BioReliance®
Contract Testing Services

White Paper

Assessing the Blazar® CHO AOF Assay Against Traditional *In Vitro* Testing for Adventitious Virus Detection

A Demonstration of Equivalence

Detection of adventitious viruses in Chinese Hamster Ovary (CHO) bulk harvest material has historically been examined using classical *in vitro* adventitious agent tests, but the advent of molecular-based technologies has enabled testing to become more rapid whilst retaining sensitivity. The degenerate PCR-based Blazar® CHO Animal Origin-Free (AOF) assay is one such technology where targeted virus detection has been employed to streamline testing. As a targeted molecular method, this assay provides robust, sensitive detection of viruses in less than half the time of a traditional, culture-based *in vitro* assay. The Blazar® CHO AOF assay therefore brings a clear advantage in terms of speed. However, prior to implementation of any new method for biosafety testing, it is important to perform a risk-benefit analysis comparing all aspects of the incumbent and new assays, to determine whether adoption of the new assay is appropriate for each manufacturer's unique process and will meet regulatory expectations. Early discussion with regulatory authorities is recommended before introducing novel technologies into a testing package. This white paper provides supporting information on the Blazar® CHO AOF assay to assist developers and manufacturers with such assessments and help inform adoption of this rapid alternative method.

Technology

The Blazar® CHO AOF assay uses our established degenerate PCR platform, which employs multiplexed primer sets to target multiple virus families in a single reaction. Automated extraction of the nucleic acids in a sample is followed by two rounds of PCR; this nested PCR approach improves the assay sensitivity. The final step in the workflow is separation of amplicons generated in the PCR using capillary electrophoresis, with the amplicons from each virus family designed to fall within a designated size range to allow for straightforward identification of that virus family.

The molecular technology used for the Blazar® CHO AOF assay differs greatly from the traditional *in vitro* method, which relies on culture-based amplification of any viral contaminants in the sample over a 14- or 28-day time period. In the case of the 28-day assay, a passage is typically performed at 14 days to improve sensitivity of detection, especially for slower growing viruses. The outcome of the assay relies on manual observation of cultures for cytopathic effects during the incubation period, as well as hemagglutination and hemadsorption end-points. This has the advantage that only viable viruses are detected but suffers from a degree of subjectivity, as well as the limitation that the virus must grow well in culture. Viruses that are not able to infect the selected detector cell lines, or to produce a discernible effect in one of the end-points, will remain undetected. PCR-based methods cannot distinguish between viable and non-viable virus, but the Blazar® platform allows identification of the virus family and subsequent sequencing of the amplicon, so that any follow-up investigational testing required can be executed efficiently. A summary of the technologies and their features is shown in **Table 1**.

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Table 1: Key features of the Blazar® CHO AOF assay compared with the *in vitro* method.

	Traditional *in vitro* assay	Blazar® CHO AOF assay
Technology	Culture-based with three or more detector cell lines	Degenerate PCR
End-point (s)	Cytopathic effect, hemadsorption, hemagglutination	Amplicon detection by capillary electrophoresis
Incubation period required to allow viral replication	14 or 28 days	None
Virus detection capabilities	Dependent on susceptibility of cell lines used; viable viruses only	All viruses and variants encompassed by degenerate primers (directed to virus families selected by expert panel); viable and non-viable viruses
Subjectivity of result interpretation	Cell health and presence of cytopathic effects can be subjective. Hemagglutination and hemadsorption assays are less subjective	Generally unbiased detection due to the use of a single consistent threshold between panels
Ability to identify the contaminant	None	Virus family is ascertained based on amplicon size; amplicon can be sequenced as part of follow-up investigation to enable identification

Given the significant differences in methodology and end-points between the Blazar® CHO AOF assay technology and the traditional *in vitro* assay, a head-to-head comparison between the two assays does not have significant value. However, there is still a regulatory expectation that the Blazar® CHO AOF assay is fully validated and can demonstrate better or equivalent selectivity and sensitivity to the *in vitro* method. This is discussed in the following sections.

Detection of virus families

The Blazar® CHO AOF assay is a targeted molecular method designed specifically to address the risk of adventitious virus in unprocessed bulk harvest, provided that the following conditions are met:

- The bulk harvest material is derived from CHO cells
- The production process uses only animal origin-free materials (our experience indicates that this applies to the majority of CHO-based processes)
- The master and working cell banks (and the end of production cells, where available) have been fully characterized, including testing (typically on the master cell bank) for specific rodent, bovine and porcine viruses

Based on the above conditions being met, the virus families for the Blazar® CHO AOF assay have been selected based on viruses that are relevant to CHO-based processes at the bulk harvest stage. Thus, the Blazar® CHO AOF assay is not intended as a replacement for *in vitro* testing in all scenarios, since it does not cover all of the families that are encompassed by a typical *in vitro* assay when MRC-5, Vero, and CHO are used as detector cell lines. Rather, the targeted approach of the Blazar® CHO AOF assay makes it appropriate for the specific scenario defined above, in line with a risk-based approach for detection of specific viruses and virus families in bulk harvest samples[1].

Virus families were selected for inclusion by a group of experts with decades of experience in the industry, comprising our own virology specialists and three external consultants. All of these expert virologists share extensive knowledge of viral risks in biologics processes and familiarity with the global regulatory landscape, having spent many years working with manufacturers and developers around the world in the field of biosafety and QC testing. Virus family selection was based on several considerations, including the known virus susceptibility of CHO cells, their potential to infect human cells, emerging virus families, and historical CHO contamination events (see selected references 2-6).

The chosen virus families give broad coverage relevant to bulk harvest samples, and the degenerate nature of the primers allows variants to be detected, further increasing coverage (**Table 2**). In addition, the Blazar® CHO AOF assay targets viruses that are not readily detectable in a standard *in vitro* assay (using MRC-5, Vero, and CHO as the detector cell lines), including all currently known strains of Mouse Minute Virus (MMV). This avoids the need to supplement testing with additional PCRs for these viruses, some of which have previously been responsible for contamination events in CHO bioreactors[2].

Table 2: 15 virus families are covered by the Blazar® CHO AOF assay, including some that are not reliably picked up by a standard *in vitro* assay using MRC-5, Vero, and CHO as the detector cell lines.

	Virus family	Primary reason(s) for inclusion	Reliable detection of viruses of concern using standard *in vitro* assay	Viruses associated with historical contaminations of CHO-based processes[2,3]
DNA viruses	Adenoviridae	Replicates in CHO cells / Potential to infect human cells	Yes	
	Anelloviridae	Emerging	No	
	Circoviridae	Replicates in CHO cells / Emerging	No	
	Parvoviridae	Replicates in CHO cells / Emerging	No	MMV
	Polyomaviridae	Potential to infect human cells	Yes	
RNA viruses	Bornaviridae	Potential to infect human cells / Emerging	No	
	Caliciviridae	Replicates in CHO cells / Emerging	Yes	Vesivirus 2117
	Coronaviridae	Replicates in CHO cells / Emerging / Potential to infect human cells	Yes	
	Hepeviridae	Emerging / Potential to infect human cells	No	
	Orthomyxoviridae	Replicates in CHO cells / Potential to infect human cells	Yes	
	Paramyxoviridae	Replicates in CHO cells / Emerging / Potential to infect human cells	Yes	
	Picornaviridae	Replicates in CHO cells	Yes	
	Reoviridae	Replicates in CHO cells	Yes	EHDV, Reovirus type 2
	Rhabdoviridae	Replicates in CHO cells	Yes	
	Togaviridae	Replicates in CHO cells / Emerging / Potential to infect human cells	Yes	

Validation and use of positive controls

The Blazar® CHO AOF assay has been validated as a limit test according to ICH Q2 guidelines[7]. Validation was performed using a series of synthetic nucleic acid positive controls corresponding to each virus family included in the panel (27 control sequences in total). This represents a much more extensive validation than is typically performed for an *in vitro* assay, which generates data for only a small number of positive control viruses. As a result, the limit of detection can be provided for every virus family covered by the Blazar® CHO AOF assay (**Table 3**).

Table 3: Validated limits of detection for virus families included in the Blazar® CHO AOF assay.

	Virus family	Detection limit (gc/reaction)
DNA viruses	Internal control (PhiX-174)	10
	Adenoviridae	10
	Anelloviridae	10
	Circoviridae	10
	Parvoviridae	10
	Polyomaviridae	100
RNA viruses	Internal control (MS2)	10
	Bornaviridae	10
	Caliciviridae	10
	Coronaviridae	10
	Hepeviridae	10
	Orthomyxoviridae	10
	Paramyxoviridae	10
	Picornaviridae	10
	Reoviridae	10
	Rhabdoviridae	10
	Togaviridae	10

Every GMP-compliant run of the Blazar® CHO AOF assay includes internal positive controls, which are spiked at the validated limit of detection (10 genomic copies/reaction) into (**a**) PBS, and (**b**) the test sample prior to extraction. PhiX-174 and MS2 intact bacteriophages (representing DNA and RNA genomes, respectively) have been selected as internal controls and are used to confirm that all stages of the assay (nucleic acid extraction, cDNA synthesis and PCR, and amplicon separation) are controlled and reproducible. Importantly, they also provide information during each assay run to determine whether there is any interference with detection of the DNA and RNA virus controls arising from the test article matrix. Since this evaluation of the assay performance and any matrix interference uses the same aliquot of test sample as the actual adventitious agent detection, this offers a superior design to the *in vitro* assay, where spiked test article aliquots must be run as separate samples.

Sensitivity

When moving to an alternate technology for contaminant detection, it is important to demonstrate that it achieves equivalent sensitivity to the incumbent assay. To generate data to compare the relative sensitivities of the *in vitro* and Blazar® CHO AOF assays, we performed a study using a panel of live viruses. The viruses (either commercially available, or from our own Virology department stocks) were selected to represent a variety of genome composition and physical characteristics (**Table 4**). Droplet digital PCR was used to determine the genomic copy (gc) concentration of each virus stock. To mimic representative test samples, studies used bioreactor material from CHO-K1 cells producing Human IgG1 Monoclonal antibody (acquired from Merck site in Bedford, MA) as the sample matrix.

Table 4: Characteristics of viruses used in the proof-of-concept and equivalency studies.

Virus Family	Virus	Size (nm)	Genome Structure	Physical Structure
Parvoviridae	Mouse minute virus (MMV)	26	ssDNA	Icosahedral capsid, non-enveloped
Circoviridae	Porcine circovirus type 1 (PCV-1)	17	ssDNA	Icosahedral capsid, non-enveloped
Paramyxoviridae	Simian virus 5 (SV5)	150 - 250	ssRNA(-)	Helical nucleocapsid, lipid envelope
Paramyxoviridae	Parainfluenza virus 3 (PI3)	150 - 200	ssRNA(-)	Helical nucleocapsid, lipid envelope
Sedoreoviridae	Reovirus Type 3 (Reo)	60 - 100	dsRNA	Icosahedral capsid, non-enveloped

For initial proof-of-concept studies, 1 mL of unprocessed bioreactor sample was spiked with viruses at various Median Tissue Culture Infectious Dose concentrations ($TCID_{50}$/mL, allowing comparison to the validated detection limit of the *in vitro* assay) and absolute genomic concentrations (gc/mL, allowing comparison to the validated detection limit of the Blazar® CHO AOF assay). Bioreactor material was used at a cell density of 1 x 10^7 cells/mL to represent a 'worst-case scenario' in terms of background that may impact assay sensitivity (the maximum cell density of bioreactor material used for routine testing in the validated GMP assay is 1 x 10^6 cells/mL).

Each bioreactor sample was dually spiked with the virus of interest and the appropriate Blazar® CHO AOF assay internal control (PhiX-174 or MS2 bacteriophage at a final concentration of 10 gc/reaction). Results of the proof-of-concept study are summarized in **Table 5** and demonstrate that the assay is fit-for-purpose in terms of its ability to detect live viruses in a complex CHO bulk harvest matrix.

Table 5: Detection of live viruses in the Blazar® CHO AOF assay. Viruses are detectable at 10 gc/reaction (the validated limit of detection for the Blazar® CHO AOF assay) and at 1 $TCID_{50}$/reaction (at or below the validated limit of detection for the *in vitro* assay), even in the presence of a high concentration of bioreactor material (1 x 10^7 cells/mL).

	Number of samples in which virus spike was detected (out of 5 independent biological replicates)			
	Model virus	Internal control	Model virus	Internal control
Spike level per reaction	10 gc	10 gc	1 $TCID_{50}$	10 gc
PI3	5	5	5	5
SV5	4	5	5	5
MMV	5	5	5	5
PCV-1	5	5	5	5

To demonstrate equivalency with the *in vitro* assay, spiked bioreactor samples were tested in parallel in the two assay formats. It should be noted that the two assays differ in terms of initial handling of the samples. To maximize the chances of observing any contaminants, the Blazar® CHO AOF assay has been designed so that bulk harvest samples can be tested without further processing. This contrasts with the *in vitro* assay procedure, where bulk harvest samples must be clarified by low speed centrifugation prior to testing (since cell debris may interfere with detector cell growth).

Based on historical validation data and >20 years of data generated through regulated testing, we were able to leverage prior knowledge for the *in vitro* assay, allowing us to identify the most susceptible cell line available for testing of each virus and give the highest likelihood of detecting the spiked virus. In routine testing, detectability in the *in vitro* assay may be lower, depending on the detector cell lines employed. Prior knowledge also enabled the use of a single sample (generating six technical replicates) for *in vitro* testing, in contrast to the Blazar® CHO AOF assay, for which six independent replicates (each having three technical replicates) were tested to ensure reproducibility. The experimental outline is shown in **Figure 1**.

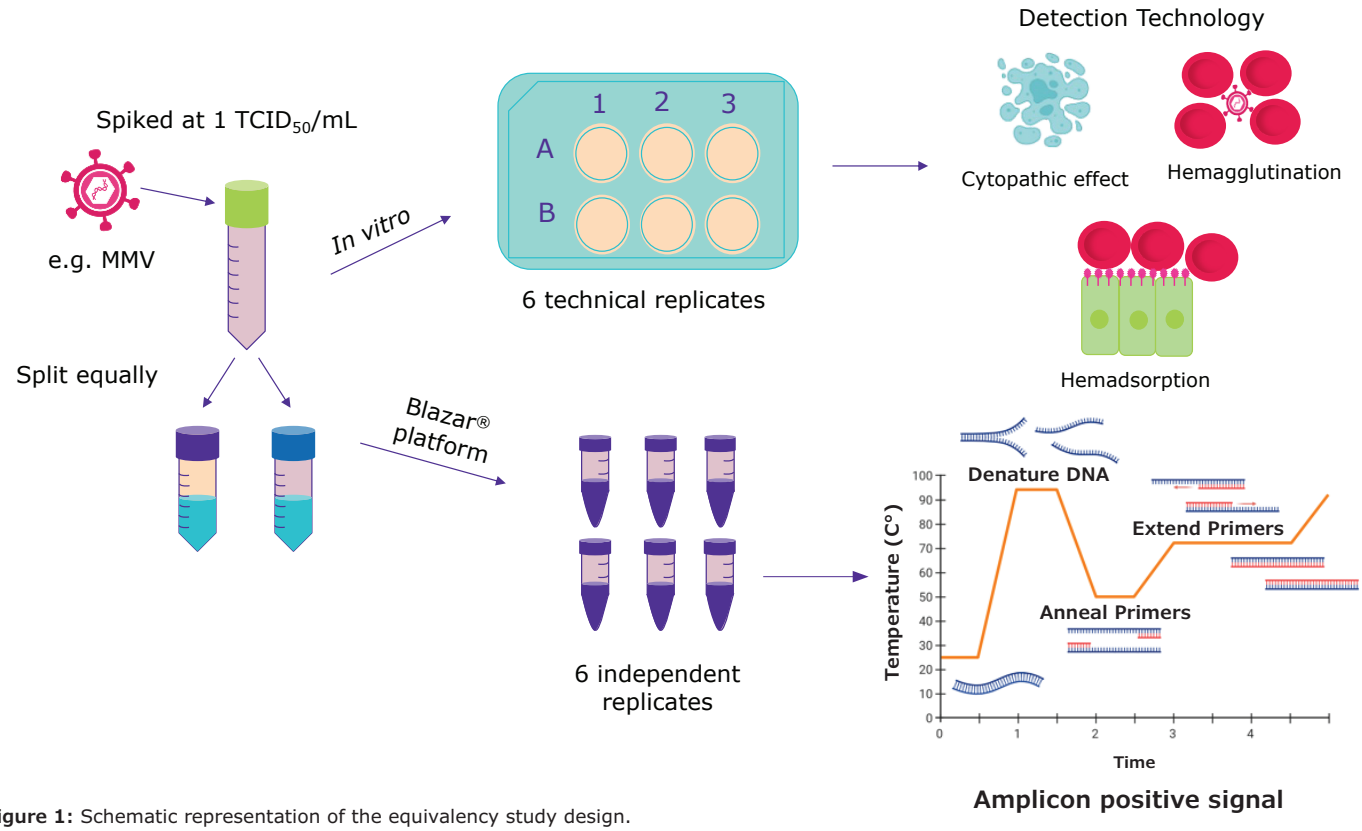

Figure 1: Schematic representation of the equivalency study design.

The results are summarized in **Table 6** and clearly show that the two assay formats produce equivalent results at the validated limit of detection for the *in vitro* assay (or below limit of detection, in the case of SV5). Therefore, the key objective of this study, which was to demonstrate that the Blazar® CHO AOF assay achieves at least equivalent sensitivity to the *in vitro* assay, has been met.

Table 6: The Blazar® CHO AOF assay produces equivalent results to the *in vitro* assay when challenged at the validated *in vitro* assay limit of detection (note: *in vitro* assay validated detection limit for SV5 in CHO cells is 10 $TCID_{50}$/mL).

Spike Level	Virus	Blazar® CHO AOF Assay Final Disposition	*in vitro* Final Disposition	Equivalency
1 $TCID^{50}$/mL	PI3	Detected	Detected (Vero cells)	Yes
1 $TCID^{50}$/mL	MMV	Detected	Detected (324K cells)	Yes
1 $TCID^{50}$/mL	SV5	Detected	Detected (CHO cells)	Yes
1 $TCID^{50}$/mL	PCV-1	Detected	Detected (PT cells)	Yes
1 $TCID^{50}$/mL	Reo	Detected	Detected (Vero cells)	Yes

Overall conclusion

The Blazar® CHO AOF assay provides rapid detection of relevant adventitious viral contaminants in bulk harvest material generated from CHO cells using animal origin-free processes. In appropriate situations, there is a strong justification for it to serve as a suitable replacement for the traditional *in vitro* assay due to its appropriate breadth of virus detection (plus variants) and sensitivity, plus a range of advantages as outlined in **Table 7**. Data generated to show equivalent sensitivity with the *in vitro* assay supports the regulatory expectations that any alternative assay must show equal or better performance when compared with the current method.

Table 7: Comparison of the traditional *in vitro* assay and Blazar® CHO AOF assay for bulk harvest testing.

	Traditional *in vitro* assay	Blazar® CHO AOF assay
Technology	Culture-based with multiple observational end-points	Degenerate PCR with capillary electrophoresis end-point
Compliance level	GMP	GMP
Viruses detected	Viable only; specificity based on susceptibility / permissiveness of detector cell lines and ability of the virus to produce a positive result in one or more end-points	Viable and non-viable; specificity based on design of primers against selected families
Spiked test article controls	May not always be included (requires separate aliquot of test sample)	Included in every run
Coverage of virus families of concern* for CHO bulk harvest (animal origin-free processes)	10/15 (additional PCR assays may be required to mitigate specific contamination risks)	15/15 (no additional PCR assays required)
Detection limit	1-10 $TCID_{50}$/mL based on selected live viruses (not validated for all families)	≤1 $TCID_{50}$/mL based on selected live viruses (not validated for all families) 10 gc/reaction based on synthetic controls (validated for all families**)
Total turnaround time	35 days	12 days
Sample requirements	15 mL bulk harvest (unprocessed)	1 mL bulk harvest (clarified by low speed centrifugation to remove debris)

* As selected by our experts for the Blazar® CHO AOF assay.

** Validated detection limit for polyomaviridae is 100 gc/reaction.

Use of the Blazar® CHO AOF assay also aligns with industry and regulatory trends towards increased use of molecular methods, particularly at downstream process steps, since rapid results facilitate faster decision-making[1]. It can be combined with other rapid methods to significantly reduce the overall turnaround time for a bulk harvest testing package (**Figure 2**). For implementation of any alternate technology, a risk assessment should be conducted and early dialogue with regulators is strongly encouraged to ensure that expectations are met for each developer's specific product and situation.

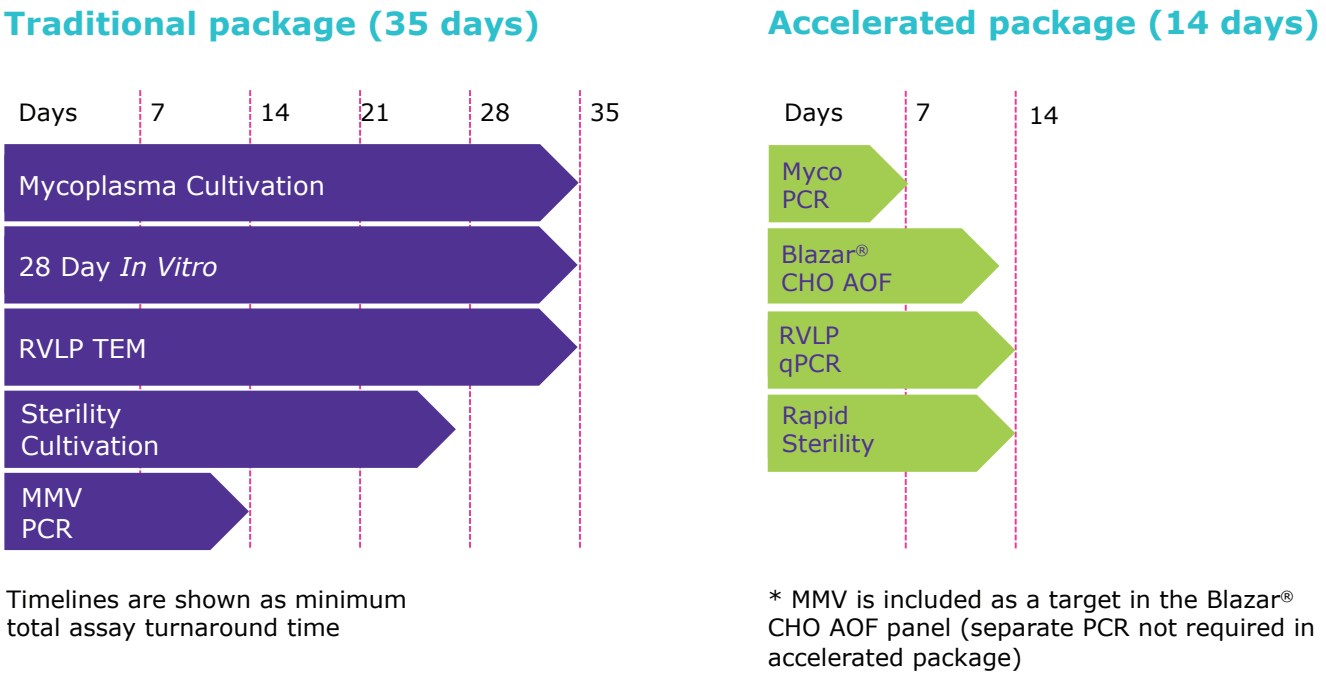

Figure 2: Implementation of the Blazar® CHO AOF assay, together with other rapid methods for sterility, mycoplasma detection, and quantitation of Retrovirus-Like Particles (RVLP), can decrease CHO bulk harvest testing time by up to three weeks. RVLP quantitation is typically only required on the first three batches.

References

1. ICH Q5A(R2) (2023) Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin.
2. Barone, P.W., Wiebe, M.E., Leung, J.C. *et al.* (2020) Viral contamination in biologic manufacture and implications for emerging therapies. *Nat. Biotechnol.* 38 563–572.
3. Nims, R. (2011) Adventitious Viral Contamination of Biopharmaceuticals: Who is at Risk? *Bioprocessing J.* 10 (1) 4-10.
4. Berting, A., Farcet, M.R., Kreil, T.R. (2010): Virus susceptibility of Chinese Hamster Ovary (CHO) cells and detection of viral contaminations by adventitious agent testing. Biotechnol. Bioeng. 106 (4) 598–607.
5. Poiley, J.A., Nelson, R.E., Hillesund, T., Raineri, R. (1991) Susceptibility of CHO - K1 cells to infection by eight adventitious viruses and four retroviruses. *In Vitro* Toxicology 4 (1) 1-12.
6. Firth, C., Bhat, M., Firth, M.A. *et al.* (2014): Detection of zoonotic pathogens and characterization of novel viruses carried by commensal *Rattus norvegicus* in New York City. mBio. 5 (5) e01933-14.
7. ICH Q2 (R1) (2005) Validation of Analytical Procedures: Text and Methodology (effective at time of assay validation).

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