PathoQuest

Transcriptomic NGS assay of cells as a substitute for conventional virus testing techniques

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PathoQuest

IABS
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Viral nucleic acids in infected cells

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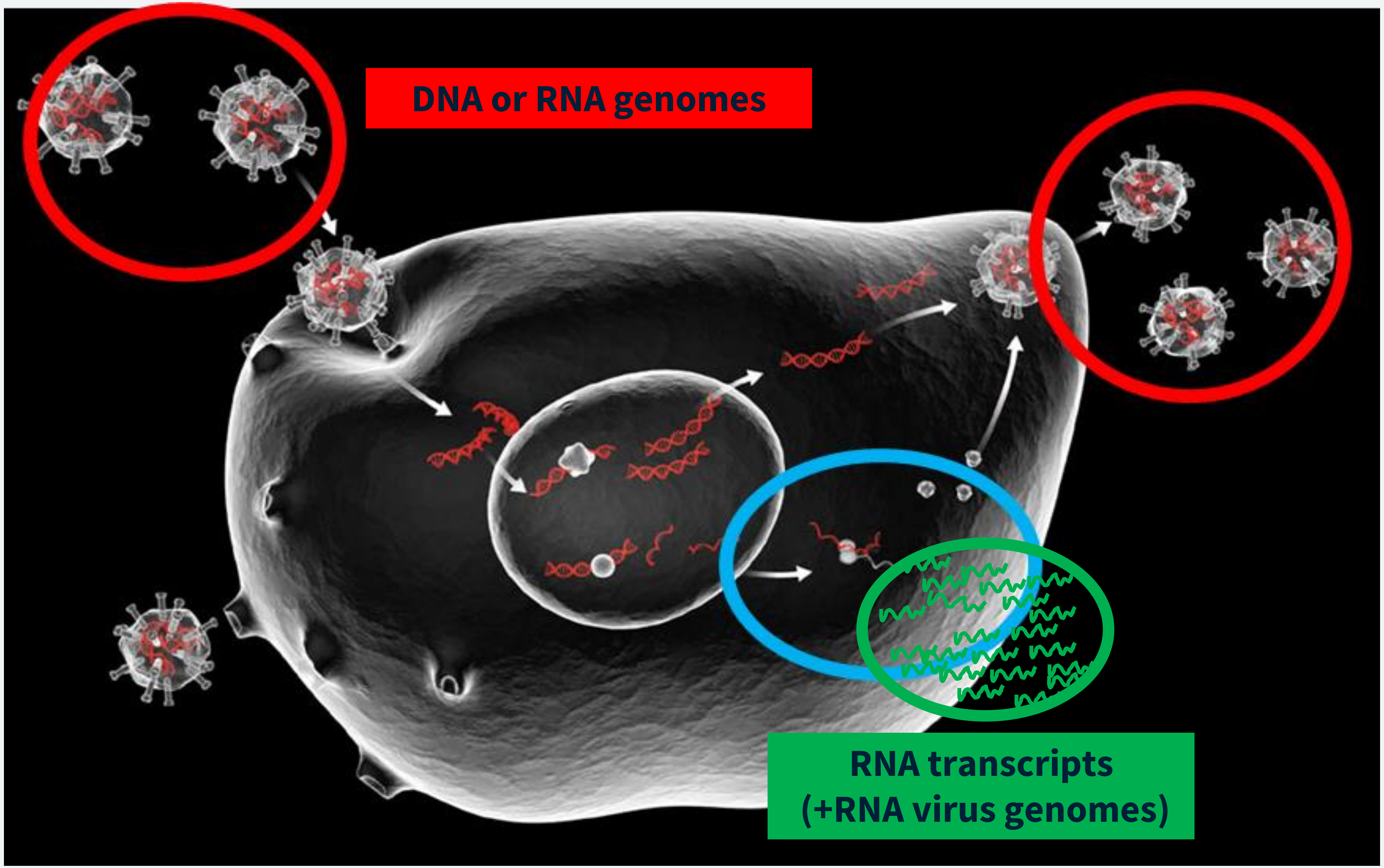

DNA or RNA genomes

RNA transcripts
(+RNA virus genomes)

Why sequence only RNAs when testing cells for DNA and RNA virus infection?

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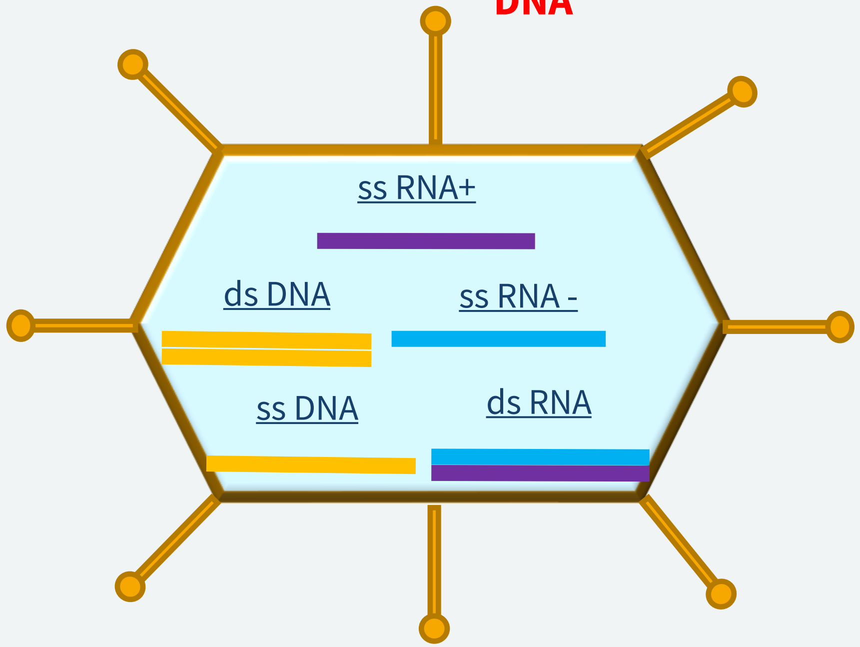
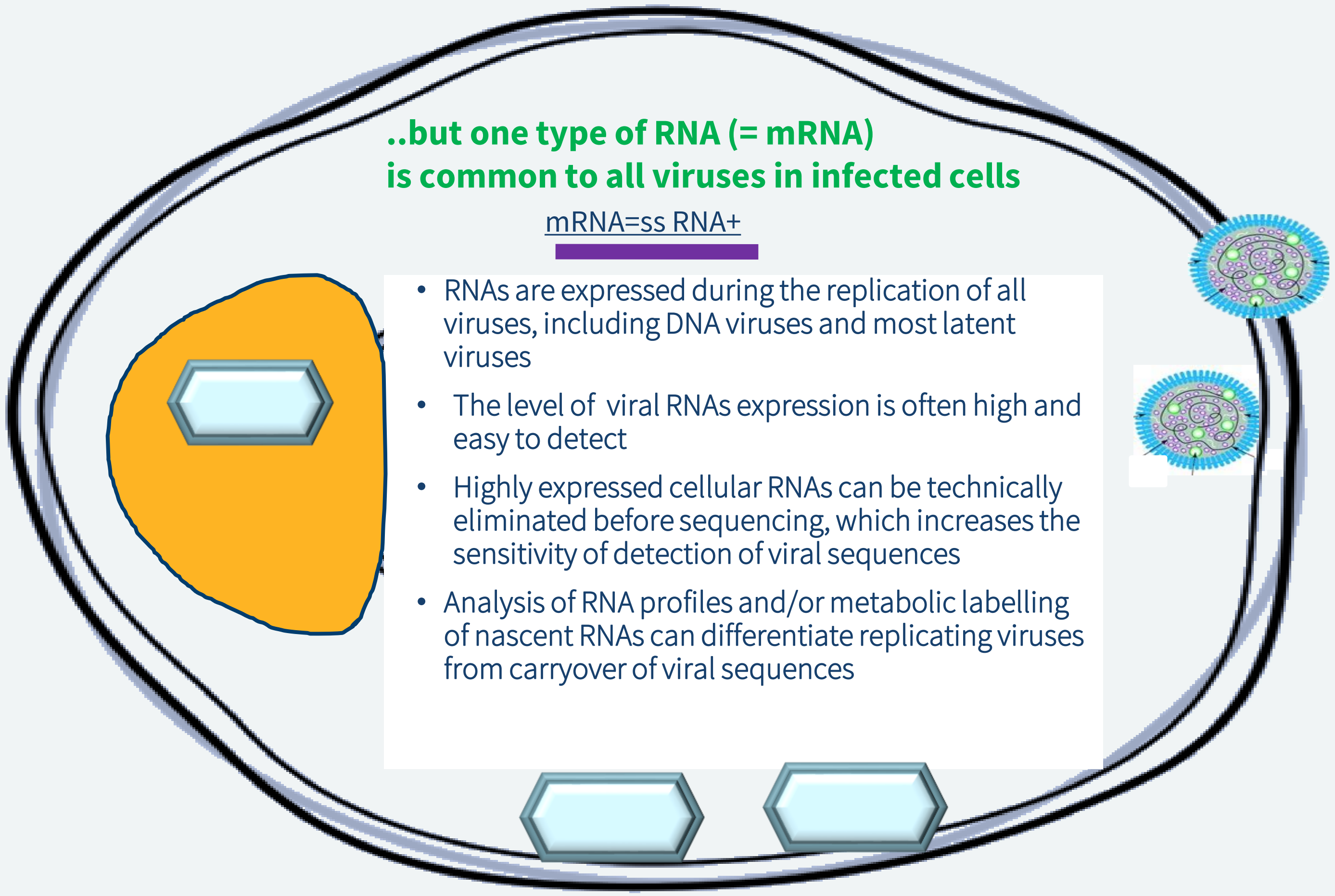
Different genomes types in virus particles…
DNA virus genomes hidden by host cellular DNA





..but one type of RNA (= mRNA)
is common to all viruses in infected cells

- RNAs are expressed during the replication of all viruses, including DNA viruses and most latent viruses
- The level of viral RNAs expression is often high and easy to detect
- Highly expressed cellular RNAs can be technically eliminated before sequencing, which increases the sensitivity of detection of viral sequences
- Analysis of RNA profiles and/or metabolic labelling of nascent RNAs can differentiate replicating viruses from carryover of viral sequences

Objective

We have previously shown that this GMP **NGS transcriptomic assay can replace *in vivo* tests**:

Vaccine 41 (2023) 5383–5391

Contents lists available at ScienceDirect

Vaccine

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

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

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Context: ICH Q5A(R2) : NGS (targeted or non-targeted) **can replace virus-specific PCR assays and rodent antibody production tests** (Section 3.2.4) without a head-to-head comparison.

Can this NGS transcriptomic assay replace targeted PCRs, MAP/RAP/HAP and 9CFR assays for the testing of cells ?

- analytical sensitivity , ability to detect distant strains in each targeted species ?

Discussion of performances for the testing of cell banks, bulk of recombinant proteins, cellular therapy DP

Virus selection

BOVINE and PORCINE

- **9CFR:** Title 9 Chapter I Subchapter E Part 113 Standard Procedures § 113.47
- **All cells:** (i) Bovine virus diarrhea virus; (ii) Reovirus; and (iii) Rabies virus.
- **Bovine, caprine, ovine cells:** (i) Bluetongue virus; (ii) Bovine adenoviruses; (iii) Bovine parvovirus; and (iv) Bovine respiratory syncytial virus.
- **Porcine cells:** (i) Porcine adenovirus; (ii) Porcine parvovirus; (iii) transmissible gastroenteritis virus; and (iv) Porcine hemagglutinating encephalitis virus.

- **RODENTS (ICH Q5A R2)**: MAP or RAP or HAP

- **HUMAN:** typically: HIV types 1 and 2; HTLV types 1 and 2; hepatitis virus types A, B and C; human bocavirus, human herpes simplex virus types 1 and 2 human herpes virus types 6, 7 and 8; CMV; VZV; EBV; parvovirus B19;SRV-1 and 2; simian foamy virus; human papilloma types 16 and 18; human polyomavirus JC and BK; enterovirus, rubella, West Nile virus

Table 3. Viruses detected in antibody production tests[d]

MAP	HAP	RAP
Ectromelia Virus[b,c]	Lymphocytic Choriomeningitis Virus (LCM)[a,c]	Hantaan Virus[a,c]
Hantaan Virus[a,c]	Pneumonia Virus of Mice (PVM)[b,c]	Kilham Rat Virus (KRV)[b,c]
K Virus[b]	Reovirus Type 3 (Reo3)[a,c]	Mouse Encephalomyelitis Virus (Theilers, GDVII)[b]
Lactic Dehydrogenase Virus (LDM)[a,c]	Sendai Virus (SV)[a,c]	Pneumonia Virus of Mice (PVM)[b,c]
Lymphocytic Choriomeningitis Virus (LCM)[a,c]	SV5[a,c]	Rat Coronavirus[b]
Minute Virus of Mice[b,c]		Reovirus Type 3 (Reo3)[a,c]
Mouse Adenovirus (MAV)[b,c]		Sendai Virus[a,c]
Mouse Cytomegalovirus (MCMV)[b,c]		Sialodacryoadenitis Virus (SDAV)[b]
		Toolan's H-1 Virus[b,c]
Mouse Encephalomyelitis Virus (Theilers, GDVII)[b]		
Mouse Hepatitis Virus (MHV)[b]		
Mouse Rotavirus (EDIM)[b,c]		
Pneumonia Virus of Mice (PVM)[b,c]		
Polyoma Virus[b]		
Reovirus Type 3 (Reo3)[a,c]		
Sendai Virus[a,c]		
Thymic Virus[b,e]		

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b. Viruses for which there is evidence of capacity for infecting humans or primates.

c. Viruses for which there is no evidence of capacity for infecting humans.

d. Virus capable of replicating in vitro in cells of human or primate origin.

e. NAT such as PCR assays or targeted or non-targeted NGS or other molecular methods can be used instead.

f. Also called murid herpesvirus 3.

Method: samples

TEST SAMPLES

RODENT VIRUSES TARGETED BY MAP/RAP/HAP in CHO-K1 cells

Pool #1: 25 homolog synthetic RNAs + 1 mirror RNA
Pool#2: 25 Distant synthetic RNAs + 1 mirror RNA

BOVINE and PORCINE VIRUSES TARGETED BY 9CFR in BT cells

Pool #1: 24 homolog synthetic RNAs + 1 mirror RNA

HUMAN AND SIMIAN VIRUSES TESTED BY PCR in HEK-293 cells

Pool #1: 29 homolog synthetic RNAs + 1 mirror RNA

Cell pellets lysed in RNeasy extraction kit

Spiking at 0,1 to 0,00001 RNA copies /cell

CONTROL SAMPLES

Negative control: MRC-5
Positive control: B95-8 (EBV+) diluted 10^{-4} in RAMOS cells (MuLV+)

SPIKE-IN CONTROLS

ERCC: RNA-spike In Control mixes (n=7) spiked in each test sample (matrix interference): coverage ≥ 80% for at least 4 of 7 ERCC targets compared to the coverage observed in the positive control

Simulation of sequences of distant strains : example of « Murine Rotavirus »

Species demarcation criteria

Rotaviruses grouped into nine species (Rotavirus A to J),

Species may be identified by serological cross-reactivity by ELISA targeting VP6,

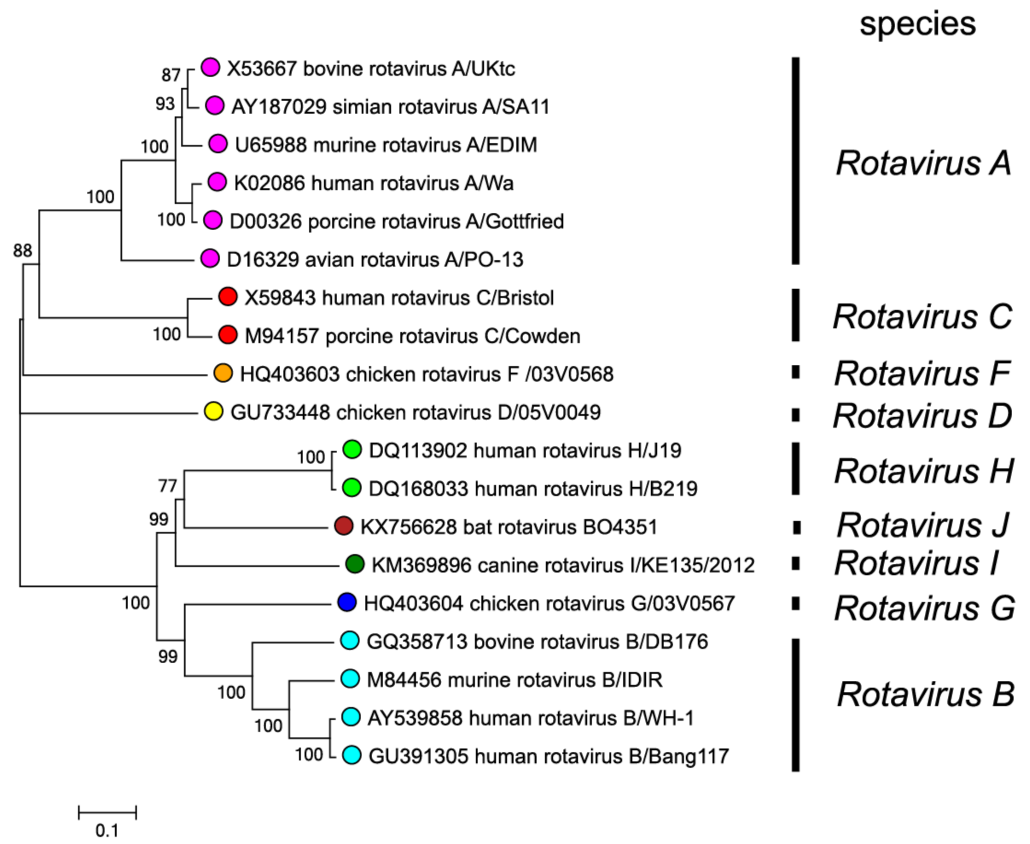
Sequence of conserved genome segments (*e.g.,* Seg1 and Seg6). A cut-off value of 53% identity of the VP6 amino acid sequence has been used to differentiate species (Matthijnssens *et al.*, 2012).

Definition of RNAS used for testing

Close (homolog) synthetic RNAs : representative sequences in GenBank.

Distant RNAs : IQ-Tree2 using parameter "Model" set to "random" or based on a given phylogenetic tree, and with a worst-case percentage of similarity within the species defined by ICTV

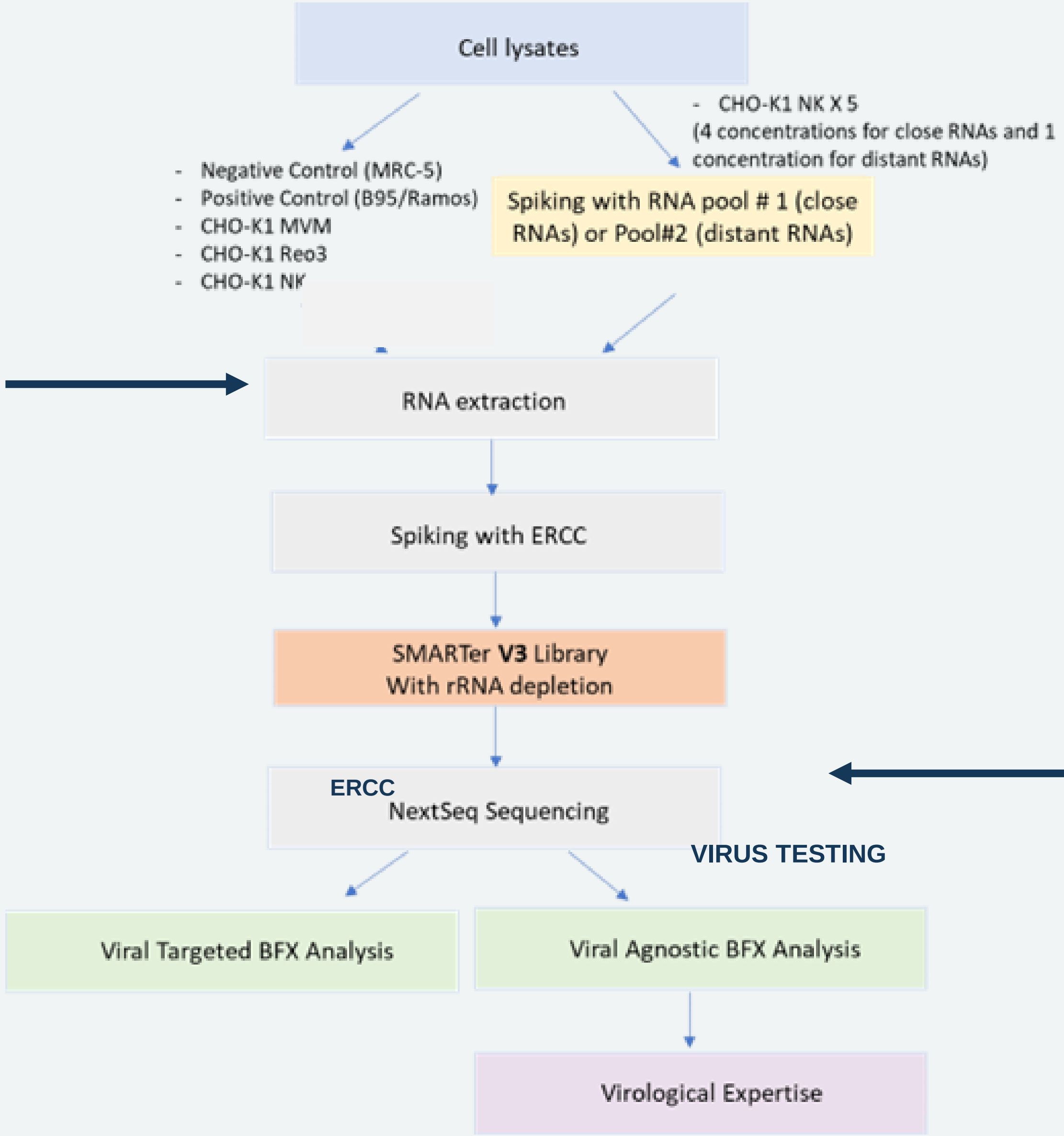
Mirror RNA : reverse of one of the RNAs. This RNA keeps some properties of the sequence (*e.g.,* the percentage of GC) but it is not expected to match to anything known.



Rotavirus Phylogenetic relationships between members of different rotavirus species using the amino acid sequences of the VP6 inner capsid protein. Bootstrapping values (1000 replicates) above 70 % are shown.

Method: sample processing and sequencing

RNA extraction, Quality testing



Cell lysates

- Negative Control (MRC-5)
- Positive Control (B95/Ramos)
- CHO-K1 MVM
- CHO-K1 Reo3
- CHO-K1 NK

- CHO-K1 NK X 5
(4 concentrations for close RNAs and 1 concentration for distant RNAs)

Spiking with RNA pool # 1 (close RNAs) or Pool#2 (distant RNAs)

RNA extraction

Spiking with ERCC

SMARTer **V3** Library
With rRNA depletion

ERCC

NextSeq Sequencing

VIRUS TESTING

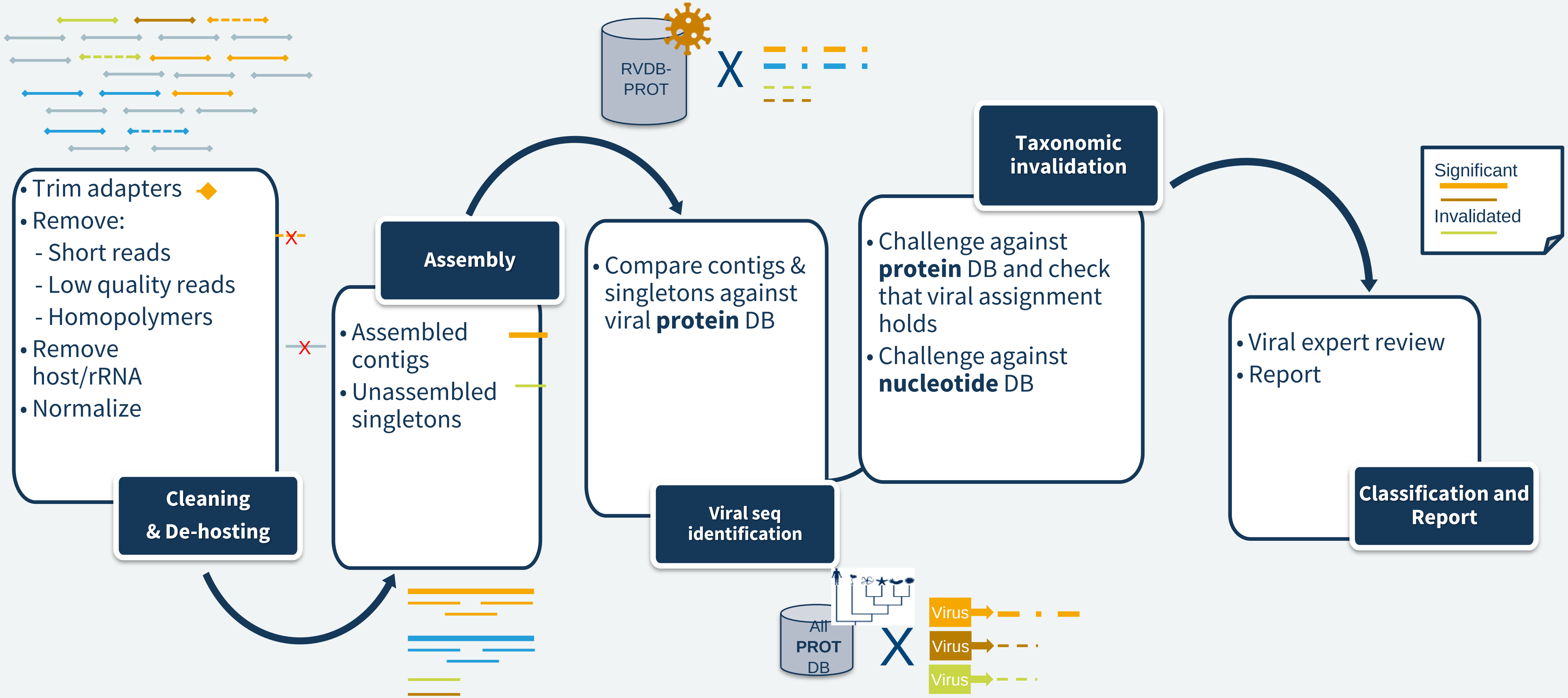
Viral Targeted BFX Analysis

Viral Agnostic BFX Analysis

Virological Expertise

NextSeq 500/550 High Output kit v2.5, single-read 150 nucleotides
> 65 million reads per library

Agnostic bioinformatics pipeline



- Trim adapters ◆
- Remove:
  - Short reads
  - Low quality reads
  - Homopolymers
- Remove host/rRNA
- Normalize

Cleaning & De-hosting

Assembly

- Assembled contigs
- Unassembled singletons

- Compare contigs & singletons against viral **protein** DB

Viral seq identification

Taxonomic invalidation

- Challenge against **protein** DB and check that viral assignment holds
- Challenge against **nucleotide** DB

- Viral expert review
- Report

Classification and Report

*Pérot, P., Bigot, T., Temmam, S., Regnault, B. & Eloit, M. Microseek: A protein-based metagenomic pipeline for virus diagnostic and discovery. Viruses **14**, 1990 (2022)*

HAP/MAP/RAP panels : detection close and distant strains

PathoQuest

Detects all close &

Virus		Spiked CHO-K1 cells (copies/cell)				
Family	**Species**	**0.1** *Distant and Close transcripts*	**0.01** *Close transcripts*	**0.001** *Close transcripts*	**0.0001** *Close transcripts*	**0.00001** *Close transcripts*
Adenoviridae	Mouse Adenovirus (MAV) MAdV1	🟩	🟩	🟩	🟩	🟩
	MAdV2	🟩	🟩	🟩	🟩	🟩
Arenaviridae	Lymphocytic choriomeningitis virus (LCMV)	🟩	🟩	🟩	🟩	🟩
Arteriviridae	Lactate Dehydrogenase elevating virus (LDV)	🟩	🟩			
Coronaviridae	Mouse Hepatitis virus (MHV)	🟩	🟩			
	Sialodacryoadenitis virus (SDAV)	🟩	🟩			
	Rat coronavirus (RCV)	🟩	🟩			
Hantaviridae	Hantaan virus (HANT)	🟩	🟩	🟩		
	Prospect Hill virus (PHV)	🟩	🟩			
Herpesviridae	Mouse Cytomegalovirus (MCMV)	🟩	🟩	🟩		
	Mouse thymic virus (MTLV)	🟩	🟩	🟩		
Paramyxoviridae	Pneumonia virus of mice (PVM)	🟩	🟩			
	Sendai virus (SEND)	🟩	🟩			
	Simian virus 5 (SV5)	🟩	🟩			
Parvoviridae	Minute virus of mice (MVM)	🟩	🟩	🟩		
	Kilham's rat virus (KRV)	🟩	🟩	🟩		
	Mouse Parvovirus (MPV)	🟩	🟩	🟩		
	Toolan's H-1 virus (H-1)	🟩	🟩	🟩		
Picornaviridae	Theiler's murine encephalomyelitis virus (GDVII)	🟩	🟩	🟩		
	Rat Theilovirus (RTV)	🟩	🟩	🟩		
Polyomaviridae	Mouse K virus	🟩	🟩			
Poxviridae	Ectromelia (ECTRO)	🟩	🟩			
Reoviridae	Reovirus type 3	🟩	🟩			
	Reovirus type 1	🟩	🟩			
Rotaviridae	Mouse Rotavirus (A/EDIM)	🟩	🟩	🟩		

HAP/MAP/RAP panels : coverage close strains

High coverage for close strains @ 0,1 copy per cell for all viruses tested

Virus Family	Virus	Protein selected	RNA Transcript Coverage
Adenoviridae	Mouse Adenovirus (MAV) MAdV1	Polymerase	99.8
	MAdV2	Polymerase	100.0
Arenaviridae	Lymphocytic choriomeningitis virus LCMV	Nucleoprotein	98.5
Arteriviridae	Lactate Dehydrogenase elevating virus (LDV)	Polyprotein 1b	98.0
Coronaviridae	Mouse Hepatitis virus (MHV)	Spike Protein	78.7
	Sialodacryoadenitis virus (SDAV)	Nucleocapsid protein	95.2
	Rat coronavirus (RCV) = Murine coronavirus	Nucleocapsid protein	100.0
Hantaviridae	Hantaan virus (HANT)	Nucleocapsid protein	98.0
	Prospect Hill virus (PHV)	Nucleocapsid protein	99.8
Herpesviridae	Mouse Cytomegalovirus (MCMV)	Envelope glycoprotein B	93.0
	Mouse thymic virus (MTLV)	MCP	75.0
Paramyxoviridae	Pneumonia virus of mice (PVM)	Nucleocapsid protein	91.5
	Sendai virus (SEND)	Nucleocapsid protein	73.7
	Simian virus 5 (SV5)	Nucleocapsid protein	97.2
Parvoviridae	Minute virus of mice (MVM)	Proteine NS1	100.0
	Kilham's rat virus (KRV)	NS1	83.7
	Mouse Parvovirus (MPV)	NS1	96.5%
	Toolan's H-1 virus (H-1)	NS1	97.8%
Picomaviridae	Theiler's murine encephalomyelitis virus (GDVII)	Polyprotein (P1 correspond to genomic region 1296 to 4259 corresponding to VP4, VP2, VP3 and VP1/ regions proteiques: 91 -898)	94.7%
	Rat Theilovirus (RTV)	Polyprotein (P1 corresponds to genomic region encoding for VP4, VP2, VP3 and VP1/ regions proteiques: 92-092)	83.8%
Polyomaviridae	Mouse K virus	Capsid protein VP1	65.8%
Poxviridae	Ectromelia (ECTRO)	Hemagglutinin	42.8%
Reoviridae	Reovirus Type 3	Outer capsid protein sigma 3	91.3%
	Reovirus Type 1	Outer capsid protein sigma 3	98.0%
Rotaviridae	Mouse Rotavirus (A/EDIM)	inner capside protein VP6	80.2%

HAP/MAP/RAP panels by NGS: coverage distant strains

High coverage for distant strains @ 0,1 copy per cell for all viruses tested

Virus Family	Virus	Protein selected	RNA Transcript Coverage
Adenoviridae	Mouse Adenovirus (MAV) MAdV1	Polymerase	74.7
	MAdV2	Polymerase	100.0%
Arenaviridae	Lymphocytic choriomeningitis virus LCMV	Nucleoprotein	97.5%
Arteriviridae	Lactate Dehydrogenase elevating virus (LDV)	Polyprotein 1b	99.0%
Coronaviridae	Mouse hepatitis virus (MHV)	Spike protein	92.2%
	Sialodacryoadenitis virus (SDAV)	Nucleocapsid protein	90.1%
	Rat coronavirus (RCV) = Murine coronavirus	Nucleocapsid protein	96.3%
Hantanviridae	Hantaan virus (HANT)	Nucleocapsid protein	98.5%
	Prospect Hill virus (PHV)	Nucleocapsid protein	96.7%
Herpesviridae	Mouse Cytomegalovirus (MCMV)	Envelope glycoprotein B	88.5%
	Mouse thymic virus (MTLV)	MCP	99.0%
Paramyxoviridae	Pneumonia virus of mice (PVM)	Nucleocapsid protein	91.5%
	Sendai virus (SEND)	Nucleocapsid protein	99.2%
	Simian virus 5 (SV5)	Nucleocapsid protein	97.7%
Parvoviridae	Minute virus of mice (MVM)	Proteine NS1	96.2%
	Kilham's rat virus (KRV)	NS1	99.8%
	Mouse Parvovirus (MPV)	NS1	98.0%
	Toolan's H-1 virus (H-1)	NS1	98.2%
Picomaviridae	Theiler's murine encephalomyelitis virus (GDVII)	Polyprotein (P1 correspond to genomic region 1296 to 4259 corresponding to VP4, VP2, VP3 and VP1/ regions proteiques: 91 -898)	100.0%
	Rat Theilovirus (RTV)	Polyprotein (P1 corresponds to genomic region encoding for VP4, VP2, VP3 and VP1/ regions proteiques: 92-092)	100.0%
Polyomaviridae	Mouse K virus	Capsid protein VP1	87.5%
Poxviridae	Ectromelia (ECTRO)	Hemagglutinin	77.8%
Reoviridae	Reovirus type 3	Reovirus type 3	99.5%
	Reovirus type 1	Reovirus type 1	100.0%
Rotaviridae	Mouse Rotavirus (A/EDIM)	Mouse Rotavirus (AVEDIM)	95.8%

Human/simian panels : detection close strains

Detects all close strains @ 0,1 copy per cell for all viruses tested

Virus		Spiked HEK293 cells (copies/cell)		
Family	**Species**	**0.1** *Distant and Close transcripts*	**0.01** *Close transcripts*	**0.001** *Close transcripts*
Human Viruses	Adenovirus (Broad range A - G)	🟩	🟩	🟩
	Human immunodeficiency Virus type I (HIV-1)	🟩		
	Human immunodeficiency Virus type II (HIV-2)	🟩	🟩	
	Human T Lymphotropic virus type 1 and 2 (HTLV-1/2)	🟩		
	Human Foamy virus (HFV)	🟩	🟩	
	Human Papillomavirus types 16 and 18 (HPV-16/18)	🟩	🟩	
	Epstein Bar virus (EBV-HHV4)	🟩	🟩	
	Human Cytomegalovirus (HCMV; HHV5)	🟩	🟩	
	Human Herpesvirus 6 (HHV-6)	🟩	🟩	
	Human Herpesvirus 7 (HHV-7)	🟩	🟩	
	Human Herpesvirus 8 (HHV-8)	🟩	🟩	
	Herpes Simplex virus type 1 (HSV-1)	🟩		
	Herpes Simplex virus type 2 (A-G) (HSV-2)	🟩	🟩	🟩
	Human Polyomavirus (JCV)	🟩		
	Human Polyomavirus (BK)	🟩		
	Human Parvovirus (B19)	🟩		
	Adeno Associated Virus (AAV)	🟩		
	Hepatitis A virus (HAV)	🟩		
	Hepatitis B virus (HBV)	🟩	🟩	
	Hepatitis C virus (HCV)	🟩	🟩	
	West Nile virus (WNV)	🟩		
	Hepatitis E virus (HEV)	🟩		
	Severe Acute Respiratory Syndrome Virus 2 (SARS-CoV2)	🟩		
Simian Viruses	Simian Immunodeficiency Virus (SIV)	🟩		
	Simian T-lymphotropic virus (STLV)	🟩		
	Squirrel Monkey Retrovirus (SMRV)	🟩		
	Simian Foamy virus (SFV)	🟩	🟩	
	Simian Retrovirus-1/2/3 (SRV-1/2/3)	🟩	🟩	
	Simian Virus 40 (SV-40)	🟩		

Human/simian panels close strains: coverage

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High coverage for close strains @ 0,1 copy per cell for all viruses tested

Family	Virus	Protein Selected	Spiked with 105 copies per 106 cells of close RNA	Number of Contigs	Number of Singletons	RNA Transcript Coverage (%)
Human Viruses	Adenovirus (Broad range A-G)	Polymerase	🟩	9	61	100.0
	Human immunodeficiency Virus type I (HIV-1)	Envelope		1	0	57.5
	Human immunodeficiency Virus type II (HIV-2)	Polymerase		1	0	100.0
	Human T Lymphotropic virus type 1 and 2 (HTLV-1/2)	Envelope		2	1	66.2
	Human Foamy virus (HFV)	Envelope		0	1	77.8
	Human Papillomavirus types 16 and 18 (HPV-16/18)	L1		1	1	94.7
	Epstein Barr virus (EBV-HHV4)	MCP		1	5	89.2
	Human Cytomegalovirus (HCMV; HHV5)	MCP		0	4	83.3
	Human Herpes virus 6 (HHV-6)	MCP		1	0	84.2
	Human Herpes virus 7 (HHV-7)	MCP		1	2	26.5
	Human Herpes virus 8 (HHV-8)	MCP		1	1	76.7
	Herpes Simplex virus type 1 (HSV-1)	MCP		1	1	45.8
	Herpes Simplex virus type 2 (HSV-2)	MCP		2	4	81.8
	Human Polyomavirus (JCV)	Large T Antigen		1	1	52.8
	Human Polyomavirus (BK)	Large T Antigen		1	1	72.7
	Human Parvovirus (B19)	NS1		1	1	98.8
	Adeno Associated Virus (AAV)	Rep 78		1	3	67.3
	Hepatitis A virus (HAV)	3C-3D		1	0	82.8
	Hepatitis B virus (HBV)	Polymerase		1	0	52.5
	Hepatitis C virus (HCV)	NS3		1	1	42.5
	West Nile virus (WNV)	NS5-Polymerase		1	2	71.5
	Hepatitis E virus (HEV)	ORF1		2	0	98.3
	Severe Acute Respiratory Syndrome Virus 2 (SARS-CoV2)	Spike protein		0	2	53.8
Simian Viruses	Simian Immunodeficiency Virus (SIV)	Envelope		1	6	76.0
	Simian T-lymphotropic virus (STLV)	Envelope		1	0	98.7
	Squirrel Monkey Retrovirus (SMRV)	Envelope		1	1	91.3
	Simian Foamy virus (SFV)	Envelope		0	1	25.2
	Simian Retrovirus-1/2/3 (SRV-1/2/3)	Polymerase		0	2	49.8
	Simian Virus 40 (SV-40)	Large T Antigen		1	0	86.2

Bovine and porcine viruses (9CFR) : detection

	Virus	Spiked BT cells (1 copy/cell)	Spiked BT cells (0,1 copy/cell)
Both bovine and porcine	Bovine viral diarrhea virus	🟩	🟩
	Reovirus	🟩	🟩
	Rabies virus	🟩	🟩
Bovine viruses	Bluetongue virus	🟩	🟩
	Bovine Adenovirus	🟩	🟩
	Bovine Parvovirus	🟩	🟩
	Bovine respiratory syncytial virus	🟩	🟩
	Bovine Parainfluenza virus type 3	🟩	🟩
	Infectious Bovine Rhinotracheitis (IBR/BHV-1)	🟩	🟩
	Bovine polyomavirus (BPyV)	🟩	🟩
Porcine viruses	Porcine Adenovirus A (PAdV-3)	🟩	🟩
	Porcine Adenovirus B (PAdV-B)	🟩	🟩
	Porcine Adenovirus C (PAdV-C)	🟩	🟩
	Porcine Parvovirus (parvo_KRESSE)	🟩	🟩
	Transmissible gastroenteritis virus (TGEV)	🟩	🟩
	Porcine hemagglutinating encephalitis virus (HEV)	🟩	🟩
	Pseudorabies (PRV)	🟩	
	Encephalomyocarditis virus (cardio)	🟩	🟩
	Porcine respiratory and reproductive syndrome virus (PRRS)	🟩	🟩
	Porcine Circovirus (PCV-1)	🟩	🟩
	Porcine Circovirus (PCV-2)	🟩	🟩
	Porcine Circovirus (PCV-3)	🟩	🟩
	HEV genotype 3a (HEV-3a)	🟩 At the genus level only	🟩
	HEV genotype 4a (HEV-4a)	🟩	🟩

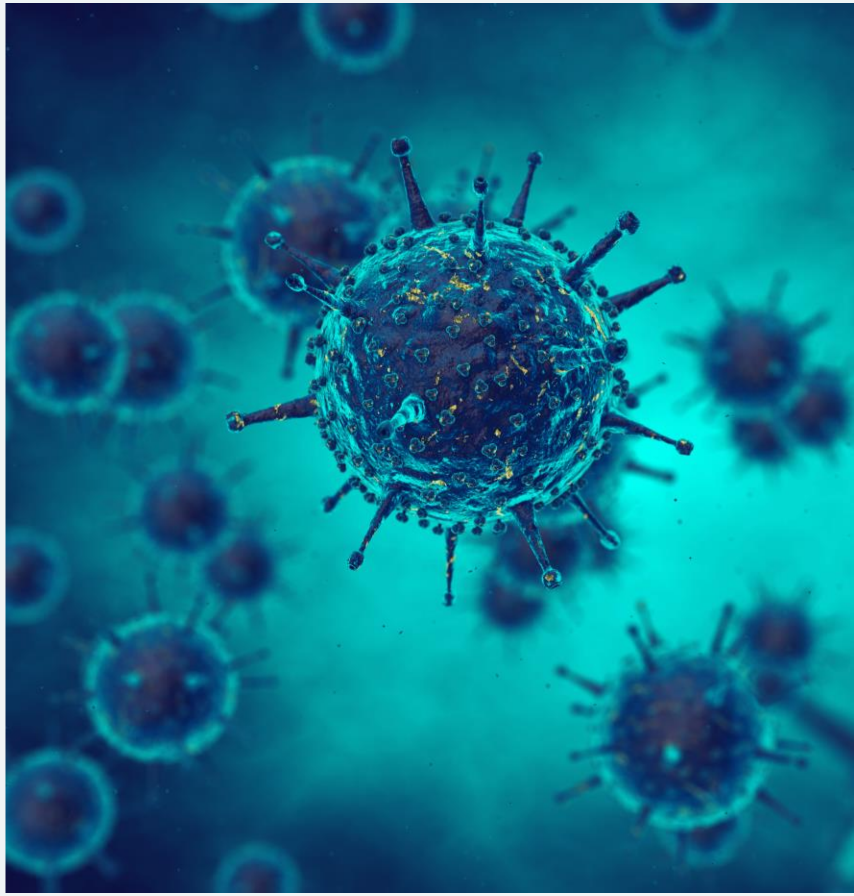
Bovine and porcine viruses (9CFR): coverage

High coverage for close strains @ 1 and 0.1 copy per cell for all viruses tested

	Virus	Selected Protein	coverage (1 copy/cell)	Coverage (0.1 copy/cell)
Both bovine and porcine	**Bovine viral diarrhea virus**	Polyprotein	99.0	87.5
	Reovirus	outer capsid protein Sigma 3	>90	84.8
	Rabies virus	N protein	92.5	64.2
Bovine viruses	**Bluetongue virus**	VP3	100.0	70.2
	Bovine Adenovirus	polymerase	96.3	58.2
	Bovine Parvovirus	NS1	99.0	50.0
	Bovine respiratory syncytial virus	Nucleocapsid protein	99.2	51.0
	Bovine Parainfluenza virus type 3	Nucleocapsid protein	98.0	65.0
	Infectious Bovine Rhinotracheitis (IBR/BHV-1)	major capsid protein	98.8	48.7
	Bovine polyomavirus (BPyV)	VP1	99.0	75.2
Porcine viruses	**Porcine Adenovirus A (PAdV-3)**	polymerase	100.0	58.7
	Porcine Adenovirus B (PAdV-B)	polymerase	100.0	83.0
	Porcine Adenovirus C (PAdV-C)	Polymerase	98.0	70.3
	Porcine Parvovirus (parvo_KRESSE)	Proteine NS1	98.0	75.2
	Transmissible gastroenteritis virus (TGEV)	spike protein	90.0	25.2
	Porcine hemagglutinating encephalitis virus (HEV)	spike protein	98.5	53.0
	Pseudorabies (PRV)	MCP	93.8	49.7
	Encephalomyocarditis virus (cardio)	Polyprotein (P1)	99.2	23.7
	Porcine respiratory and reproductive syndrome virus (PRRS)	Polyprotein 1b	87.5	64.7
	Porcine Circovirus (PCV-1)	Capsid	96.7	83.8
	Porcine Circovirus (PCV-2)	Capsid	99.8	49.0
	Porcine Circovirus (PCV-3)	Capsid	98.8	69.2
	HEV genotype 3a (HEV-3a)	ORF1	100.0	19.5
	HEV genotype 4a (HEV-4a)	ORF1	99.8	ND

Discussion 1: analytical sensitivity

- The LOD of this agnostic transcriptomic NGS test is estimated at **0.1 viral RNA copies** per cell.
- Detects 100% of **78 viruses** across **Human, Simian** (n=29), **Rodent** (n=25), **Bovine** and **Porcine** (n=24) species targeted by conventional tests (MAP/HAP/RAP, 9CFR, and extended PCR panels).
- This LOD is also valid for **distant strains** aligning with the definition of these virus species from the ICTV, giving high assurance of reliability.



Discussion 2: application to the testing of cell banks

- Risk analysis based on published viral RNA loads in infected cells:

Family	Virus	Genome Type	RNA Copies (log10, minim.)	Reference
Herpesviridae	Herpes Simplex Virus 1 (HSV-1)	DNA (Double-stranded)	**4**	Iwami et al, 2012
	EBV (HHV4)		**1-2 during latency** **4 during active infection**	Tierney et al, 2015
Paramyxoviridae	Measles Virus	RNA (Single-stranded, negative sense)	**4**	Plumet et al, 2005
Retroviridae	HIV	RNA (Single-stranded, positive sense)	**3**	Pasternak et al, 2013; Hockett et al, 1999
Flaviviridae	Hepatitis C Virus (HCV)		**4**	Zitzmann et al, 2023
Picornaviridae	Coxaxkie B virus		**5**	Zitzmann et al, 2023
	Foot and mouth disease		**2-4**	Xin et al, 2018
	Poliovirus		**4-5**	Lee et al, 2022
Coronaviridae	SARS-CoV-		**2-5**	Schulte,et al, 2014

- Worst-case LOD = 0.1 RNA copy/cell, equating to (for worst case infected cells): 1 infected in 1000 non-infected cells (**10^{-3}**).
- Coherent with experimental data using infected cells (Vaccine, 2024): LOD = **10^{-3} to 10^{-7}** infected in non-infected cells

Discussion 3: testing of the bulk of recombinant proteins produced in eukaryotic cells

- **Eukaryotic cell cultures**, unlike bacterial cultures or chemical synthesis processes, can support the replication and amplification of **eukaryotic viruses**….regulatory guidelines require **virological control** specifically for eukaryotic cell culture-derived products to mitigate this risk,
- **Bacterial or chemically synthesized** products do not mandate viral testing of the bulk because the risk of **viral carryover from raw materials is considered ne**gligible,
- What are effective testing NGS strategies to detect any **viral amplification** that may have occurred during cell culture ?
  - **viromic analysis on both intact or disrupted cells and supernatant** (as viral particles can may be predominantly present in one or the other compartment) : sequencing must target both DNA and RNA: comprehensive, but this dual-target approach may dilute sensitivity compared to a transcriptomic approach focused solely on RNA.
  - **Transcriptomic analysis on intact cells** if they are available for the reasons explained before.

We advocate for the use of agnostic transcriptomic analysis in routine virological testing of bulk samples for which intact cells are accessible at the end of production

Discussion 4: strategy for testing CAR-T cells for HHV-6/7: background

- A **high proportion of adults are chronically infected with HHV-6B**, and many with HHV-6A.
- HHV-6 DNA in healthy adults source of CAR-T cells (peripheral lymphocytes or blood by PCR) : 9% -90%... detection of HHV-6 and HHV-7 DNA among CAR-T cells batches is high.
- Virus genome is **episomal or integrated in a fraction of the body cells**
- Sometimes, the **genome integrates in the germline: inherited chromosomally integrated HHV-6** (iciHHV-6). This leads to its presence in every cell of the offspring. This is the case in around 1% of the human population
- The latent virus can reactivate, leading to infectious virions: In vitro, a population of HHV-6“super-expressors” (**about 1 in 300-10,000**) CAR-T cells possesses high viral transcriptional activity combined with an overexpression of the HHV-6 receptor (OX40)

Article

Latent human herpesvirus 6 is reactivated in CAR T cells

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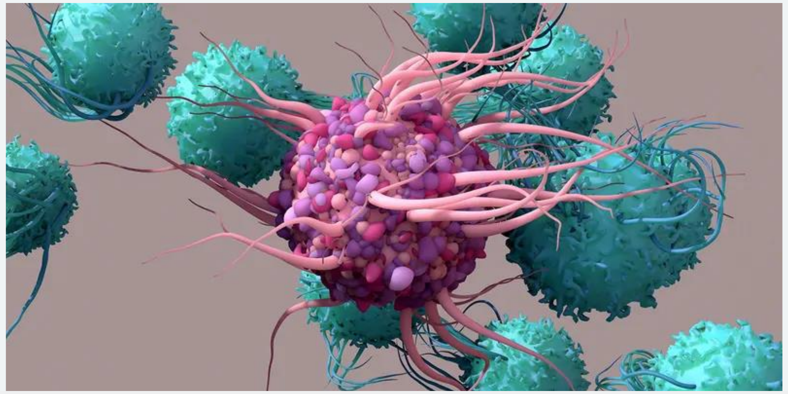


drugdiscoverynews.com/viral-reactivation-may-cause-car-t-cell-induced-neurotoxicity-15530

Discussion 4: strategy for testing CAR-T cells for HHV-6/7: proposition

- **Background: LATENCY** No RNAseq reads aligning to any specific HHV-6B transcripts can be identified in:

  - PBMCs from patients without viremia,
  - lymphoblastoid cell lines with latent HHV-6B infection
  - PBMC from iciHHV-6 patients (despite the high DNA load of the latent virus because of its presence in every cell)

  ➔ highlighting the specificity of transcriptomics for active infections.

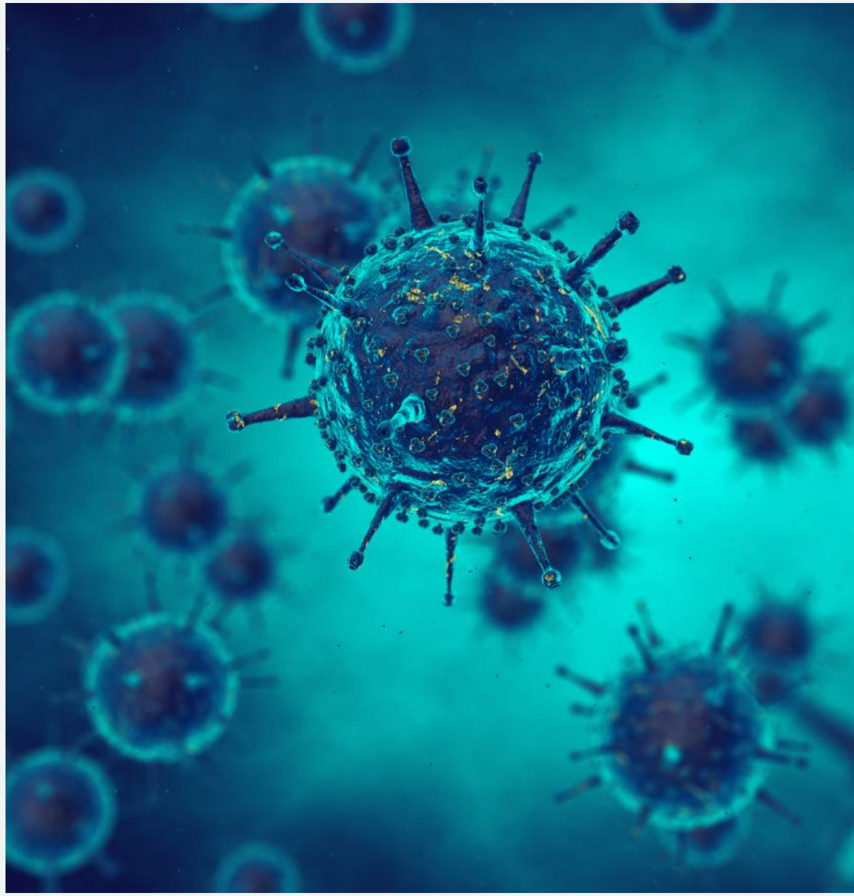
- **ACTIVE INFECTION** wide range of transcripts , the most preeminent one often being the polymerase transcript (U38),

- **Strategy for testing:** Culture Period on DP, then RNA-Seq, with an acceptance criterion of “no detectable HHV6/7 RNAs after extended culture time” (simulating 2 weeks post-infusion time, therefore typically 3 to 4 weeks),

- **Reliability (NPV)** function of the LOD of the RNAseq used: 1 infected cell in 1000-10,000 non-infected cells, a satisfactory order of magnitude considering the ratio of cells that reactivate the virus: 1 in 300-10,000.

TAKE HOME MESSAGEs

- The LOD **obtained from non-regulated R&D testing of this GMP transcriptomic assays technology** is estimated at **0.1 viral RNA copies** per cell or during active infections in **≤1 infected cell diluted in 1000 non-infected cells**
- Detects all **viruses including distant strains** across **Human, Simian Rodent**, **Bovine** and **Porcine** species targeted by conventional tests (MAP/HAP/RAP, 9CFR, and extended PCR panels).
- Application to **cell banks, cell therapy DP and bulk routine testing of recombinant proteins**



KK

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