HAVE WE FINALLY REACHED THE END OF *IN VITRO* TESTING?

POSITIONING NGS IN YOUR BIOSAFETY TESTING STRATEGY

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iDTECT® TRANSCRIPTOME

Introduction

For decades, traditional biosafety testing packages for biologics have consisted of a complex collection of aging, low resolution assays with poor specificity and extended timelines. These assays have struggled to keep pace with the complexity and speed of development (and subsequently, the race to market) of today's biologics. Recent revisions to the guidance documents by global regulatory authorities have made great strides in encouraging the **replacement of many of these legacy assays with next-generation sequencing (NGS)-based strategies**. For example, **ICH Q5A (R2) clearly endorses the use of NGS to replace both animal and quasi-animal-based tests in support of a faster, safer and more ethical testing package**. This endorsement has two distinct advantages: **1)** it overcomes many of the known shortfalls in animal-based testing, including limited susceptibility of the test system to viral agents and poor resolution/sensitivity of these assays, and **2)** it supports the 3Rs principles of refinement, reduction and the replacement of animal model systems. **Despite similar shortcomings in the specificity and susceptibility of *in vitro*, 9 CFR and PCR-based biosafety tests, full acceptance by both the industry and, to some extent, regulatory authorities of NGS to comprehensively replace this latter collection of tests continues to lag**.

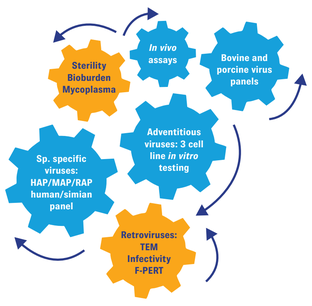
Traditional biosafety testing

Test package depends on product and regulatory guidance

- Multiple, aging assays
- Includes animal-based testing
- Low resolution tests
- Extended timelines
- Increased cost

Classical test concerns

- Resolution
- Specificity
- Lack of test system susceptibility
- Assay interference by the test matrix



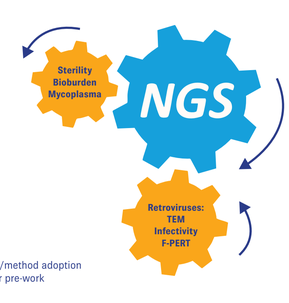
Simplified biosafety testing

Goal: one test package that combines and replaces several assays

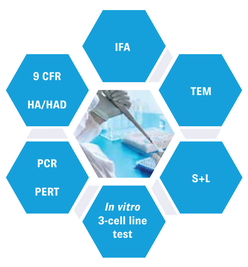
- Animal and quasi-animal-based testing (*in vivo*, HAP/MAP/RAP)
- PCR tests
- *In vitro* tests
- Immunoassays (9 CFR)

Advantages

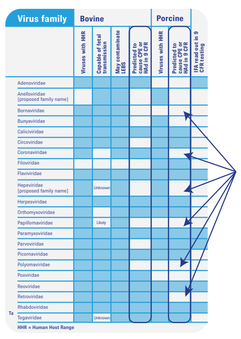
- Improved resolution
- Exceptional range of detection
- Very good analytical sensitivity
- Improved specificity
- Simplified matrix interference assessments/method adoption
- No need for neutralizing antibodies or other pre-work



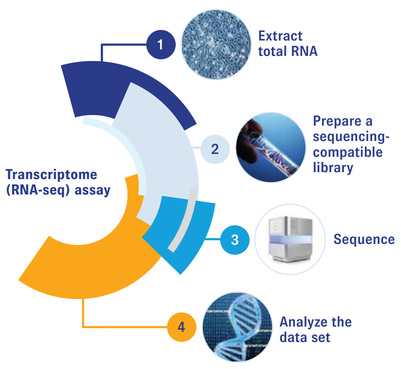
NGS advances viral safety testing by providing a broad, unbiased method of detecting all viral contaminants in biologics. NGS is unmatched in terms of streamlined, accelerated testing that is not encumbered by target specificity requirements or test system susceptibility constraints that existing assays experience.



Traditional biosafety tests



Virus families not detected by traditional methods are readily detectable by NGS



So why the hesitation to use NGS? A lack of publicly available use cases is the most common response. To overcome this perceived limitation, PathoQuest undertook a joint study with Charles River Laboratories to **directly assess the use of NGS, specifically PathoQuest's iDTECT® Transcriptome assay platform, as a direct replacement for the HAP/MAP/RAP, 9 CFR bovine/porcine virus tests and human virus PCR panels**.

Methodology

To assess the ability of NGS to detect the virus families targeted by traditional tests (detailed below), synthetic RNA transcripts (each 600 base pairs in length) corresponding to both close and distantly related viral sequences were spiked at decreasing concentrations into representative cell lysates shortly after cell disruption and prior to nucleic acid purification (illustrated below). Libraries were prepared from each test sample and sequenced using an Illumina® NGS platform. The data was then analyzed using PathoQuest's proprietary data analysis pipelines for adventitious agent detection.

HAP/MAP/RAP panels (rodent viruses)

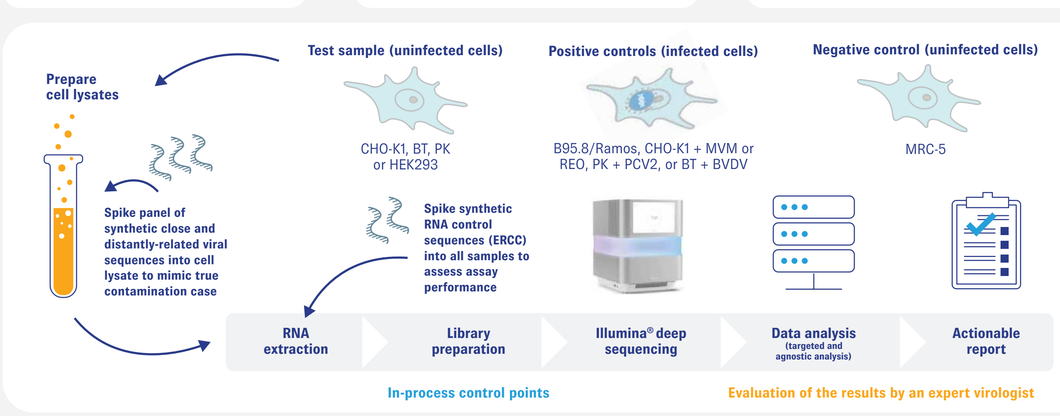
Virus		
	Family	**Species**
Rodent viruses	Adenoviridae	Mouse adenovirus MAV MAdV1 Mouse adenovirus MAV MAdV2
	Arenaviridae	Lymphocytic choriomeningitis virus (LCMV)
	Arteriviridae	Lactate dehydrogenase elevating virus (LDV)
	Coronaviridae	Mouse hepatitis virus (MHV) Sialodacryadentitis virus (SDAV) Rat coronavirus (RCV)
	Hantaviridae	Hantaanvirus (HANT) Prospect hill virus
	Herpesviridae	Mouse cytomegalovirus (MCMV) Mouse thymic virus (MTLV)
	Pnemoviridae	Pneumonia virus of mice (PVM)
	Paramyxoviridae	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)

9 CFR 113 panels (bovine/porcine viruses)

Virus		
	Family	**Species**
Bovine/porcine viruses	Flaviviridae	Bovina viral diarrhea virus
	Reoviridae	Reovirus
	Rhabdoviridae	Rabies virus
	Adenoviridae	Bovine adenovirus Porcine adenovirus A Porcine adenovirus B Porcine adenovirus C
	Sedoreoviridae	Bluetongue virus
	Pneumoviridae	Bovine respiratory syncytial virus
	Paramyxoiridae	Bovine parainfluenza virus type 3
	Herpesviridae	Infectious bovine rhinotracheitis (BoHV-1) Pseudorabies virus
	Polyomaviridae	Bovine polyomavirus
	Coronaviridae	Transmissible gastroenteritis virus Porcine hemagglutinating encephalomyelitis virus
	Picornaviridae	Porcine encephalomyocarditis virus
	Arteriviridae	Porcines reproductive and respiratory syndrom virus
	Circoviridae	Porcine circovirus - 1 Porcine circovirus - 2 Porcine circovirus - 3
	Parvoviridae	Bovine parvovirus Porcine parvovirus
	Hepeviridae	Hepatitis E virus genotype 3a Hepatitis E virus genotype 4a

Human/simian virus panels

Virus		
	Family	**Species**
Human viruses	Adenoviridae	Human adenovirus (broad range A-G)
	Retroviridae	Human immunodeficiency virus type (HIV-1) Human immunodeficiency virus type (HIV-2) Human T-lymphotropic virus type 1 and 2 (HTLV-1/2)
	Papillomaviridae	Human papillomavirus types 16 and 18 (HPV-16/18)
	Herpesviridae	Epstein barr virus (EBV; HHV4) Human cytomegalovirus (HCMV; HHV5) Human herpesvirus 6 (HHV-6) Human herpesvirus 7 (HHV-7) Human herpesvirus 8 (HHV-8; KSV) Herpes simplex virus type 1 (HSV-1) Herpes simplex virus type 2 (A-G) (HSV-2)
	Polyomaviridae	Human polyomavirus JCV Human polyomavirus BK
	Parvoviridae	Human parvovirus B19 Adeno associated virus
	Picornaviridae	Hepatitis A virus
	Hepadnaviridae	Hepatitis B virus
	Flaviviridae	Hepatitis C virus West nile virus
	Hepeviridae	Hepatitis E virus
	Coronaviridae	Severe acute respiratory syndrome virus 2 (SARS-CoV2)
Simian viruses	Retroviridae	Simian immunodeficiency virus (SIV) Simian T-lymphotropic virus (STLV) Squirrel monkey retrovirus (SMRV)
	Polyomaviridae	Simian virus 40 (SV-40)



Results

Comprehensive screening for replication-competent viruses is crucial. Transcriptome-based NGS offers a broad, agnostic method that detects both known and novel viral variants that traditional assays may miss.

HAP/MAP/RAP tests

HAP/MAP/RAP assays have been the standard for rodent cell line characterization for over 50 years, but their sensitivity and validation have never been rigorously established, mainly due to ethical constraints.

In this study, **PathoQuest's transcriptome-based NGS assay detected not only all 19 ICH Q5A (R2) rodent viruses, but also six (6) additional viruses, including both close and distant sequence variants. These results clearly demonstrate the wide range of detection by NGS of distant viral strains within the species listed by regulatory guidance, therefore ensuring an exceptional range of detection**.

The HAP/MAP/RAP test
Viral transcripts spiked into CHO-K1 cell lysate

Virus		Sensitivity (copies/cell)		
	Family	**Species**	**0.1 Close Transcripts**	**0.1 Distant Transcripts**	**0.01 Close Transcripts**
Rodent viruses	Adenoviridae	Mouse adenovirus MAV MAdV1	•	•	•
		Mouse adenovirus MAV MAdV2	•	•	•
	Arenaviridae	Lymphocytic choriomeningitis virus (LCMV)	•	•	•
	Arteriviridae	Lactate dehydrogenase elevating virus (LDV)	•	•	
	Coronaviridae	Mouse hepatitis virus (MHV)	•	•	
		Sialodacryadentitis virus (SDAV)	•	•	
		Rat coronavirus (RCV)	•	•	
	Hantaviridae	Hantaanvirus (HANT)	•	•	•
		Prospect hill virus	•	•	
	Herpesviridae	Mouse cytomegalovirus (MCMV)	•	•	•
		Mouse thymic virus (MTLV)	•	•	•
	Pnemoviridae	Pneumonia virus of mice (PVM)	•	•	
	Paramyxoviridae	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)	•	•	•

Sensitivity: 1 copy per 10 cells for close and distant transcripts

9 CFR 113 tests

While the **9 CFR test** is designed to detect specific bovine and porcine viruses, it has a limited scope (screens for 11 bovine/porcine viruses) and therefore does not guarantee detection of virus variants or other porcine or bovine viruses. To mitigate this, it is often supplemented with PCR tests to broaden detection.

In this study, **PathoQuest's transcriptome-based NGS assay detected not only the full 9 CFR panel of bovine and porcine viruses, but also 13 additional bovine and porcine viruses beyond those required by regulatory guidance**.

The 9 CFR 113 test (bovine/porcine viruses)
Viral transcripts spiked into BT cell lysate

Virus		Sensitivity (copies/cell)	
	Family	**Species**	**1.0**	**0.1**
Bovine/porcine viruses	Flaviviridae	Bovina viral diarrhea virus	•	•
	Reoviridae	Reovirus	•	•
	Rhabdoviridae	Rabies virus	•	•
	Adenoviridae	Bovine adenovirus	•	•
		Porcine adenovirus A	•	•
		Porcine adenovirus B	•	•
		Porcine adenovirus C	•	•
	Sedoreoviridae	Bluetongue virus	•	•
	Pneumoviridae	Bovine respiratory syncytial virus	•	•
	Paramyxoiridae	Bovine parainfluenza virus type 3	•	•
	Herpesviridae	Infectious bovine rhinotracheitis (BoHV-1)	•	•
		Pseudorabies virus	•	
	Polyomaviridae	Bovine polyomavirus	•	•
	Coronaviridae	Transmissible gastroenteritis virus	•	•
		Porcine hemagglutinating encephalomyelitis virus	•	•
	Picornaviridae	Porcine encephalomyocarditis virus	•	•
	Arteriviridae	Porcines reproductive and respiratory syndrom virus	•	•
	Circoviridae	Porcine circovirus - 1	•	•
		Porcine circovirus - 2	•	•
		Porcine circovirus - 3	•	•
	Parvoviridae	Bovine parvovirus	•	•
		Porcine parvovirus	•	•
	Hepeviridae	Hepatitis E virus genotype 3a	•	•
		Hepatitis E virus genotype 4a	•	•

Sensitivity: 1 copy per 10 cells for most close transcripts - 1 copy per cell for all transcripts

The human virus test panel (for human cell substrates)
Virus transcripts spiked into HEK293 cell lysate

Virus		Sensitivity (copies/cell)		
	Family	**Species**	**0.1**	**0.01**	**0.001**
Human viruses	Adenoviridae	Human adenovirus (broad range A-G)	•	•	•
	Retroviridae	Human immunodeficiency virus type (HIV-1)	•		
		Human immunodeficiency virus type (HIV-2)	•	•	
		Human T-lymphotropic virus type 1 and 2 (HTLV-1/2)	•		
		Human foamy virus	•	•	
	Papillomaviridae	Human papillomavirus types 16 and 18 (HPV-16/18)	•	•	
	Herpesviridae	Epstein barr virus (EBV; HHV4)	•	•	
		Human cytomegalovirus (HCMV; HHV5)	•	•	
		Human herpesvirus 6 (HHV-6)	•	•	
		Human herpesvirus 7 (HHV-7)	•	•	
		Human herpesvirus 8 (HHV-8; KSV)	•	•	
		Herpes simplex virus type 1 (HSV-1)	•		
		Herpes simplex virus type 2 (A-G) (HSV-2)	•	•	•
	Polyomaviridae	Human polyomavirus JCV	•		
		Human polyomavirus BK	•	•	
	Parvoviridae	Human parvovirus B19	•		
		Adeno associated virus	•		
	Picornaviridae	Hepatitis A virus	•		
	Hepadnaviridae	Hepatitis B virus	•	•	
	Flaviviridae	Hepatitis C virus	•		
		West nile virus	•		
	Hepeviridae	Hepatitis E virus	•		
	Coronaviridae	Severe acute respiratory syndrome virus 2 (SARS-CoV2)	•		
Simian viruses	Retroviridae	Simian immunodeficiency virus (SIV)	•		
		Simian T-lymphotropic virus (STLV)	•		
		Squirrel monkey retrovirus (SMRV)	•		
		Simian foamy virus	•	•	
		Simian retrovirus-1/2/3 (SRV-1/2/3)	•		
	Polyomaviridae	Simian virus 40 (SV-40)	•	•	

Sensitivity: 1 copy per 10 cells for close transcripts

Human/simian virus PCR panels

PCR in general is both rapid and robust, but it detects only known sequences and might not detect viral variants. Additionally, PCR assays may register non-infectious fragments as positives. In contrast, NGS targets viral RNAs, enabling detection of both known and novel viral species, thereby facilitating the differentiation of active infections from inert nucleic acids.

In this study, **PathoQuest's transcriptome-based NGS assay detected all simian and human viruses tested, including 13 viruses requested by regulatory agencies and 15 additional human and simian viruses**.

Conclusion

This study leveraged PathoQuest's iDTECT® Transcriptome assay – an agnostic, animal-free and regulatory-aligned NGS platform – to benchmark its performance against traditional viral safety assays, including panel-based HAP/MAP/RAP, PCR and 9 CFR tests.

A minimal sensitivity of one (1) RNA copy per 10 cells, which can be translated into a sensitivity of roughly one (1) infected cell per 1,000 non-infected cells, was demonstrated for a total of 78 analyzed viruses covering a broad range of virus families and viral genome types. This sensitivity can be regarded as a 'worst-case' scenario as, in real contamination cases (based on published data for different virus families), the detection sensitivity will likely be higher, due to higher viral copy load, as well as the ability of NGS to detect broadly across the viral genome. Given the broad detection range demonstrated here, one final question to ask is '**what is the likelihood that a specific virus contaminant will only be detected by *in vitro* cell assays and not the iDTECT® Transcriptome assay?**' Conversely, what is the likelihood that a virus will not be detected by the *in vitro* cell assay but by this transcriptomic approach? In either case, NGS is the clear winner.

In all, these results illustrate that **not only does NGS have comparable or superior detection sensitivity, but also has broader viral detection capabilities than traditional assays, enabling confident detection of known as well as novel or emerging viruses.**

Contact us

PathoQuest's GMP-compliant NGS solutions are designed to meet and exceed regulatory expectations and accelerate adventitious agent detection as part of your biosafety testing strategy to help ensure safe manufacturing.

Contact our experts to hear more about how PathoQuest's GMP-compliant NGS testing portfolio can simplify and secure your virus safety testing strategy and accelerate your path to market… compliant, faster and animal-free.

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