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Selecting and developing approaches for adventitious virus detection by HTS, contrasting release testing with investigational follow-up

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Agenda

- Context of Sanofi Vaccines HTS for adventitious virus detection
- Our choice for *in vivo* test substitition
- Overview of our test , controls and validity criteria
- Method development / Matrix assessment
- Generic validation / Evaluation of new matrices
- Follow up investigation tools
- Conclusions

How to Detect Viral Contaminants

the “traditional” test methods [before 2010]

Non-specific test methods
(broad-range of detection)

Tests in animals
(mouse, eggs, guinea pig, rabbit)

Direct observation & haemadsorption test (cell cultures)

Infectivity assay
(retrovirus)

Live infectious virus

Viral components

Test for reverse transcriptase (RT)

Transmission Electron Microscopy (TEM)

in vivo Antibody production tests (MAP, RAP, HAP)

PCRs

9CFR for Bovine & Porcine viruses

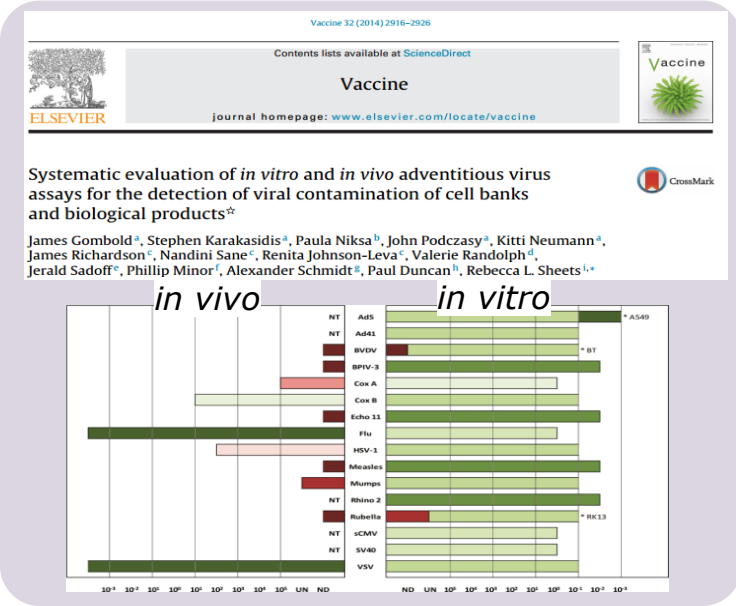
Virus-specific test method

Tests combination for retroviruses

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

The conventional testing package could miss viral contaminant

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0022-538X/10/$12.00 doi:10.1128/JVI.02690-09

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]

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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.**

- 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

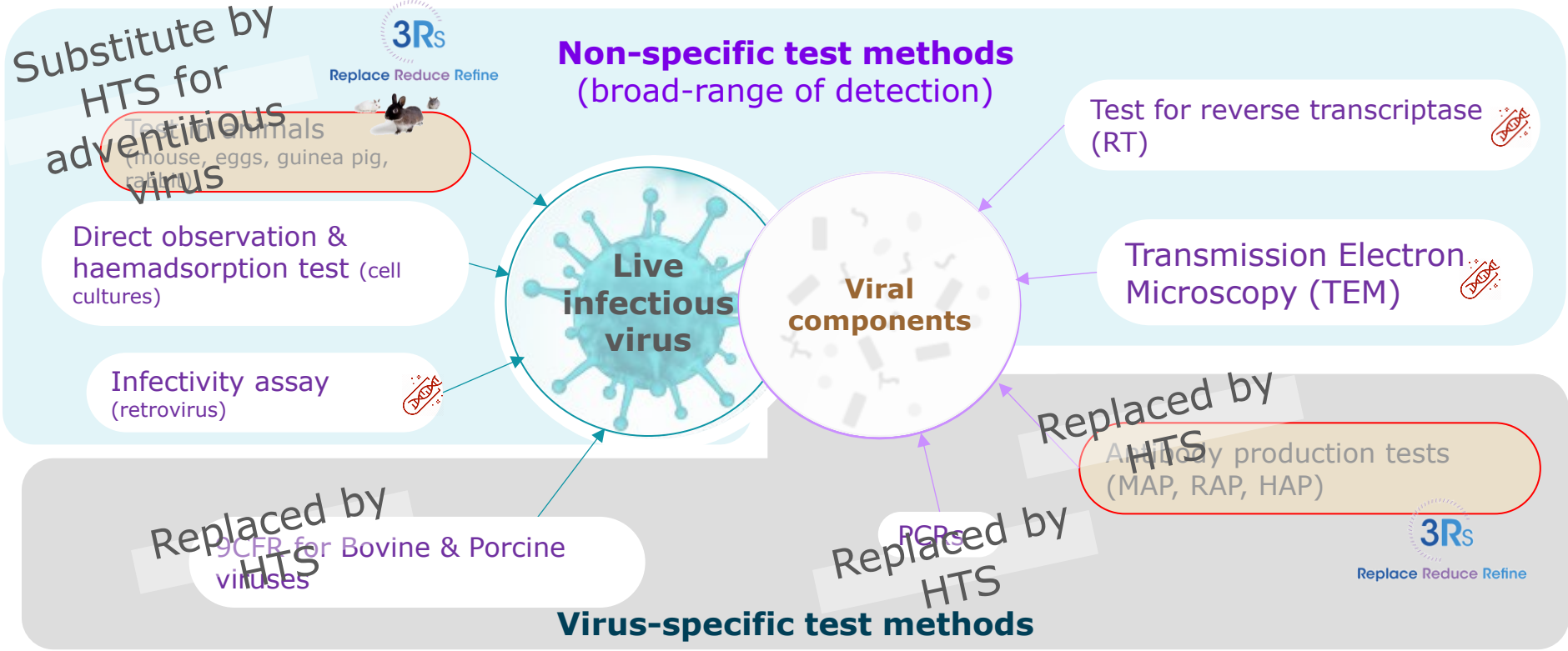
- Aug 2010 : FDA letter to licensed vaccine manufacturers

> 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.

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

Our Goal : to Substitute *in Vivo* Test for Adv Virus Detection

And to strengthen our adventitious virus detection



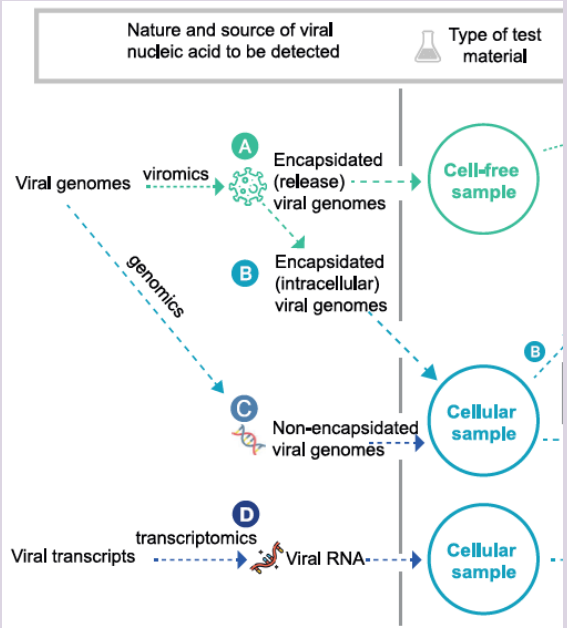
Our Choice for *In Vivo* Test Substitution (1/3)

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

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.



Extract from Figure 2.6.41-1

Our Choice for *In Vivo* Test Substitution (2/3)

01/2018:50214

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

... 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. ...*

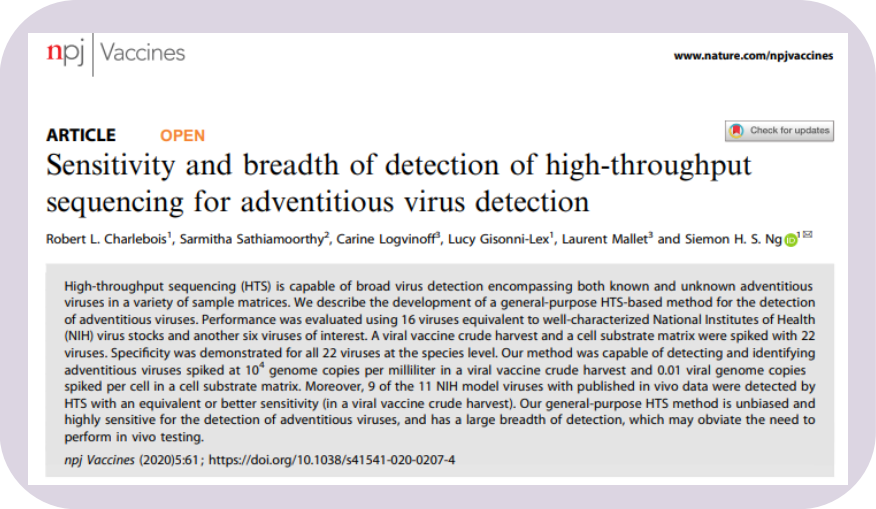
Viral Family	Virus	Strain	Enveloped	Viral Genome	Genome Size (Kb)
Adenoviridae	Adenovirus 5	Adenoid 75	No	dsDNA	36
	Adenovirus 41	N/A	No	dsDNA	34
Flaviviridae	Bovine Viral Diarrhea Virus	NY-1	Yes	ssRNA (+ve)	12.4
Herpesviridae	Herpes Simplex Virus Type 1	MacIntyre	Yes	dsDNA	150
	Simian Cytomegalovirus	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

Viral Stocks

- The 16 NIH viruses represent potential contaminants that could be introduced during vaccine production, includes human and animal viruses from a variety of families, both RNA and DNA genomes as well as enveloped and non-enveloped virus
- The NIH protocols for virus propagation and titration
- Characterization of viral stocks includes Infectious Titer and GEQ

=> Allows to link our HTS data to NIH *in vivo* data from Gombold *et al*, 2014), as proposed by Ph. Eur. 5.2.14

Our Choice for *In Vivo* Test Substitution (3/3)



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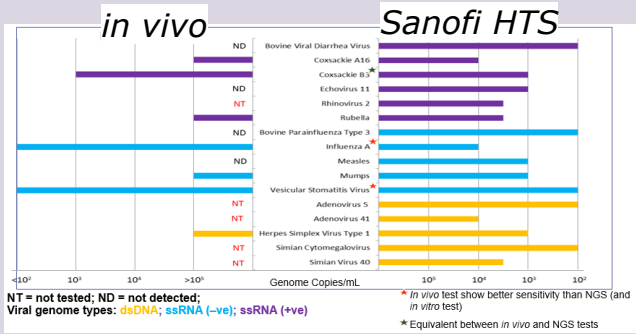
Check for updates

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]✉

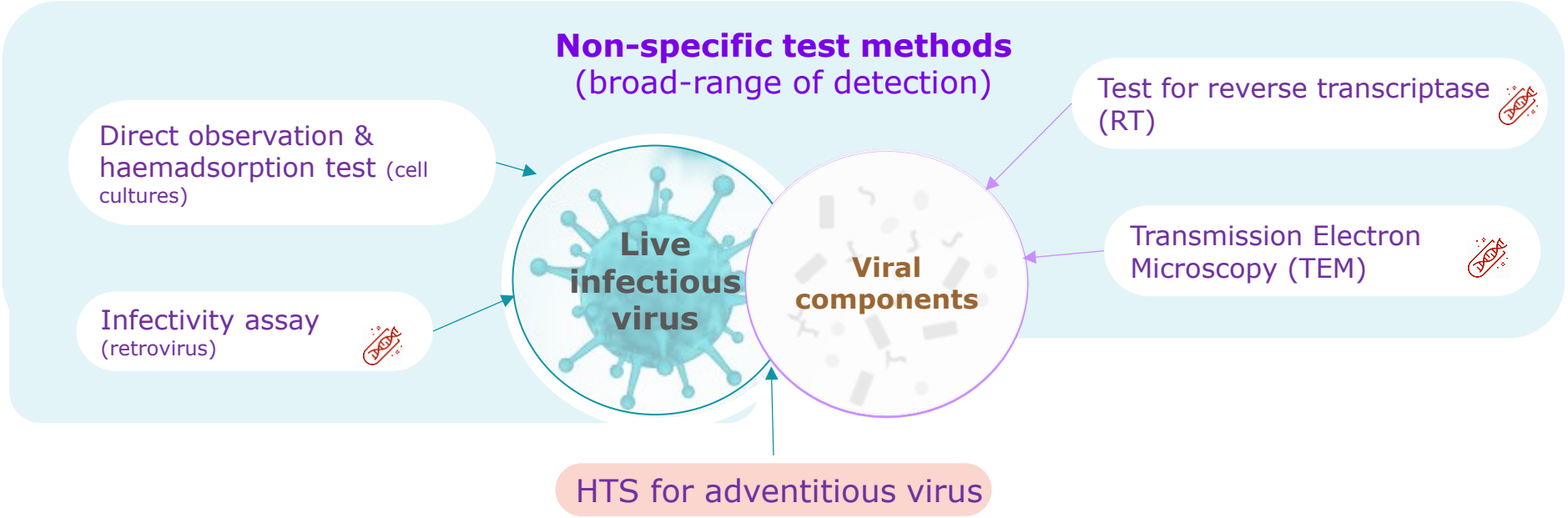
High-throughput sequencing (HTS) is capable of broad virus detection encompassing both known and unknown adventitious viruses in a variety of sample matrices. We describe the development of a general-purpose HTS-based method for the detection of adventitious viruses. Performance was evaluated using 16 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

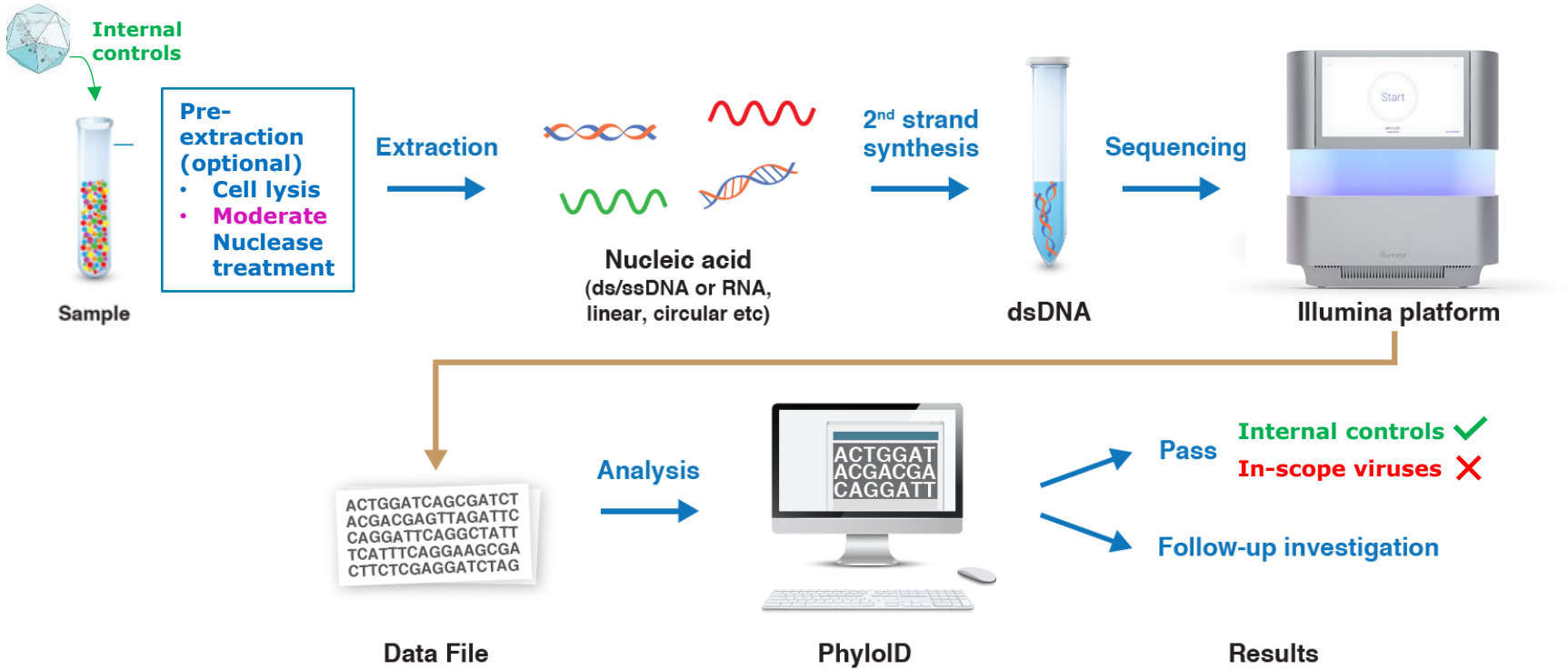


Using viral stocks as representative spiking materials in our detection pipeline, we were able to document the breadth of detection and sensitivity of our HTS pipeline.
Our data package supports the substitution of *in vivo* test by HTS for adv virus detection test, as recommended within Ph.Eur. 5.2.14

HTS to Streamline the Adventitious Virus Testing Package
and to Strengthen Product Viral Safety



Overview of our Test



Controls and Validity Criteria Selection

General:

- Measures to avoid cross-contamination: segregate lab rooms with dedicated biosafety cabinets for nucleic acid extraction and library preparation and unidirectional workflow; dual-indexed sequencing library
- Quantify input DNA for sequencing library preparation
- Assessment of sequencing library: validity criteria for size and concentration
- Sequencing: validity criteria for read quality (minimum % Bases Q ≥ 30)

Matrix-specific:

- Sequencing: validity criteria for total output per sample (number of reads)
- Internal controls (pseudo-viral particles for both DNA and RNA): validity criteria for the recovery of the internal controls (number of reads detected as the two internal controls)

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
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WHO/BS/2024.2471
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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
- Define validation characteristics (LOD and specificity)
- Study Design
- Qualify internal controls as critical reagents
- Define validity and acceptance criteria

Validation Execution
- Validation execution (LOD and specificity)
  - ✓ Two (or three) spiked samples
    - ✓ (16 model virus or WHO viruses as spike-ins)
  - ✓ One un-spiked sample

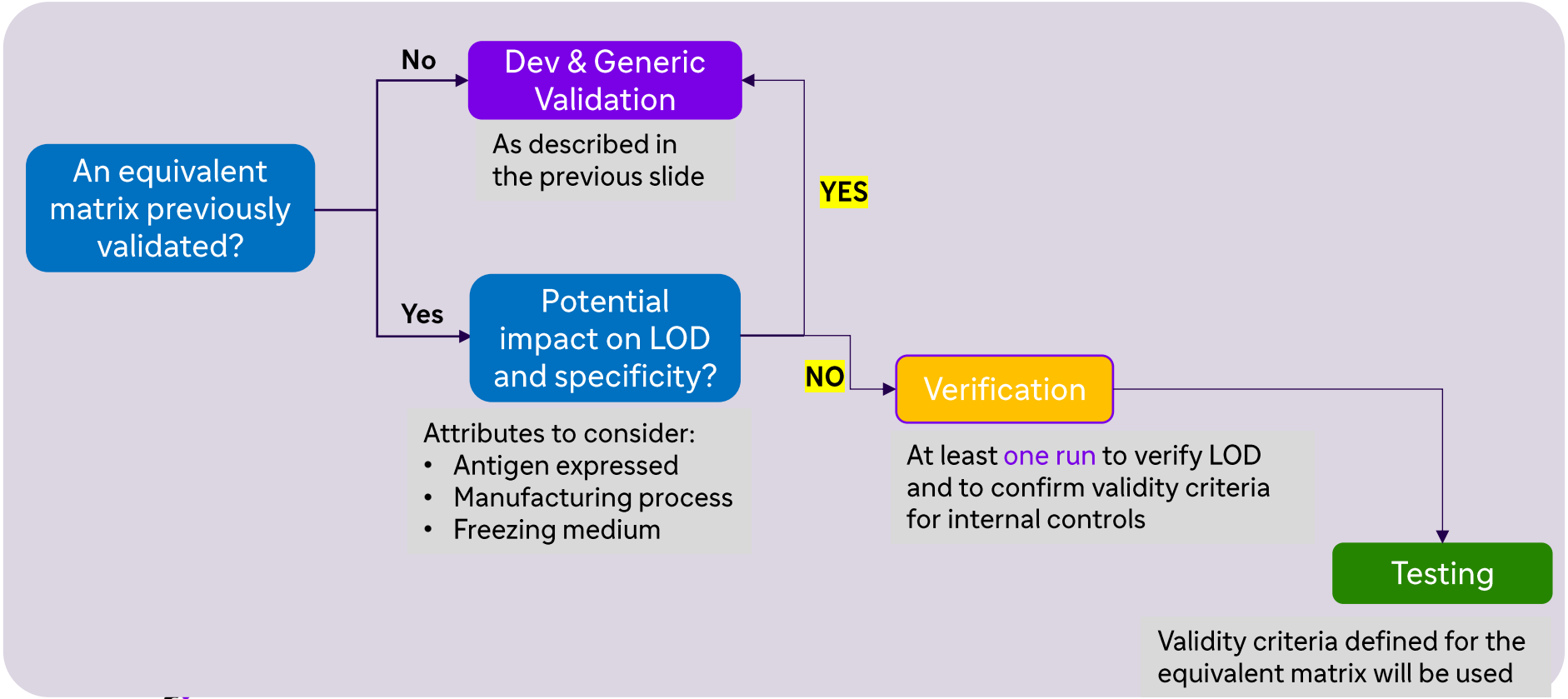
Generic Validation
(an example for a Viral Crude Harvest Matrix with 2 runs)

- As a Limit test
  - not a quantitative assay
- Validation parameters :
  - **Specificity (breadth of virus detection)**
    - Specificity demonstrated by a negative control extracted and sequenced in parallel
  - **Sensitivity (LOD)**
    - 16 NIH viruses spiked in at 10^4 genome copies into 1 mL of viral harvest seed matrix
- Controls for extractions and recovery for viral nucleic acids
- Validation was done with two analysts over 2 days with 1 run each; plus a negative control.

Viruses Spiked into the Sample Matrix (at 10^4 copies / mL)	Spiked Sample #1	Spiked Sample #2	Unspiked control
Adenovirus 5	+	+	-
Adenovirus 41	+	+	-
Bovine Viral Diarrhea Virus	+	+	-
Herpes Simplex Virus Type 1	+	+	-
Simian Cytomegalovirus	+	+	-
Influenza A virus	+	+	-
Mumps virus	+	+	-
Bovine Parainfluenza Type 3	+	+	-
Measles virus	+	+	-
Coxsackievirus A16	+	+	-
Coxsackievirus B3	+	+	-
Echovirus 11	+	+	-
Rhinovirus 2	+	+	-
Simian Virus 40	+	+	-
Vesicular Stomatitis Virus	+	+	-
Rubella virus	+	+	-
DNA Control	+	+	+
RNA Control	+	+	+

+ = confirmed detection

Evaluation of New Matrices



Follow Up Investigation

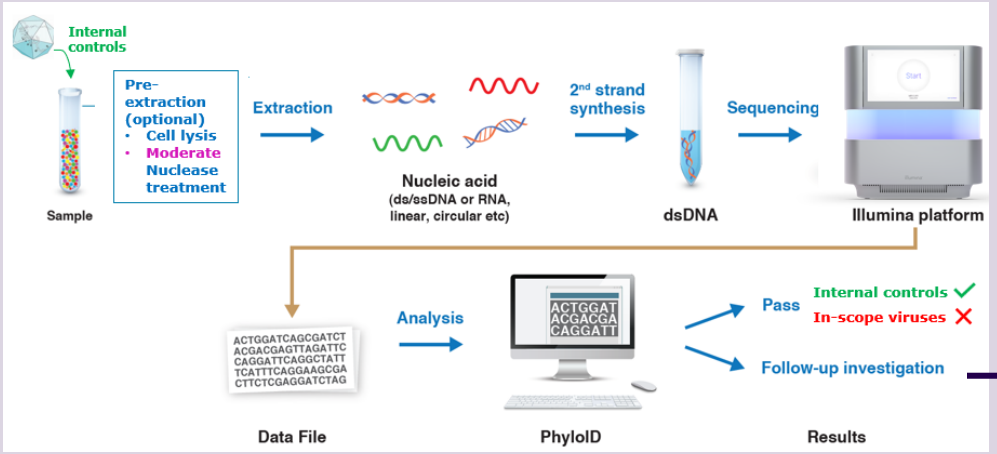


Table 2.6.41.-3 – *Follow-up investigation*

Question to be addressed	Example approaches
Are the nucleic acids that were found associated with full-length, intact genomes?	– Long-range or overlapping PCR, – Sequencing with long-read technology
Are the nucleic acids particle-associated?	Nuclease digestion and/or ultracentrifugation followed by virus testing: – PCR, – Transmission electron microscopy (TEM), – Detection by antibodies (e.g. immunoprecipitation, immunostaining, immunoblot, ELISA), – Mass spectrometry
Are the particles infectious in cell cultures?	Infectivity assay including readouts such as: – Cytopathic effect, haemadsorption, haemagglutination, – Viral replication by qPCR, – Transmission electron microscopy (TEM), – Additional HTS (e.g. transcriptomics), – Stranded sequencing, – Detection by antibodies (e.g. immunoprecipitation, immunostaining, immunoblot, ELISA), – Product-enhanced reverse transcriptase assay (PERT)

The follow up investigation is to be built based on the identified signal(s) with dedicated tools to conclude on the replicative status of this potential viral contaminant.

Tools for Investigational Follow-up : PCR

Developing **a generic PCR method (including long range PCR)**

- An orthogonal method with comparable sensitivity to confirm the source of the viral signal
- Leverage the sequencing data to design primers/probes
- Sensitivity can be estimated using synthetic templates
- Multiple samples can be tested in parallel with short turnaround time

Tools for Investigational Follow-up : ONT Long Read HTS

Evaluating Oxford Nanopore sequencing technology to assess genome integrity

- Capable of generating very long reads
- Can sequence native DNA and RNA without the need for amplification
- Real-time data analysis
- Potential considerations:
  - ✓ High yield of input DNA/RNA is required for a sequencing run with good quality and output

Tools for Investigational Follow-up : Stranded RNA seq

Evaluating a stranded RNA-seq method

- to detect viral RNA with directionality and to determine viral replication based on the type of viral nucleic acid
- Potential considerations:
  - ✓ Transcripts may be degraded in some matrices
  - ✓ High yield of input RNA is required to generate sequencing library

Conclusion

Our choices

Release Test : to detect (mainly encapsidated) viral nucleic acid
With comprehensive validation packages using well characterized representative spiked-in viruses (NIH & WHO reference standard) in cell bank or harvest matrices

NB: LOD might be underestimated , a true contaminant would present additional viral nucleic acid (transcript, replicative intermediate, ...) that are not spiked-in within our validation with viral stocks

Follow Up Investigation : to conclude on the replicative status of the potential viral contaminant
Any other tools , including those for which representative validation as release test could be a challenge (in the absence of representative reference standard)

Thank

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- Laurent Mallet (currently at EDQM)

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Carine Logvinoff and Song Sun are Sanofi employees and may hold shares and/or stock options in the company

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Submitted abstract

Multiple approaches for adventitious virus detection by HTS (e.g., viromics, genomics, transcriptomics) are described in Ph.Eur 2.6.41. Within this presentation, we will discuss Sanofi Vaccines' rationale for approach selection based on sample type, considering both validated release testing as well as investigational testing.

We will also discuss how we designed and configured our HTS pipeline for testing under both scenarios.