BioReliance®
Pharma & Biopharma
Manufacturing &
Testing Services

White Paper

Accelerate CHO Cell Characterization with the Blazar™ Rodent Panel: A Guide for Adoption

Summary

The development of the Blazar™ Rodent Panel allows biopharma to move away from traditional *in vivo* antibody production assays when characterizing cell banks of rodent origin used in the production of biologicals. This degenerate PCR-based method accelerates characterization by reducing timelines by up to 80%, and replaces the use of animals. As described in various regulations and guidelines, sensitive molecular techniques with broad detection capabilities such as NGS and multiplex PCR approaches may be used as alternatives to *in vivo* based methods. The regulators have provided positive feedback on the use of the Blazar™ Rodent Panel as an alternative to the antibody production assays which enables timely the transition to this alternative method as soon as practicable.

Introduction

As part of the characterization package for biological products derived from cell lines, the manufacturing cell bank must be assessed for the presence of viral contamination. The ICH harmonized guideline Q5A (R1) states: "*Such contamination… can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production.*" For the characterization of the master cell bank (MCB), Q5A indicates that testing for viruses should include "*species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.*"

The mouse antibody production (MAP) test has been used extensively for more than 30 years as the primary method for detecting adventitious murine viruses in cell lines. The MAP test was first developed by Rowe and co-workers in 1959[1] for the quantitation and detection of Polyomavirus. Subsequently, they and other investigators used the MAP test to detect additional murine viruses[2-7]. The hamster antibody production (HAP) test is a modification of the MAP test designed for the detection of adventitious viruses that are capable of infecting hamster tissues.

Both the MAP and HAP tests are based on the detection of antibodies in test article inoculated mice, or hamsters, raised against viruses that are present in the test article. The target antibodies are selected for viruses that may not be detected using more general *in vivo* or *in vitro* methods due to their lack of clinical, pathological or cytopathic effects or their inability to replicate in other *in vivo* or *in vitro* test systems. Indeed, MAP and HAP test methods are described in various regulations including the E.P., U.S. 21CFR and U.S. FDA guidance (see **Table 1** for recommended viral coverage).

Three routes of inoculation are used for both tests to assure maximum opportunity for adventitious viruses to infect and replicate in the test animal:

- The *per os* route provides enteric viruses access to the alimentary canal
- The intranasal route provides respiratory viruses entry into the respiratory system and mucosa
- The intraperitoneal route provides access to internal organs while by-passing the virucidal substances found on and in mucous membranes of the alimentary canal

In addition, the MAP test inoculation via the intracerebral route is used to detect avirulent strains of Lymphocytic choriomeningitis virus (LCMV), for which a lethal strain of LCMV is used as a challenge.

Following a defined observation period, enzymatic and immunological endpoint assays are used to indicate the presence or absence of specified viruses as outlined in the guidance documents (**Table 1**).

Mouse Antibody Production Test	Hamster Antibody Production Test
Ectromelia	Sendai
Mouse encephalomyelitis	Pneumonia virus of mice
Lactate dehydrogenase elevating virus	Reovirus 3
Lymphocytic choriomeningitis virus	Lymphocytic choriomeningitis virus
Hantaan virus	Simian virus 5
Murine minute virus	
Mouse adenovirus	
Mouse hepatitis virus	
Pneumonia virus of mice	
Polyomavirus	
Sendai	
Epizootic diarrhea of infant mice	
Mouse cytomegalovirus	
Reovirus type 3	
Mouse pneumonitis virus	
Mouse thymic virus	
Mouse parvovirus	

Table 1 – Viruses tested for in the MAP and HAP tests

While the antibody production test has stood the test of time, there are several considerations that make an alternative detection method more desirable. The use of any biological system for the detection of virus is often very lengthy, as well as potentially susceptible to adverse effects of the test article. For example, the test article may induce an adverse response within the biological system making the detection of specific viral threats more challenging. However, perhaps the most important consideration is the guiding ethical and legal principals of the 3Rs (replacement, reduction and refinement), where the whole industry is obliged to seek out and implement alternatives to the use of animals when and where possible. Specifically for *in vivo* MAP and HAP tests, there is provision within the E.P. section 5.2.3. and U.S. FDA guidance to use nucleic acid-based tests such as PCR as an alternative to *in vivo* MAP and HAP tests and to use broad detection methods (e.g. NGS) as an alternative to directed nucleic acid tests. Further, ICH Q5A notes that: "Numerous assays can be used for the detection of endogenous and adventitious viruses. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable."

The Blazar™ Rodent Panel was specifically developed to help biopharma reduce animal use as well as accelerate cell line characterization. This is a unique PCR-based platform that uses a multiplexed degenerate PCR approach for the sensitive detection of viruses. The Blazar™ Rodent Panel can be used as an alternative to the mouse, hamster and rat antibody production tests.

Technical Summary

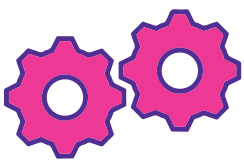
The use of degenerate primers in PCR for the detection of virus families is well-established in both the literature as well as regulatory documents. Primer degeneracy allows for amplification of multiple targets (e.g. variants) within a conserved region of the viral family genome. Therefore, to broaden the range of more traditional molecular approaches the Blazar™ platform from the BioReliance® portfolio uses a nested degenerate PCR method. The amplified regions for the targeted virus are relatively large, between 300 and 600 bp, which allow for multiplex amplification and detection by highly accurate endpoint sizing with capillary electrophoresis (CE). Subsequent analysis and identification is undertaken by validated peak-calling software based on sizing ranges.

In more detail, the assay is comprised of three steps (**Figure 1**):

1. **Extraction of nucleic acid material from the test cells.** 1 to 2x10^6 cells are extracted for either DNA or RNA using automated protocols on liquid handling instruments. RNA material is extracted separately from DNA as this has the additional reverse transcriptase step to create cDNA. Each extraction has a spike recovery nucleic acid

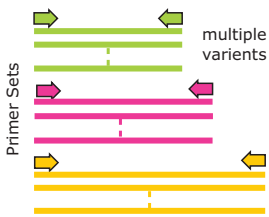
3 Step Process

1 Automated Extraction
(1-2 x10^6 cells)



e.g. EZ1® Advanced XL workstation

2 Multiplex Amplification
(degenerate primer target virus families)



3 Detection & Reporting
(sequencing confirmation for positives)

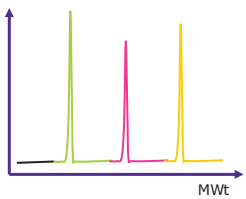


Figure 1 – Overview of the Blazar™ platform.

sequence added at the detection limit (10 genomic copies) prior to extraction as a positive control for extraction and PCR. Prior to amplification the sample is split into three technical replicates.

2. **Nested multiplex PCR amplification with degenerate primers.** 134 degenerate primers are employed across separate DNA and RNA nested PCR amplification steps. The use of a degenerate PCR approach enables the detection of the 45 target viruses, and closely-related viruses of the targets. A nested approach allows for targets to be amplified at the validated detection limit of 10 genomic copies across the three replicates.
3. **Detection and sizing of amplicons.** Any amplified viral signal is detected and sized using the CE capability of the Genetic Analyzer System (Applied Biosystems™/Thermo Fisher Scientific). In the unlikely event of a viral contamination, a peak-calling software tool will identify the viral family based on the size of the amplicon. The internal control template must be identified in 1 or more of the replicates. The pass criteria for the assay is that no target is observed across any of the replicates, including the control wells.

The central engine of the Blazar™ platform is the degenerate primer set. As discussed, this allows for the detection of the specified viruses as well as the ability to detect closely related, but unknown viruses. For example, a primer pair which can target the parvovirus MMV can also amplify other parvovirus members such as mouse parvovirus 4a and 4b, rat parvovirus and Kilham rat virus. This broad specificity is achieved through the specific targeting of conserved protein motifs across the virus family. The primer design pipeline (**Figure 2**) identifies the associated genetic sequences so that degenerate primers can be fabricated. For RNA viruses, the target amplified region is checked for the presence of secondary structures as these are known to inhibit PCR amplification. The final step of the pipeline is the optimization of the amplification step, thus determining the most robust primer sequences, PCR conditions and primer concentrations for this multiplex PCR.

As indicated above, the degenerate primers are designed to target and amplify across closely-related members of viral families via conserved protein motifs. The platform, therefore, has the ability to detect unknown or uncharacterized viral variants as well as new viruses where the protein motif is sufficiently conserved. For example, the MMV primer set mentioned previously has been demonstrated to detect a newly-characterized mouse parvovirus (mouse kidney parvovirus)[8] where the sequence was only made available in 2018 after the degenerate primers were established. This greatly expands the ability of the traditional PCR approach and provides additional risk mitigation as traditional primers must match the target sequence. Mismatch in the binding region, for example in a viral variant, may prevent a primer from annealing to the target and lead to a failure to detect a virus – even if that virus is present within the sample.

A frequently highlighted concern is the risk of false positives coming from non-specific amplification when using a degenerate primer PCR approach. Non-specific amplification is not only a concern for degenerate PCR, traditional PCR can also show non-specific amplification. In addition, there is a concern that environmental nucleic acid sequences could cause a false positive. The Blazar™ platform has been extensively tested with high µg backgrounds of DNA from multiple sources, including CHO cell DNA without seeing any false positives. This is because apart from a careful primer design, a number of steps have been taken in the assay to eliminate the risk of issues like these giving a positive result. Firstly, the amplicons are relatively large – ranging from 300-600 bp. This reduces the risk of detection of environmental sequences, as these tend to be much smaller. Secondly, the CE separates out the specific amplicons from issues such as primer dimers and larger non-specific amplification. As part of the analysis the CE trace is visually examined as specific amplification generates sharp peaks. Broad peaks outside the analysis window are automatically discounted. Any sharp peaks outside the analysis window would be subject to an investigation, as this could be caused by a target virus or closely related virus having an insertion event within the amplicon. Any positives would spark an immediate investigation,

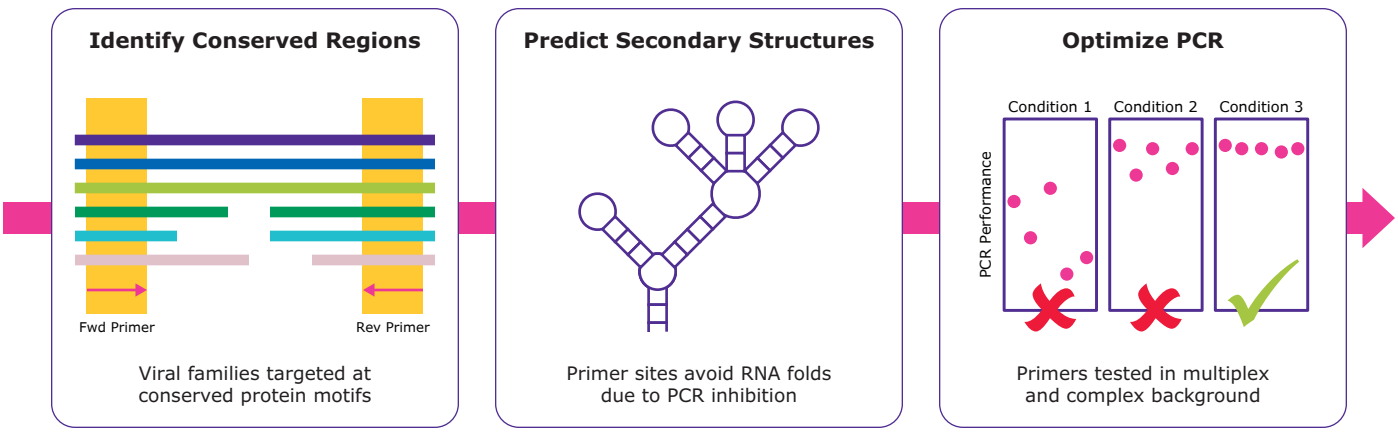


Figure 2 – Design pipeline for the central degenerate PCR part of the Blazar™ platform.

Specifications for BioReliance® Blazar™ Rodent Panel	
Sample format	1 to 2 x 10^6 cells - tested in triplicate wells
Internal controls	Spike recovery of DNA or RNA at LOD in the cell pellet prior to extraction demonstrates extraction and PCR efficiency
Sensitivity	10 genomic copies* per reaction
False positive rate	<1%
True positive rate	>99%
Specificity (pass criteria)	No target peaks observed in 3/3 wells
	Spike recovery control spiked at LOD detected in ≥1/3 wells
System suitability	No Template Control (NTC) signal detected in 0/3 wells
	Spike recovery must fall within specified sizing window
Virus coverage (Q5A recommendations shown in bold)	**Murid Adenovirus 2*-3**, **Lymphocytic Choriomeningitis virus**, **Lactic Dehydrogenase virus**, **Hantaan virus**, Dobrava virus, Seoul virus, Puumala virus, Vesivirus 2117, Calicivirus Allston-2008/2009, Calicivirus Geel-2008, Calicivirus Bari, Murine Norovirus 1-6, Murine Norovirus GV, Murine Norovirus Guangzhou, Mouse Hepatitis virus, Murine Coronavirus SA59, **Rat Coronavirus**, **Sialodacryoadenitis Virus**, **Murid herpesvirus 1 & 3**, Murid herpesvirus 2/4/7/8/68, **Pneumonia Virus of Mice**, **Sendai virus**, **Parainfluenza virus 5**, **Kilham Rat virus**, **Minute Virus of Mice**, **Toolan virus**, **Mouse Encephalomyelitis virus**, **Murid Polyomavirus**, Rat Polyomavirus 1, **K virus**, **Ectromelia virus**, **Mouse Rotavirus**, Rat Rotavirus, **Reovirus Type 3**
Assay duration	14 days

*murid adenovirus 2 (Mad2) was validated to 12 genomic copies per reaction.

Table 2 – Specifications for the Blazar™ Rodent Panel.

with the first course of action being Sanger sequencing of the amplified region. Other investigation routes are available and can be discussed in further detail upon request.

The Blazar™ Rodent Panel has been validated in accordance with ICH Q2 (R1) for the detection of 45 rodent viruses (internal reference #VPPO0394). This includes the 22 viruses from ICH Q5A (R1) guidance[9] (17 MAP viruses and 5 HAP viruses) plus an additional 23 emerging viruses of concern. The detection of 15 DNA viruses was represented by 6 target reference controls and detection of 30 RNA viruses was represented by 10 target reference controls. The DL was established at 10 copies/PCR for all targets except Mad2 where 12 copies/PCR were validated. This DL was also established in the presence of CHO-K1 test matrix. Spiking studies with a representative DNA virus and a representative RNA virus with known 50% tissue culture infectious dose ($TCID_{50}$) titres normalized to genomic copies, in mock CHO cell lines, showed the limit of detection to be less than 1 $TCID_{50}$/ml per extracted sample as analyzed by Chi-squared test of comparing expected frequencies to observed frequencies. A summary report highlighting the parameters challenged during validation of the method is available upon request or via our iNet portal. The full validation reports have been included in our U.S. FDA Master File (No. 3493) which can be referenced by the U.S. FDA during review of regulatory submissions.

The Blazar™ Rodent Panel expands the viral coverage of the recommendations provided in the guidance for the incumbent antibody production test (see **Table 2**). Clearly, industry and regulatory understanding of the viral threats posed to CHO and other rodent-based cell manufacturing have evolved considerably since the development of the recommendations for ICH Q5A in the early 1990s. For example, there is no recommendation to screen for vesivirus 2117 (also known as calicivirus 2117) despite a number of contamination events of CHO manufacturing cells[11]. Obviously broadening the coverage in this way helps to reduce the manufacturers' risk given today's understanding. Another benefit is that viral coverage in this rodent panel removes the need to test for specific viruses separately in a cell line characterization package, e.g. vesivirus 2117 would not require a separate PCR assay as is currently recommended.

Comparability to Antibody Production Test

A common question we receive is "what comparability studies have been conducted to support a migration from the incumbent antibody production test to the Blazar™ rodent panel?" The following extracts are taken from various regulatory documents in regards to alternative methods:

- ICH Q5A (R1) states that *"Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable."*
- EP 5.2.14 *'Novel sensitive molecular techniques with broad detection capabilities are available including ……degenerate PCR for whole virus families……..The implementation of such new molecular methods as substitutes for in vivo methods requires a comparison of the specificity (breadth and detection) and the sensitivity of the new and existing methods'*
- U.S. FDA CBER: *'If you use alternative methods (to antibody production tests), such as PCR, you should demonstrate sensitivity comparable to that of the described test'*

A number of existing PCR methods for the detection of rodent viruses have been compared with the *in vivo* MAP test method in three published comparability studies[12-14]. Bootz[12] and Bauer[13] conducted direct head-to-head comparison studies in mice and by PCR to compare the sensitivity of the molecular based assays with the MAP assay using a range of virus concentrations. A third publication[14] uses the Bootz[12] data to compare an alternative PCR method with the MAP test, altogether avoiding the inoculation of animals with infectious viruses and the associated distress.

The viruses tested in all three studies provide good coverage of the standard MAP targets. The Detection Limit (DL) established for each virus showed the PCR methods to have equal or greater sensitivity as compared to the *in vivo* MAP test method.

As an organization, we are committed to the 3Rs. As such, performing a further set of experimental *in vivo* comparability studies will not provide any additional useful information above what has already been published, not least due to the variability in using *in vivo* test systems. Further, we advocate that this published data is a suitable comparison for reference in any submission to the regulatory authorities. This approach has been presented with a favourable response from the U.S. FDA.

Regulatory Position and Acceptance

Regulatory agencies accept that as science progresses, assays that were once the standard may be replaced or augmented by more sensitive, accurate, and reproducible assays. ICH Q5A (R1) notes that, *"Numerous assays can be used for the detection of endogenous and adventitious viruses. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive."* Indeed, a final concept paper produced in November 2019 by the ICH to revise the Q5A guidance has been published[15]. This paper indicates that "nucleic acid-based tests such as PCR and NGS provide sensitive detection of adventitious and endogenous viruses…" There is further provision within the E.P. section 5.2.3. and U.S. FDA guidance[16] to use NAT tests as an alternative to *in vivo* antibody production tests.

The Blazar™ platform generally and the Blazar™ Rodent Panel specifically, have been presented on several occasions in scientific conference with very positive feedback. Most notably presentations at the PDA Virus Safety Forum in Florence, Italy in 2018 and Long Beach, California in 2019 where several relevant regulatory agencies were present, including the U.S. FDA and Germany's Paul Ehrlich Institute (PEI). In addition to these conference presentations, we have also delivered an open scientific presentation to the U.S. FDA Bethesda, Maryland site in April, 2019. Here we presented the development approach, showing intermediate data as well as the results from assay validation. We also shared our approach on utilizing existing *in vivo* comparability data. Overall there was very positive feedback on the Blazar™ platform, with an affirmation on the strong desire to move away from the incumbent *in vivo* antibody production test. Therefore, the use of this alternative method should be undertaken in agreement with the competent authority and documented within the submitted viral safety risk assessment.

As of September 2020, 10 different biopharma companies have transitioned testing using the Blazar™ Rodent Panel. Three of these were from top 10 pharma companies, with one currently known approved BLA submission for a Phase I clinical trial of a mAb therapy. More regulatory approvals for mAb therapies using this alternative method are expected through 2020 and into 2021. As always, we are available to supply information in support of regulatory submissions.

Conclusion

The Blazar™ Rodent Panel allows mAbs producers in the biopharmaceutical industry using rodent-based manufacturing systems to move away from the *in vivo* antibody production test. The mouse, hamster, and rat antibody production tests are used for the detection of specific rodent viral threats in rodent cell banks as part of the cell line characterization package. However, new technologies such as the Blazar™ Rodent Panel, a degenerate PCR based method, are able to detect the specific viral threats indicated within the guidance, as well as expand coverage to emerging threats which were not conceived in the original documents from the early 1980s. This expanded coverage helps to reduce risk in a cell line characterization package. Further, the use of this molecular based alternative not only helps the industry meet their ethical obligations to reduce the use of animal models, in combination with other rapid approaches it can significantly reduce the time taken to complete a cell line characterization package by as much as 80%.

Our position, that has been confirmed in conversation with regulatory bodies, is that the use of existing published studies as detailed above provide sufficient comparative data to support replacement of the *in vivo* MAP and HAP tests with the Blazar™ Rodent Panel in a cell line characterization package. Biopharmaceutical manufacturers have already transitioned to this alternative and are in the process of submitting testing packages to the regulators. We are aware of one therapeutic mAb that has been granted BLA approval for Phase I clinical trials using the Blazar™ Rodent Panel.

As this suitable alternative is available, we have announced our intention to discontinue the mouse, hamster and rat antibody production assays from the 31 December 2022.

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