PathoQuest

NGS Transcriptome Analysis in Cell Banking

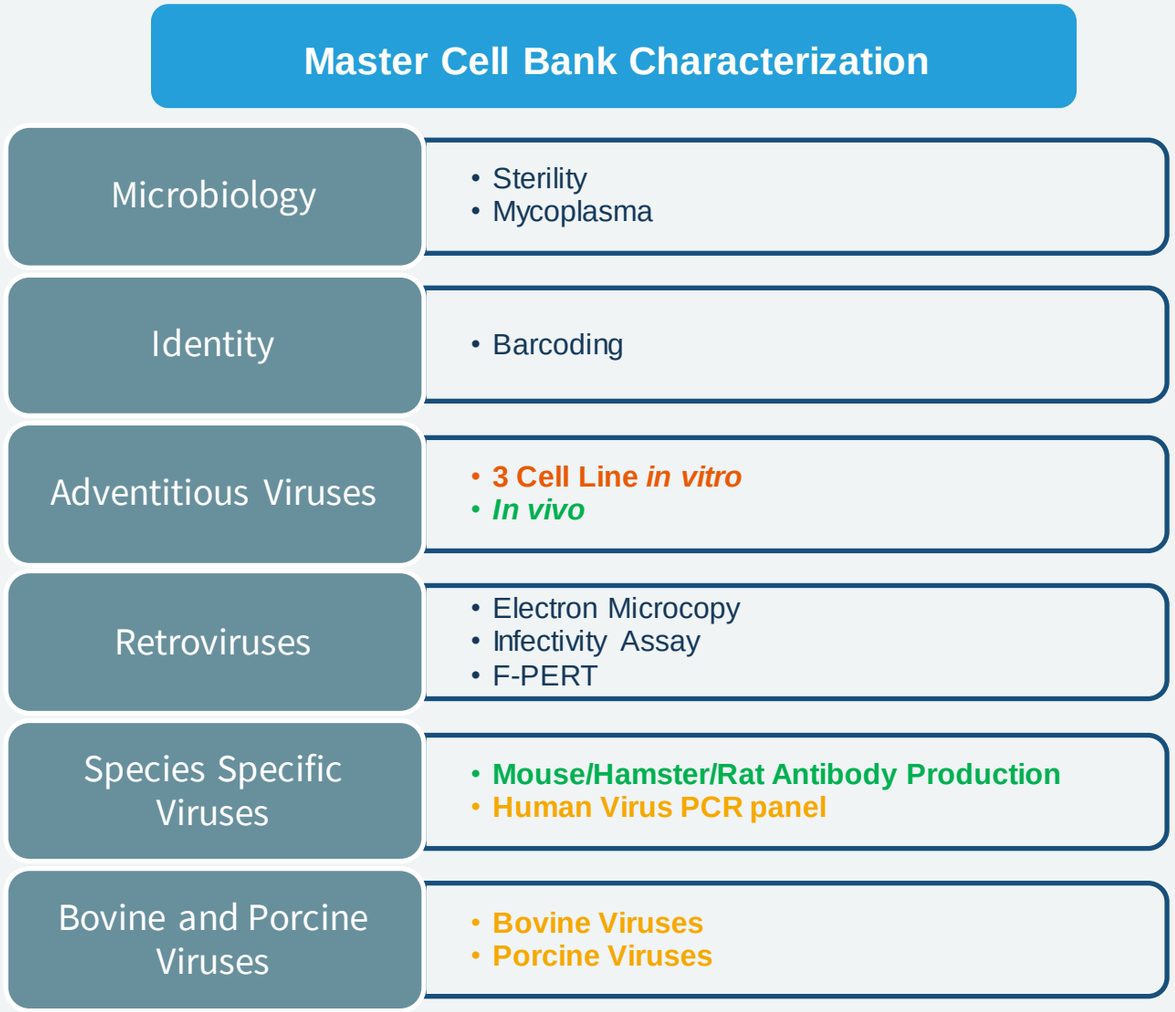
Colette Côté

Chief Scientific & Portfolio Officer and US General Manager

FEB 2024

ICHQ5A(R2) Guideline & MCB Characterization

Adopted November 2023; Coming into effect June 2024



3.2.3: "NGS is encouraged as a **replacement for *in vivo* assays** because it can overcome the limitations of the breadth and sensitivity of virus detection of the in vivo assays. Furthermore this promotes the global objective to replace, reduce, and refine the use of animal testing."

The guideline also refers to NGS as a replacement for:

- **Mouse, Rat and Hamster Antibody Tests (3.2.4)**
- **PCR assays for virus specific detection. This can also help overcome the limitation of detection of virus variants (3.2.5)**
- **Targeted detection of known viruses based upon availability of their sequence (3.2.5.2).**
- ***In vitro* adventitious virus tests (3.2.5)**

ICH Q5A(R2) clearly supports NGS as:

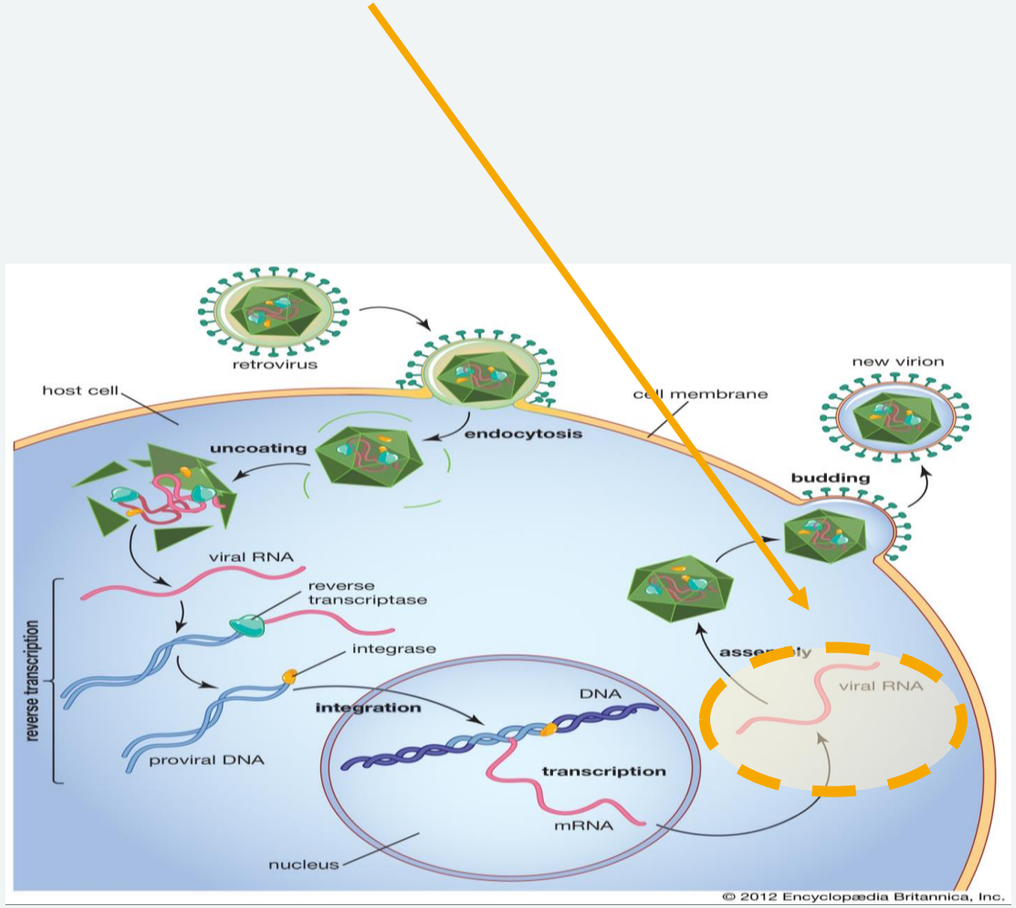
- **Replacement for *in vivo* tests (supporting the 3Rs principle) for detection of inapparent viruses**
- **Replacement for HAP/RAP/MAP tests**
- **Supplement for 9CFR PCR panels**
- **Supplement for *in vitro* assays**

- Focus on cell-based systems -

NGS Transcriptomic Analysis

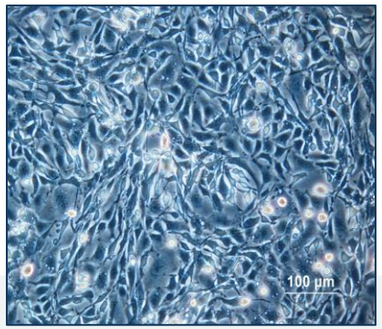
The Key to Success

- **Transcriptome = analysis of expressed RNAs within cells**
- **Rapid & simple way to detect & identify any viral sequence in a sample**
- **Takes advantage of RNA phase of viral replication**
  - **All viruses go through an RNA phase during lifecycle**
  - **Minimizes background levels (host nucleic acid) making detection easier**
- **Detects all types of viruses**
  - **RNA/DNA**
  - **Circular/linear**
  - **Enveloped/non-enveloped**
  - **Single & double-stranded**
- **Analysis can be agnostic or targeted**
- **Use strand info to assess active replication**
- **Use in-process controls to assess matrix effects**



Assay Basics

1



Starting material is a cell line or purified RNA:

- In-process controls (negative and positive) processed in parallel
  Neg = MRC5 cells; Pos = TPA-treated B95-8:Ramos (1:10^4)
- Use spiked-in collection of synthetic RNAs to track matrix effects
- Extract total RNA
- QC checks for quality/quantity (go/no-go gates)

2

Prepare a sequencing-compatible library:

- Convert material to dsDNA
- Add sequencing-compatible adapters
- Add tag to identify originating strand
- Remove rRNA to minimize background levels
- QC checks for quality/quantity (go/no-go gates)

3

Sequence using Illumina platform:

- NextSeq is the preferred instrument
- Multiple samples can be batched together

4

Analyze the data set:

- Proprietary algorithms (targeted and agnostic)
- Custom databases (viral/targeted)
- Output assessed for biological significance (secondary qualification & viral expert review)

Bioinformatics Basics

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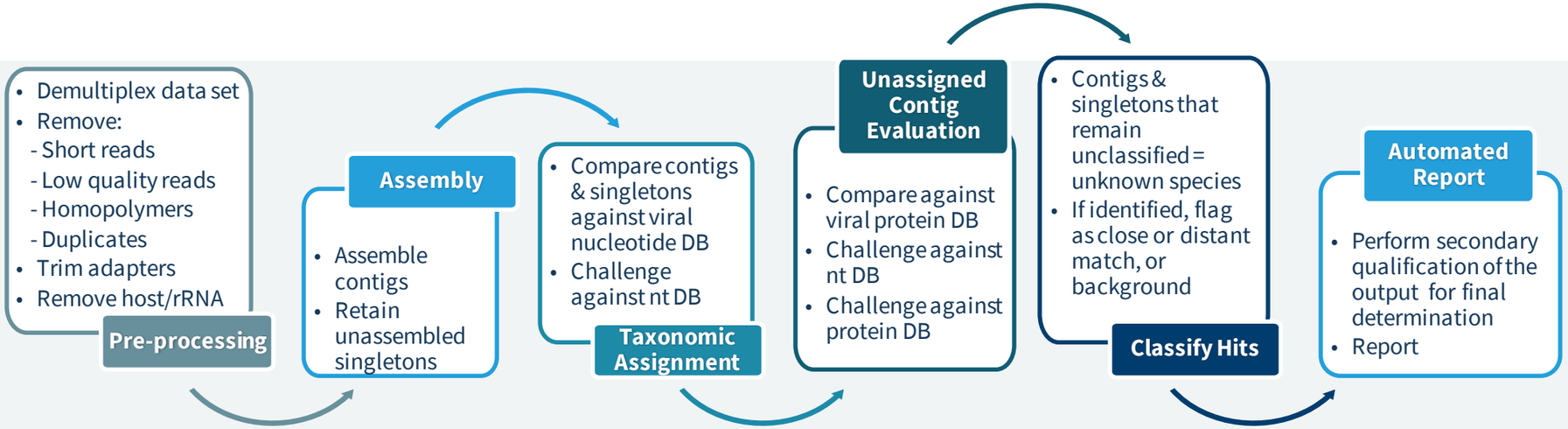
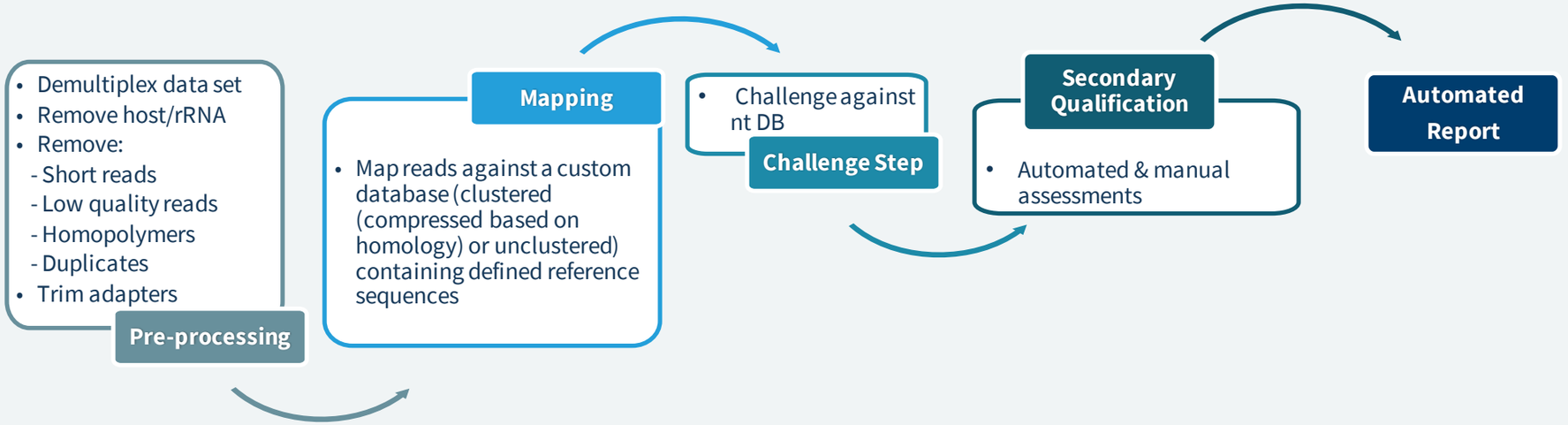
Agnostic Analysis



Targeted Analysis

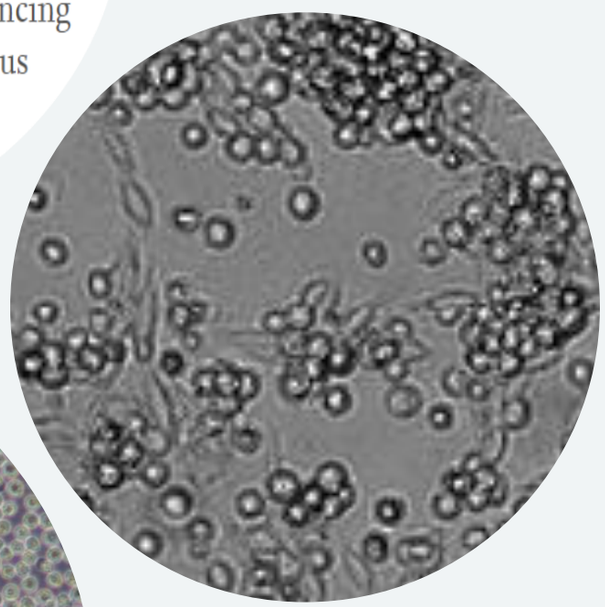


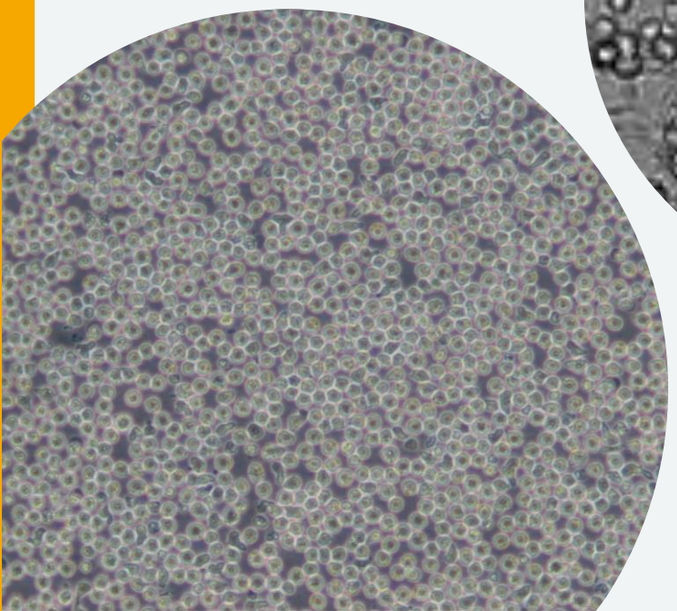
Secondary Qualification is Critical

- **Assay focuses on:**
  - Detection and strandedness of viral RNA sequences to assess presence of replicating viruses
  - Reduces impact of benign/free viral nucleic acids; minimizes false positives
- **Dedicated controls built into the assay to:**
  - Verify performance of the assay
  - Assess test sample matrix effects (*e.g.*, inhibition)
  - Reduce false positives and false negatives
- **Expertise provides:**
  - Biological significance and context of any identified viral hits
  - Recommendations for mitigation/investigation if needed

Assay Sensitivity Similar to PCR

Brussel *et al.*, 2019; Biologicals





- **Serial dilution of persistently infected B cell lymphocytes (B95-8 w/latent HHV4 (EBV)) in uninfected cells (Ramos)**
- **Mimics low level infection**
  - Prepared 10X serial dilutions of B95-8 in Ramos (up to 1:10^8)
  - Cultured each in presence of TPA to express HHV4
  - Counted cells & prepared fresh 1:10^3 to 1:10^8 dilutions in Ramos cells
  - TPA treated B95-8 cells alone → positive control
  - Untreated Ramos cells → negative control
  - Sequenced & compared with HHV4 PCR results

NGS detected down to 1:10^5 (same as PCR)

Table 3
RT-PCR and NGS agnostic analysis results of Ramos and B95-8 infected HHV-4 cells.

Sample description	PCR HHV-4	Next Generation Sequencing		
	Mean number of copies/ reaction	Total number of reads (per library)	Number of reads post filtration	Number of HHV-4 reads
Ramos (negative control)	negative	164 604 917	6 014 833	0
B95-8/Ramos [(1/10^3)]	24 691	N/A	N/A	N/A
B95-8/Ramos [(1/10^4)]	1604	169 304 831	6 362 936	317
B95-8/Ramos [(1/10^5)]	802	168 907 048	6 531 975	31
B95-8/Ramos [(1/10^6)]	negative	168 718 801	6 588 113	0
B95-8/Ramos [(1/10^7)]	negative	N/A	N/A	N/A
B95-8/Ramos [(1/10^8)]	negative	N/A	N/A	N/A
B95-8 + TPA/Ramos [(1/10^4)]	11 234	161 142 284	6 323 478	2657
B95-8 (positive control)	1‖ 049 382	N/A	N/A	N/A
B95-8 + TPA (positive control)	60 432 098	N/A	N/A	N/A

Table 4
Targeted stranded HHV-4 analysis: number of HHV-4 reads and genome horizontal coverage.

Sample description	Number of HHV-4 reads[a] positive strand (genome coverage)	Number of HHV-4 reads[a] negative strand (genome coverage)
B95-8/Ramos [(1/10^4)]	97 (3.6%)	211 (3.7%)
B95-8/Ramos [(1/10^5)]	17 (0.7%)	2 (0.2%)
B95-8 + TPA/Ramos [(1/10^4)]	591 (17.2%)	2137 (19.6%)

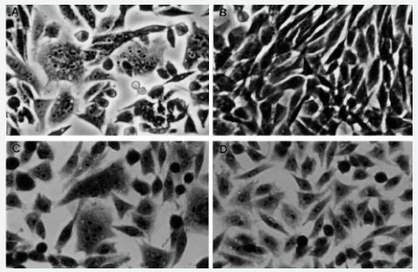
[a] Reads mapped on HHV-4, reference NC_007605.1.

Comparability with *in vivo* and *in vitro* tests?

A quick note about spiking controls…

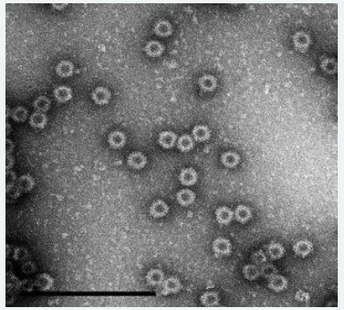
Infected Cell Lines

- Better reflects typical test matrix in the industry
- Reflects natural virus infection patterns & replication lifecycle
- Variable MOI but can prepare ratios of infected to non-infected cells to assess assay sensitivity/LOD



Intact Purified Viral Particles

- Broader spectrum of purified, characterized stocks available
- Quicker/simpler to use & most common spiking strategy
- Impact and kinetics of an active infection are lost
- Cannot be used to assess replication



Nucleic Acids

- Readily available stocks
- Easy to purify or synthesize & characterize
- Quick & simple to use
- Can be used to assess matrix effects
- Cannot be used to assess replication

Replacement for Animal Testing

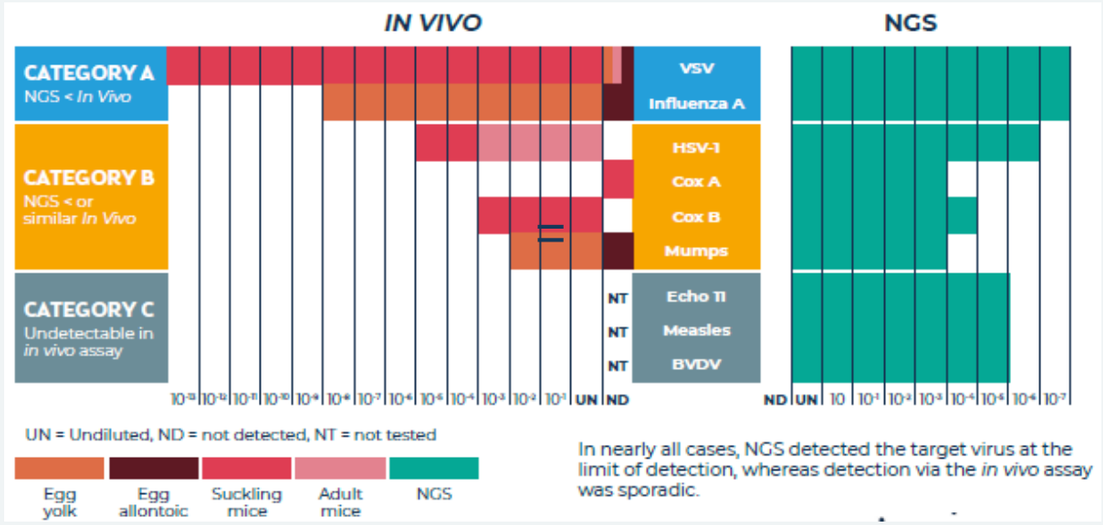
ICH Q5A(R2): NGS is encouraged as a replacement for *in vivo* assays

KEY CRITERIA FOR VIRAL SAFETY TESTING TO EVALUATE NGS ADVANTAGES OVER ANIMAL TESTING

	Animal Testing	NGS
Detection range	✗	✓
Specificity for virus identification	✗	✓
Sensitivity	✗	✓
Turnaround time	✗	✓
3Rs compliance	✗	✓
Validation	✗	✓
Regulatory acceptance	✓	✓

Vaccine
Volume 41, Issue 37, 23 August 2023, Pages 5383-5391

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



Beurdeley *et al.*, 2023: Head-to-head comparison providing supporting data for the industry

Replacement for *in vivo* HAP/MAP/RAP Testing

ICH Q5A(R2) refers to replacement with NGS

Virus		Spiked CHO-K1 NK Cells (Copy Number)					
		Close Species					Distant
Family	Species	10	100	1000	10,000	100,000	
Adenoviridae	Mouse Adenovirus (MAV) MAdV1	Not detected	Not detected	Not detected	Detected	Detected	Detected
	MAdV2				Detected	Detected	Detected
Adenoviridae	Lymphocytic choriomeningitis virus LCMV				Detected	Detected	Detected
Arteriviridae	Lactate Dehydrogenase elevating virus (LDV)				Not detected	Detected	Detected
Coronaviridae	Mouse Hepatitis virus (MHV)				Not detected	Detected	Detected
	Sialodacryoadenitis virus (SDAV)				Not detected	Detected	Detected
	Rat coronavirus (RCV) = Murine coronavirus				Not detected	Detected	Detected
Hantaviridae	Hantaan virus (HANT)				Detected	Detected	Detected
	Prospect Hill virus (PHV)				Not detected	Detected	Detected
Herpesviridae	Mouse Cytomegalovirus (MCMV)				Detected	Detected	Detected
	Mouse thymic virus (MTLV)				Detected	Detected	Detected
Paramyxoviridae	Pneumonia virus of mice (PVM)				Not detected	Detected	Detected
	Sendai virus (SEND)				Not detected	Detected	Detected
	Simian virus 5 (SV5)				Not detected	Detected	Detected
Parvoviridae	Minute virus of mice (MVM)				Detected	Detected	Detected
	Mouse Parvovirus (MPV)				Detected	Detected	Detected
	Kilham's rat virus (KRV)				Detected	Detected	Detected
	Toolan's H-1 virus (H-1)				Detected	Detected	Detected
Picornaviridae	Theiler's murine encephalomyelitis virus (GDVII)				Detected	Detected	Detected
	Rat Theilovirus (RTV)				Detected	Detected	Detected
Polyomaviridae	Mouse K virus				Not detected	Detected	Detected
Poxviridae	Ectromelia (ECTRO)				Not detected	Detected	Detected
Reoviridae	Reovirus type 3				Not detected	Detected	Detected
	Reovirus type 1				Not detected	Detected	Detected
Rotaviridae	Mouse Rotavirus (A/EDIM)				Detected	Detected	Detected

Control RNA ID	NEGATIVE CONTROL	POSITIVE CONTROL	Acceptance Criteria	SAMPLE			
	% Coverage			% Coverage	Ratio Sample:NC	Ratio Sample:PC	Conclusion
#00058	60.44	59.04	The coverage of test item ≥ 80% of the coverage of the NC or the PC for each Control ID	68.68	114	116	PASS
#00126	95.73	94.84		89.15	93	94	PASS
#00033	82.85	73.85		81.25	98	110	PASS
#00014	94.01	82.45		90.66	96	110	PASS
#00039	89.73	96.76		84.32	94	87	PASS
#00150	82.23	67.70		78.87	96	117	PASS
#00085	78.79	82.70		96.09	122	116	PASS
			> 50% Control ID PASS				PASS

	MAP/HAP/RAP	Degenerate PCR panel	NGS
Speed	★☆☆☆☆	★★★★★	★★★★★
Detection of live virus	★★★★☆	☆☆☆☆☆	★★★★★
Identification of unknowns	★☆☆☆☆	★★☆☆☆	★★★★★
Volume required	★☆☆☆☆	★★★★★	★★★★★
Meet 3Rs	☆☆☆☆☆	★★★★★	★★★★★

- Faster
- Animal-free
- More robust
- Fit for purpose
- Efficient

Mouse Antibody Production Test

Ectromelia
Mouse encephalomyelitis
Lactate dehydrogenase elevating virus
Hantaan
Murine minute virus
Mouse adenovirus
Mouse hepatitis
Pneumonia virus of mice
Polyomavirus
Sendai
Epizootic diarrhea of infant mice
Mouse cytomegalovirus
Reovirus type 3
Mouse pneumonitis virus
Mouse thymic virus
Mouse parvovirus

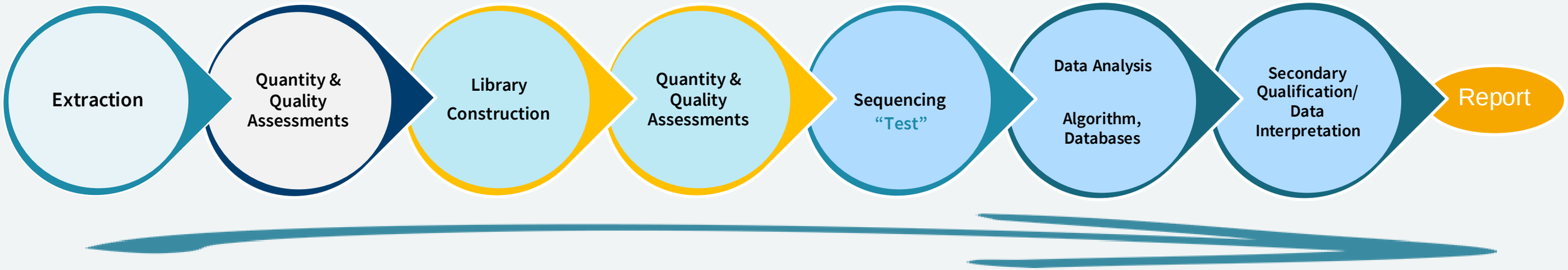
Hamster Antibody Production Test

Sendai
Pneumonia virus of mice
Reovirus 3
Lymphocytic choriomeningitis
Simian virus 5

Rat Antibody Production Test

Hantaan
Kilham rat
Mouse encephalomyelitis
Pneumonia virus of mice
Rat coronavirus
Reovirus 3
Sendai
Sialoacryoadenitis
Toolan

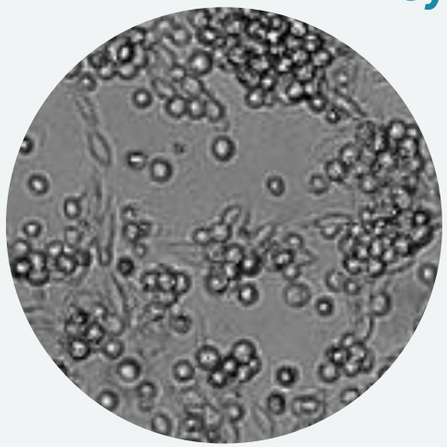
Ensuring a GMP-compliant Assay

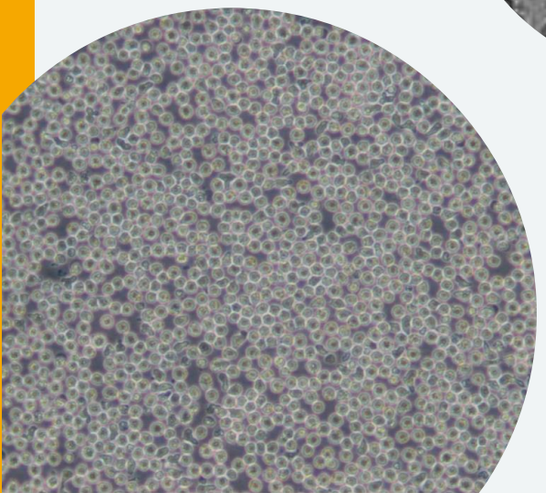


"System Validation" broadly encompasses a range of method validations
(*e.g.* extraction through data generation & reporting; including the software and its components (databases))

A validated system must be 'fit for purpose' -- in this case, must detect replicative adventitious viruses

Transcriptome Validation





Laboratory Method Validation:

- Mixture of infected: uninfected cells (EBV infected B95-8:Ramos or Raji:Ramos mixtures) spiked with known concentration (copy numbers) of a synthetic collection of representative target RNAs

- In-process controls included:
  - TPA treated B95-8 cells → EBV positive control
  - Raji cells → EBV positive control
  - Ramos cells → MuLV positive companion control
  - MRC-5 cells → Negative (uninfected) control

- Validation also included:
  - MRC-5 cells infected with Ad5, PI3, & Cox3
  - MDBK cells infected with BVDV

Bioinformatics/Software Validation:

- Used agnostic & targeted pipelines
- Screen for presence of EBV, MuLV, & spiked RNA controls
- Secondary qualification of the results by viral expert to verify hits

Criteria for a Valid Test

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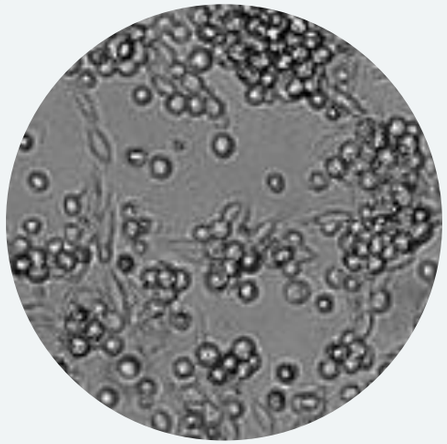
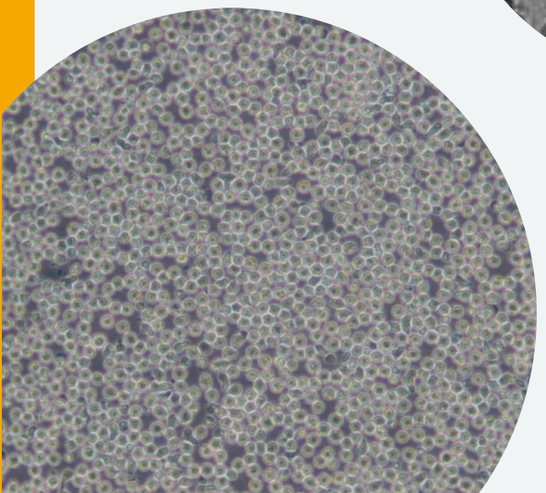
EXAMPLE





Method's step	Quality control	Quality acceptance criteria
RNA Extraction	Quality of extract (by Nanodrop):	Absorbance 260 / Absorbance 280 ≥ 1,8
	Quality of RNAs (by Bioanalyzer):	- RIN (RNA Integrity Number) ≥ 5 - Major features of a successful ladder
	Quantification of extract (by Qubit):	≥ 0,25 ng/µL
Library Preparation	Quantification of libraries (by Qubit):	≥ 3,00 ng/µL
	Qualification of libraries (by Bioanalyzer):	Fragments range between 200bp and 1000bp with a local maximum at ~300–400bp
Sequencing	Illumina metrics: - Min read per library - Percent bases higher than Q30 (for flowcell High output 150 cycles) - Homogeneous repartition of read by libraries - Data generation	- 65 million of reads at least per library - Q30 > 80% - Chi2 test of homogeneity p-value > 0,05 - Output data at a minimum of 50gb
Data Analysis	Negative control analysis (MRC-5 Cell Line (ATCC CCL-171)	- No replicative viruses are detected
	Positive control analysis (TPA induced B95-8 cells (EBV infected) + Ramos cells (MuLV infected) ratio $1/10^4$)	-Replicative HHV4 virus must be detected -MuLV virus genome has to be complete (>95% coverage) -No RNA control sequences identified as a positive viral hit

Enabling Regulatory Agency Support

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ansm
Agence nationale de sécurité du médicament et des produits de santé

ANSM review of PathoQuest's Transcriptome Assay published 2023

Modality	Process stage by	Assays that can be substituted for PathoQuest NGS
Recombinant proteins	• Unpurified bulk (with intact cells) • Master Cell Bank • Working Cell Bank • End of Production Cells	• *In vitro* adventitious agents • *In vivo* adventitious agents

"The wide detection spectrum of NGS assays allows the identification of viruses that cannot be detected by [traditional] assays, thus improving the diagnostic sensitivity of virological controls. This comparative study confirms the potential of the NGS transcriptomic assay as replacing traditional testing strategies, providing better assurance of safety and opening up new possibilities for safer innovative biologics and therapies."

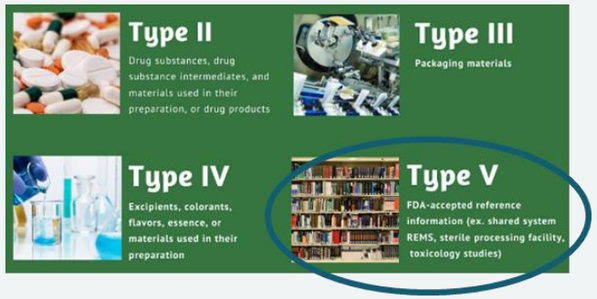
PENDING

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

- **Innovation Task Force (ITF)**

DEPARTMENT OF HEALTH & HUMAN SERVICES • USA
FDA

- **CBER Advanced Technologies Team (CATT)**
- **CDER Emerging Technologies Program (ETP)**
- **Drug or Biologics Master File**



Cell Bank Characterization

Application of an NGS Transcriptome Assay

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Production Systems for:
mAb's, r-proteins, Vaccines, Gene Therapy, Cell Therapy

Master Cell Bank Characterization

Microbiology	• Sterility • Mycoplasma
Identity	• Barcoding
Adventitious Viruses	• **3 Cell Line *in vitro*** • ***In vivo***
Retroviruses	• Electron Microcopy • Infectivity Assay • F-PERT
Species Specific Viruses	• **Mouse/Hamster/Rat Antibody Production** • **Human Virus PCR panel**
Bovine and Porcine Viruses	• **Bovine Viruses** • **Porcine Viruses**

Faster, Safer, More Ethical Cell Bank Characterization

Rapid Testing

- ✓ Complete in as little as 2-3 weeks.
- ✓ Quicker release to manufacturing
- ✓ Faster to patients

3Rs Compliant

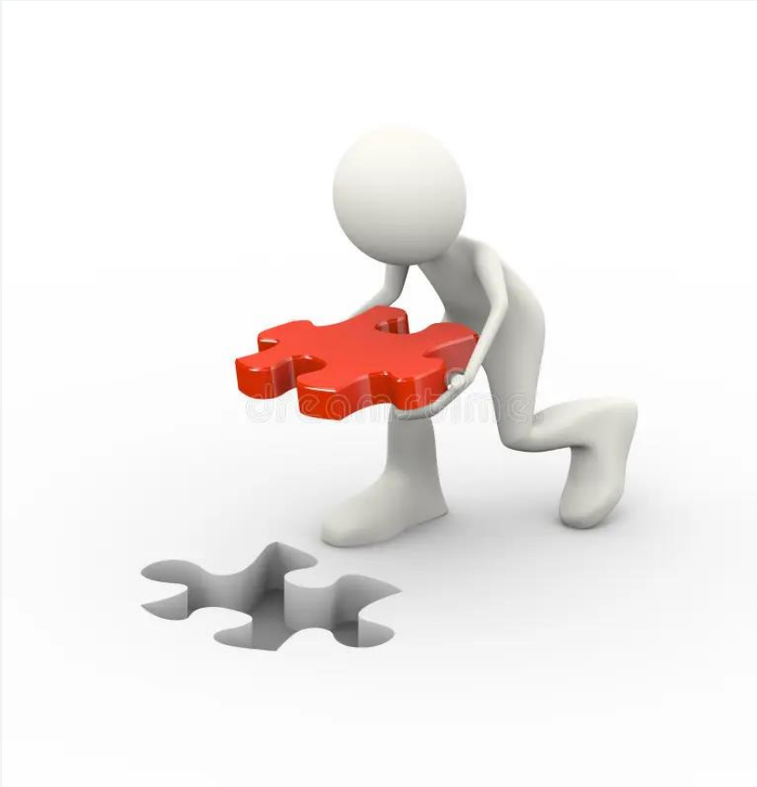
- ✓ Replacement, Reduction, Refinement
- ✓ In accordance with Directive 2010/63/EU

Integrated Solution

- ✓ Broad breadth and depth of coverage
- ✓ Replaces multiple assays
- ✓ Head-to-head comparisons completed
- ✓ Reduction in false positives

20 Years After Arriving on the Scene, NGS is Now:

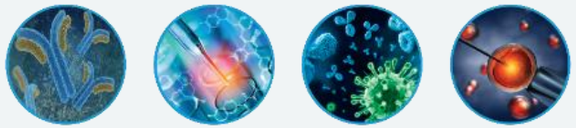
- **Robust, fit-for-purpose assay enabling targeted and agnostic approaches for contaminant detection**
- **Already integrated into guidance documents**
- **Supported by the agencies as a replacement assay for select classical tests (*in vivo*; HAP/MAP/RAP)**
- **Faster, safer, and animal-free testing**



Thank you!

PathoQuest

Biologics Quality Testing. Faster, Safer, Animal Free.



contact@pathoquest.com