High-Throughput Sequencing for Adventitious Virus Detection in Biologics

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Overview

- Considerations for using HTS for adventitious virus detection in biologics
  - What is the intended purpose of the application (*complementing, supplementing or replacing a current assay)*?
  - Which platform would be most suitable (*for obtaining the data to support the claim*)?
- Readiness of HTS for implementation
  - FDA and other efforts
  - Availability of reference materials for HTS qualification/validation
- Acceleration of HTS applications for adventitious virus testing

  - *COVID-19 Era*

Next Generation Sequencing Platforms

454 Roche FLX+
A Pyrosequencing System

Yield per Run:0.4-0.7Gb
Reads Lenght: up to 700bp
Instrumental Time: 5h
Equipment Cost:$500K

Illumina Platforms
High throughput Sequencing

Yield per Run:7-350Gb
Reads Lenght: up to 300bp
Instrumental Time: 2-10days
Equipment Cost:$128-654K

PacBIO RS II
Long-read Sequencer

Yield per Run:0.5-3Gb
Reads Lenght: up to 20kbp
Instrumental Time: 2h
Equipment Cost:$695K

Oxford Nanopore MinION
Miniaturised USB Device

Yield per Run:5Gb
Reads Lenght: up to 200kbp
Instrumental Time: 1-48h
Equipment Cost:$1K

Short-read sequencing

Long-read sequencing

2nd HTS

3rd HTS

Goals of a Well-Characterized Product

✓ **Adventitious viruses are a major safety concern in all biological products**

❖ **Obtaining knowledge of the product**

- Assuring product safety by demonstrating the absence of unexpected or unintended components
  - Adventitious agents

- Characterizing the expected, intended components
  - Product
  - Some cellular components (host proteins and nucleic acids)

Origins of Adventitious Virus Contamination in Biologics

- **Viruses in donor species of source materials** *(virus seed, cell substrate, or animal-derived cell culture reagents)*
  - Naturally-occurring exogenous and endogenous viruses
  - Acquired by specific-exposure to infectious agents (e.g., animal vaccines, environmental)

- **Cell substrate passage history**
  - Cross-contamination in previous facilities (*if applicable*)
    - Propagation in different labs
    - Other viruses or cell lines used
  - Raw materials (serum, trypsin, etc.)
  - *De novo* generation of novel recombinant viruses

- **During product manufacturing**
  - Potential introduction due to: Handling; Equipment; Raw materials
  - Potential activation of endogenous retroviruses or reactivation of latent DNA viruses due to production conditions

Recent, Unexpected Cases of AV Detection Using Advanced Technologies: *Detection of Known and Novel Viruses*

BIOLOGICAL RAW MATERIALS

- **BOVINE SERUM** *(suspected)* **- June 17, 2009: Vesivirus 2117 detected CHO cell bioreactor production runs of Cerezyme and Fabrazyme by Genzyme: MS/LC detection of peptides, RT-PCR confirmation (3 events, different processes at 2 different plants** (*2009 Cell Substrate Workshop, Genzyme*)

- **PORCINE TRYPSIN- April 7, 2010** (*online*): **Porcine circovirus Type 1 (PCV1) sequences detected by UCSF lab and LLNL in Rotarix using virus microarrays and high throughput sequence analysis 2014:** (*Victoria et al., J. Virol. pub. June 2010, vol. 84,. 6033-6040*)

- **HOST CELL LINE- March 26, 2014** (*online*): **Discovery of novel rhabdovirus in Sf9 cell line using degenerated PCR and high throughput sequencing** (*Ma et al., J. Virol. Pub. June 2014, vol. 88, 6576-6585*)

❖ ***Conventional testing using currently recommended assays failed to demonstrate absence of these adventitious viruses.***

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
    - Know the spectrum of infectious viruses that could potentially be in the host species of source materials (naturally-occurring, animal vaccines)
    - Gain cell culture passage history and characterization
    - Examine potential for virus exposure in the supplier's facilities (*including chemically-derived materials*)
  - **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
    - Product purity: reduction of residual cellular materials (DNA, RNA, proteins)
- **TESTING**
  - **Detection of known and unknown agents** in the starting materials (*cell substrate, virus seeds, vector virus preparation*)
  - **Different stages** in the manufacturing process and at **steps with the greatest potential for contamination**
  - **Sensitive and broad detection assays**

Conventional Testing for Detection of Adventitious Viruses

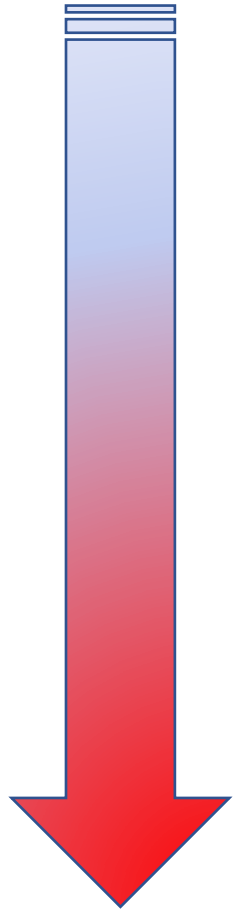
- **General Detection Assays**
  - *In vitro* cell culture tests in cell lines of 3 species (same as cell substrate, monkey, and human)
  - *In vivo* assays (adult mice, suckling mice, embryonated hens' eggs)
  - Transmission electron microscopy (TEM)
  - Reverse transcriptase assay for retroviruses (PERT)
- **Species-specific Assays**
  - Tests for animal viruses *e.g.* bovine, porcine (9CFR 113.47 and 113.53)
  - Antibody-production assays for rodent viruses (MAP *including LCMV challenge*, HAP, RAP)
  - Assays for known viruses (PCR, DNA hybridization, Infectivity, antibody detection)
- **Additional assays for novel cell substrates** *(OVRR/CBER: recommended case-by-case)*
  - Extended PCR assays
  - Oncogenicity assays: Tumor-inducing viruses
  - Chemical induction assays: Endogenous retroviruses, latent DNA viruses

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**
  - 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

Applying HTS for Adventitious Virus Detection of Known and Novel Viruses

ASSAY QUALIFICATION AND VALIDATION



- To provide complementary data for added confidence in demonstrating absence of adventitious viruses using the conventional assays
  - In case of enhanced safety concerns for adventitious viruses *e.g.* in case of novel cell substrates or new manufacturing platforms; unknown cell passage history
- To provide supplementary results for addressing limitations of the conventional virus detection assays
  - In case of limited assay interference due to the test sample *e.g.* due to sample-related cell toxicity or virus-induced cytopathic effect
  - Cell substrate characterization *e.g*. where there are added concerns for occult viruses due to cell passage history or using a novel cell substrate
- For replacement of one or more of the conventional assays
  - In case of extensive assay interference that prevents use of cell-based and animal assays
  - Reduce animal use
  - Replace using numerous PCR assays with one assay

General Challenges of NGS Applications for Virus Detection

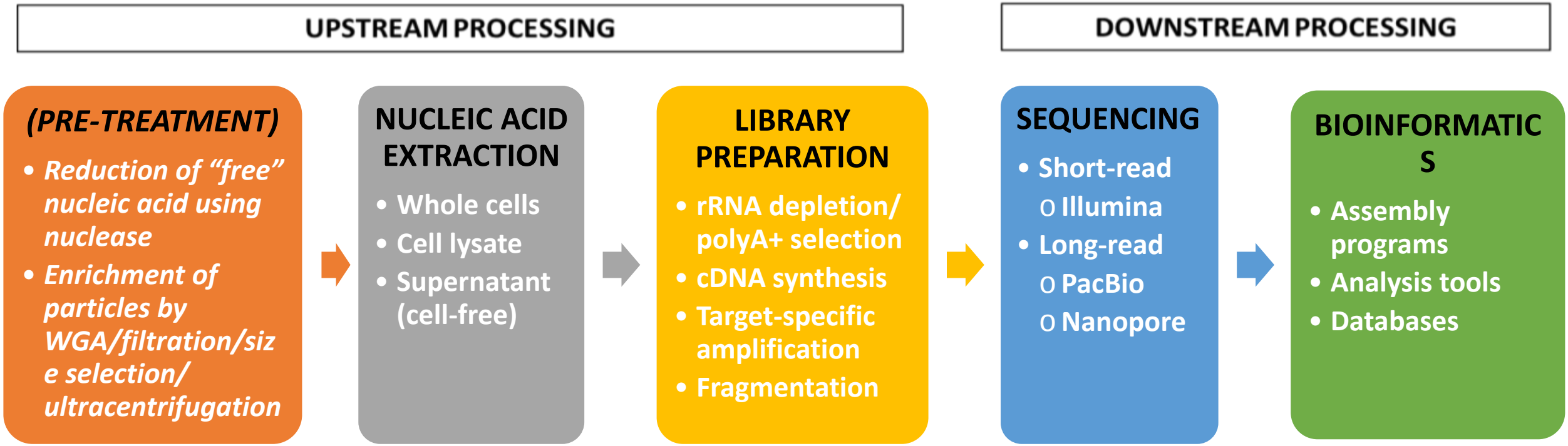
- **Qualification and validation**
  - Appropriate reference viruses and other standards (for spiking studies)
    - Efficiency of the different steps involved in the methodology
    - Sensitivity and specificity
- **Bioinformatics**
  - Data analysis
    - Pipeline optimization
      - Reference datasets
      - 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

Introducing New Assays for Regulatory Applications

- **Assay qualification or validation**
  - Development of appropriate standards
  - Inclusion of relevant controls (*positive and negative)*
  - Determination of sensitivity and specificity
  - Demonstration of precision (*reproducibility and repeatability*)
  - Evaluate assay robustness (*change of assay conditions and reagents*)
  - Demonstrate the reliability of the assays (*e.g. interference of sample matrix to the assay's intended use*)
  - More details on the validation of analytical assays and statistical analyses are described in ICH Q2(R1)

- **Availability of assay (establishment of method in-house or through CROs)**

HTS Workflow



FDA Efforts on HTS for Adventitious Virus Detection in Biologics

- Establishment of FDA and CBER Genomic WGs to support a research and regulatory infrastructure to support policy development and decision-making related to applications of HTS
- Strengthen in-house FDA laboratory and bioinformatics expertise for HTS analysis
- Establishment of the FDA/Industry led PDA-Advanced Virus Detection Technologies Interest Group (AVDTIG) with focused efforts on HTS standardization and implementation for adventitious virus detection in biologics (*PDA J Pharm Sci and Tech 2016, 70 591-595)*

Advanced Virus Detection Technologies Interest Group (AVDTIG)

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

"Mission" – To advance next generation sequencing for viral risk evaluation by providing an informal, scientific forum for discussions and scientific collaborations

Co-chairs

- Arifa Khan: FDA, U.S.A.
- Dominick Vacante: Janssen R & D, U.S.A.
- Jean-Pol Cassart: GSK, Belgium
- Keisuke Yusa: National Institute of Health Science, Japan

➢ **More than 180 participants (> 80 organizations): industry *(vaccines, cell and gene therapies, therapeutics)*, regulatory and other government agencies and national authorities, academia, CROs, and others**

- Meetings/discussions by t-con every other month
- Five focus subgroups on identified priority areas, with additional meetings

AVDTIG - Subgroups

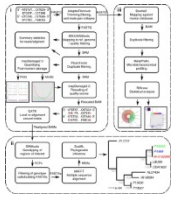
Subgroup A
Sample selection/ preparation/processing
Siemon.Ng@sanofipasteur.com

Subgroup B
Virus standards and reference materials
Megan.cleveland@nist.gov

Subgroup AB

Open platform for discussion and initiation of new studies

Subgroup C
Complete and correctly annotated, virus reference database
Arifa.Khan@fda.hhs.gov



Subgroup D
Bioinformatics pipelines analysis
Christophe.G.Lambert@gsk.com

Subgroup E
Follow-up strategies to confirm the identity of a "hit"
Robert.Charlebois@sanofipasteur.com

Subgroup DE

16

Development of HTS Reference Virus Stocks

➢ **Based on results of the 1st collaborative spiking study with CBER, GSK, Sanofi (Khan et al., 2017, mSphere) and discussions in the AVDTIG, 5 large scale Reference Virus Stocks with distinct physical, chemical, and genome properties were made at ATCC by the Khan Lab**

❖ **Well-Characterized**
- Sterility, Mycoplasma
- Infectious titer
- Viral genome copy number (ddPCR)
- Reference virus genome (HTS)
- Residual host cell nucleic acids (ddPCR)

❖ **Stability studies**
- 24-month

❖ **Vialed individually**
- To allow freedom for custom-mixing by user

Virus Name	Total vials prepared
Porcine circovirus type 1	392
Human orthoreovirus type 1	403
Feline leukemia virus	503
Human respiratory syncytial virus	388
Epstein-Barr virus	490

WHO Reference Virus Reagents for HTS

Currently Available:

- Based on data from the recent AVDTIG #2B Spiking Study, CBER's 5 virus stocks were established as WHO International Virus Reference Reagents for Adventitious Virus Detection in Biological Products by HTS technologies (*ECBS, Oct. 2020*)
- Virus Stocks are currently being used for spiking studies in the AVDIG to evaluate performance of HTS in different matrices mimicking various biological sample materials relevant for vaccines and therapeutics (cell substrate, virus seed, bulk harvest)
- Provided upon request for HTS qualification and validation studies for adventitious virus detection (*and other relevant studies with justification*)
- Contact: arifa.khan@fda.hhs.gov

Ongoing Work:

❖ 1000 vials of each virus is under production by CBER at ATCC to replenish the current reference virus reagents and to expand the virus families to include a seasonal coronavirus (OC43) and a parvovirus (MMV)

KHAN LAB: Development of a New Reference Viral DataBase (RVDB)

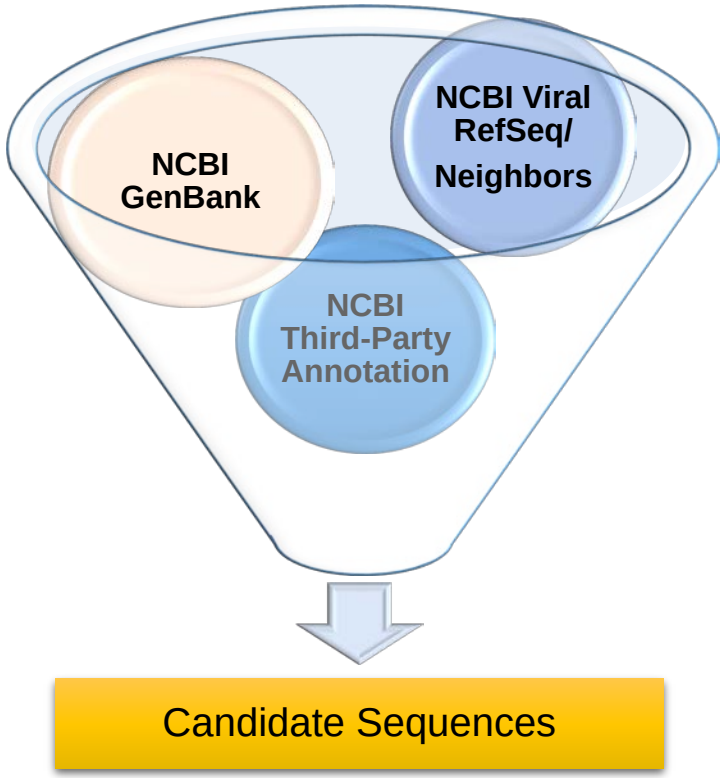
- Limitations of the public databases were recognized by several including the Khan lab during discovery of the novel Sf9-rhabdovirus *(Ma et al., 2014*)
  - NCBI Viral Genomes Resource (RefSeq and Neighbors) had under-representation of partial viral sequences and endogenous retroviruses
  - Nr/nt (non-redundant nucleotide collection) had viral diversity and included full and partial viral genomes but had abundant cellular and uncharacterized sequences and many were mis-annotated
  - NCBI protein database (non-redundant protein sequences) only contains complete protein sequence

Generation of RVDB for Known and Novel virus detection

RVDB includes sequences of all virus species from eukaryotes (based on cell substrates used in biologics), regardless of size

Semantic-Refine (SEM-R) Filter

"rRNA"
"Phage"
"Receptor"
"Cytochrome"
"non autonomous"
"Nuclear envelope"
"endogenous tripeptide"
……≈700 Negative Keywords



NCBI GenBank

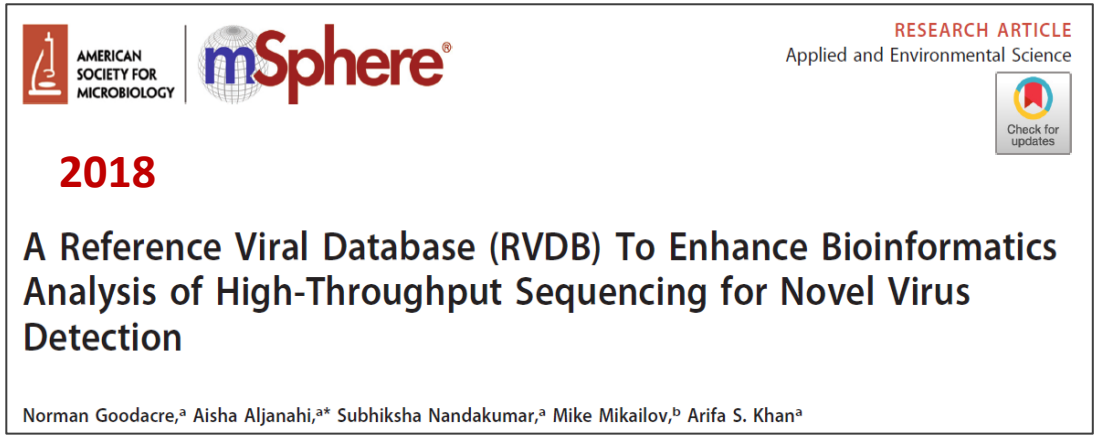
Environmental Sampling (ENV)
HTS Sampling(HTC)
Invertebrate (INV)
Mammalian (MAM)
Plant (PLN)
Primate (PRI)
Rodent (ROD)
Viral (VRL)
Vertebrate (VRT)
Third-party Annotation (TPA)
Phage (PHG)

A Reference Viral Database (RVDB) to enhance HTS bioinformatics analysis for novel virus detection

❖ ***A comprehensive database for broad virus detection (known and distantly-related sequences)***

- **Performs HTS bioinformatics with less computational effort and time due to the reduced nonspecific cellular hits**

➢ **Publicly available in four formats provided for use in various bioinformatics strategies**
  - Unclustered U-RVDB
  - Clustered C-RVDB (collapsed at 98% identity)
  - SQLite DB Script
  - Proteic RVDB



➢ **RVDB is updated quarterly and coincident with a GenBank/RefSeq updates to include new viral sequences deposited in GenBank along with an annotation file to aid in detection of emerging viruses**

➢ **Current RVDB version 23.0 is available by the Khan Lab at Univ Delaware:**
  - **https://rvdb.dbi.udel.edu/**

Potential Applications of NGS 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

HTS Can Enhance Adventitious Virus Testing

Generally recommended testing (Results > 28 days)

- ***In vitro* virus tests**
  - General
    - AV testing in 3 cell lines
    - Retrovirus testing: PERT/infectivity
    - TEM
  - Species-specific
    - Testing for bovine and porcine viruses
    - PCR assays
- ***In vivo* virus tests**
  - General
    - AV testing in 3 animal species
  - Species-specific
    - Antibody production assays

- **HTS testing can reduce the number of tests and the testing time (*One-Stop Shop*)**
- **Results -> 1 – 4 weeks depending on the sample type and analysis**
  - Targeted vs agnostic for detection of known vs novel virus detection
  - Volume of data for detection of low vs high level of virus
- ***Ongoing refinements will improve testing time***
  - *Bioinformatics pipelines for agnostic analysis*
  - *Databases*

HTS for Virus Detection in Biologics

➢Replacement assay

- ***In vivo* AV assays** – HTS can provide defined sensitivity and breadth of virus detection
  - Reduce use of animals – meet 3R's objectives
    - Minimum numbers for 1 Test Article = 30 eggs; 20 adult mice; 40 suckling mice
  - Reduce testing time - months to days
- **PCR assays** – HTS can have similar or greater sensitivity than PCR assays
  - Single assay with broader virus detection

➢Complementary/Supplementary/Replacement assay

- **Cell substrate characterization** – particularly in case where there are concerns for occult and novel viruses
- ***In vitro* AV assays** – can shorten testing time from weeks to days
  - In case of assay interference due to lack of effective neutralization of vaccine virus or cell toxicity
  - As a read-out method for early and broad virus detection in cell culture assays

Introducing HTS for Improving Viral Safety Testing

- Increased efficiency (reduce number of tests, effort, time)
- Ethical (reduce animal use)
- Superiority (LOD, specificity, repeatability, accuracy)

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

- US FDA (2010)
- WHO (2010, pub. 2013)
- Ph. Eur. (2017)
- ICH Q5A(R2): (*in revision*)

Potential Approach for Follow-up of an HTS signal

- **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?*

- Determine **biological relevance and significance** (*as with any nucleic acid-based assay*)

- HTS data can aid in design of a “custom” infectivity assay for risk management

The Changing Landscape of HTS Applications in OVRR: *COVID-19 Era*

- OVRR has been receiving submissions requesting use of HTS as a broad adventitious virus test (*pre-COVID*).
- The number of requests have increased in 2020 for using HTS an alternative adventitious virus detection assay to accelerate SARS-CoV-2 vaccine development.
  - Increase in the number of sponsors using HTS
    - Increased in-house capabilities and commercial availability
  - Expanded use of HTS for product characterization and testing
    - Cell substrate characterization
    - Testing of Master and Working Virus Seeds and DS Harvest
    - Genetic stability of vaccine virus
  - Extended use of HTS for a virus detection
    - Complementary or supplementary assay -> Replacement of one or more conventional virus detection assays

HTS Review and Ongoing Efforts in OVRR

- HTS may be considered for supplementing or substituting the conventional assays for adventitious virus detection based upon justification for suitability and fit for purpose
- HTS data is considered on a case-by-case basis
  - Efforts are directed toward method qualification and assay validation
- Within OVRR we highly recommend that sponsors request a technical working group discussion related to the use of HTS and their product characterization
  - Non-regulatory meeting to discuss “plans” for use of HTS
  - Reach consensus prior to initiating lengthy, expensive studies
- In-house efforts for broad implementation of HTS
  - Development of viral standards and pipelines for adventitious virus detection
  - Generation of databases that are complete and correctly annotated to facilitate virus-specific detection
- Continue international discussions to harmonization of requirements for HTS

Summary

- Collaborative studies in the AVDTIG and externally can provide data and experience for using HTS technologies as a rapid alternative method to supplement or replace current AV tests
- Continued efforts for generating reference materials for HTS qualification and validation can facilitate HTS implementation and broader use
- It should be recognized that some aspects of HTS are still evolving and the infrastructure for HTS may not be established in all organizations. Therefore, currently, we need to maintain flexibility for using the conventional tests and the advanced HTS technologies
- Early discussions among regulatory authorities can help harmonize expectations/recommendations for using HTS by manufacturers

3rd NGS Conference – 2022

3rd Conference on Next Generation Sequencing for Adventitious Virus Detection in Biologics for Humans and Animals

Sept 27 - 28, 2022

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Vaccines, Gene Therapy Products, Therapeutics

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