IABS-HSI Webinar- Global availability of critical reagents for biologicals testing: Current status, challenges and possible solutions.

Reference Materials for Detection of Adventitious Viruses in Biologics by HTS/NGS

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July 2, 2024

Disclaimer

Routine Adventitious Virus Tests for Vaccine 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 novel cell substrates *(OVRR/CBER: recommended case-by-case)***
  - Extended PCR assays
  - Expanded cell culture assays (*adding more target cell lines*)
  - Oncogenicity assays: Tumor-inducing viruses
  - Chemical induction assays: Latent viruses (endogenous and episomes)

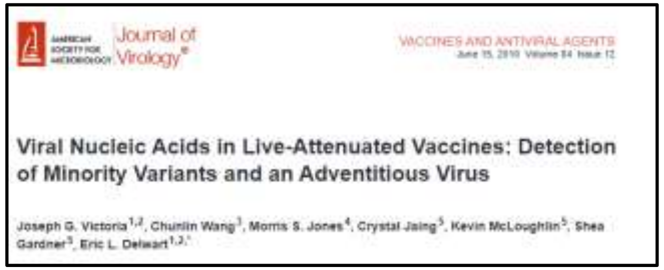
The currently recommended assays have been generally effective in demonstrating the absence of adventitious viruses for product safety

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) or 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

HTS for Broad Detection of Adventitious Viruses

- HTS was initially recognized as a powerful advanced technology for adventitious virus detection by **the identification of 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

Received 16 March 2014 Accepted 21 March 2014
Published ahead of print 26 March 2014

June 2014 Volume 88 Number 12

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

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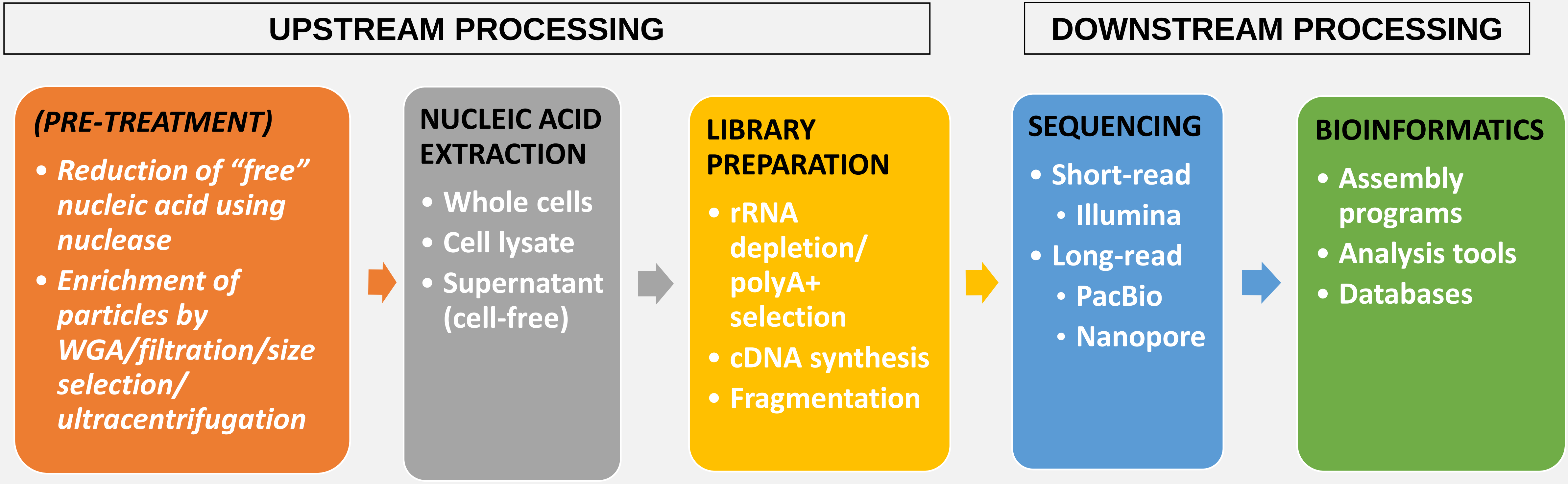
  - ➢In both cases, routine testing had been done. Additional testing using degenerate PCR assays to detect various insect virus families was done for the Sf9 cells since it was a novel cell substrate.

- The testing gaps and complexity of the HTS technologies were recognized in CBER and by industry leading to their combined efforts for establishing the Advanced Virus Detection Technologies Interest Group in 2014, supported by the Paternal Drug Association (PDA)

HTS Workflow for 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

General Challenges of HTS Applications for Virus Detection

- **Standardization and validation**
  - Appropriate reference viruses and other standards (*for spiking studies*)
    - Efficiency of the different steps involved in the methodology
    - Sensitivity and specificity
- **Bioinformatics**
  - Data analysis
    - Pipeline optimization
      - Reference datasets
      - Criteria for acceptable quality of reads
      - Parameters for short read assembly; hybrid assembly to correct high error-rate currently seen in long-read sequencing
    - Development of a complete and correctly annotated, publicly available, Reference Virus Database
    - Develop strategies for novel virus detection
  - Data submission, storage, and transfer
    - Format
    - Security
- **Follow-up strategy**
  - Confirmation of a “true” hit
  - Determination of biological relevance and significance of a positive signal

Advanced Virus Detection Technologies WG

Mission: To facilitate the use of advanced technologies (such as NGS) for the detection of adventitious viruses in biologics by providing an informal, scientific forum for knowledge exchange, scientific discussions and collaborations among scientists across different organizations

Organization:

> 200 participants

> 60 organizations

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

Meeting/discussions virtually once every 2 months

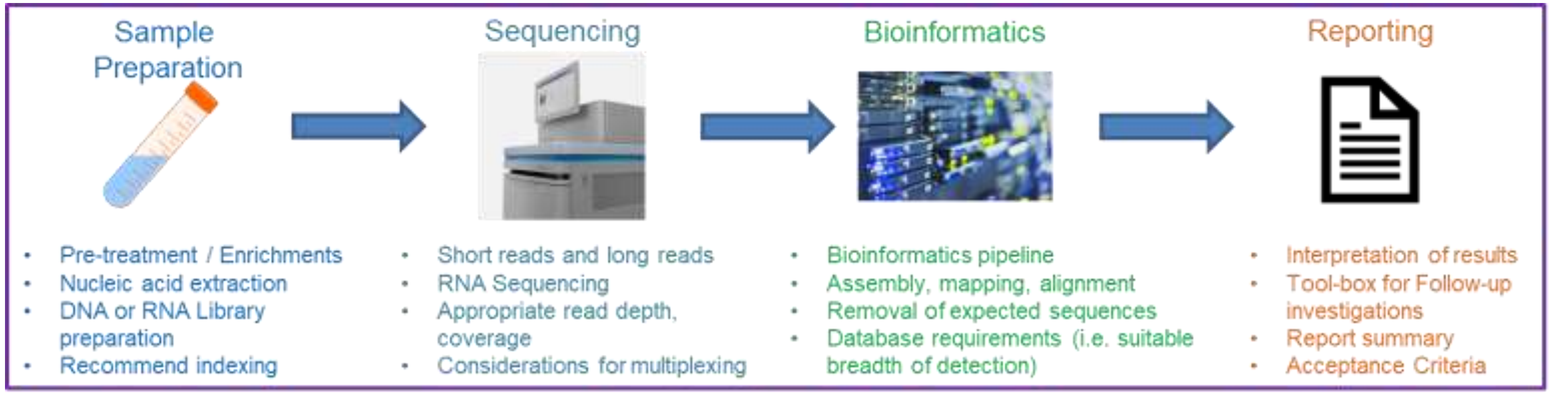
Co-chairs:

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

AVDTIG

AVDTWG Subgroups

Multiple key challenges requiring additional scientific data



Sample Preparation

- Pre-treatment / Enrichments
- Nucleic acid extraction
- DNA or RNA Library preparation
- Recommend indexing

Sequencing

- Short reads and long reads
- RNA Sequencing
- Appropriate read depth, coverage
- Considerations for multiplexing

Bioinformatics

- Bioinformatics pipeline
- Assembly, mapping, alignment
- Removal of expected sequences
- Database requirements (i.e. suitable breadth of detection)

Reporting

- Interpretation of results
- Tool-box for Follow-up investigations
- Report summary
- Acceptance Criteria

2012

Subgroup A

Sample selection/ preparation/processing

Subgroup B

Virus standards and reference materials

Subgroup C

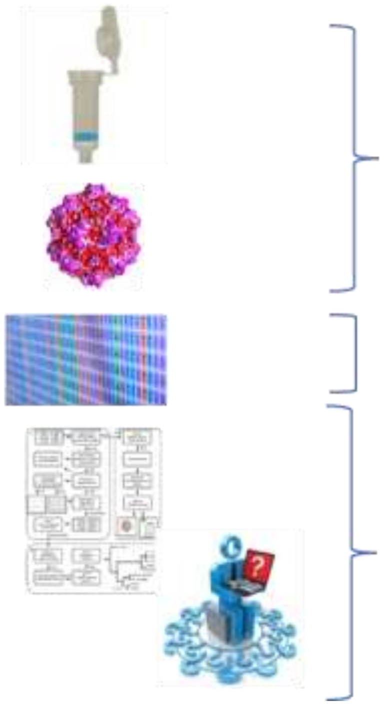
Complete and correctly annotated, virus reference database

Subgroup D

Bioinformatics pipelines analysis

Subgroup E

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



2024

Subgroup AB

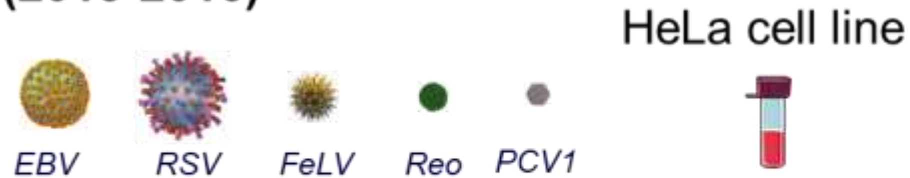
Subgroup C

Subgroup DE

AVDTWG – Subgroup AB
Collaborative Spiking Studies (Matrix Mimics)

- **For performance evaluation and standardization of NGS**
  - Evaluate reference standards and bioinformatics tools
  - Compare and optimize experimental protocols

Spiking study 1 (*short-read NGS*) (2013-2016)




mSphere

A Multicenter Study To Evaluate the Performance of High-Throughput Sequencing for Virus Detection

Arifa S. Khan, Siemon H. S. Ng, Olivier Vandeputte, Aisha Aljanahi, Avisek Deyati, Jean-Pol Cassart, Robert L. Charlebois, Lanyn P. Taliaferro

Short-read NGS

Spiking study 2A Started 2017	MVM in *CHO cell background*
Spiking study 2B Started 2016	WHO 5 viruses in high titer virus background: *Viral seed/Viral vector*
Spiking study 3 Started 2019	Transcriptomics: infected cells in uninfected cell background

Long-read NGS

Spiking study 4 Started 2020	WHO 5 viruses in high titer virus background: *Viral seed/Viral vector*
Spiking study 5 Discussions- 2023	Cell substrate/high cellular background
Spiking study 6 Discussions-2024	Transcriptomics: virus replication in cell culture

International Standards are Needed for Implementation of HTS for Adventitious Virus Detection in Biologics

- For qualification and validation of HTS*
  - Provide consistency in review of regulatory submissions
  - Needed for HTS applications for replacing or supplementing the currently recommended *in vivo* and *in vitro* assays for adventitious virus detection
    - -> Reduce use of animals (meet the global 3Rs objectives)
    - -> Shorten the testing time (current assays $\geq$ 28 days)
    - -> Expand the breadth of virus detection to include known, unexpected viruses and unknown, novel viruses
    - -> Accelerate vaccine development
- Intact viruses are particularly useful to evaluate the performance of all the different steps in the entire HTS workflow

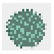
***Draft Ph. Eur. 2.6.41**

CBER's Reference Materials for HTS Validation and Qualification

- ❑ **Development of virus reference stocks**
  - 5 large scale, well-characterized, live virus stocks were prepared at ATCC
    - Adopted as WHO International Ref Reagents for adventitious virus detection by HTS in October 2020
    - Distributed upon request to industry, CROs, and others for HTS validation studies and assay qualification
  - 7 larger scale, virus stocks were prepared at ATCC, including 5 original ones plus 2 new families
    - Adopted as the 1st WHO International Virus Panel for AV detection by HTS (*March 2024*)
- ❑ **Generation of a Comprehensive Reference Virus Database (RVDB)**
  - In-house lab efforts, in consultation with AVDTIG scientists, resulted in development of a viral database that includes all viral, viral-related, and viral-like sequences selected from all species of cell substrates used for biologics (*Goodacre, et al., mSphere, 2018*)

WHO Reference Virus Panel for NGS/HTS Adventitious Virus Detection

CBER Virus Reagents - Currently Available

		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	linear 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 Virus Panel – adopted March 2024

https://www.niaid.nih.gov/research/bei-resources-repository BEI NR-59622

(*new ones expected to be available July-Aug 2024 BEI NR-59630*)

Development of Reference Viruses

❖ **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
- Stability studies: infectious titer; genome copy number

➢ **Vialed individually to allow freedom for custom-mixing, as needed by user**

- About 300 vials remaining of the currently distributed 5 viruses
- 1000 vials stored for each of the new 7 viruses (*expected distribution in Q2/2024*)

○ CBER has supported storage and distribution at ATCC. Transferred to NIH/NIAID BEI from Sept 29, 2023 for long-term storage and distribution

A Comprehensive Reference Virus Database (RVDB) for HTS 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)*
  - Contained all viral sequences regardless of size
  - Included endogenous viral and retroelements
  - Has a reduced cellular content

- The latest version RVDBv28.0 (*Nov 22, 2023*) 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!)*
- *Updated quarterly by the Khan Lab (Pei-Ju Chin)*
- *Ongoing work on annotation of sequences to remove misannotated sequences and enhance virus-specific detection (Pei-Ju Chin & Trent Bosma)*

RVDB Provides 4 Formats to Adapt Various Application Scenarios

- U-RVDB *fasta* file
  - Un-clustered, contain all viral sequences with redundancy
  - Higher computation-demanded. **Suitable for virus detection by *blastn/nhmmer***
- C-RVDB *fasta* file
  - Clustered, sequences share 98% similarity are collapse to one representative sequence for each clade
  - Lower computation-demanded. **Suitable for virus detection by *tblastx***
- SQLite DB Script
  - Create the entries (*fasta* header and the corresponding information) for advanced bioinformatic pipelines/workflows
- Proteic RVDB
  - **Hidden Markov Model (HMM)** profile of viral protein domains
  - Unknown viruses with remote homology by ***hmmsearch*** / ***hmmscan***
  - provided by Institut Pasteur: https://rvdb-prot.pasteur.fr/

Further Efforts towards HTS Implementation

ICH Q5A Revision (published in Nov. 2023)

- Includes HTS as a potential approach for specific (targeted) and broad (non-targeted) adventitious virus detection.
  - Replace in vivo assays without a head-to-head comparison
  - Supplement or replace cell-based infectivity assays
  - Positive results should be investigated to determine whether the detected signal is from an infectious virus.

Creation of a Ph. Eur General Chapter (public comment period ended June 30, 2024)

- A newly formed HTS working party has drafted a new Ph. Eur. general chapter (2.6.41) on “High Throughput Sequencing for the detection of extraneous agents”
  - Provide details on important considerations when implementing a HTS for adventitious virus detection assay
  - A strategic frame-work for the HTS validation approach

Outcomes of collaborative HTS spiking studies - BMGF/NIIMBL projects (CBER/GSK/Merck-MilliporeSigma)

- **3.1G-305:** Completed study with head-to-head comparison evaluating HTS, in vivo, and in vitro assays. Identical material used in all of the assays! Supports recommendations for replacing the in vivo adventitious virus detection assays.
- **GHF-04:** Initiated study (June 1) for optimization of virus detection by HTS in a complex matrix and further refinements of RVDB to enhance efficiency of database generation and accuracy of adventitious and endogenous virus detection and

Moving HTS into LMICs

- Discussions involve IABS, FDA, EDQM, WHO, and AVDTWG
  - HTS-focused conferences, workshops, and webinars to provide training in LMICs to facilitate technology implementation for global reduction in animal use (3Rs objectives) are being planned for 2024

ICH Q5A(R2): New Nucleic Acid-Based Test Methods

- Guideline encourages use of new alternative tests (includes Next Generation Sequencing and PCR in discussion) -> **Aligns with the 3Rs initiative to reduce animals for testing**
- Specific opportunities to replace existing methods with targeted test (e.g. PCR or HTS) for replacing antibody production tests or non-targeted (agnostic/broad; such as HTS) for replacing in vivo/in vitro.
  - Antibody Production Tests in Rodents (MAP, HAP, RAP)
  - *In Vivo* Assays
  - *In Vitro* Assay
  - ➢ Highlights that direct head-to-head comparison with existing methods is generally not expected (*in vivo and in vitro*)
- It is recognized that these nucleic acid-based assays have limitations as they cannot distinguish between infectious and noninfectious particles and therefore detection of a signal may need a confirmatory test with an infectivity assay for risk-assessment.

ICH Q5A(R2): New Test Methods

- NGS is considered a “**limit test**”. This means that qualification /validation should consider the “**detection limit**” and “**specificity**” (breadth of viruses detected).
- NAT should be appropriately validated for their intended use. This includes the method validation and matrix-specific verification, as applicable.
- Validation of NGS includes suitable reference materials such as virus standards and comprehensive viral database
- Method **validation** requires predefined performance criteria while method **qualification** only evaluates the performance characteristics of the method.
- Clarifies that head-to-head comparison of NGS with existing methods **is not recommended for both,** *in vivo* and *in vitro* virus testing.

Ph. Eur. General Chapter: 2.6.41

HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

SELECTION OF SPIKING MATERIAL FOR VALIDATION

- “The spiking material used for validation of the HTS method must be relevant for the intended purpose and the chosen strategy for detection. The use of model viruses as spike is relevant for the viromics and genomics approaches, whereas the use of infected cells is more appropriate for the transcriptomics approach. “

- “The WHO *International Reference Panel for Adventitious Virus Detection by High-Throughput Sequencing* (hereinafter referred to as ‘WHO virus reference panel’) provide diverse virus families: EBV, FeLV, RSV, PCV, Reovirus, MVM, and HCoV-OC43 and is recommended as the minimum panel of model viruses for validation. Model viruses may be spiked individually or combined as one single mixture. “

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General Acceptance of HTS for Adventitious Virus Detection in Biologics

- **Cell substrate characterization** – particularly for novel cell substrates or in case where there are concerns for occult and novel viruses
- **Supplementary to or replacement of *In vitro* AV assays** – particularly in case of assay interference due to lack of effective neutralization of vaccine virus
  - Potentially supplement *in vitro* AV assays as a read-out assay to broaden virus detection
- **Replacement assay (*in vivo* and PCR assays)**
  - *In vivo* AV assays – HTS can provide defined sensitivity and breadth of virus detection
  - Antibody Production Assays – MAP, RAP, HAP
    - ➢ *Reduce use of animals – meet the global objectives for 3Rs (reduction, refinement, replacement)*
  - PCR assays – HTS can have similar sensitivity to PCR assays
    - Single assay with broader virus detection

CBER/OVRR: Examples of Submissions Using HTS 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			

Ongoing Work on Reference Materials (Khan Lab)

- **Development of a viral reference reagent for cell-based biologics**
  - Human cell line with stably integrated retrovirus (cell clones with latent and active infection)
  - Well-characterized for viral genome copy number and integration site(s)
  - Can be used for spiking into an uninfected cell background to evaluate sensitivity of virus detection by NGS in cell substrates and cell-based products

Still More Work for Routine Implementation!

- Optimization of pre-treatment conditions to increase sensitivity of virus detection in complex matrices
- Development of SOPs and test datasets for establishing HTS by an early user of the technology
- Development of other types of standard materials for those who do not want to handle live viruses (e.g., VLPs)

Thank You!