of the cells is counted from the master cell banks. Each working cell bank is prepared from one or more containers of the master cell banks. Diploid cell lines such as MRC-5 and WI 38 can be used for production upto a level of 10 generations before senescence.

Cell seed. Cell seed shall have the information on source, history and characterization. For characterization of the cell seed the properties such as the identity of the cells (for example; isoenzymes, DNA fingerprinting), karyotype, growth characteristics, viral susceptibility and viability during storage and finite life span of the cells.

Test for extraneous agents. Cell lines for vaccine production shall be free from infectious agents. Test will depend on the origin and culture history of the cell line particularly those which are known to infect latently the species of origin such as simian virus 40 (SV40) in cell line derived from monkeys and other viruses in case of cell lines of rodent origin.

Cell lines that show the presence of infectious retroviruses are not acceptable for production of vaccines, unless otherwise justified and authorised.

Table 2.7.2.-1. – Testing of Cell Lines

Test	Cell seed	Master cell bank (MCB)	Working cell bank (WCB)	EOPC/ECB (Cells at or beyond the maximum population doubling level used for production)
1. Identity and Purity				
Morphology	+	+	+	+
Identification	+	+	+	+
Karyotype (diploid cell lines)	+	+	+(1)	+(1)
Life span (diploid cell lines)	–	+	+	–
2. Extraneous Agents				
Bacterial and fungal contamination	–	+	+	–
Mycobacteria	–	+(2)	+(2)	–
Mycoplasmas	–	+	+	–
Spiroplasmas[2]	–	+	+	–
Electron microscopy	–	+(3)	–	+(3)
Tests for extraneous agents in cell cultures (with viable cells or equivalent cell lysate)	–	+	+	+
Tests in suckling mice and eggs	–	–	+(3)	+(3)
Test for specific viruses by NAT	–	+(3)	+(3)	+(3)
Test for viruses using broad molecular methods	+(3)	+(3)	+(3)	+(3)
Retroviruses	–	+(3)	–	+(3)
3. Tumorigenicity				
Tumorigenicity	+(3,4)	–	–	+(3)

(1) The diploid character is established for each WCB but using cells at or beyond the maximum population doubling level used for production.

WHO Collaborative Study – Status update?

World Health Organization

WHO/BS/2020.2394
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 19 to 23 October 2020

Proposed 1st International Virus Reference Standards for Adventitious Virus Detection in Biological Products by Next-Generation Sequencing (NGS) Technologies (CBER-5)

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….. Continued

1. Biogen, Cambridge, MA
2. Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD
3. Eurofins Genomics, Konstanz
4. Eurofins Lancaster Laboratories, Lancaster, PA
5. GSK Vaccines, Rixensart
6. Millipore Sigma
7. National Institute for Biological Standards and Control, South Mimms, Hertfordshire
8. Pathoquest, Paris Institut Pasteur, Paris
9. Sanofi Pasteur Ltd., Toronto, Ontario
10. American Type Culture Collection, Herndon, VA

APPENDIX D. Reference genome accession numbers for mapping reads

Virus	Accession number
Epstein Barr Virus	V01555.2
Respiratory Syncytial Virus	JF920069.1
Reovirus 1	M24734.1 (REO1LAM3P) AF378003.1 (L2) AF129820.1 AF461682.1 AF490617.1 AF174382.1 M10260.1 (REO1S1A) L19774.1 (REO1MCPS2A) M18389.1 (REOS3NSA) X61586.1
Feline leukemia virus	NC_001940.1
Porcine circovirus 1	NC_001792.2
Adenovirus-5	AY339865.1

❑ Questions for Regulatory Check and discussion

- Though NGS platform technology has advantages in comparison to the classical methods (both *in-vivo* and *in-vitro* techniques). Is it required to generate Correlation data?
- Method to check the **integrity, quality** and **quantity** of the extracted DNA
- Method to check **contamination during library preparation**
- Controls to check unintended introduction of viral sequences through sample handling or reagent handling and primer hopping
- **Validation Next Generation Sequencing (NGS),** Bioinformatics and Computer System Validation (CSV)
- **Use of validated standards and its availability?**
- To address variation caused by different read lengths
- **Strengthen in-house laboratory** and bioinformatics expertise for NGS analysis before regulatory submission

❑ References

1. Cleveland, Megan H et al. “Report of the 2019 NIST-FDA workshop on standards for next generation sequencing detection of viral adventitious agents in biologics and biomanufacturing.” *Biologicals : journal of the International Association of Biological Standardization* vol. 64 (2020): 76-82. doi:10.1016/j.biologicals.2020.02.003
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3. Khan, Arifa S et al. “Report of the third conference on next-generation sequencing for adventitious virus detection in biologics for humans and animals.” *Biologicals : journal of the International Association of Biological Standardization* vol. 83 (2023): 101696. doi:10.1016/j.biologicals.2023.101696
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Thank You

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