IABS 4th Conference on NGS for Adventitious Virus Detection in Biologics for Humans and Animals: Validation and Implementation of NGS, Dec. 4-5, 2024; Frankfurt, Germany

NGS for Adventitious Virus Testing of Biological Products: FDA's Efforts, Experience and Perspective

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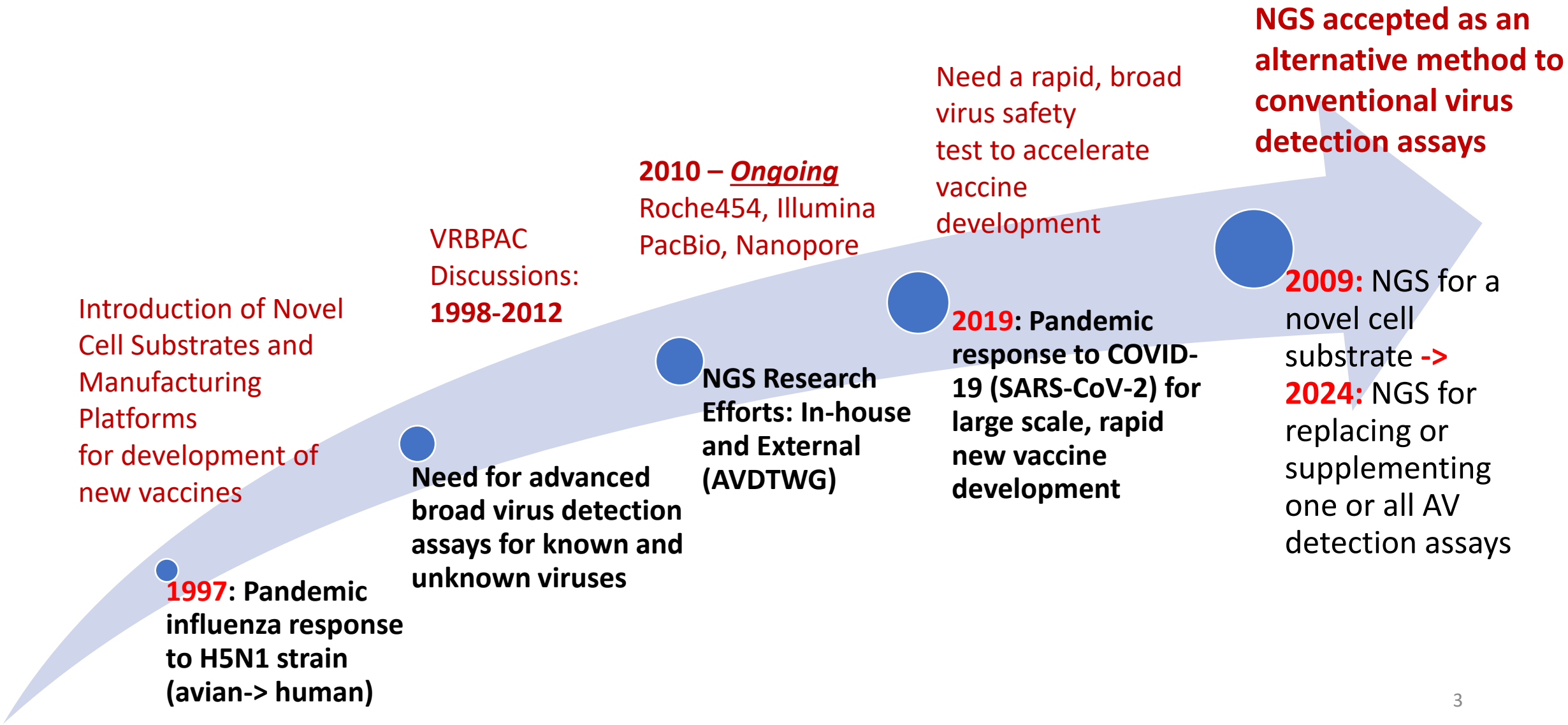
Disclaimer

This presentation is an informal communication and represents my own best judgment.

The material in this presentation and my comments do not bind or obligate FDA.

NGS Journey (in OVRR):
Research – Implementation - Acceptance



“Early/Traditional” Animal-derived Cell Substrates Used for U.S. Licensed Vaccines

Cell Substrate	Type	Species	Viral Vaccine	
			Inactivated	Live / Live, Attenuated
Embryonated eggs	Tissue	Chicken	Influenza virus	Influenza virus, Yellow fever virus
Embryo fibroblasts	Primary cell culture	Chicken	Rabies virus	Measles virus Mumps virus
MRC-5 (lung)	Diploid cells	Human	Hepatitis A virus Rabies virus	Varicella-zoster virus
WI-38 (lung)	Diploid cells	Human		Rubella virus Adenovirus
Vero	Continuous cell line	African green monkey	Poliovirus Japanese encephalitis virus	Smallpox virus Rotavirus

Needs/Demands of New Product Development
(AIDS, Pandemic influenza .....)

- **Novel cell substrates**
  - Designer cells to provide complementary genes for viral vectors
  - Eggs -> cell lines for Large-scale, rapid production
  - New expression technologies
  - New manufacturing platforms

Some Novel Cell Substrates for Viral Vaccines

- **Mammalian**
  - MDCK (canine; non-tumorigenic and tumorigenic)
  - 293; PER.C6; HER96 (human; transformed, tumorigenic)
  - A549; HeLa; CEM (human; tumor-derived)
  - CHO (rodent; tumorigenic, retroviral particles)
- **Avian**
  - EB66 (duck; embryonic stem cell)
- **Insect cell lines**
  - Hi-5 (*Trichoplusia ni)*
  - Sf9 *(Spodoptera frugiperda)*
- **Plants**
  - Tobacco
- **Bacteria**
  - *E. coli*

CBER Discussions on Novel Cell Substrates for Vaccines

- **1998:** CBER engages Vaccines and Related Biological Products Advisory Committee (VRBPAC) on topic of neoplastic and tumorigenic cells for vaccine manufacture
- **2000:** VRBPAC discussion: Vero cells (non-tumorigenic passage) for live-attenuated vaccines
- **2001:** VRBPAC discussion: *In vitro* transformed human cells (293, PER.C6) for defective adenovirus-vectored vaccines
- **2005:** VRBPAC discussion: Tumorigenic MDCK cells for inactivated influenza virus vaccine
- **2008:** VRBPAC discussion: MDCK cells for live, influenza virus vaccine
- **2012:** VRBPAC discussion: Human tumor-derived cell lines; A549 for adenovirus-vectored influenza and HIV vaccines, HeLa for AAV-vectored HIV vaccine, and CEM for an inactivated HIV vaccine

Routine Viral Adventitious Agents Tests for Product Safety

- **General virus detection assays**
  - ***In vivo* assays (adult mice, suckling mice, embryonated hens' eggs, guinea pigs)**
  - *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 (PERT)
- **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 (MAP, including LCMV challenge; HAP; RAP)**
  - Assays for known viruses (PCR, Infectivity)
- **Additional assays for virus detection**
  - Extended and broader PCR-based assays
  - Expanded cell culture assays (*adding more target cell lines*)
  - Chemical induction assays: Latent viruses (endogenous and episomes)

The currently recommended assays have been generally effective in demonstrating the absence of adventitious viruses for product safety

Limitations of Currently Recommended Adventitious Virus Tests

- **Cell-culture assays**
  - Based upon susceptibility of target cells to virus infection
  - Assay read-out is a visible effect due to virus replication, such as cytopathic effect (CPE), hemadsorption, hemagglutination
  - Sample-related interference
  - 28-day observation period
- **Animal-based assays**
  - Unknown sensitivity for virus detection
  - Detection depends on susceptibility of animal species to virus infection
  - Based upon a measurable pathological effect due to a replicating virus
  - Sample-related interference
  - > 18 day-observation period depending upon the species
  - Use of animals globally discouraged (3 R's initiative!)
- **Molecular assays (PCR)**
  - Designed based upon available known virus sequences
  - Large number of assays needed for detection of different viruses
- **Additional assays: Chemical induction**
  - Can activate latent viruses, but detection of induced, unknown viruses would be missed due to using the conventional methods for virus detection

Specific Challenges Regarding Potential Adventitious Viruses in Viral Vaccines

- For live, viral vaccines, cannot include steps for adventitious virus inactivation and removal during manufacturing
  - Need to retain vaccine potency
  - Vaccine virus can cause false positive results in the biological assays

- Some purification steps may be used for some viral vaccines (*e.g., subunit, VLP, Nanoparticle*), but cannot achieve high virus clearance
  - Need to retain vaccine function
  - The vaccine product may be similar in size to a model virus (e.g. MVM)

Need for Advanced Adventitious Virus Detection Methods

Currently recommended assays may not be sufficiently broad to address safety concerns associated with novel cell substrates

- **Known, unknown, and unexpected viruses**
  - Tumor-inducing viruses (*in case of tumor cell lines*)
  - Latent viruses (*infectious but silent and can become active*)
    - DNA and RNA viruses
    - Endogenous retroviruses
  - Occult viruses (*known, unexpected*)
  - Novel viruses (*unknown*)

- **Reverse transcriptase (RT) activity**
  - Produced constitutively from avian and insect cells (endogenous retroviruses: retroviral particles/retrotransposons?)

Early Public Discussions on New Technologies for Adventitious Virus Detection in Biologics

- **2009:** PDA Cell Substrate Workshop. Discussed **broad virus detection technologies**. Conference Proceedings in PDA J Pharm Sci and Tech, 2010; PDA Technical Report on Emerging Methods for Virus Detection (71) 2015

- **2011:** IABS: Adventitious Agents, **New Technologies** and Risk Assessment. Conference Summary in Biologicals, 2011

- **Nov. 2011:** PDA/FDA Adventitious Agents and Novel Cell Substrates: Emerging Technologies and New challenges. Discussed applications and identified **knowledge and technical gaps** that needed to be addressed for further development and applications. Conference Proceedings in PDA Journal, 2012

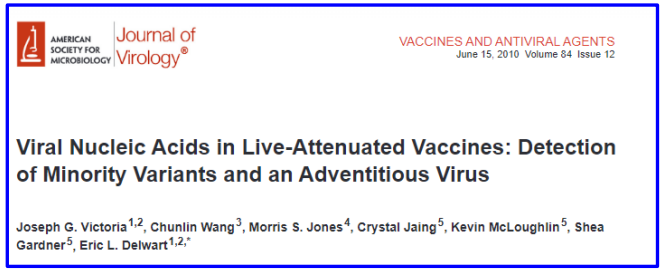
- **Sept. 2012**: FDA VRBPAC discussions on use of human tumor cell lines and potential use of **advanced virus detection technologies** *Transcript pdf available: www.fda.gov*

- **May 2013:** WHO Cell Substrate Study Group (Beijing) discussed **DNA-based detection** of adventitious agents in cell substrates

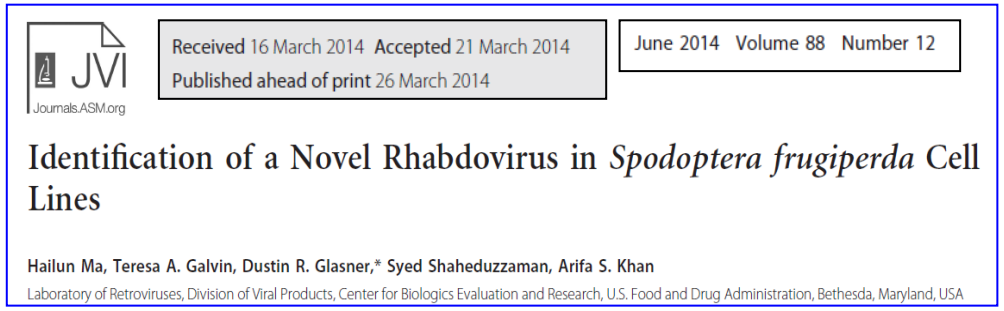
- **Nov. 2013:** PDA/FDA Advanced Technologies for Virus Detection in the Evaluation of Biologicals: Applications and Challenges. **Identified challenges and priority areas** to be addressed for applications to biological products. Conference Proceedings in PDA Journal, 2014

Potential of NGS for Known and Novel Virus Detection in Biologics

- **Unexpected finding of a known porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine *(Victoria et al., 2010)***



- **Discovery of a novel rhabdovirus in the Sf9 insect cell line used for baculovirus-expressed products *(Ma et al., 2014)***



- ***In both cases, extensive testing was done using currently recommended assays to demonstrate absence of adventitious viruses.***

FDA 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 NGS

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

- Establishment of the PDA-Advanced Virus Detection Technologies Interest Group with focused efforts on addressing the challenges with NGS standardization and implementation for adventitious virus detection in biologics
    - *AVDTIG: PDA J Pharm Sci and Tech 2016, 70 591-595*

Advanced Virus Detection Technologies WG

(PDA sponsored Users Group - Oct. 2012; Interest Group - 2014; "Working Group-2022)

Mission: **To advance next generation for viral risk evaluation by providing an informal, scientific forum for discussions and scientific collaborations** ***(ongoing focus: NGS)***

Co-chairs

Arifa S. Khan (FDA, U.S.; October, 2012)
Siemon Ng (Notch Therapeutics, Canada; June, 2022)
Ken Kono (National Institute of Health Sciences, Japan; October, 2023
Noémie Deneyer (GSK, Belgium; November, 2023)

More than 240 participants from over >60 organizations:

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

***Meeting/discussions* by t-con once every 2 months**

AVDTWG – Subgroups

❖ Addressing multiple key challenges requiring additional scientific data

2012

Subgroup A

Sample selection/ preparation/processing

Subgroup B

Virus standards and reference materials

Subgroup C

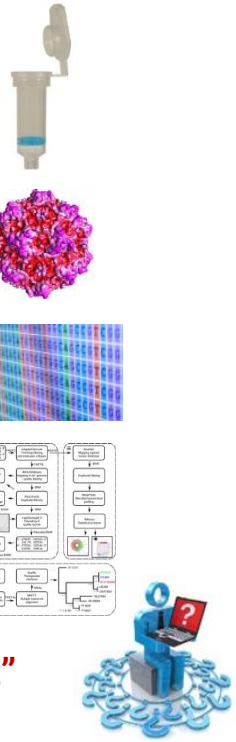
Complete and correctly annotated, virus reference database

Subgroup D

Bioinformatics pipelines analysis

Subgroup E

Follow-up strategies to confirm the identity of a "hit"



2024

Subgroup AB

Subgroup C

Subgroup DE

CBER/OVRR Efforts Towards NGS Implementation (*Khan Lab*)

- Collaborative studies to determine breadth and sensitivity of virus detection by short-read and long-read NGS platforms (AVDTWG and NIIMBL/GHF)

- Development of reference virus stocks
  - CBER NGS Virus Reagents to support NGS development and advancement (NIAID BEI cat no. NR-59622) (*previous WHO reference reagents established based on collaborative study 2B*)
  - First WHO International Reference Panel for Adventitious Virus Detection in Biological Products for NGS qualification and validation studies ((NIAID BEI cat. no. NR-59630) (*established based on new collaborative study*)
  - Available for distribution; to be used as a panel to demonstrate breadth and sensitivity of virus detection

- Generation of a comprehensive Reference Virus Database (RVDB); *ongoing consultation with AVDTWG subgroup C members*
  - Currently, RVDB v29.0 is available at https://rvdb.dbi.udel.edu/

AVDTWG Subgroup AB: *Collaborative Spiking Studies (Matrix Mimics)*

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

Short-read NGS	
Spiking study 1 *Published in 2017*	5 viruses in HeLa cell line background
Spiking study 2A *Started in 2017*	MVM in CHO cell background
Spiking study 2B *Started in 2016-paper in progress*	5 viruses (*CBER NGS Virus Reagents*) in high titer virus background: Viral seed/Viral vector
Spiking study 3 *Started in in 2019*	Transcriptomics: infected cells in uninfected cell background: Cell Bank
Long-read NGS	
Spiking study 4 *Started in 2020*	5 viruses (*CBER NGS Virus Reagents*) in high titer virus background: Viral seed/Viral vector
Spiking study 5 *Started in 2024*	7 viruses (*WHO international Reference Panel)* in high cellular background: Unprocessed bulk
Spiking study 6 *Started in 2024*	Transcriptomics: infected cellular transcriptome in uninfected cellular transcriptomic background

Goals

- Evaluate reference standards and bioinformatics tools
- Compare and optimize experimental protocols

Reference Viruses for Adventitious Virus Detection by NGS

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

		Particle size (nm)	Envelope	Genome topology	Genome size (bp/b)	Physical chemical resistance	
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**
Human respiratory syncytial virus type A		150-300	YES	ss-RNA linear	15,158	Low to Medium	**Paramyxovirus**
Human reovirus type 1		60-80	NO	ds-RNA segmented	1,196 3,915	Medium to High	**Reovirus**
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**
Minute Virus of Mice		26	NO	ss-DNA linear	5,100	High	**Parvovirus**

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

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

Development of Reference Virus Stocks

- **Characterization**
  - Infectious titer per mL (>10^6 $TCID_{50}$ per mL)
  - Number of particles : TEM
  - Genome copy number: ddPCR (>10^8 gc per mL)
  - Adventitious virus analysis: Illumina HTS
  - Host DNA copy number: ddPCR (*different species*)
  - Reference virus genome sequence and variant analysis: HTS
  - *Due to the production cell lines, the EBV stock contains squirrel monkey retrovirus; PCV-1 stock contains porcine endogenous virus*
- **Stability studies**: infectious titer; genome copy number
- **Vialed**: Each virus individually vialed to allow freedom for custom-mixing, as needed by user

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

- To address the deficiencies in the public databases, we developed a new reference virus database based upon semantic selection from GenBank and NCBI RefSeq + Neighbor Genomes *(Goodacre et al., mSphere, 2018. doi: 10.1128/mSphereDirect.00069-18)*
  - Contained all viral sequences regardless of size
  - Included endogenous viral and retroelements
  - Has a reduced cellular content
- The latest version RVDBv29.0 (*Aug 8, 2024*) 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 to increase likelihood of novel virus detection, with reduced nonspecific cellular hits resulting in less data volume for bioinformatics analysis (and less computational time!)*
- *Maintained and updated quarterly by Pei-Ju Chin (Khan Lab) and available through the University of Delaware (Shawn Polson's group).*
- *Continued work on annotation of sequences to remove misannotated sequences and enhance virus-specific detection (Pei-Ju Chin and Trent Bosma)*

Ongoing Efforts for NGS Implementation

- Collaborative studies for increasing sensitivity of virus detection in complex matrices and development of SOPs and test datasets to facilitate routine NGS implementation (*Talks on* ***NIIMBL-3.1-305: Alison Armstrong and AVDTWG Spiking Study 4: Valeria Zanda)***
- In-house studies for evaluating performance of different NGS platforms for virus detection and characterization in cell substrates
- Development of cell-based standards for testing sensitivity of virus detection in cell substrates and cell therapy products (*Poster by* ***Sandra Fuentes***)
- Database enhancements for increasing accuracy in adventitious virus detection and reducing follow up of false positive signals (*Talk by* ***Pei-Ju Chin***)

Status of NGS Applications in the FDA

- NGS data is currently under review at **CBER**
  - Adventitious virus testing of Cell Banks, Virus Seeds, and Bulk Harvests
  - Genetic stability of vaccine virus
  - Cell substrate characterization
  - The CBER Advanced Technologies Team (CATT) coordinates scientific discussions on new technologies including NGS
  - Within CBER/OVRR we highly recommend that sponsors request a technical working group discussion related to the use of NGS for vaccine safety and characterization
    - Non-regulatory meeting to discuss "plans" for use of NGS
    - Reach consensus prior to initiating lengthy, expensive studies
  - NGS for AV testing is being applied in gene therapies (CBER/OTP)
- The Emerging Technologies Team (ETT) in **CDER** is involved in discussions on NGS for adventitious virus detection in biotherapeutics
- Interest in considering NGS for AV detection has also been initiated for animal-based biologics (**CVM**)

General Acceptance of NGS for Adventitious Virus Detection

➢ **Replacement assay (*in vivo* assays; PCR assays)**

- *In vivo* AV assays – NGS can provide defined sensitivity and breadth of virus detection
  - *Reduce use of animals and meet the global objectives for 3Rs*
- PCR assays – NGS can have similar sensitivity than PCR assays; broader virus detection; single assay

➢ **Supplementary or Replacement assay (*in vitro cell culture* assays)**

- Cell substrate characterization – particularly in case where there are concerns for occult and novel viruses
- *In vitro* AV assays – particularly in case of assay interference due to lack of effective neutralization of vaccine virus; as a read-out to reduce assay time

❖ ***Note***

- *Follow-up of a positive result is critical to determine biological significance of a signal for decision-making (as for any nucleic acid-based detection assay)*
- *NGS data may help design a "custom" assay to determine if signal due to infectious virus*

NGS Applications for Adventitious Virus Detection in Biologics

- Strategy to mitigate risk of AV introduction
  - Raw materials used for cell culture
  - Cell banks
  - Virus seeds

- Monitor/test absence of AV during production
  - Bulk harvest
  - Final product

CBER/OVRR: Examples of Submissions Using NGS for Adventitious Virus Detection

Sample Type	NGS Application	Role of NGS	Outcome
2009- Novel CS- EOP cells and supernatant	To broaden detection of unexpected and unknown/novel viruses	Complementary – Additional; Full conventional testing done	2013-Results used for supporting submission
2011- Novel CS-WCB, EOPC	To broaden detection of unexpected and unknown/novel viruses	Complementary – Additional; Full conventional testing done	2015- Re-analysis of data with updated database
2019- MVS, Bulk Harvest	To potentially replace PCR assays	Supplementary – to fill gaps in vitro AV assays due to assay interference	Results as FYI.
2020- 2022 > 25; Cell Banks, MVS, WVS, Bulk Harvest	To replace *in vivo* or/and *in vitro* assays	Complementary, Supplementary, or Replacement due to assay interference	Method validation, Assay qualification/ validation
2023 – *Live, viral vaccine, License: MVS, Bulk DS	To replace *in vivo* adventitious virus assays	NGS for Replacement of the *in vivo* adv virus testing due to challenges of the in vivo assays	Performed acceptable assay qualification and validation

2024: NGS Submissions for Adventitious Virus Detection

FDA CENTER/OFFICE	NGS Consideration
CBER / OVRR and OTP **NGS in regulatory submissions**	➢ **Genomics, Transcriptomics, Viromics** ➢ **Short-read NGS platforms** ➢ **Variety of biological materials: cell bank, viral seed, bulk harvest** ➢ **Replacing *in vivo* or *in vitro* for one ore more stage in the testing**
CDER / ETT **NGS discussion**	**Replacing *in vivo* and *in vitro* assays for testing of biotherapeutic products**
CVM **NGS considerations**	**Discussion for using NGS for detecting agents in Animal Cells, Tissues, and Cell- and Tissue based products (ACTPs):** *intended for animal recipient*

General NGS Expectations in *OVRR* Submissions

- Use of references viruses: WHO Reference Virus Panel or equivalent
  - Cross-references or provide details
- Use of non-targeted NGS bioinformatics analysis for broad virus detection for replacing general virus detection assays (*in vivo* and *in vitro*)
- Details for sample preparation protocols, including sample pre-treatment if any, cDNA synthesis, library preparation
  - **SOPs and results**
  - Assay qualification and/or validation report (extent of data *flexible based on submission package; demonstrated by virus spiking studies conducted in product-specific or relevant material)*
- Details for NGS platform(s) used
  - Total reads, quality reads, number of reads analyzed
  - Method validation report to demonstrate the throughput is sufficient to reach satisfactory detection limit for breadth and sensitivity of virus detection

General NGS Expectations in *OVRR* Submissions (*cont.*)

- Details for the bioinformatics approaches and pipelines used including
  - Host cell removal – genome accession no. (*need to do testing for retroviruses*)
  - Subtraction of expected sequences (viral vector, known virus, ..)
  - Non-targeted analysis for broad AV detection; Targeted analysis for specific viruses
  - With and w/o *de novo* assembly
  - Different data formats
    - Excel sheets or any structural data formats with list of hits, justification for calling a hit "expected" (background, low complexity, etc)
    - Mapping data is useful for easy visualization
  - Viral Reference Database – RVDB or other
- Details of all tools and program used, parameters applied
- Cut-off criteria for positive/negative

General NGS Expectations in *OVRR* Submissions (*cont.*)

- Follow up of NGS signal to determine biological relevance and significance
  - Calling criteria for including or excluding unexpected hit(s)
  - Verification of results
    - *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?*

❖ *Review of various submissions is being used to develop a knowledge database and create a general format as reference for expected information to be included in submissions*

Implementing NGS for Adventitious Virus Testing of Biologics

- Increased efficiency (time)
- Ethical (reduce animal use; 3Rs)
- Superiority (LOD, specificity, repeatability, accuracy)

❖ Current cell substrate and viral safety guidelines and regulatory guidances provide flexibility for using alternative approaches with broad virus detection capabilities and “fit-for-purpose”

- *US FDA (2010)*
- *WHO (2010, 2013)*
- *Ph. Eur. (2017)*
- *ICH Q5A(R2) (2023)*
- *Ph. Eur. (2.6.41; expected 2025)*

Some Expectations for Using NGS for Adventitious Virus Testing

- **NGS applications for adventitious virus testing include replacing or supplementing the conventional adventitious virus detection methods**
  - NGS is considered a limit test [ICH Q2(R2): *Validation of Analytical Procedures]*
  - The method should be appropriately validated for its intended use. This includes the method **validation** and **matrix-specific qualification/verification** using suitable reference materials such using virus standards and a comprehensive viral database to demonstrate the limit and breadth of virus detection.
  - Method **validation** requires predefined performance criteria while method **qualification** only evaluates the performance characteristics of the method.

- *A head-to-head comparison of NGS with existing methods is not required*

Expectations for NGS Reports

- Generic method validation report to demonstrate the capacity of NGS for breadth and sensitivity of virus detection
- Product/Matrix specific qualification report to demonstrate absence of interference in NGS virus detection (*if generic validation is not done in the matrix*)
- Study Report of the test sample with SOPs and results (including controls)

Still More Work for Routine Implementation!

- Continued in-house efforts and external work (AVDTWG members and NIIMBL/BMG project)
- Optimization of pre-treatment conditions to increase sensitivity of virus detection in complex matrices
- Development of SOPs and reference datasets for broader NGS implementation
- Development of other types of standard materials (e.g., VLPs, virus-infected cell lines)
- Database enhancements for increasing accurate virus detection and reducing follow up of false positive signals

U.S. Department of Health and Human Services Food and Drug Administration
Center for Biologics Evaluation and Research [February 2010]

Guidance for Industry

Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

- Provides guidance for the characterization and qualification of **cell substrates, viral seeds, and other biological materials (including vaccine intermediates)** used in manufacture of viral vaccines for human use.

https://www.fda.gov/regulatory-information/search-fda-guidance-documents/characterization-and-qualification-cell-substrates-and-other-biological-materials-used-production

THANK YOU FOR YOUR ATTENTION!