Detection of Adventitious Viruses Using Transcriptome NGS

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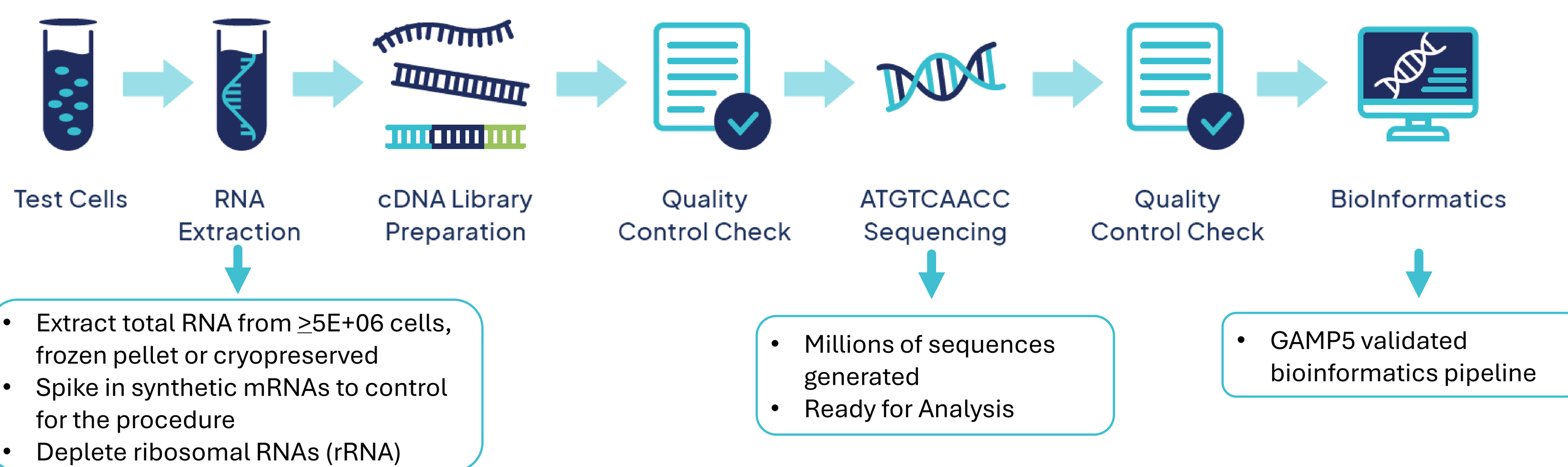
Poster

1) Introduction: NGS Transcriptome Method for Detection of Adventitious Viruses

Next Generation Sequencing (NGS) is a powerful method for detecting a broad range of adventitious viruses in biological samples without prior knowledge of the agents present. Updated ICH Q5A(R2) guidance encourages NGS as a replacement for in vivo assays due to its breadth and sensitivity of virus detection and the limitations of animal-based assays. The use of NGS promotes the global initiative to replace, reduce and refine the use of animal testing.

Minaris has developed a NGS transcriptome method to test cell banks and other cell samples for the presence of adventitious viruses. All viruses have an mRNA stage in their lifecycle. During an active viral infection, viral transcripts are expressed with the host mRNA. The transcriptome method is designed to examine all mRNA transcripts to detect any active viral infection by RNA and DNA viruses.

NGS Transcriptome Workflow for Cell Bank and Other Cell Samples



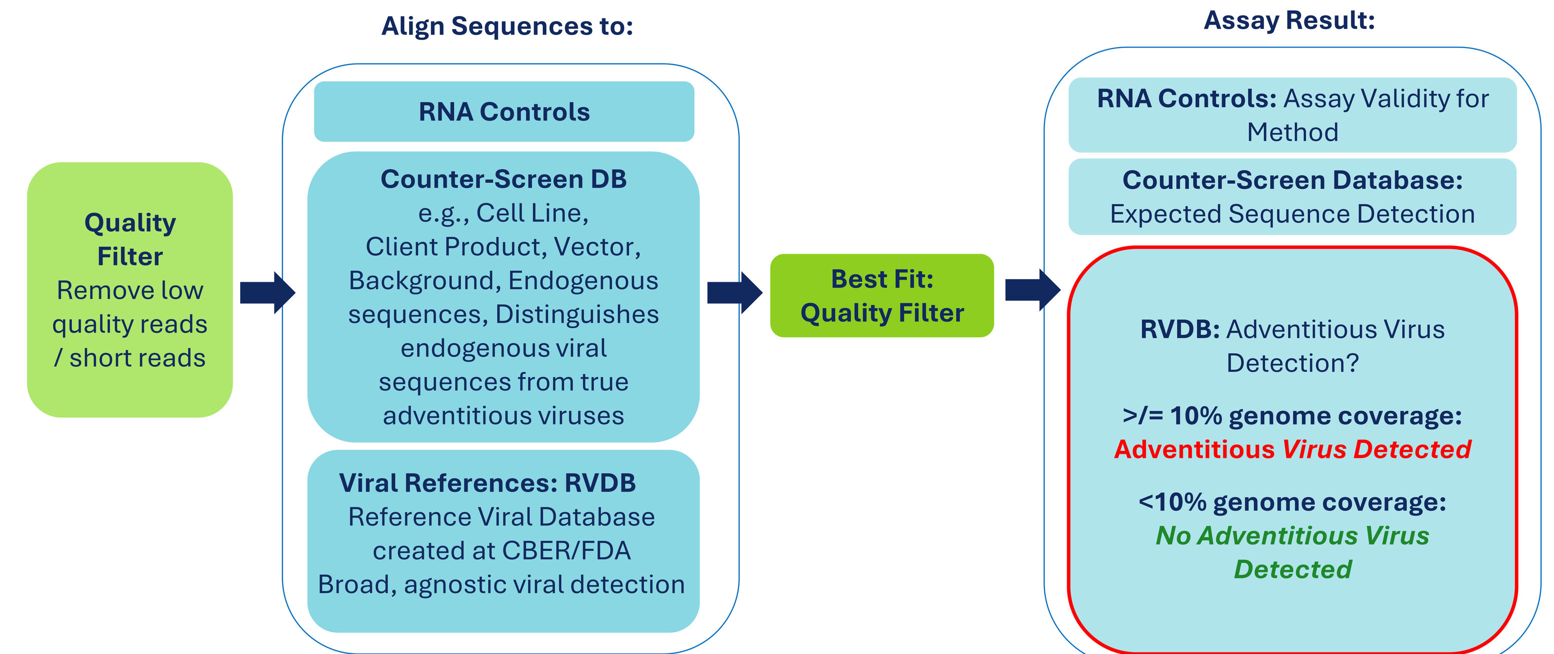
- Extract total RNA from >5E+06 cells, frozen pellet or cryopreserved
- Spike in synthetic mRNAs to control for the procedure
- Deplete ribosomal RNAs (rRNA)

- Millions of sequences generated
- Ready for Analysis

- GAMP5 validated bioinformatics pipeline

2) Minaris Transcriptome Bioinformatics Pipeline

Sequence data are analyzed using Minaris Bioinformatics pipeline. The pipeline is validated as per GAMP5 guidelines with recommended levels of data integrity and security. Data are handled and analyzed by 21 CFR part 11 compliant systems.



Filtered sequence data aligned against the Viral Reference Database (RVDB) are analyzed to evaluate adventitious virus detection.

% of Virus Genome Covered: Aligns to Viral Reference Database (e.g., RVDB)	Sequence aligns to Counter-screen Database	Result
10% or greater	No	Adventitious Virus Detected
N/A	Yes	Expected Sequence
Less than 10%	No	No Adventitious Virus Detected

3) Transcriptome Verification Study – Simulation of a Master Cell Bank Contamination

Study to evaluate detection of an active low-level contamination in a CHO cell bank

CHO cells served as the cell bank sample. Mus dunni cells infected with Amphotropic Murine Leukemia Virus (A-MuLV) were added to CHO cells to create 3 infection levels.

Cells:	Case 1	Case 2	Case 3
CHO	99%	99.9%	99.99%
Mus dunni with A-MuLV	1%	0.1%	0.01%

Two analysts processed the spiked CHO samples in independent runs of the transcriptome NGS workflow.

Cells:	Case 1	Case 2	Case 3
% A-MuLV Genome Sequenced Detected			
Analyst 1	78%	58%	41%
Analyst 2	81%	63%	41%

Results: Robust detection of at least 41% of the A-MuLV genome by separate analysts during independent runs.

4) Transcriptome Qualification Study Design

Transcriptome NGS Method is considered a limit test. As per ICH guidelines, Minaris evaluated specificity (breadth of detection and discrimination) & Limit of Detection (sensitivity) parameters

To demonstrate detection of diverse virus types, 6 viruses from six different virus Taxon Families were analyzed in the study, which included viruses with RNA and DNA genomes, single-stranded and double-stranded genomes, enveloped and non-enveloped viruses. ICH guidelines state that specificity is demonstrated by the ability of the method to discriminate between analytes. To show discrimination specificity of the transcriptome NGS method, the 6 viruses were divided into 2 groups: Group 1 viruses consisted of Ad-5, PI-3, and GaLV. Group 2 viruses included TGEV, PPV, and Reo. As per ICH guidelines, another demonstration of specificity is the ability to assess the analyte in the presence of impurities or excipients. Two cell lines, HEK293 and ST, served as matrices for the qualification to evaluate detection of viral mRNA in a background of cellular RNA.

Virus		Genome	Enveloped / non-enveloped	Taxon Family
Human Adenovirus type 5	Ad5	dsDNA	Non-Enveloped	*Adenoviridae*
Bovine parainfluenza-3 virus	PI-3	ssRNA-	Enveloped	*Paramyxoviridae*
Gibbon ape leukemia virus	GaLV	ssRNA+	Enveloped	*Retroviridae*
Transmissible gastroenteritis virus	TGEV	ssRNA+	Enveloped	*Coronaviridae*
Porcine parvovirus	PPV	ssDNA	Non-Enveloped	*Parvoviridae*
Mammalian orthoreovirus	Reo	dsRNA	Non-Enveloped	*Spinareoviridae*

Qualification Study Design

To demonstrate the method can detect active viral infections in a cell bank sample, independent active viral infections were established for each of the 6 viruses.

- Host cells inoculated with virus. Cells incubated to allow virus to go through its lifecycle of transcription & replication
- Cells harvested and total RNA extracted
- Quantified viral mRNA transcripts by reverse-transcriptase - ddPCR

Cell Line Matrices

- Negative cultures of HEK293 and ST cells
- Cell count performed at harvest
- Total RNA extracted from each negative culture

Created test articles to mimic a true contamination of a cell bank.

- Spiked HEK293 RNA with group 1 (Ad5, PI-3, GaLV) viral mRNAs @ 2 levels
  - 1 viral transcript copy per cell
  - 0.1 viral transcript copy per cell (1 copy per 10 cells)
- Spiked ST RNA with group 2 (TGEV, PPV, Reo) viral mRNAs @ 2 levels
  - 1 viral transcript copy per cell
  - 0.1 viral transcript copy per cell (1 copy per 10 cells)
- Created technical replicates for each test article

Test Articles Created for the Qualification			
Test Article #	Total RNA Matrix	Virus	Copies of viral mRNA per cell
1	HEK293 Human Kidney cells	Ad-5	1 copy/ cell
		PI-3	1 copy/ cell
		GaLV	1 copy/ cell
2	HEK293 Human Kidney cells	Ad-5	0.1 copy/ cell
		PI-3	0.1 copy/ cell
		GaLV	0.1 copy/ cell
3	ST Swine Testis cells	TGEV	1 copy/ cell
		PPV	1 copy/ cell
		Reo	1 copy/ cell
4	ST Swine Testis cells	TGEV	0.1 copy/ cell
		PPV	0.1 copy/ cell
		Reo	0.1 copy/ cell

Three (3) independent runs were performed by two (2) analysts and in each run the analyst:

- Prepared a cDNA library for each Test Article for a total of 4 libraries per run
- Attached a unique barcode to each cDNA library to allow multiplexing
- Combined the 4 libraries together and sequence in one run
- Performed bioinformatics

One (1) run was performed by one (1) analyst to establish host cell background sequences.

- Prepared cDNA library for negative HEK293 RNA and cDNA library for negative ST RNA
- Attached a unique barcode to each cDNA library to allow multiplexing
- Combined the 2 libraries together and sequence in one run
- Performed bioinformatics

Test Evaluation

- Virus detected if sequences cover >/=10% of virus genome

5) Transcriptome Qualification Study Results

Assay Validity Acceptance Criterion

Synthetic mRNAs are added to the test article at the start of the procedure to serve as a positive control. Detection of the synthetic mRNAs is an assay validity criterion. Coverage of the synthetic mRNA Controls must be at least 10%.

Results: coverage of the synthetic mRNA controls were greater than 10% in all test articles in each run, to meet acceptance criteria.

Test Article #	% Coverage of mRNA Controls			Validity Acceptance Criteria Met?
	Analyst 1		Analyst 2	
	Run 1	Run 2	Run 3	
1	>10%	>10%	>10%	☒ Yes ☐ No
2	>10%	>10%	>10%	☒ Yes ☐ No
3	>10%	>10%	>10%	☒ Yes ☐ No
4	>10%	>10%	>10%	☒ Yes ☐ No

Specificity Evaluation

Specificity Acceptance Criteria:

- Transcripts from PPV, Reo, and TGEV must not be detected in HEK293 spiked test articles
- Transcripts from Ad5, PI-3, and GalV must not be detected in ST spiked test articles

Results:

- No transcripts from PPV, Reo, and TGEV were detected in HEK293 spiked test articles
- No transcripts from Ad5, PI-3 and GaLV were detected in ST spiked test articles
- Acceptance criteria met in all 3 runs

Test Article #	Test Article RNA	Virus RNAs			Specificity Acceptance Criteria Met?
		PPV	Reo	TGEV	
1	HEK293	Not Detected	Not Detected	Not Detected	☒ Yes ☐ No
2		Not Detected	Not Detected	Not Detected	☒ Yes ☐ No

Test Article #	Test Article RNA	Virus RNAs			Specificity Acceptance Criteria Met?
		Ad4	PI-3	GaLV	
3	ST	Not Detected	Not Detected	Not Detected	☒ Yes ☐ No
4		Not Detected	Not Detected	Not Detected	☒ Yes ☐ No

Limit of Detection Evaluation

A virus is considered detected if sequence reads cover at least 10% of the full-length virus genome. Note: Reo virus has a segmented genome comprised of 10 RNA segments. The lengths of the 10 segments were added together to calculate the length of the complete Reo genome.

% Coverage of Viral Genome Results

Test Article #	Virus	Total RNA sample	Copies of viral mRNA per cell	% Coverage of Virus Genome		
				Analyst 1		Analyst 2
				Run 1	Run 2	Run 3
1	Ad5	HEK293	1	>10%	>10%	>10%
	PI-3		1	>10%	>10%	>10%
	GaLV		1	>10%	>10%	>10%
2	Ad5	HEK293	0.1	>10%	>10%	>10%
	PI-3		0.1	>10%	>10%	>10%
	GaLV		0.1	>10%	>10%	>10%
3	TGEV	ST	1	>10%	>10%	>10%
	PPV		1	>10%	>10%	>10%
	Reo		1	>10%	>10%	>10%
4	TGEV	ST	0.1	>10%	>10%	>10%
	PPV		0.1	<10%	>10%	<10%
	Reo		0.1	<10%	<10%	<10

Limit of Detection Results

Virus	Taxon Family	Total RNA sample	Limit of Detection: Viral mRNA copies per cell		
			Analyst 1		Analyst 2
			Run 1	Run 2	Run 3
Ad5	*Adenoviridae*	HEK293	0.1	0.1	0.1
PI-3	*Paramyxoviridae*		0.1	0.1	0.1
GaLV	*Retroviridae*		0.1	0.1	0.1
TGEV	*Coronaviridae*	ST	0.1	0.1	0.1
PPV	*Parvoviridae*		1	0.1	1
Reo	*Spinareoviridae*		1	1	1

Conclusion

The transcriptome NGS method is specific. Viruses were correctly identified to the species level.
mRNA from each virus (Ad-5, PI-3, GaLV) spiked into HEK293 total RNA and mRNA from each virus (TGEV, PPV, Reo) spiked into ST total RNA were identified to the species level.

The method is sensitive and can detect a low-level contaminant.
The test evaluation for the qualification required at least 10% coverage of a viral genome to consider a virus detected. In 3 out of 3 runs, greater than 10% of the Ad5, PI-3 and GalV genomes were detected in a background of HEK293 total RNA, and greater than 10% of the TGEV genome was detected in a background of ST total RNA. Limit of Detection for Ad5, PI-3, GalV and TGEV was 0.1 viral mRNA copy per cell. In at least 2 out of 3 runs, Limit of Detection for PPV and Reo was 1 viral mRNA copy per cell in a background of ST total RNA.

To assess discrimination specificity of the transcriptome NGS method, ST RNA samples spiked with TGEV, PPV, Reo viral transcripts were analyzed together in the same sequencing run as HEK293 RNA samples spiked with Ad5, PI-3 and GaLV viral transcripts. This strategy generated intra-run results to evaluate specificity during the method qualification. Sequencing a single test article per run is expected to provide increased sensitivity and a lower limit of detection, relative to results generated for multiplexed test articles.

6) Summary

Updated ICH Q5A(R2) guidance encourages NGS as a replacement for in vivo assays due to its breadth and sensitivity of virus detection and the limitations of animal-based assays.

NGS detects adventitious viruses in biological samples without prior knowledge of the viruses present.

Transcriptome NGS Method

- ✓ Detects an active viral infection in a cell bank or other cell sample
- ✓ Analyzes sequence data using a GAMP5 validated Minaris bioinformatics pipeline that ensures data integrity and security. Systems are 21 CFR part 11
- ✓ Detects a broad range of viruses, including RNA and DNA viruses, viruses with single-stranded and double stranded genomes, enveloped and non-enveloped viruses
- ✓ Is specific, can discriminate between different viruses at the species or family level
- ✓ Is sensitive, can detect a low-level contaminant in a background of cellular RNA

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For information about our technologies, please contact us at www.minaris.com.

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