BioReliance®
Pharma & Biopharma
Manufacturing &
Testing Services

White Paper

Alternatives to *in vivo* assays for biosafety testing of biologics

Replace, Reduce, Refine

Summary

The use of animal models for the detection of adventitious agents has been a feature of biologic testing packages for many decades. However, as alternative methods such as PCR and NGS have emerged these *in vivo* tests have stubbornly remained a central part of testing. Here we examine the current *in vivo* methods and explore alternatives which can be employed today. We also propose that while the industry may be some years away from removing *in vivo* testing completely, a case can be made for removing animal use from well-characterized production systems such as CHO.

A critical aspect of developing biologics-based medicinal products is the biosafety evaluation of materials and intermediates used in manufacturing. Evaluation includes qualification of starting materials, testing for the presence of adventitious agents in intermediates and characterization of the final product. Tests employed for biosafety evaluation encompass a wide variety of methods, test systems and technologies from *in vivo* models to state of the art molecular methods.

The use of *in vivo* test systems is prescribed in safety testing regulations and guidelines globally, some of which were established several decades ago. Generic *in vivo* methods are used for the detection of adventitious agents in starting materials, including virus and cell seeds, and in intermediates such as bulk harvests, as well as detection of toxins and pyrogens in the drug product. Multiple species are used in these methods and each may be administered with test material via several different routes of inoculation to enhance detection of adventitious agents, or other contaminants, with a limited host range or tissue tropism.

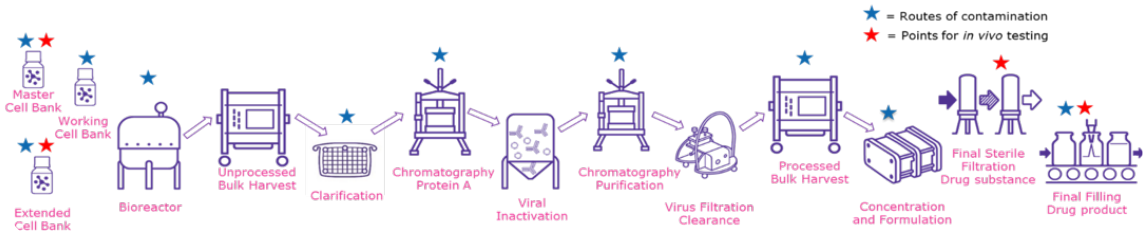


Figure 1

Overview of the biomanufacturing process for a monoclonal antibody

Table 1. Overview of *in vivo* test systems used in biosafety testing of biologicals

In vivo assay	Species used	Assay target	Test application
Rabbit pyrogen test	 Rabbit	Pyrogens	**Drug Substance / Drug Product**
Abnormal Toxicity / General Safety	 Guinea pig	Toxic contaminants	**Drug Substance / Drug Product**
	 Mouse		
General adventitious agent test	 Guinea pig	Wide range of viruses including Paramyxoviruses & Reoviruses as well as Mycobacteria	**Master & Extended Cell Banks**
	 Mouse	Wide range of viruses including Coxsackieviruses & members of the Flavivirus group	
	 Suckling mouse	Wide range of viruses including Togaviruses, Bunyaviruses, Flaviviruses, Picornaviruses & Herpesviruses	
	Embryonated chicken eggs	Orthomyxoviruses, Paramyxoviruses and Herpesviruses as well as rickettsiae, mycoplasmas & bacteria	
Mouse antibody production test	 Mouse	Wide range of viruses including Parvoviruses & Paramyxoviruses	**Master Cell Bank**
Hamster antibody production test	 Hamster	Range of viruses including Paramyxo- & Parainfluenza viruses	

The 3Rs

Background

The use of animals in scientific procedures, including biosafety testing, is regulated through national and international legislation globally. Embedded in these legislations are guiding principles for more ethical use of animals known as the 3Rs (replacement, reduction and refinement). Developed nearly 60 years ago as a framework for humane animal research, the 3Rs were first described by Russell and Burch in 1959.[1]

Today, the 3Rs are recognized as a framework for high quality science in academia and industry with an increasing emphasis on developing alternative approaches which reduce or avoid the use of animals. Within the biosafety testing sector, examples of this can be seen with the introduction of novel technologies and methods that can achieve equivalent or greater test sensitivity than the traditional *in vivo* test systems. Key to supporting the introduction of such novel technologies and methods is regulatory acceptance of comparability, demonstrating equivalence or greater sensitivity compared to the *in vivo* method. In recognition of this a number of organizations have been established, such as the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM); these organizations have brought about the validation of various alternative assays.

Legal Basis for the 3Rs

EU Directive 2010/63/EU (2013)[2], revising Directive 86/609/EEC[3] on the protection of animals used for scientific purposes, builds a common framework and promotes the collaboration of Member States with the European Commission to promote animal welfare in the European Union. It is firmly established on the 3Rs.

In October 2014 the CHMP and CVMP published a draft guideline on regulatory acceptance of 3R testing approaches (EMA/CHMP/CVMP/JEG-3Rs/450091/2012)[4] which was adopted in 2016. As a follow-up, a draft reflection paper that provides an overview of the main animal tests required for medicinal products for human use and opportunities for implementation of the 3Rs, has been developed (EMA/CHMP/CVMP/JEG-3Rs/742466/2015)[5].

The reflection paper includes information on opportunities for limiting animal testing that can already be implemented as well as information on opportunities that may become available in the future. Examples include:

- Guidance on the use of the Rabbit Pyrogen Test (RPT) in situations where neither the monocyte-activation test (MAT) or endotoxin test can be performed
- Guidance on the use of the Abnormal Toxicity Test (ATT), where it should not be used routinely

In the U.S., the Animal Welfare Act 1966, and subsequent amendments, regulates the treatment of animals in research. It requires that minimum standards of care and treatment be provided for specified animals used in research.

Within Europe and the U.S. as well as Japan and Korea there are a number of Committees, International Co-operations and Conventions for establishing guidelines and principles supporting the 3Rs.

1. Pyrogen Test

1.1 Background

The purpose of the pyrogen test is to detect contaminants in preparations for parenteral administration that can cause fever, hypotension and shock resulting in organ failure and death. The first test was established by Hort and Penfold using the rabbit model, following the identification of pyrogens in 1912. At that time, the pyrogenic agent was identified as endotoxin in preparations of Gram-negative bacteria which were frequently identified in the water used for production of parenteral preparations. Interestingly, it was shown that live and dead micro-organisms presented the same pyrogenic potential which led to the inclusion of a pyrogen test, distinct from a sterility test, in the 12th edition of the *United States Pharmacopoeia* (USP) in 1942.

Over subsequent years, development of alternative test methods was driven by the introduction of the 3Rs and a recognition of the variable sensitivity of the rabbit test system (e.g. by development of pyrogen tolerance in rabbits after repeated injections). The first and most successful of these new tests was the bacterial endotoxin test (BET) or Limulus Amoebocyte Lysate (LAL) test based on the lysate of amoebocytes isolated from the blood of the horseshoe crab (Limulus polyphemus). The test became commercially available in the 1970s and has been widely used as a replacement for the rabbit pyrogen test (RPT).

Today, while the quality of water systems used in the production of biologicals no longer presents such a high risk of endotoxin contamination, updated processes for the production of biologicals bring new risks of Non-Endotoxin Pyrogens (NEPs) entering the final product. NEPs, such as viruses from animal based raw materials or Gram-positive bacteria, are undetectable by the BET, thereby underlining the need for development of alternative test methods.

In 2016 the general chapter for endotoxin testing in the *European Pharmacopoeia* (E.P.) (chapter 5.1.10) introduced the requirement for an evaluation of the product, production process and raw materials with respect to the risk for pyrogens that are non-detectable by the BET.

1.2 *In vivo* rabbit pyrogen test

The RPT is designed to test drug products for the presence of pyrogens originating from:

- Endotoxins or lipopolysaccharides; cell wall components of Gram-negative bacteria such as E.coli
- Lipoteichoic acids from the cell walls of Gram-positive bacteria such as Staphylococcus

The test involves measuring the rise in temperature of three rabbits following the intravenous injection of a test solution (figure 2). The RPT is designed for products that can be tolerated by the test rabbit at a dose that does not exceed 10 ml per kg injected intravenously within a period of no more than 10 minutes. It is a qualitative test that detects both endotoxin and non-endotoxin pyrogens. The test is described in the E.P. and U.S.P.

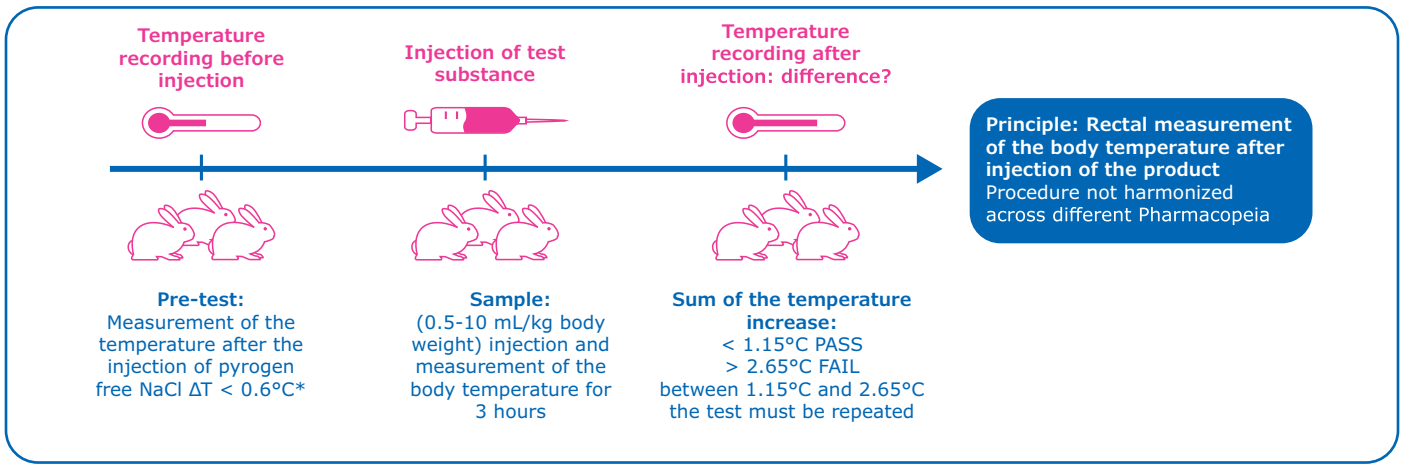


Figure 2

Example procedure of the RPT

1.3 Alternative methods

1.3.1 Endotoxin tests

The BET, more commonly referred to as the LAL test, is designed to detect endotoxins from Gram-negative bacteria (figure 3). The test is based on the clotting reaction of hemolymph from the horseshoe crab in the presence of lipid A portion of endotoxin. There are three basic methodologies: Gel-clot, Turbidimetric and Chromogenic. The test is simple, easy to perform, has high sensitivity and is cost effective. As it only detects endotoxin, not NEPs, it cannot be used as a complete alternative to the RPT for pyrogen testing. There are also a number of products which it cannot be used to test.

A non-animal derived alternative to the LAL test is one based on a genetically engineered protein, recombinant Factor C (rFC). In the test, this protein is activated by endotoxin to produce a fluorescent end product which is quantifiable. Like the LAL test, it is cost effective, easy to perform and has high sensitivity. It has similar drawbacks with respect to NEPs and is unsuitable for testing certain product types.

1.3.2 Monocyte activation test

The monocyte activation test (MAT) is designed to detect pyrogens from Gram-positive bacteria as well as endotoxins and as such represents a true alternative to the RPT (figure 4). The test is based on the principle that monocytes, activated by pyrogens, produce cytokines/interleukins (IL) that can be detected by an immunological assay (e.g. ELISA). This pathway mimics the human fever reaction. There are different variants of the MAT depending on:

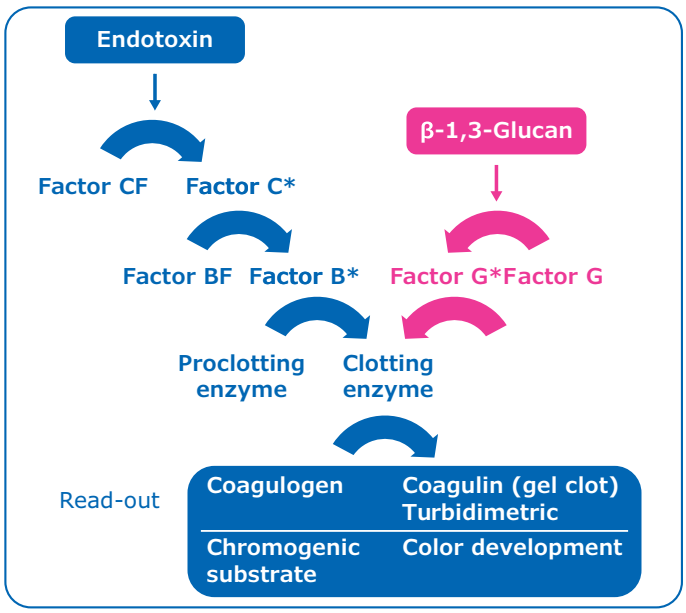
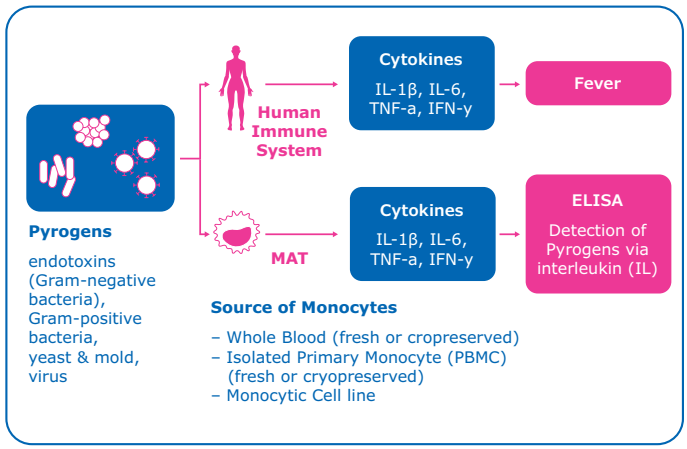


Figure 3

Example procedure of the BET

- Source of human monocytes: whole blood, isolated primary monocytes (e.g. PBMC) or a monocytic cell line
- Immunological endpoint assay read-out e.g. IL-6, IL-1β or TNF-α



Since January 2010, the MAT has been described as a compendial method for pyrogen detection in the E.P. (chapter 2.6.30) and since the 2016 revision, recommendations have been given to replace tests on rabbits with the MAT wherever possible and after product specific validation (E.P. 2.6.8, Rev. July 2016).

Table 2. Comparison of pyrogen test methods

		RPT	LAL	MAT
				
Test type		*In vivo*	*Ex vivo*	*In vitro*
Animal consumption		++	+	-
Pyrogen detection	**Gram-negative (LPS)**	+	+	+
	Gram-positive (LTA)	+	-	+
	Yeasts & Molds	+	-	+
	Viruses	+/-	-	+
Application	**Pharmaceuticals**	+	+	+
	Biologicals	+	+/-	+
	Medical devices	+	+/-	+
	Cellular products	-	-/+	+
	Blood products	-	-	+

2. Abnormal Toxicity Test/General Safety Test

2.1 Background

The purpose of the Abnormal Toxicity Test (ATT) (E.P.) or General Safety Test (GST) (U.S.P.) is to detect extraneous toxic contaminants in drug products. The test was developed in the early 1900s when production processes and quality control for biological products were poorly established compared with current day and licensing procedures didn't exist.

At the time of implementation, it was designed to ensure safe and consistent production of serum samples; mice were used for the detection of phenol levels in diphtheria antiserum and guinea pigs for the detection of tetanus toxin in antiserum preparations.

Today, a number of reviews of historical data, including a review performed by the Paul Ehrlich Institute, have revealed that no reliable conclusions could be drawn from the ATT and that the test does not serve its purpose.[6] The ability of the ATT to identify potentially harmful batches is scientifically highly questionable, the test is variable, non-reproducible and non-specific. Under GMP, where adequate measures using state of the art techniques for product control and release are in place, the ATT is not considered to add information of any value.

2.2 *In vivo* abnormal toxicity and general safety test methods

Methods for the ATT and GST have been described in the E.P. (chapter 2.6.9) and US Code of Federal Regulations (21 CFR chapter 610.11) respectively.

2.3 Alternative methods

It has been established that the *in vivo* ATT does not serve its purpose or add any value to QC release testing of current day biologicals using state of the art analytical techniques. Accordingly, no alternative methods to the ATT have been developed.

In recognition of this, the ATT has been removed from over 80 product monographs of the E.P. in recent amendments and removed from E.P. supplement 9.6 (implementation date 01 January 2019). Furthermore, the GST was revoked from the U.S. 21CFR in 2015 for biological products in an amendment to the biologics regulations, where particular safety tests are only required for certain products that present specific safety concerns if set forth in an approved Biologics License Application (BLA).

These changes serve as clear examples of commitment to the 3Rs at a regulatory level:

- Revision of outdated requirements
- Adoption of new and evolving technology
- Acceptance of new and evolving testing capabilities

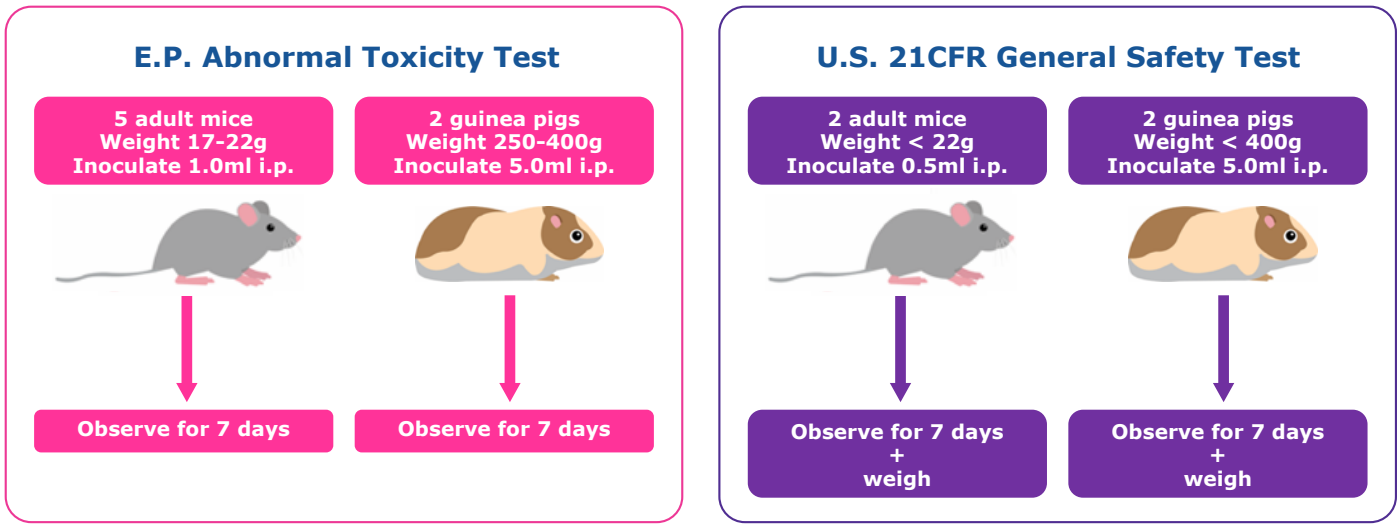


Figure 5

Method overview of the ATT and GST

3. *In vivo* Adventitious Agent Test

3.1 Background

The *in vivo* adventitious agent test was developed more than 50 years ago for the detection of adventitious agents in vaccine preparations that may not be detected using *in vitro* methods. Over the years the assay has been applied more broadly for the detection of adventitious agents in a wide variety of biological preparations from vaccines and monoclonal antibodies to advanced therapy medicinal products.

The assay is used for the detection of known and unknown adventitious agents where clinical signs and / or pathology are the key indicators for the presence of such adventitious agents, capable of replication in one or more of the test systems used.

3.2 *In vivo* adventitious agent test method

Methods are described in various regulations including the E.P., U.S. 21CFR and U.S. F.D.A. guidance (see figure 6). Typically up to four test systems are inoculated with test material via multiple routes of injection followed by a defined period during which clinical observations are made for the presence of adventitious agents. Endpoint tests such as the haemagglutination test or general pathology may also be performed.

In 2018 relevant sections of the E.P. (sections 5.2.3 and 2.6.16)[7,8] were revised to significantly reduce the number of animals required for *in vivo* adventitious agent testing of vaccines and cell substrates. This change was triggered by ongoing evaluation and challenge of the *in vivo* adventitious agent test in light of historic data as well as new data comparing sensitivity of *in vivo* and *in vitro* test systems, Gombold *et al* (2014)[9]. The following points capture the key changes:

Chapter 5.2.3 ”Cell substrates for the production of vaccines for human use“ formerly required the use of embryonated eggs if the cell substrate was of avian origin, suckling mice and adult mice. For 2018, the revised chapter no longer requires the use of adult mice and the tests in suckling mice and embryonated eggs are carried out if a risk assessment indicates that they provide risk mitigation, taking into account the overall testing package applied.

Chapter 2.6.16 ”Tests for extraneous agents in viral vaccines for human use“ formerly required the use of embryonated eggs if the cell substrate was of avian origin, suckling mice, adult mice and guinea pigs. For 2018, the revised chapter no longer requires the use of adult mice and guinea pigs and the tests in suckling mice and eggs are carried out if a risk assessment indicates that they provide risk mitigation, taking into account the overall testing package applied.

Global harmonization on the number of animals required for *in vivo* adventitious agent testing is however yet to be achieved. Therefore if compliance with multi-national regulatory requirements (e.g. U.S. FDA combined with European requirements) is desired, a recommended approach is to meet the requirements of the most stringent method in a single adventitious agent study.

This global approach avoids the excessive use of animals where multiple studies are performed to meet the requirements of individual regulations and guidelines. The practice of using a single 'global' *in vivo* assay to generate data for review by multiple regulatory authorities has been in use for several decades, supporting the safety of a wide variety of biologicals while at the same time, observing and promoting the principles of the 3Rs.

Table 3. Susceptibility of *in vivo* test systems to known pathogens

Adult mice	Suckling mice	Guinea pigs	Embryonated chicken eggs
			
Arboviruses	Arboviruses	Arboviruses	Arboviruses
Eastern equine encephalomyelitis	Colorado tick fever	Ebola	Eastern equine encephalomyelitis
Encephalomyocarditis	Coxsackie A 1-24	Encephalomyocarditis	Herpes Simplex type 1
Herpes Simplex type 1	Coxsackie B 1-6	Junin	Herpes Simplex type 2
Herpes Simplex type 2	Dengue type 1-4	Lassa	Influenza
Japanese B encephalitis	Foot and mouth disease	Lymphocytic choriomeningitis	Lymphogranula venereum
Lassa	Herpes Simplex type 1	Marburg	Mumps
Lymphocytic choriomeningitis	Herpes Simplex type 2	Monkey B	Newcastle Disease
Lymphogranula venereum	Junin	Rabies	Chlamydia psittaci
Chlamydia psittaci	Monkey B		Parainfluenza type 1 (Sendai)
Rabies	Machupo		Parainfluenza type 2
Saint Louis encephalitis	Variola		Rabies
Venezuelan equine encephalomyelitis			Vaccinia
Western equine encephalomyelitis			Variola
			Venezuelan equine encephalomyelitis
			Western equine encephalomyelitis

3.3 Alternative methods

In recent years the scientific value of the *in vivo* adventitious agent test has been evaluated and challenged in the light of historic *in vivo* data. Additionally, the advent of new test methods with equivalent or greater sensitivity and broader specificity, has also challenged the value of the *in vivo* adventitious agent test.

Sensitive molecular techniques with broad detection capabilities, performed at the required quality standard, are now widely available with a range of applications. As alternatives to *in vivo* adventitious agent testing, current methods include next generation sequencing (NGS), single target polymerase chain reaction (PCR) and multiplexed PCR panels. These PCR panels can have various endpoints detection routes, such as sequencing, as well as amplification strategies, such as the use of degenerate PCR for the broad detection of virus families.

Since *in vivo* assay methods are not generally validated, primarily on account of ethical concerns, it is often not possible to perform a direct comparison of *in vivo* and alternative methods for adventitious agent detection. Instead, current initiatives to evaluate alternatives are focusing on scientific rationale behind *in vivo* test methods, relative to what is provided from the *in vivo*. This position is reflected in the recent update to E.P. chapter 5.2.14. entitled "Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines."[10]

E.P. chapter 5.2.14 has been developed by the E.P. group of experts on vaccines and provides guidance to facilitate the implementation of *in vitro* methods as substitutes for existing *in vivo* methods where a direct head to head comparison is not appropriate. The chapter applies primarily to vaccines for human or veterinary use, however the principles described may also apply to other biologicals. It explains that the use of alternative methods requires a comparison of the specificity and sensitivity of the new and existing methods. For this purpose the ability of the new method to detect adventitious agents that are (or are not) detected by the *in vivo* method, should be assessed using an appropriate panel of representative, well-characterized model viruses. Furthermore it should be determined if the sensitivity of the new method is at least equivalent to the sensitivity of the *in vivo* method. Since new molecular methods do not detect the same characteristic of the adventitious agent (genome for molecular methods versus infectious agent for *in vivo* methods) and since no or limited validation data exist for the *in vivo* methods, determining the sensitivity can be particularly complex. It should also be kept in mind that the outcome obtained from the new molecular methods is not necessarily the final result, since the detection of a genome or a genome fragment does not always confirm the presence of an infectious agent. As such, while new molecular methods have high potential as a tool for detection of adventitious agents in regulatory safety testing, their use as an adjunct to traditional adventitious agent tests, capable of distinguishing infectious agents from non-infectious sequences, should be considered.

As an example, in the 2018 revised E.P. sections 2.6.16 and 5.2.3, it describes that in agreement with the competent authorities, broad molecular methods may be used as an alternative to *in vivo* tests, based on a risk assessment. In the case of positive results with broad molecular methods, a follow up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious agents and / or are known to constitute a risk to human health.

So too, there is a place for targeted molecular based methods (nucleic acid tests) as an alternative to *in vivo* methods for the detection of specific, known adventitious agents. A risk assessment should define the specific agents to be tested for, taking into account the origin of the material and the potential sources of contamination through sourcing and production as well as other relevant factors. Examples of applying nucleic acid tests (NAT) as an alternative to *in vivo* tests include detection of mycobacterium, where guinea pigs have historically been used, and detection of viruses of rodent origin (see also section 4.0).

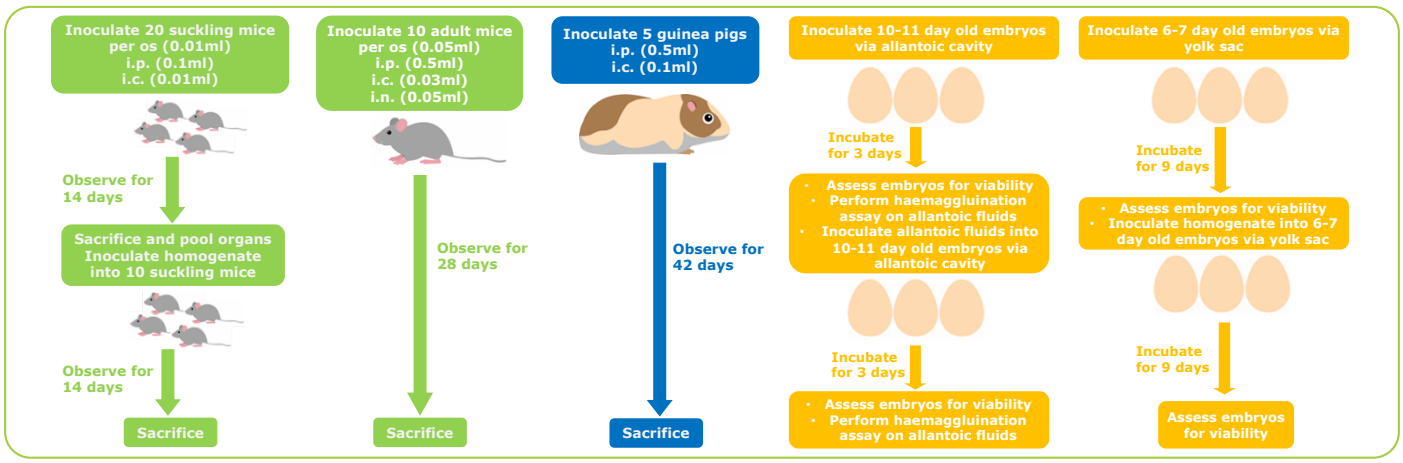


Figure 6

Example method overview of the *in vivo* adventitious agent test

The same caveats apply as for broad molecular based methods in terms of follow up in the event nucleic acid sequences are detected, for example, as described in E.P. sections 2.6.16 and 5.2.3.

The above approaches and concerns are reflected within the U.S. F.D.A. Vaccines Guidance for Industry 2010[11] where it is described that if alternative methods are used, such as PCR, sensitivity comparable to the prescribed (*in vivo*) test should be demonstrated.

It is clear then that there is an increasing role expected by regulatory authorities for both broad and specific molecular methods in adventitious agent testing. In parallel with updated regulations and guidance described above, dialogue is ongoing between regulators and manufacturers on the use of these novel methods. One such example is the Advanced Virus Detection Technologies Interest Group (AVDTIG) formed in 2014 in support of regulatory acceptance of NGS whose members span global representatives from industry and regulatory agencies.

4. MAP and HAP test

4.1 Background

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 was first developed by Rowe and co-workers in 1959[12] for the quantitation and detection of Polyomavirus. Subsequently, they and other investigators used the MAP test to detect additional murine viruses[13-18].

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.

Table 4. Viruses tested for in the MAP and HAP tests

Adult mice	Hamsters
	
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 diarrhoea of infant mice	
Mouse cytomegalovirus	
Reovirus type 3	
Mouse pneumonitis virus	
Mouse thymic virus	
Mouse parvovirus	

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 indeed their inability to replicate in other *in vivo* or *in vitro* test systems.

4.2 *In vivo* MAP and HAP test methods

In vivo MAP and HAP test methods are described in various regulations including the E.P., U.S. 21CFR and U.S. F.D.A. guidance. 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

For the MAP test inoculation via the intracerebral route is also used, purposefully for the detection of 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.

4.3 Alternative methods

In the absence of cytopathic effects or inability to replicate in commonly used *in vitro* test systems, the viruses of concern in the MAP and HAP test may

Table 5. Alternative tests to MAP and HAP tests

PCR panel	BioReliance® Blazar™ Rodent Virus Panel
A panel of specific PCR assays each targeting a virus in the MAP or HAP list viruses (table 2)	Degenerate primers in a single reaction target virus families corresponding to all the MAP or HAP viruses (Table 4)
Offers high sensitivity and specificity for detection of viruses	Offers high sensitivity (10 genomic copies) and specificity for detection of viruses
Inability to distinguish between infectious virus versus 'free' DNA/RNA	Ability to detect targets that have not been formally identified e.g. new variants
	DNA/RNA detection versus infectious virus

alternatively be tested for using nucleic acid-based tests. Example tests include single target PCR assays[19,20] as well as the BioReliance® Blazar™ rodent virus panel.

For *in vivo* MAP and HAP tests, there is provision within the E.P. section 5.2.3. and U.S. F.D.A. guidance to use NAT tests as an alternative to *in vivo* MAP and HAP tests and to use broad detection methods (e.g. NGS) as an alternative to NAT tests. The challenges with using these alternative methods are as discussed in some detail for the general *in vivo* adventitious agent test (section 3.0). To reiterate, their use requires a comparison of the specificity and sensitivity of the new and existing methods and a follow up investigation must be conducted if nucleic acids are detected. These alternative methods should be used in agreement with the competent authority and based on a risk assessment.

Summary and Conclusions

Historically, the use of *in vivo* assays in combination with classical *in vitro* cell culture detection systems has been used as a key strategy in safety evaluation of biopharmaceuticals. In the last decade, new assay technologies, scientific evaluation of *in vitro* and *in vivo* assay methods, and review of historic *in vivo* assay data trends have enabled a significant reduction in the use of animals in biosafety testing of biologicals. In particular, with the advent of sensitive molecular techniques with broad detection capabilities and following a head-to-head evaluation of *in vitro* and *in vivo* assay sensitives, the number of animals and range of species required by some compendial methods for detection of adventitious viruses have been significantly reduced. Apart from these compendial changes, other regulatory guidance describes how alternative methods may be used to replace or supplement *in vivo* methods. Of particular note, a review of historic *in vivo* data trends has been pivotal in bringing about removal of the Abnormal Toxicity test from E.P. monographs and revocation of the General Safety test from U.S. 21CFR regulations. Furthermore, in light of biopharmaceutical manufacturing production changes presenting new risks of pyrogen contamination, non-animal derived test methods have been established for the detection of NEPs.

There is still much that can be done to further reduce and replace the use of animals in biosafety testing of biopharmaceuticals. Significant changes have been made to compendial methods for the biosafety testing of vaccines within the E.P. and to U.S. FDA regulatory guidance. While there may not be such clear direction within guidance for other biopharmaceutical products, there is global recognition that the most appropriate techniques may change with scientific progress.[21] We have seen that historic *in vivo* assay data has been pivotal in bringing about changes in the use of animals in biosafety testing. Generation of data using alternative methods will also be key in bringing about the complete removal of *in vivo* test systems. It is incumbent on the industry to generate this data and shape animal-free biosafety testing regulations of the future.

With new technologies, such as the BioReliance® Blazar Platform as well as changing regulatory expectations, clear pathways for animal-free biosafety testing can be mapped out. One such pathway is outlined in figure 7. This reflects the points discussed within this paper and how the current and future adoption of *in vivo* alternatives can influence the shape of an evolving testing regime, not just reducing time to market but making a clear commitment to the 3Rs in the biosafety testing of biologicals.

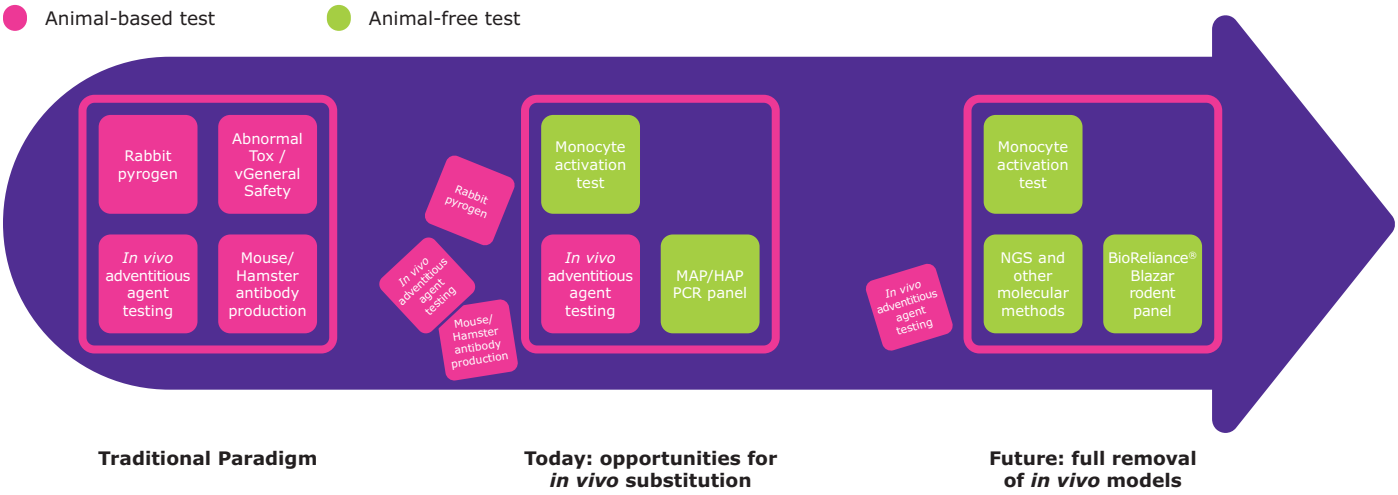


Figure 7

Pathway to a biosafety testing regime for biologicals without the use of *in vivo* assays

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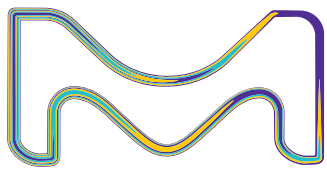

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