

Webinar 3: Reference Materials for HTS/NGS-Based Adventitious Virus Detection

Teams / 12:00-14:00 CET 10th November 2026


sanofi

HTS for Adventitious Virus Detection :

Reference materials considered throughout Sanofi Vaccine test set up

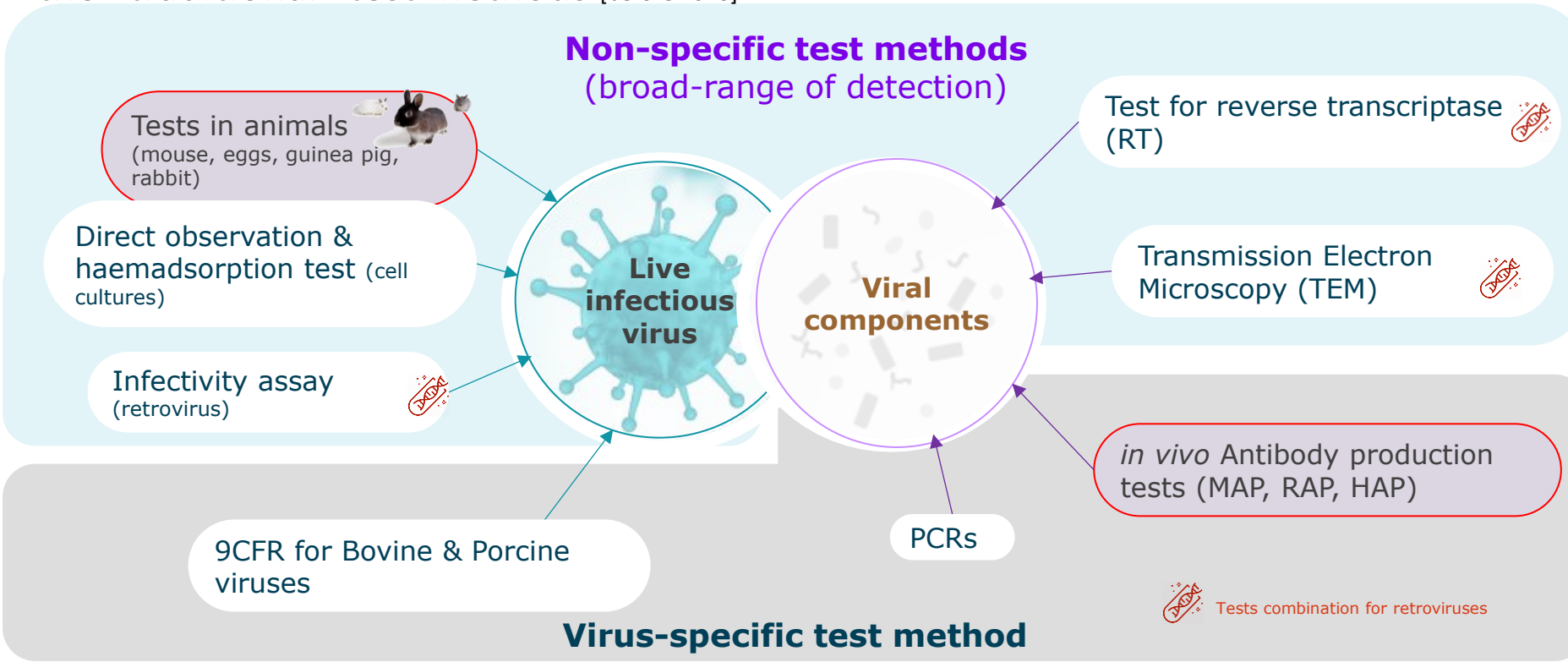
Carine LOGVINOFF

Virology Global Analytical Expert, Global AS & Regulatory Compliance, Analytical Sciences, Sanofi Vaccines (Marcy L' Etoile , France)

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How to Detect Viral Contaminants

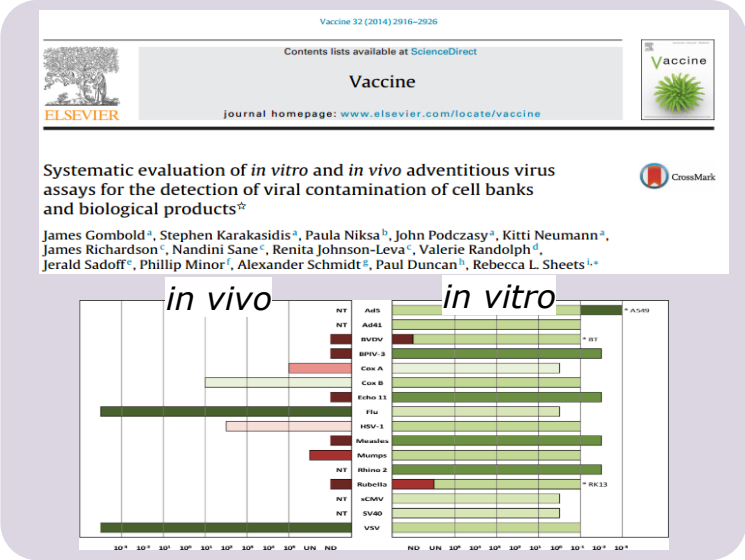
the “traditional” test methods [before 2010]



Some Limitations of the Traditional Testing Package

For non-specific test methods

Test	Test Read out	To be detected, the contaminant virus has
In vivo – Tests in animals	Compromised animal model health	to replicate in the animal model & to alter animal health
In vitro - Direct observation & haemadsorption (HAD) test	CPE and/ or HAD on indicator cells	to replicate on indicator cells & to induce CPE or HAD



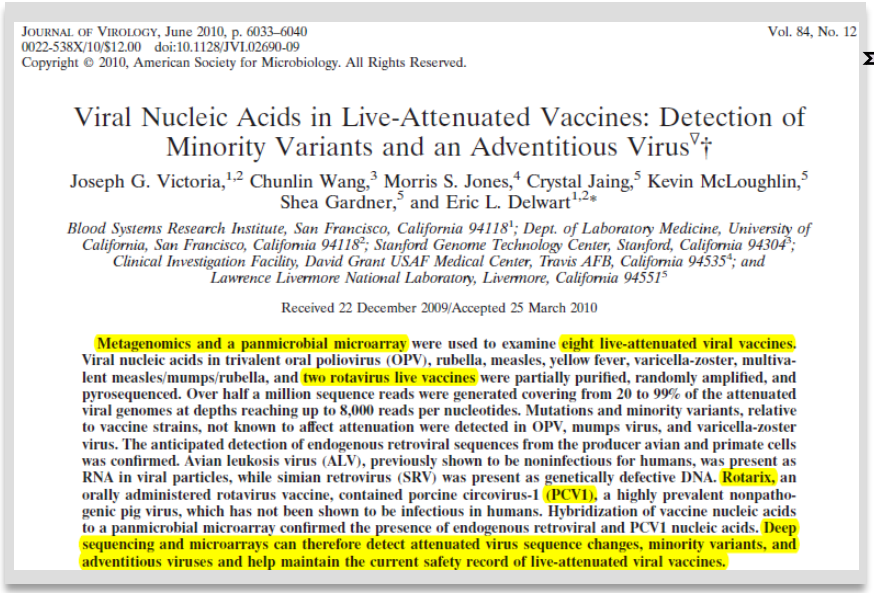
For virus specific test methods

- Need to select **which virus** to specifically look for

=> Viral Risk Assessment is to be conducted to define the adventitious testing package , and the specific testings

- Viral variants are to be considered, especially when designing a specific test such as PCR

A *Noisy* Entry of HTS for Adv. Virus in the Vaccine World



JOURNAL OF VIROLOGY, June 2010, p. 6033–6040 Vol. 84, No. 12
0022-538X/10/$12.00 doi:10.1128/JVI.02690-09
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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] and Eric L. Delwart[1,2]*

Blood Systems Research Institute, San Francisco, California 94118[1]; Dept. of Laboratory Medicine, University of California, San Francisco, California 94118[2]; Stanford Genome Technology Center, Stanford, California 94304[3]; Clinical Investigation Facility, David Grant USAF Medical Center, Travis AFB, California 94535[4]; and Lawrence Livermore National Laboratory, Livermore, California 94551[5]

Received 22 December 2009/Accepted 25 March 2010

Metagenomics and a panmicrobial microarray were used to examine eight live-attenuated viral vaccines. Viral nucleic acids in trivalent oral poliovirus (OPV), rubella, measles, yellow fever, varicella-zoster, multivalent measles/mumps/rubella, and two rotavirus live vaccines were partially purified, randomly amplified, and pyrosequenced. Over half a million sequence reads were generated covering from 20 to 99% of the attenuated viral genomes at depths reaching up to 8,000 reads per nucleotides. Mutations and minority variants, relative to vaccine strains, not known to affect attenuation were detected in OPV, mumps virus, and varicella-zoster virus. The anticipated detection of endogenous retroviral sequences from the producer avian and primate cells was confirmed. Avian leukosis virus (ALV), previously shown to be noninfectious for humans, was present as RNA in viral particles, while simian retrovirus (SRV) was present as genetically defective DNA. Rotarix, an orally administered rotavirus vaccine, contained porcine circovirus-1 (PCV1), a highly prevalent nonpathogenic pig virus, which has not been shown to be infectious in humans. Hybridization of vaccine nucleic acids to a panmicrobial microarray confirmed the presence of endogenous retroviral and PCV1 nucleic acids. Deep sequencing and microarrays can therefore detect attenuated virus sequence changes, minority variants, and adventitious viruses and help maintain the current safety record of live-attenuated viral vaccines.

- March 2010 : HTS revealed the presence of Circovirus in the final product of a commercial vaccine.
- May 2010 : VRBPAC Meeting
- Aug 2010 : FDA letter to licensed vaccine manufacturers

> Please describe any plans you may have to implement additional adventitious agent testing methods as part of your manufacturing process as these methods become available including, but not limited to, screening for PCV and PCV DNA as well as any additional in-process testing for adventitious agents that you may have recently added, but not reported to the agency. In this regard, please consider any animal derived materials (e.g., culture medium, albumin, enzymes, lipids, etc) and the point at which they are used in your product manufacture, any adventitious agent related quality control testing performed by the material vendor or done in-house, and any applicable viral clearance or inactivation steps provided by your manufacturing process.

- The conventional testing package could miss viral contaminant
- High-throughput sequencing (HTS) has the potential to identify both known and unknown adventitious viruses by sequencing all the nucleic acid within a sample without needing prior knowledge of the contaminating agents whatever the virus

⇒ Development of Adventitious Virus detection by HTS at Sanofi Vaccines started in 2010

Our Choice : *In Vivo* Tests Substitution

In vivo tests limitations as highlighted by Gombold *et al*, 2014

3Rs : EMA *Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches*

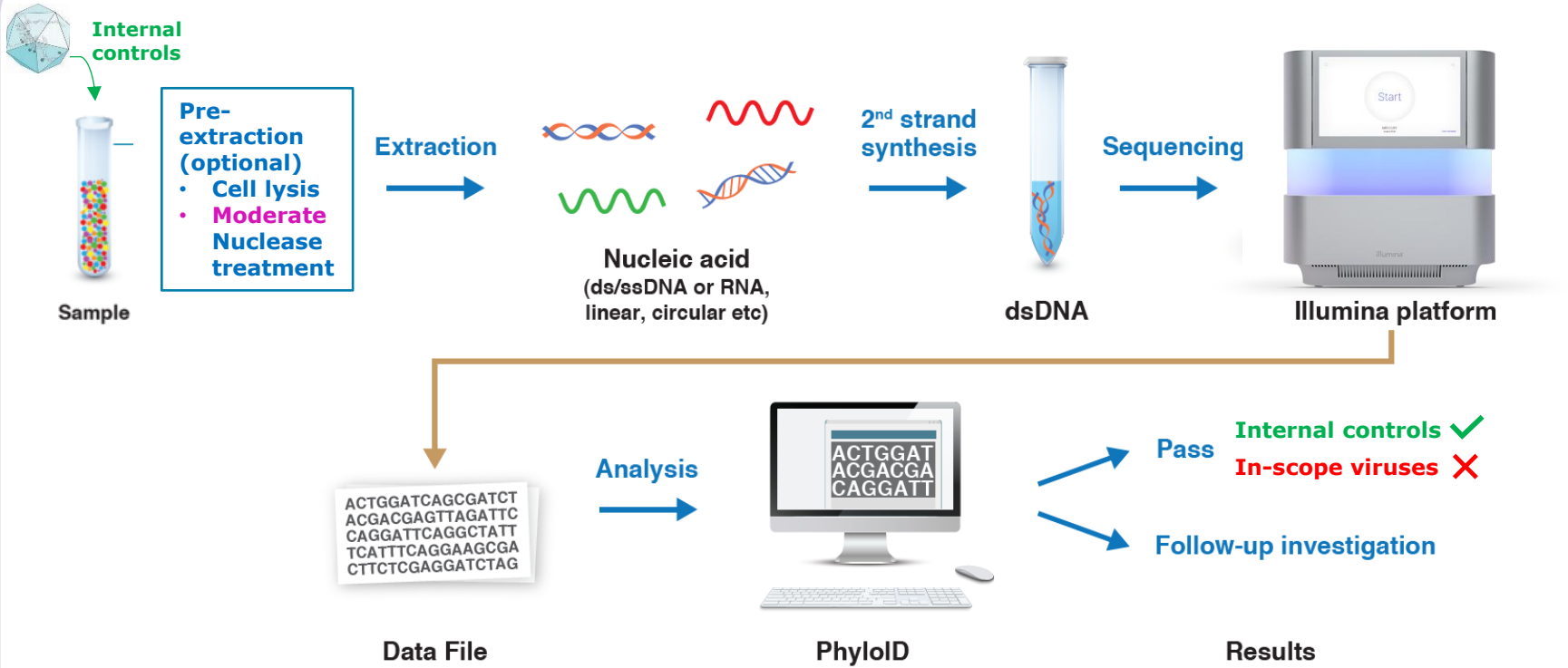
In vivo tests are typically performed on Cell Banks and Viral Seed Lots

Matrices for virus detection are cells as well as harvest (with more or less cellular components)

Our goal: One approach to fit all types of matrices , with documentable breadth of detection and sensitivity to support *in vivo* test substitution

Since our new vaccines are produced with animal-free materials, we chose to develop a pipeline capable of detecting any viral nucleic acids, preferentially those that are encapsidated and potentially also non-encapsidated.

Overview of our Test



HTS as Specification Test in Substitution of *in Vivo* Tests



01/2018:50214

5.2.14. SUBSTITUTION OF *IN VIVO* METHOD(S) BY *IN VITRO* METHOD(S) FOR THE QUALITY CONTROL OF VACCINES

Ph. Eur. 5.2.14

.... These new molecular methods (e.g. deep sequencing or high-throughput sequencing) detect genomes while the existing in vivo methods are based on observations of the effects viruses have on experimental animals. *The implementation of such new molecular methods as substitutes for in vivo methods requires* *a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods. For this purpose, an* ***appropriate panel of representative, well-characterised model viruses*** *should be used to assess the ability of the new method to detect viruses that are (or are not) detected by the in vivo methods, and to determine if the sensitivity is at least equivalent to the sensitivity of the in vivo methods.* *This last element is particularly complex since these new molecular methods do not detect the same characteristic of the viral contaminant (genome for molecular methods versus infectious virus for in vivo methods) and also since no or limited validation data exist for the in vivo methods. It should also be emphasized that the outcome of the new molecular methods is not the final result since the detection of a genome or fragments of a genome does not necessarily indicate the presence of an infectious virus.*

Model Viruses to Substitute *in Vivo* Tests


Vaccine 32 (2014) 2916–2926

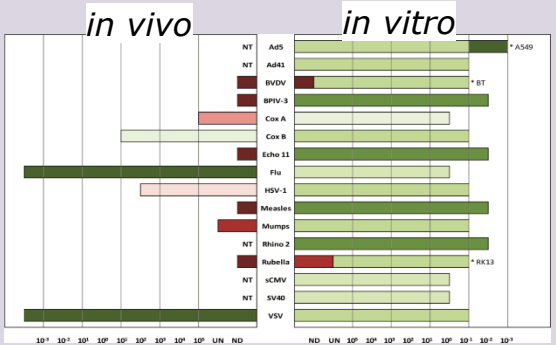
Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Systematic evaluation of *in vitro* and *in vivo* adventitious virus assays for the detection of viral contamination of cell banks and biological products☆

James Gombold[a], Stephen Karakasidis[a], Paula Niksa[b], John Podczasy[a], Kitti Neumann[a], James Richardson[c], Nandini Sane[c], Renita Johnson-Leva[c], Valerie Randolph[d], Jerald Sadoff[e], Phillip Minor[f], Alexander Schmidt[g], Paul Duncan[h], Rebecca L. Sheets[i,*]



NIH assessed the sensitivity of *in vivo* vs. *in vitro* tests by using a panel of **16 viruses** across 9 viral families (Gombold *et al*, Vaccine, 2014)

Viral Family	Virus	Strain	Enveloped	Viral Genome	Genome Size (Kb)
Adenoviridae	Adenovirus 5	Adenoid 75	No	dsDNA	36
	Adenovirus 41	N/A	No	dsDNA	34
Flaviviridae	Bovine Viral Diarrhea Virus	NY-1	Yes	ssRNA (+ve)	12.4
Herpesviridae	Herpes Simplex Virus Type 1	MacIntyre	Yes	dsDNA	150
	Simian Cytomegalovirus	CS6	Yes	dsDNA	221
Orthomyxoviridae	Influenza A	A/PR/8/34 (H1N1)	Yes	8 ssRNA (-ve)	12.5
Paramyxoviridae	Mumps	Enders	Yes	ssRNA (-ve)	15.4
	Bovine Parainfluenza Type 3	N/A	Yes	ssRNA (-ve)	15.5
	Measles	Edmonston	Yes	ssRNA (-ve)	15.9
Picornaviridae	Coxsackie A16	N/A	No	ssRNA (+ve)	7.4
	Coxsackie B3	N/A	No	ssRNA (+ve)	7.4
	Echovirus 11	Gregory	No	ssRNA (+ve)	7.4
	Rhinovirus 2	HGP	No	ssRNA (+ve)	7.1
Polyomaviridae	Simian Virus 40	Pa-57	No	dsDNA	5.2
Rhabdoviridae	Vesicular Stomatitis Virus	Indiana	Yes	ssRNA (-ve)	11.2
Togaviridae	Rubella	M-33	Yes	ssRNA (+ve)	9.7

The 16 NIH viruses/ sanofi viral stocks

- represent potential contaminants
- Includes human and animal viruses from a variety of viral families,
- Genomes of different **type** (DNA and RNA , ds and ss) and size
- enveloped and non-enveloped

The **NIH protocols** for virus propagation and titration were followed as close as possible

- **Infectious titers** and **GEQ** for both the Sanofi Vaccine and NIH stocks were determined to confirm that the ratio is about the same as that for the original stock

HTS as Specification Test in Substitution of *in Vivo* Tests



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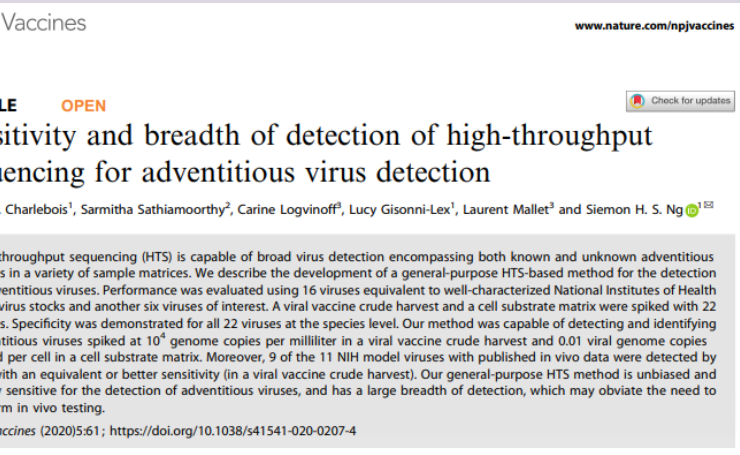
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... The implementation of such new molecular methods as substitutes for in vivo methods requires ***a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods****.*

For this purpose, ***an appropriate panel of representative, well-characterised model viruses*** *should be used to assess the ability of the new method to detect viruses that are (or are not) detected by the in vivo methods, and to determine if the sensitivity is at least equivalent to the sensitivity of the in vivo methods. ...*

Use of the same panel of viruses as NIH to compare HTS to *in vivo* data (Charlebois *et al*, npj Vaccines, 2020)

This data package supports the substitution of *in vivo* testing by HTS for adv virus detection, as recommended within Ph. Eur. 5.2.14



npj | Vaccines

www.nature.com/npjvaccines

ARTICLE OPEN

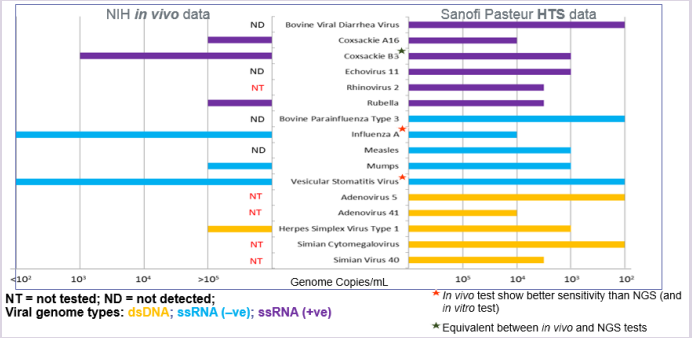
Check for updates

Sensitivity and breadth of detection of high-throughput sequencing for adventitious virus detection

Robert L. Charlebois[1], Sarmitha Sathiamoorthy[2], Carine Logvinoff[3], Lucy Gisonni-Lex[1], Laurent Mallet[3] and Siemon H. S. Ng[1]✉

High-throughput sequencing (HTS) is capable of broad virus detection encompassing both known and unknown adventitious viruses in a variety of sample matrices. We describe the development of a general-purpose HTS-based method for the detection of adventitious viruses. Performance was evaluated using 16 viruses equivalent to well-characterized National Institutes of Health (NIH) virus stocks and another six viruses of interest. A viral vaccine crude harvest and a cell substrate matrix were spiked with 22 viruses. Specificity was demonstrated for all 22 viruses at the species level. Our method was capable of detecting and identifying adventitious viruses spiked at 10^4 genome copies per milliliter in a viral vaccine crude harvest and 0.01 viral genome copies spiked per cell in a cell substrate matrix. Moreover, 9 of the 11 NIH model viruses with published in vivo data were detected by HTS with an equivalent or better sensitivity (in a viral vaccine crude harvest). Our general-purpose HTS method is unbiased and highly sensitive for the detection of adventitious viruses, and has a large breadth of detection, which may obviate the need to perform in vivo testing.

npj Vaccines (2020)5:61 ; https://doi.org/10.1038/s41541-020-0207-4



HTS for Adv. Virus Detection to Substitute *In Vivo* Test

Yesterday…
(up to 2023)

Specification test for release & compendial test substitution

MCB, WCB, ECB
MSL, WSL (DS)

Ph. Eur 5.2.3
Ph. Eur. 2.6.16
Ph. Eur 5.2.14

GMP ICH validation package + **Comparison bridging study**

ICH HARMONISED GUIDELINE

VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

Q5A(R2)

3.2.3 In Vivo Assays

NGS is encouraged as a replacement for *in vivo* assays because of the breadth of viruses it detects and because its use promotes the global objective to replace, reduce, and refine the use of animal testing. Use of NGS to replace *in vivo* assays may be justified by submitting a validation package. Based on risk assessment and on the overall testing strategy, the use of the

Charlebois *et al*, NPJ Vaccine 2020


Sensitivity and breadth of detection of high-throughput sequencing for adventitious virus detection

Beurdeley-Fehlbaum *et al*, Vaccine 2023


Vaccine

Evaluation of a viral transcriptome Next Generation Sequencing assay as an alternative to animal assays for viral safety testing of cell substrates

….*et al*

AVDTWG collaborative studies

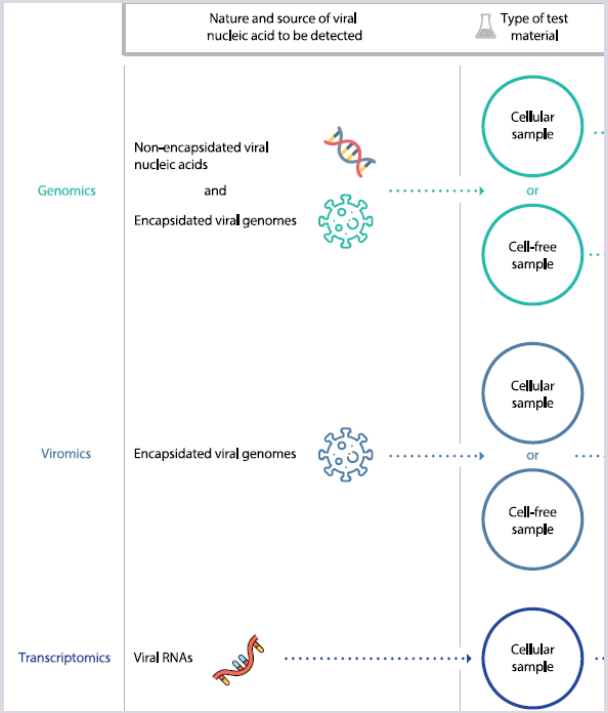
… Today

Specification test for release & compendial test substitution

MCB, WCB, ECB
MSL, WSL (DS)
ICH Q5A (R2)
Ph. Eur. 2.6.41
Ph. Eur 5.2.3
Ph. Eur. 2.6.16
Ph. Eur 5.2.14

GMP ICH validation package **w/o head to head comparaison**

HTS Approaches for Detecting Adventitious Viruses



Extract from Figure 2.6.41-1

Genomics: all viral nucleic acids (DNA and RNA, encapsidated and non-encapsidated) in cells or extracellular

Viromics : detection of encapsidated viral genomes

Transcriptomics : detection of viral RNAs in infected cells

Our choice at Sanofi Vaccine :

Mainly **Viromics approach**, to suit any matrix for which *in vivo* test are to be susbstituted , ie cell banks & (viral) harvest

As specification test

Sensitivity established throughout the validation (generic & matrix specific)

Considered in the context of follow up investigation

Spiking Materials for Method Development and Validation

*Our choice with **characterized viral stocks***

- The chosen **reference viruses** must represent **viral diversity** in terms of structure (enveloped, non-enveloped), nucleic acid type (RNA or DNA genome, single-stranded or double-stranded genome, linear or circular or segmented), genome size, virus morphology and chemical resistance.

- These reference **model virus stocks** are **characterized** for genome copy number, infectious titre, viral genome sequence(s) including any variants, and any additional expected background signals (e.g. from production substrates).
  - An equivalent panel of 16 NIH viruses used by NIH (Gombold *et al*. 2014)
  - The WHO International Reference panel

Viral Family	Virus	Strain	Enveloped	Viral Genome	Genome Size (Kb)
Adenoviridae	Adenovirus 5	Adenoid 75	No	dsDNA	36
	Adenovirus 41	N/A	No	dsDNA	34
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WHO/BS/2024.2471
ENGLISH ONLY

World Health Organization

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 11 to 14 March 2024

A Collaborative Study to Evaluate the Proposed First WHO International Reference Panel for Adventitious Virus Detection in Biological Products by High-throughput Sequencing (HTS) Technologies

Arifa S. Khan[1,2], Pei-Ju Chin[1], Sandra M. Fuentes[1], Jen-Hui Tsou[1], and Study Group*

Virus	Genome type	Genome size	Particle size	Envelope	Chemical resistance
REO	RNA, double-strand; Linear (segmented)	23.6 kb (1,196 – 3,915 nt	80 nm	No	Medium - high
FeLV	RNA; single-strand; Linear (dimeric)	8.5 kb	80-100 nm	Yes	Low
RSV	RNA; single-strand; linear	15 kb	150-200 nm	Yes	Low - medium
PCV	DNA, single-strand; circular	1.8 kb	16-18 nm	No	High
EBV	DNA, double-strand; Linear	172 kb	122-180 nm	Yes	Low - medium
hCOV	RNA, single-strand; Linear	30.7 kb	80-120 nm	Yes	Low
MVM	DNA, single-strand; Linear	5.1 kb	26 nm	No	Medium-High

Method Development / Matrix Assesment

Matrix assessment using representative matrix samples

- Evaluate optional pre-extraction steps
- Evaluate potential procedure modifications for method improvement
- Determine target LOD and specificity
  - ✓ With 16 model viruses (Gombold *et al*, 2014) or WHO viruses as spike-ins
- Demonstrate assay robustness
- Determine validity criteria for internal controls
- Identify false positive generating signals from matrix or testing reagents

Considerations prior to validation

- Method improvement (e.g., add a new step or replace a reagent kit, upgrade bioinformatics software)
- Matrix-specific instructions
  - ✓ Wet lab (e.g., nuclease treatment, cell lysis)
  - ✓ Dry lab (e.g., host to prescreen)
  - ✓ Validity criteria (e.g., recovery of internal controls)

Validation

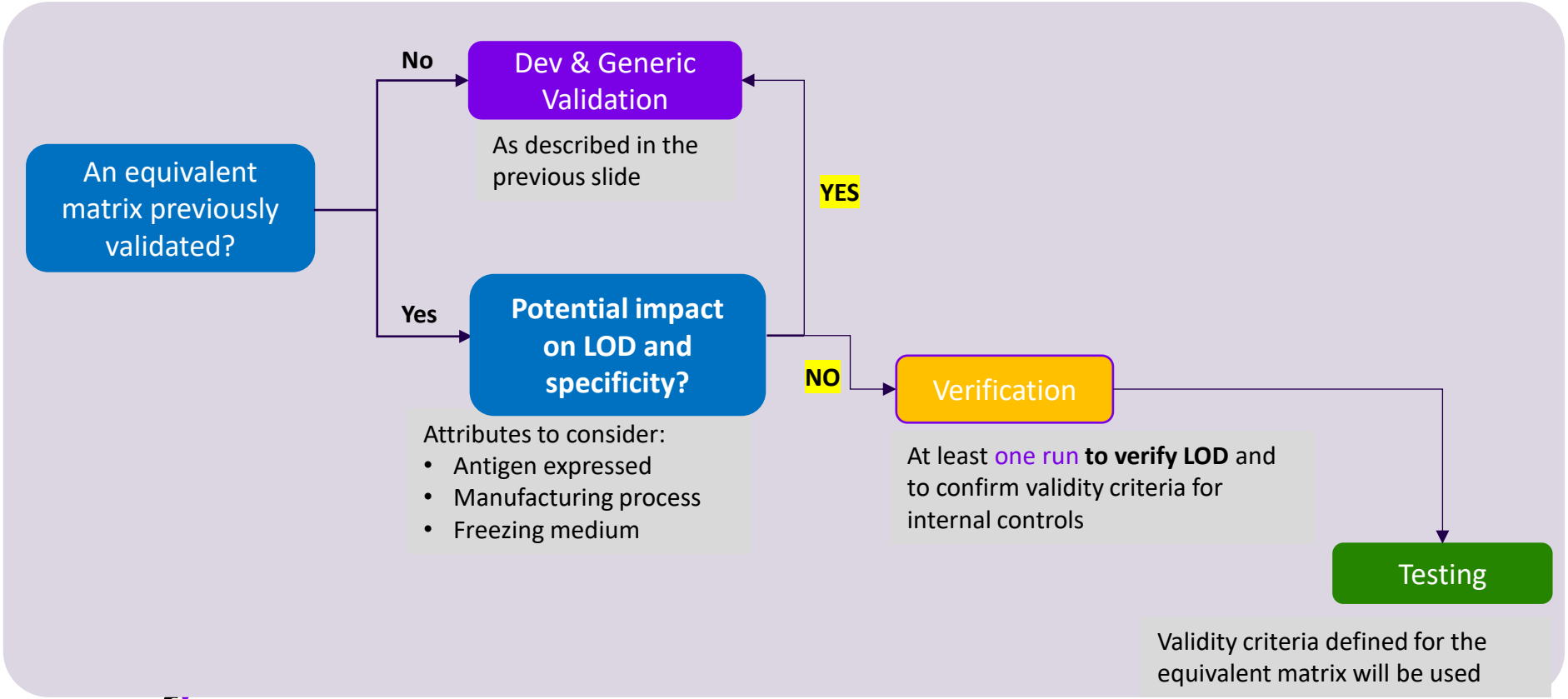
Validation Plan

- As a ***Limit test***
  - ❑not a quantitative assay
- Define validation characteristics
  - ❑**Specificity (breadth of virus detection)**
    - ❑Specificity demonstrated by a negative control extracted and sequenced in parallel
  - ❑**Sensitivity (LOD)**
    - ❑Model viruses spiked into matrix
- Study Design
- Qualify internal controls as critical reagents
- Define validity and acceptance criteria

Validation Execution

- Validation execution (LOD and specificity)
  - ✓ Prior to *Ph. Eur. 2.6.41*: Two spiked samples
    - ✓ (16 model virus or WHO viruses as spike-ins)
  - ✓ Post *Ph. Eur. 2.6.41*: nine spiked samples (three spiking levels with three replicates for each)
    - ✓ WHO viruses as spike-ins
  - ✓ One un-spiked sample

Evaluation of New Matrices



Summary

Reference Materials for HTS for Adventitious Virus Detection

The NIH equivalent virus panel was used to support HTS for adv virus detection substitution *of in vivo* test , comparing with *in vivo* described in Gombold *et al* and as per Ph. Eur. 5.2.14.

(Charlebois *et al*, npj Vaccine 2020)

For our Viromics approach, relevant reference viruses need to represent **viral diversity** in terms of structure (enveloped, non-enveloped), nucleic acid type (RNA or DNA genome, single-stranded or double-stranded genome, linear or circular or segmented), genome size, virus morphology and chemical resistance.

=> LOD is determined with **WHO reference viruses**

=> Use of the same reference material supports sensitivity comparaison , between different sample matrices or different laboratories

Thank



- Robert Charlebois
- Song Sun
- Jacek Remani
- Lauren Rodrigues
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- Patrice Riou
- *Siemon Ng*
- *Laurent Mallet*

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Carine Logvinoff is a Sanofi employee and may hold shares and/or stock options in the company