U.S. FOOD & DRUG ADMINISTRATION

Reference Materials for NGS Implementation as a New Approach Methodology for Adventitious Virus Detection

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Disclaimer: This presentation is an informal communication and represents my own best judgement. The material in this presentation and my comments do not bind or obligate FDA

Outline

- General background on viral safety and challenges
- Introduction of NGS for detection of adventitious viruses
- Development of NGS reference materials (RMs)
  - Virus panels
  - Reference viral database (RVDB)
- Using RMs for NGS applications
  - Virome and viral genome analysis
  - Case study: transcriptome analysis
  - Ongoing efforts
- Status of NGS in OVRR submissions

Adventitious Agent: Definition*

A microorganism that is unintended to be in the biological product

- Bacteria
- Fungi
- Mollicutes (Mycoplasma / Spiroplasma)
- Mycobacteria
- Rickettsia
- Protozoa
- Parasites
- TSE agents
- **Viruses**

*WHO Tech Rep Series No. 993, 2015, annex 2

An Integrated Strategy for Adventitious Virus Risk Mitigation

❑ Prevention

- **Risk assessment-** Identify potential sources of virus introduction to develop a comprehensive risk mitigation strategy and testing plan
- **Use qualified materials**
  - Well-characterized cell banks
  - Certified/tested animal-derived biological materials (e.g. serum, trypsin, antibodies)

❑ Clearance *(not applicable for all products!)*

- **Incorporate robust viral clearance steps** during manufacturing to validate the process
  - Viral inactivation and removal

❑ Testing

- **Extensive testing** for known and unknown viral agents at different stages in the manufacturing process
- Using various **improved sensitive and broad virus detection assays**

Routine Tests for Viral Adventitious Agents*

- **General virus detection assays**
  - *In vivo* assays (adult mice, suckling mice, embryonated hens' eggs)
  - *In vitro* cell culture tests in cell lines of 3 species (same as cell substrate, monkey, human)
  - Transmission electron microscopy (TEM)
  - Reverse transcriptase assay for retroviruses e.g. PCR-enhanced RT (PERT) assay
- **Species-specific assays**
  - *In vitro* tests for animal viruses e.g., bovine, porcine (9CFR 113.47 and 113.53)
  - *In vivo* antibody-production assays for rodent viruses in mice, hamsters, and rats (MAP; HAP; RAP)
  - Assays for known viruses (PCR, Infectivity)
- **Additional assays** *(high-risk:* e.g. *novel cell substrates; unknown passage history)*
  - Extended PCR assays
  - Expanded cell culture assays (adding more target cell lines)
  - Chemical induction assays: Latent viruses (endogenous and episomes)

***2010 US FDA Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infection Disease Indications (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/characterization-and-qualification-cell-substrates-and-other-biological-materials-used-production)**

Adventitious Viruses are the Most Challenging for Detection

- Large number of virus families
- High diversity in viral sequences (*especially RNA viruses*)
  - Mutations, recombination
- Enhanced complexity and size of the virome

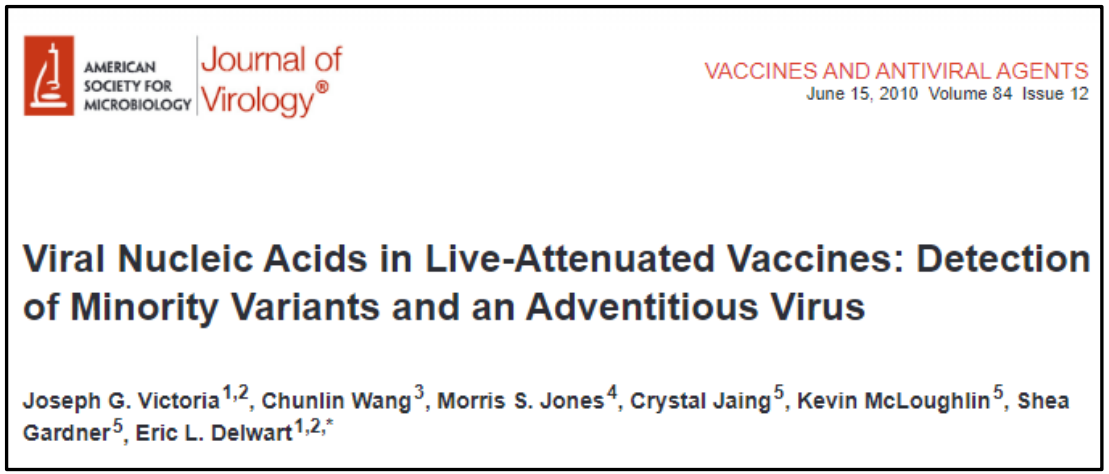
Limitations of the Currently Recommended Adventitious Virus Tests

- Conventional assays for routine testing
  - **In vitro cell-culture assays**
    - Based on virus replication in the target cells
    - Assay read-out is detection of a visible effect, such as cytopathic effect (CPE) or hemadsorption / hemagglutination
    - 28-day observation period
  - **In vivo animal assays**
    - Based on virus replication in the target species
    - Assay read-out is observable clinical/pathological effect
    - 18 – 28 d observation period based on the species
  - **Molecular (PCR) assays**
    - Primer and probe design based upon available sequences of known viruses

- Additional assays (*case-by-case in OVRR*)
  - **Chemical induction assays** can activate latent viruses, but detection of induced, unknown viruses would be missed due to using conventional methods for virus detection

Capabilities of NGS for Viral Safety of Biologics

- NGS was recognized as a powerful advanced nucleic acid-based technology for adventitious virus detection by the *identification of porcine circovirus 1 (PCV1)* in a licensed rotavirus vaccine and the subsequent *discovery of a novel Rhabdovirus* in the insect Sf9 cell line (used commonly for baculovirus-expressed vaccines and other biologics)



AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VACCINES AND ANTIVIRAL AGENTS
June 15, 2010 Volume 84 Issue 12

Viral Nucleic Acids in Live-Attenuated Vaccines: Detection of Minority Variants and an Adventitious Virus

Joseph G. Victoria[1,2], Chunlin Wang[3], Morris S. Jones[4], Crystal Jaing[5], Kevin McLoughlin[5], Shea Gardner[5], Eric L. Delwart[1,2,*]



JVI Journals.ASM.org

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Published ahead of print 26 March 2014

June 2014 Volume 88 Number 12

Identification of a Novel Rhabdovirus in *Spodoptera frugiperda* Cell Lines

Hailun Ma, Teresa A. Galvin, Dustin R. Glasner,* Syed Shaheduzzaman, Arifa S. Khan

Laboratory of Retroviruses, Division of Viral Products, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland, USA

- *In both cases, routine testing had been done. Additional testing using degenerate PCR assays to detect various insect virus families was done for the Sf9 cells since it was a novel cell substrate.*

NGS Workflow for Adventitious Virus Detection

UPSTREAM PROCESSING

DOWNSTREAM PROCESSING

(PRE-TREATMENT)
- *Reduction of "free" nucleic acid using nuclease*
- *Enrichment of particles by WGA/filtration/size selection/ ultracentrifugation*

NUCLEIC ACID EXTRACTION
- Whole cells
- Cell lysate
- Supernatant (cell-free)

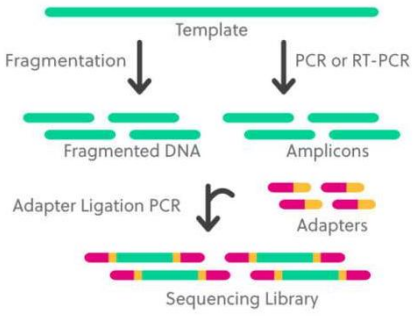
LIBRARY PREPARATION
- rRNA depletion/ polyA+ selection
- cDNA synthesis
- Fragmentation

SEQUENCING
- Short-read
  - Illumina
- Long-read
  - PacBio
  - Nanopore

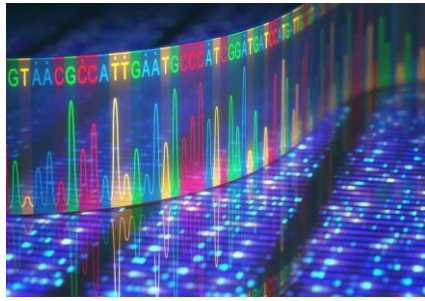
BIOINFORMATICS
- Assembly programs
- Analysis tools
- Databases











FDA In-House and External Efforts on NGS for Applications in Biologics

- Establishment of FDA and CBER Genomics WGs to create a research and regulatory infrastructure to support policy development and decision-making related to applications of HTS

- Strengthening in-house FDA laboratory and bioinformatics expertise for HTS analysis

- Establishment of the PDA-Advanced Virus Detection Technologies Working Group (**AVDTWG**) with focused efforts on HTS standardization and implementation for adventitious virus detection in biologics

Advanced Virus Detection Technologies WG

(PDA "Users Group" in Oct. 2012; "Interest Group" in 2014; "Working Group since 2022)

Mission: **To advance the tools for the next generation of viral risk evaluation by providing an informal, scientific forum for discussions, knowledge exchange, and scientific collaborations** ***(initial focus: NGS)***



More than 290 participants from over >60 organizations:

- *Regulatory agencies*
- *Government agencies*
- *Industries*
- *Service providers*
- *Technology developers*
- *Academics*

Meeting/discussions virtually once every 2 months

Co-chairs

Arifa S. Khan (FDA, U.S.A.; October, 2012)
Siemon Ng (Canadian Blood Services, Canada; June, 2022)
Ken Kono (National Institute of Health, Japan; October, 2023)
Simone Olgiati (Merck KGaA/EMD Serono, Italy; August, 2025)

AVDTIG: PDA J Pharm Sci and Tech 2016, 70 591-595

AVDTWG Subgroups

2012

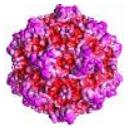
2018 – · – ▸

Subgroup A

Sample selection/ preparation/processing



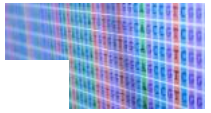
Subgroup B



- **Virus standards and reference materials**

Subgroup AB

Subgroup C

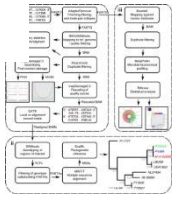


- **Complete and correctly annotated, virus reference database**

Subgroup C

Subgroup D

Bioinformatics pipelines analysis



Subgroup E

Follow-up strategies to confirm the identity of a “hit”



Subgroup DE

Development of NGS Reference Virus Panels

- Work initiated in Khan Lab in 2015

- Intact viruses were selected based on distinct properties to evaluate the performance of all the different steps in the entire NGS workflow (*discussions in the AVDTWG*)
  - WHO International Virus Reference Reagents **(2020; 500 vials) -> CBER NGS Virus Reagents**
    - Established based on results of an International Collab Study (2B) in the AVDTWG
      - *Chin et al., NPJ Vaccines, May 2025*
    - To support NGS **development and advancement** (NIAID BEI cat no. NR-59622)

  - **First WHO International Reference Panel for Adventitious Virus Detection in Biological Products by HTS (2024; 1000 vials)**
    - Established based on results of an International Collab Study of invited members from the AVDTWG
    - For **NGS qualification and validation studies (NIAID BEI cat. no. NR-59630)**

  - Available for distribution to be *used as a panel* to demonstrate breadth and sensitivity of virus detection. Limit to 1 order per year
  - Provided with a CoA with information about source of viruses and cells

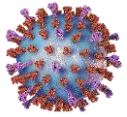
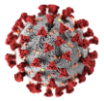
https://www.niaid.nih.gov/research/bei-resources-repos

Characterization of Reference Viruses

- **Extensively characterized and tested**
  - Infectious titer per mL
  - Number of particles
  - Genome copy number: ddPCR
  - Host DNA copy number: ddPCR (*different species*)
  - Sterility and mycoplasma
  - NGS adventitious virus analysis
  - NGS reference virus genome sequence and variant analysis
  - Stability studies: infectious titer; genome copy number
- **Vialed individually to allow freedom for custom-mixing, as needed by user**
- **Used as a panel of viruses**
- **For spiking into relevant background material for demonstrating suitability of NGS for the intended purpose**

❖ *Prepared by CBER through ATCC.*

Reference Viruses for Adventitious Virus Detection by NGS

Virus	Morph-logy	Particle size (nm)	Envelope	Genome topology	Genome size (bp/b)	Physical, Chemical resistance	Virus group	Poly (A) Tailed RNA Genome
Epstein-Barr virus type 1		122-180	YES	ds-DNA linear	172,281	Low to Medium	Herpesvirus	-
Feline leukemia virus		80-100	YES	ss-RNA dimeric	8,448	Low	Retrovirus	Positive
Human respiratory syncytial virus type A		150-300	YES	ss-RNA linear	15,158	Low to Medium	Paramyxovirus	Minus
Human reovirus type 1		60-80	NO	ds-RNA segmented	1,196 – 3,915	Medium to High	Reovirus	Minus
Porcine circovirus type 1		16-18	NO	ss-DNA circular	1,758	High	Circovirus	-
Human coronavirus HCoV-OC43		80-120	YES	ss-RNA linear	30,700	Low	Coronavirus	Positive
Minute Virus of Mice		26	NO	ss-DNA linear	5,100	High	Parvovirus	-

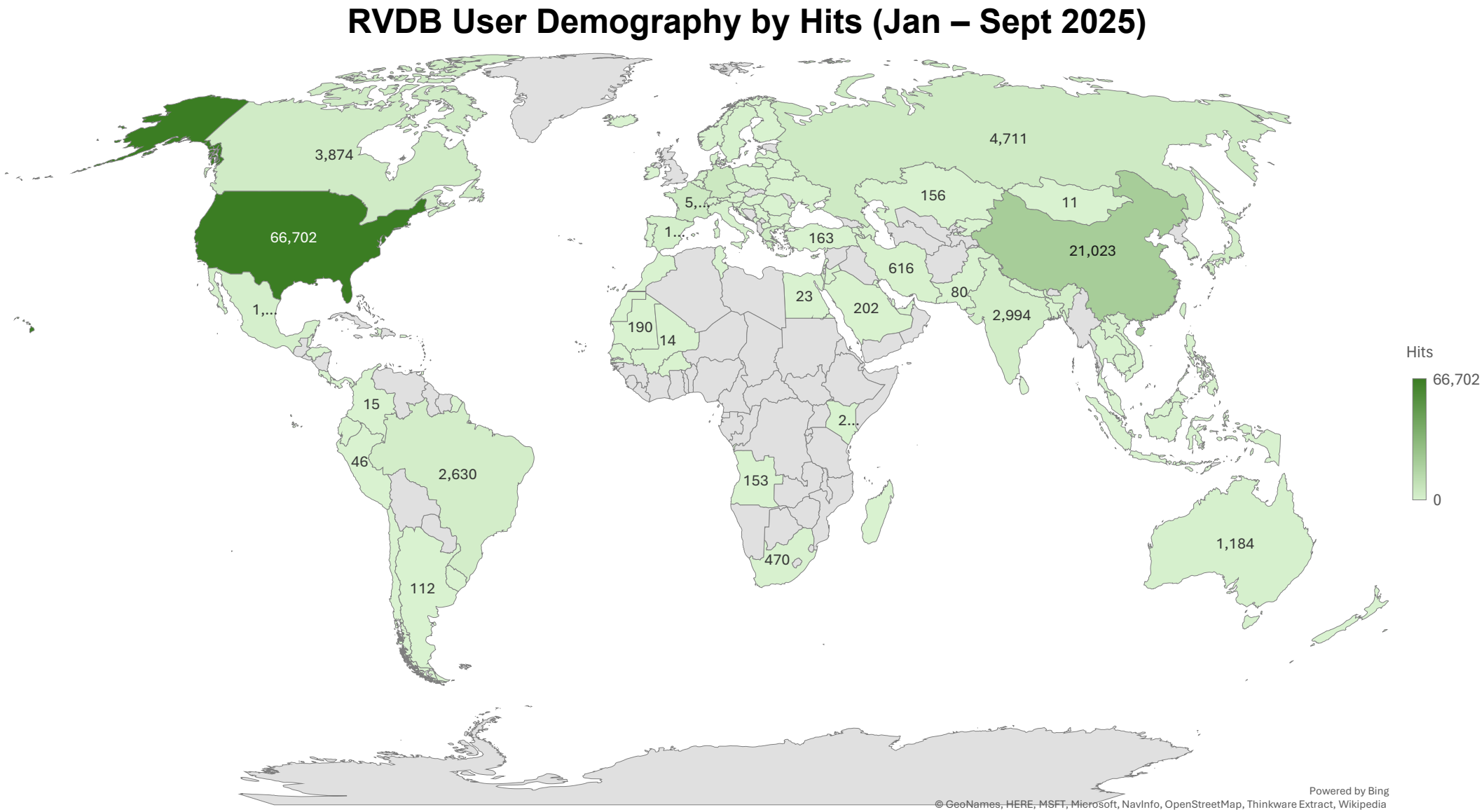
CBER NGS Virus Reagents – BEI cat.no. NR-59622

1st WHO Intl Reference Panel – BEI cat.no. NR-59630

A Comprehensive Reference Virus Database (RVDB) for NGS Broad Virus Detection

- To address the deficiencies in the public databases, we initiated work (2013) to develop a new reference virus database based upon semantic selection from GenBank and NCBI RefSeq + Neighbor Genomes *(Goodacre et al., mSphere, 2018)*
- The latest version RVDBv32.0 (*May 15, 2026*) is available at https://rvdb.dbi.udel.edu/ with link for proteic RVDBs generated by Marc Eloit and Thomas Bigot (http://rvdb-prot.pasteur.fr/).
  - Provides high diversity of viral sequences (including endogenous retroelements) to increase likelihood of novel virus detection, with a reduced cellular content, resulting in less data volume for bioinformatics analysis (and less computational time!)
  - Maintained and updated semiannually by Pei-Ju Chin (Khan Lab) and available through the University of Delaware (Shawn Polson's group).
  - Ongoing RVDB enhancement efforts include annotation of sequences to remove misannotated sequences and annotation of nonviral regions for enhancing accuracy of virus detection (*Chin et al., mSphere, 2025*)

Growing RVDB Community



Collaborative Studies to Evaluate NGS Performance

- Testing different workflows used by different sponsors/manufacturers in **international collaborative studies** for sensitivity and breadth of virus detection
  - Regulatory agencies and health authorities (US, Europe, Japan)
  - Manufacturers (large and big pharma for vaccines, CGT, biotherapeutics)
  - CROs (providing services to our sponsors!)
  - Academics (MIT; Kobe U)

- Use Reference Virus Panels and RVDB to compare results across different labs

- Test in different backgrounds representing various biologically-relevant test materials during manufacturing (Cell banks, Virus seed/Vector virus prep; Bulk harvest)
  - **Viral Genomics**: ALL viral RNA and/or DNA - encapsidated or free; located intracellular or extracellular
  - **Viromics**: ENCAPSIDATED viral RNA and/or DNA from cellular or cell-free samples
  - **Transcriptomics**: VIRAL TRANSCRIPTS – intracellular

AVDTWG Collaborative Spiking Studies

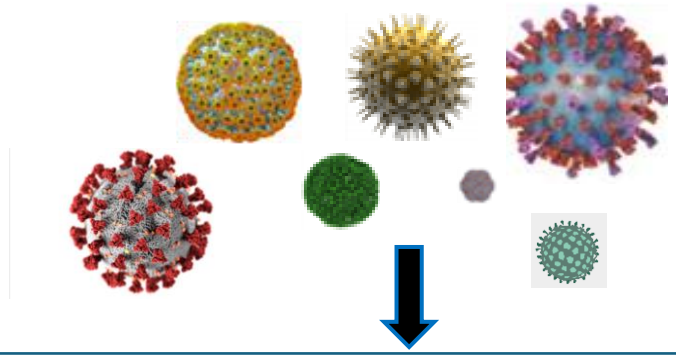
➢ **For performance evaluation and standardization of NGS technologies in different matrices**

Short-read NGS Spiking Studies	**Study 1**	5 viruses in HeLa cell line background	***Published*** *Khan et al ., 2017*
	Study 2A	MVM in a CHO cell line background	*Initiated 2017 (manuscript in prep)*
	Study 2B	5 viruses (*CBER NGS Virus Reagents*) in high titer virus background	***Published*** *Chin et al ., 2025*
	Study 3	Transcriptomics: virus infected cells in uninfected cell background	***Published*** *Deneyer, Chin et al., 2026*
Long –read NGS Spiking Studies	**Study 4**	5 viruses (*CBER NGS Virus Reagents*) in high titer virus background	*Initiated 2020 (manuscript in prep)*
	Study 5	7 viruses (WHO International Reference Panel) in high cellular background	*Initiated in 2024 CDA under review*
	Study 6	Infected cellular transcriptome in uninfected cellular transcriptomic background	*Initiated in 2024 Data analysis on-going*
Bioinformatics	**Study 7**	Assessment of different bioinformatics pipelines using common datasets.	*Initiated in 2026 CDA under review*

Goals

- Determine *minimum achievable* LOD and breadth of virus detection using different NGS workflows (Lab and bioinformatics)
  - Evaluate sample preparation, reference standards and bioinformatics tools
  - Compare and optimize experimental protocols
- Working towards a consensus protocol

Applications of Reference Virus Panel for NGS testing of Cell-Free Samples



END-TO-END VALIDATION OF NGS WORKFLOW







Short-read

"Illumina"

Long-Read

"PacBio"

"Oxford Nanopore"

MATRIX/PRODUCT-SPECIFIC VERIFICATION/QUALIFICATION

High titer virus

"Vaccine Virus Seed or Viral Vector Prep"

("low-mod" complexity)

Chin et al., 2025, NPJ Vaccines

- by different labs using different NGS workflows had LOD 10^4 gc/mL

Sup/ Whole Cells/ Cell Lysate

"Unprocessed Bulk Harvest"

("mod-high" complexity)

Chin et al., 2026, NPJ Vaccines

- NGS can replace the in vivo assays and supplement the breadth of virus detection for in vitro assays

Case Study: AVDTWG Spiking Study 3

- **Purpose and objectives of the study:**
  - Evaluated **NGS short-reads transcriptomics approach for virus detection** in a matrix mimicking adventitious virus contamination of a cell-based biological material.
  - The study used dilutions of an infected cell line expressing EBV spiked in the background of a non-infected cell line.
  - Different ratios were analyzed using both targeted and non-targeted bioinformatics analysis.
- **Participants: 8 labs (regulatory agency, industry, CRO, academic)**
- **Publication:** *Deneyer, Chin, et al. 2026; Microbiology Spectrum*



Common Starting materials:
Infected cell line *(expressing EBV RNAs and without virus production) diluted in a* ***non-infected cell line***

Cell Handling:
Different ratios with 2 common dilutions were analyzed by all participants: 0.1 and 0.01

Results / Conclusion:

Targeted analysis:
All participants detected EBV at 0.001 ratio (*infected:uninfected cells*)

Non-targeted analysis:
All participants detected EBV at 0.01 ratio

Standards for NGS testing of Cellular Samples

Khan Lab

❖ Cell clones with stable integration of a non-pathogenic retrovirus. Characterized for proviral DNA copy number, viral RNAs, virus integration sites, virus production.

SFV-K3T/A549 clones	Virus Infection	Viral RNA expression	Proposed NGS Application (Cell banks and Tissues)
55C	Latent	Basal	Viral Genomic DNA standard
51C	Chronic	Low	Transcriptomic standard
1C	Chronic	Medium	Transcriptomic standard

➢ For evaluating sensitivity of virus detection *(single virus infection)*

55C Cell Clone -Aug 2022; FDA Reference: E-2023-001; FDAInventionLicensing@fda.hhs.gov

51C and 1C Cell Clones – Characterization ongoing.

AVDTWG subgroup AB

❖ Discussions initiated for transcriptome standards and others for broad virus detection

Investigating a Putative NGS Positive Signal

- Follow up of NGS signal to determine biological relevance and significance
  - *Can the results be confirmed by PCR or another assay?*
  - *Is a complete viral genome present?*
  - *Are particles present?*
  - *Are the particles infectious?*
  - *Is there a replication-competent virus?*
  - *Can the nucleic acid/particles be quantified?*

- *Charlebois et al., Viruses (submitted; under review)*

Acceptance of NGS for Adventitious Virus Detection in OVRR

- **Replacement assay (*in vivo* assays; PCR assays)**
  - *In vivo* AV assays – **Non targeted NGS** can provide defined sensitivity and breadth of virus detection
    - *Reduce use of animals and meet the global objectives for 3Rs*
  - PCR assays – **Targeted or Non targeted NGS** can have similar sensitivity to PCR assays; broader virus detection; single assay

- **Supplementary or Replacement assay (*in vitro cell culture* assays by Non targeted NGS)**
  - Cell substrate characterization – particularly in case where there are concerns for occult and novel viruses
  - *In vitro* AV assays (virus seed or unprocessed harvest) – particularly in case of assay interference due to lack of effective neutralization of vaccine virus or cell toxicity by the product

Status of NGS for Adventitious Virus Detection by Regulatory and Public Health Authorities

- **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 (February 2010)
  - "You can use an **alternative approach** if the approach satisfies the requirements of the applicable statutes and regulations"
- **WHO: Recommendations for the evaluation of animal cell cultures as substrates** for the manufacture of biological medicinal products and for the characterization of cell banks, Annex 3, TRS No 978 (adopted 2010; revised May 2023)
  - Risk assessment strategy and new methodologies (*e.g.,* **NGS**)
- **Ph. Eur. Chapter 5.2.3** (Testing of vaccine cell substrates) **and Chapter 2.6.16 (**Tests for extraneous agent testing of viral vaccine seed lots/harvests (revised 2017)
  - Test for specific viruses by NAT (*e.g.,* PCR)
  - Test for viruses using broad molecular methods (*e.g.,* **HTS**) either as an alternative to *in vivo* tests and specific NAT, or as a supplement/alternative to *in vitro* culture tests based on the risk assessment.
- **Ph. Eur. Chapter 5.2.14**
  - Considerations for the substitution to replace *in vivo* methods by **broad molecular methods**
- **Ph. Eur. Chapter 2.6.41** *(*Oct. 2025)
  - Presents details of considerations for using High-Throughput Sequencing **(HTS**) for the Detection of Viral Extraneous Agents
- **ICH Q5A(R2):** Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin (*original 1999; revised November 2023*)
  - Encourages use of new alternative tests (**NGS/ HTS**) for replacing or supplementing the *in vivo* and *in vitro* adventitious virus detection assay, antibody production assays, and NATs (*e.g.,* PCR)
  - ➢ **Adopted by FDA Jan. 2024**

AVDTWG webpage:

https://www.pda.org/home/science-regulation/Advanced-Virus-Detection-Technologies



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Thank You!