3R INITIATIVE
Within Roche's Global QC Network

By Sven M. Deutschmann, PhD

This article describes the numerous activities in the commercial quality control (QC) network that aim to replace in vivo assays with alternative methods in the course of production and release. Specifically, three areas are considered: cell bank testing, pyrogen testing, and potency testing. For each area, examples are provided for alternative assays or control concepts with which in vivo–based assays in QC could be successfully replaced. The successfully completed and implemented 3R projects described in this article are intended to highlight alternative testing concepts to biopharmaceutical quality managers, so they can evaluate which in vivo assays in their QC network could be replaced by alternative assays.

In 1959, British scientists William Russel and Rex Burch published the book "The Principles of Human Experimental Technique" [1], in which they defined the 3R principles. These principles are aimed at completely avoiding (replacement) or reducing (reduction) the number of animals used for testing and improving animals' living conditions (refine improvement). In Europe, the objectives of the 3R principles were established more than 35 years ago in a convention for the protection of vertebrates used for experimental and other scientific purposes [2].

This agreement was ultimately the basis for the European Union (EU) Directive 2010/63/EU on the protection of animals used for scientific purposes [3]. This directive embedded the internationally recognized 3R principles into European law for the first time. Hence, all national and European authorities (e.g., the European Pharmacopoeia, a council of the EU) are bound by these requirements, and an annual reporting has been established to monitor the progress of the implementation of the 3R principles in the EU.

The ALURES Statistical EU Database contains data on the use of animals for scientific purposes collected by the member states [4]. Data extracted from the statistical reports uploaded to ALURES Statistical EU Database (see Table 1) indicate that between 1991 (the first report available on the database) and 2020, the number of in vivo assays decreased from almost 11.5 million to ~10.5 million per year. It should be noted that the number of EU member states reporting their annual data has steadily increased from 11 (in 1991) to 29 (28 member states and Norway) in 2020. It must therefore be assumed that in the years 1991–2008, when there were fewer member states reporting their data, the number of in vivo assays performed in the EU was higher than indicated in the annual reports.

Table 1: Number of in vivo assays in the EU.

Report No.	Year	Approximate Number of In Vivo Assays per Year (Million)	Number of Reporting Countries
1	1991	11.5	11 member states
2	1996	11.6	15 member states
3	1999	9.8	14 member states
4	2002	10.7	15 member states
5	2005	12.1	15 member states
6	2008	12	27 member states
7	2011	11.5	27 member states
8	2015	9.59	28 member states
	2016	9.82	28 member states
	2017	9.39	28 member states
9	2018	10.57	28 member states and Norway
10	2019	10.40	28 member states and Norway
11	2020	10.25	28 member states and Norway

In the EU, basic research and development in human and veterinary medicine are the areas that use the highest number of animals for scientific purposes. The percentage of animal experiments for regulatory use to satisfy regulatory requirements (~15%) and for routine production (~5%) has remained relatively constant.

Many pharmaceutical companies are globally active and market their products internationally. This means that approval of alternative methods to in vivo assays by regulatory authorities would be required internationally to achieve a reduction in animal consumption. As part of the harmonization of the requirements for the validation and implementation of alternative methods, alternative approaches are discussed in international forums and harmonized internationally.

These forums include groups like the Organization for Economic Co-operation and Development (OECD), the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), and the International Cooperation on Alternative Test Methods (ICATM). The overarching aim of these groups is to promote increased international cooperation and coordination in the scientific development, validation, implementation, and regulatory use of alternative approaches.

IN VIVO ASSAYS IN QC

Animal-based in vivo QC assays are typically used for the development and manufacturing of biopharmaceutical products. These include the qualification of eukaryotic cell lines, specific testing for viral clearance of products, demonstration of the absence of pyrogens to ensure patient safety, and the determination of potency of the manufactured biopharmaceuticals.

The majority of in vivo assays used in QC can be avoided without losing the appropriateness of the quality assessments. Following the 3R principles, all in vivo assays should be assessed and replaced or reduced. This would also help meet increasing regional and global sustainability goals. Novel analytical/QC technologies enable the replacement of existing in vivo assays while ensuring the efficacy of the active pharmaceutical ingredient and the absence of contamination that poses a risk to patients.

CELL BANK TESTING

In most cases, complex biopharmaceutical molecules are manufactured using eukaryotic expression systems (typically using recombinant Chinese hamster ovary [CHO] cells). To guarantee a robust manufacturing process and a constant product quality over a long period of time, so-called cell banks are used.

Cell banking is the process of preserving and storing cells for future manufacturing purposes. It typically involves a two-tier system, establishing a master cell bank first and a working cell bank from a single cell source second, expanding the cells, and cryopreserving them for long-term storage. It is crucial to comprehensively characterize and test these cell banks of the recombinant cell lines to ensure identity, stability, performance, quality, and safety.

To guarantee the quality and safety of the cell banks, various tests must be carried out to demonstrate the absence of a contamination caused by "adventitious agents" in the cell bank(s). Traditionally, in vivo assays have been used to detect viral contamination in cell banks.

In Vivo Virus Detection Assay

The in vivo virus detection assay [5] is used to detect viral contamination that could contaminate mammals—the CHO host cells but also patients. For this purpose, the cell bank cell culture fluid is injected into adult and suckling mice, as well as embryonated eggs. Depending on the source and nature of the manufacturing cell lines, additional animal species, (e.g., hamsters) may need to be tested. After injection, the health of the animals is monitored, and any abnormality is investigated to determine the cause. For the assessment of one cell bank using the in vivo virus detection assay, 75 mice (15 adult/60 suckling) and 80 embryonated eggs are required.

In Vivo Antibody Production Test

These in vivo assays should be used when the potential exists for exposure to viruses of a specific animal species. For example, murine viruses that are specific to certain rodent mammals can be detected using an immunologically based in vivo assay, the antibody production test. For this purpose, the test article is injected into virus-free, highly susceptible natural hosts (i.e., mice or hamsters) and the antibody level in the serum of the animals is assessed after a specified time using sensitive and specific serological assays. Examples of such tests that are carried out for the cell bank control are the mouse antibody production (MAP) test and the hamster antibody production (HAP) test.

Specific molecular biological assays—for example, nucleic acid amplification techniques (such as the polymerase chain reaction [PCR]) or next-generation sequencing (NGS, or massively parallel sequencing [MPS] or high-throughput sequencing [HTS])—can replace the in vivo assays described previously. The revised version of the ICH Guideline Q5A(R2) on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin [5] encourages applicants to use these alternative assays.

Roche has recently implemented a multiplexed degenerated PCR [6] into their control concept that combines the advantages of PCR, speed and sensitivity, with a broad specificity to detect a large number of virus variants. As a result, the species-specific viruses that can be detected by the in vivo MAP and HAP tests can be detected by a PCR-based in vitro assay. In recent years, the introduction of the PCR-based virus detection method reduced the need for animals by up to 90 mice and almost 45 hamsters per year.

PYROGEN TESTING

Pyrogens are fever-inducing substances of various origins, like bacteria (dead or viable), fungi, viruses, or even chemicals. The

innate human immune system reacts to pyrogens by releasing cytokines, which lead to an increase in body temperature (fever) and inflammation. Severe (over)reaction of the innate immune system (adverse reaction) can lead to an unregulated release of cytokines and ultimately to shock, multiple organ failure, and even death. For this reason, only therapeutics that are free of pyrogens may be marketed and health authorities require testing for pyrogens at various points in the production process. Three different tests are carried out for this purpose:

Rabbit Pyrogen Test

Owing to a comparable pyrogen sensitivity in humans and rabbits, the implementation of the in vivo rabbit pyrogen test (RPT) in the 1940s was able to significantly increase the safety of pharmaceutical products. The test is based on the reaction of a rabbit's innate immune system, which, like humans, reacts to pyrogenic substances by increasing body temperature and can therefore detect the majority of pyrogens. This includes endotoxin derived from gram-negative bacteria as well as non-endotoxin pyrogens derived from gram-positive bacteria, fungi, virus, or chemicals.

In the RPT, the product being tested is injected intravenously into the ear vein of rabbits. The temperature of the rabbits is monitored over a defined period of time. Finally, either the sum of the body temperature increase is calculated [7] or the increase of the body temperature of individual rabbits [8] is assessed. If the summed response does not exceed a threshold value (European Pharmacopeia acceptance criterion) or no rabbit shows an individual rise in temperature of 0.5°C or more above its respective control temperature (US Pharmacopeia acceptance criterion), the product meets the requirements for the absence of pyrogens.

The RPT currently consumes a significant number of animals. In the EU, ~50,000 animals were used for this test in 2015 and ~25,000 in 2021 [4]. The assumption for worldwide use is ~400,000 rabbits per year. In response to animal welfare objectives, multinational measures have been initiated in the EU to avoid the RPT. One notable initiative is led by the European Directorate for the Quality of Medicines & HealthCare, which aims to remove the RPT from the European Pharmacopeia [9–12]. This objective involves creating new or revising existing European Pharmacopeia General Chapters and the revision of around 60 monographs to replace the RPT with either the monocyte activation test (MAT) or the bacterial endotoxins test (BET) (described as follows).

Monocyte Activation Test

The monocyte activation test (MAT) [13] is an in vitro assay that mimics the reaction of the human innate immune system to pyrogens. A cell suspension containing monocytic cells (either peripheral blood mononuclear cells [PBMCs] or whole blood) produces cytokines (e.g., interleukins such as IL-1β or IL 6) by activation with pyrogens, and the released cytokines can be detected by an immunological assay (e.g., enzyme-linked immunosorbent assay [ELISA]).

Bacterial Endotoxins Test

Bacterial endotoxins, a subset of pyrogens, are lipopolysaccharides (LPS), a component of the outer cell wall of gram-negative bacteria. Endotoxin is a heat-stable substance and is known to be the most potent pyrogen. Because of these properties and their ubiquitous occurrence, the absence of endotoxin in the final product is of particular importance for pharmaceutical production.

In contrast to the MAT or the RPT, the BET is highly specific for endotoxins. Non-endotoxin pyrogens such as components of gram-positive bacteria, nucleic acids, proteins, or chemical components cannot be detected by the BET. Two different versions of the BET are accepted by the health authorities: the Limulus amoebocyte lysate–based BET (LAL assay) and the recombinant Factor C-based BET (rFC assay).

LAL assay

For almost 50 years, the LAL-based BET [14, 15] has been used for the determination of endotoxins, which rely on the blood from ancient and endangered horseshoe crabs (*Limulus polyphemus* or *Tachypleus tridentatus*). This test is based on the amoebocytes in the horseshoe crab's "blood," which provide the animals' natural defense mechanism against bacterial endotoxins and other pathogens. When the amoebocytes come into contact with bacterial endotoxins, an enzyme cascade is activated, which finally leads to clot formation of the LAL. Different variants of the LAL-based BET were developed: the gel clot assay and end-point or kinetic versions of a turbidimetric and a chromogenic assay.

rFC assay

The basis for this BET [16] is a synthetic rFC, the first enzyme in the LAL enzyme cascade. The activation of rFC by endotoxins leads to cleavage of a synthetic fluorogenic substrate present in the test system and thus to a fluorescent end product that can be quantified. The advantage of the rFC-based BET is that no animal-sourced reagent of an endangered species, such as the horseshoe crab, is required.

Avoiding In Vivo Pyrogen Tests

To avoid in vivo assays or the use of animal-sourced reagents for pyrogen testing, we have initiated several projects, which are discussed in the following sections.

Replacing the in vivo RPT with the BET

An analytical variation was submitted to replace the in vivo RPT assay with the LAL-based BET. It is one of our legacy products for which the RPT was originally filed many years ago as the pyrogen test method to release produced batches. The rationales for replacing the in vivo RPT were a) improved microbial control of the manufacturing process to prevent any unintentional introduction of pyrogens into the manufacturing process and b) prior knowledge based on hundreds of batches produced and released that demonstrated that the product itself was not pyrogenic. The analytical variation for lot release testing was accepted by the