Strategy for removing or replacing the rabbit pyrogen test: New pyrogenicity strategy of the European Pharmacopoeia Commission September 2022

Introduction

The Council of Europe's *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* was opened for signature in 1986. Since that time, the European Pharmacopoeia (Ph. Eur.) Commission and its experts have carried out a programme of work committed to Replacing, Reducing and Refining (3Rs) the use of animals for test purposes. At EU level, the use of animals to test medicinal products is strongly discouraged under EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

At its 170th session in June 2021, the Ph. Eur. Commission took the decision to engage on a path that should ultimately lead to the complete replacement of the rabbit pyrogen test (RPT) in the Ph. Eur. within approximately 5 years.

The Ph. Eur. test for pyrogens (general chapter *2.6.8*) consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined. It was first published in the Ph. Eur. in 1986.

The majority of pyrogens are bacterial endotoxins. These can be detected using the bacterial endotoxin test (BET) described in Ph. Eur. chapters *2.6.14. Bacterial endotoxins and 2.6.32. Test for bacterial endotoxins using recombinant factor C*. However, in some cases, non-endotoxin pyrogens may also be present and these are not detected by the BET. A test covering all types of pyrogens is therefore required to confirm the absence of non-endotoxin pyrogens.

General chapter *2.6.30. Monocyte-activation test* (MAT) was added to the Ph. Eur. in 2009, providing an *in vitro* alternative to the RPT that is capable of detecting both endotoxin and non-endotoxin pyrogens. The publication of this chapter was a significant step forward in terms of animal welfare, in accordance with the Council of Europe's *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes*.

However, despite the publication of the MAT chapter and multiple efforts since then to encourage developers to apply the MAT instead of the RPT, rabbits continue to be used extensively to detect pyrogenic substances.

The complete removal of the RPT from the Ph. Eur. is therefore necessary if the aim is to move towards the exclusive use of *in vitro* tests (i.e. BET or MAT) for the control of pyrogenicity (endotoxins and non-endotoxins).

There are currently 60 Ph. Eur. texts – covering diverse topics such as vaccines for human use, blood products, antibiotics, radiopharmaceuticals and containers – that refer to the RPT and are

affected. This document presents the Ph. Eur. strategy that will lead, for all these texts, to the replacement of the test for pyrogens with a suitable *in vitro* alternative, ultimately resulting in the complete elimination of the RPT.

New pyrogenicity strategy

1. Introducing a new general chapter, *5.1.13. Pyrogenicity*

The aim is for this new text to provide guidance to users for the selection and implementation of a suitable test to control the pyrogenicity of their products (BET or MAT), and to justify this choice. The guidance will cover the risk assessment required to support the use of the BET as the sole pyrogenicity test, based on the knowledge of the product (e.g. raw materials, manufacturing process, etc.), taking into consideration any factor that could result in the inclusion of pyrogens not detected by the BET. Some of these aspects were already covered in general chapters *5.1.10. Guidelines for using the test for bacterial endotoxins* and *2.6.30. Monocyte-activation test*; relevant information has therefore been extracted from these chapters and incorporated into the new general chapter. General chapters *5.1.10* and *2.6.30* have been revised accordingly.

New general chapter *5.1.13. Pyrogenicity* will include references to the current general chapters in which methods that can be used for the control of pyrogenicity are described (*2.6.14. Bacterial endotoxins*, *2.6.30. Monocyte-activation test*, *2.6.32. Test for bacterial endotoxins using recombinant factor C*).

2. Revision of Ph. Eur. texts in Which the RPT is mentioned

2.1 **General monograph *Substances for pharmaceutical use (2034)***: The general monograph applies to all substances for pharmaceutical use (i.e. active substances and excipients). The requirements for “Bacterial endotoxins” and “Pyrogens” have been replaced with a new single requirement for “Pyrogenicity”. The new requirement refers to new general chapter *5.1.13. Pyrogenicity* which provides guidance for the selection and implementation of a suitable test for pyrogenicity (BET or MAT, *see section 1 above*). The reference to general chapter *2.6.8. Pyrogens* (RPT) has been deleted.

In addition, the statement on labelling has been updated to reflect that substances labelled “pyrogen-free” comply with the MAT described in general chapter *2.6.30*.

2.2 **Individual monographs on substances for pharmaceutical use**: The strategy followed for individual monographs on substances is directly linked to the proposed revision of the general monograph *Substances for pharmaceutical use (2034)*. The requirement for “Pyrogens” and the reference to the RPT (under Tests) have been deleted from all the individual monographs on substances for pharmaceutical use that prescribed it. As a result, the new requirements for pyrogenicity in the revised general monograph *Substances for pharmaceutical use (2034)* will apply.

2.3 **Dosage form monographs *Parenteral preparations (0520) and Intravesical preparations (2811)***: the requirement for “Bacterial endotoxins – Pyrogens” has been replaced with a new requirement for “Pyrogenicity”, referring to new general chapter *5.1.13. Pyrogenicity* which provides guidance for the selection and implementation of a suitable test for pyrogenicity (BET or MAT), and the reference to general chapter *2.6.8* has been deleted. In addition, the statement on labelling in monograph *0520* has been updated to reflect that preparations labelled as apyrogenic comply with the MAT.

2.4 **Dosage form monograph *Preparations for irrigation (1116)* and the monograph *Solutions for organ preservation (1264)***: The requirement for “Pyrogens” has been replaced with a new requirement for “Pyrogenicity”, referring to new general chapter *5.1.13. Pyrogenicity*, and the reference to general chapter *2.6.8* has been deleted. To avoid redundancies, the requirement for “Bacterial endotoxins” and reference to general chapter *2.6.14. Bacterial endotoxins* have also been removed, as the methods given in *2.6.14* will be referred to in the new chapter *5.1.13*. In both monographs, the limit for pyrogenicity is expressed in endotoxin equivalents per mL, obtained from the previous limit in the BET.

2.5 **Vaccines for human use**: Diverse cases were encountered in human vaccine monographs, with the RPT variously prescribed as a final lot release test, a test on an intermediate, a test to be conducted during product development or as a process validation requirement. In this context, the control of pyrogenicity for vaccines was addressed through a horizontal approach, focusing on the revision of transversal texts (i.e. general monograph *Vaccines for human use (0153)*, general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use*, and monograph on *3-O-Desacyl-4'-monophosphoryl lipid A (MPL) (2537)* (adjuvant)), complemented as necessary with specific considerations at the level of individual vaccine monographs.
General monograph *0153*, which applies to all vaccines for human use, became the pivotal element of the pyrogenicity strategy for vaccines. The requirement for “Bacterial endotoxins” in the Tests section of the general monograph has been replaced with a new requirement for “Pyrogenicity”, referring to new general chapter *5.1.13* which provides guidance for the selection and implementation of a suitable test for pyrogenicity (BET or MAT). In addition, a statement has been introduced under “General provisions” in the Production section to stress the need to characterise pyrogenicity during development studies and to consider the impact of subsequent manufacturing changes on pyrogenicity.
Further to the revision of general monograph *0153*, monographs on individual vaccines have been revised to delete references to the RPT. As a result, the new requirements for pyrogenicity in the revised general monograph *0153* will apply.
In general chapter *5.2.11* on carrier proteins, the requirement for “Bacterial endotoxins” has been replaced with a new requirement for “Pyrogenicity”, referring to new chapter *5.1.13*. The provisions of the new pyrogenicity requirement in general chapter *5.2.11* apply to all carrier proteins. In consequence, the RPT, described for *N. meningitidis* group B outer membrane protein complex (OMP), could be deleted.
Finally, in the monograph on MPL (*2537*), the RPT, conducted on the trimethylamine salt of MPL, has been replaced by the MAT.

2.6 **Animal immunosera for human use**: General monograph *0084 Immunosera for human use, animal* applies to all animal sera for human use. In revised general monograph 0084, the requirement for “Pyrogens” has been replaced with a new requirement for “Pyrogenicity”, referring to new chapter *5.1.13*.

2.7 **Blood products**: In blood product monographs that include the RPT (17 texts), the requirement for “Pyrogens (*2.6.8*) or Bacterial endotoxins (*2.6.14*)” has been replaced with a new requirement for “Pyrogenicity”, referring to the new chapter *5.1.13. Pyrogenicity*.
A limit for pyrogenicity expressed in endotoxin equivalents per mL or endotoxin equivalents per IU has been introduced in 16 of the texts. This limit was obtained from the previous limit in the BET (for 15 texts), or based on 5 IU of endotoxin/kg rabbit body mass as the pyrogenic dose, taking into account the injection volume (for 1 text).

2.8 **Large-volume parenterals**: Monographs on large-volume parenterals that prescribed the RPT have been revised in a similar fashion as the monographs on blood products (*see section 2.7 above*), i.e. the requirement for "Pyrogens" has been replaced with a new requirement for "Pyrogenicity", referring to new general chapter *5.1.13. Pyrogenicity*.
In the monographs on *Glucose (0177)* and *Glucose monohydrate (0178)*, a limit for pyrogenicity expressed in endotoxin equivalents per mg has been introduced, based on 5 IU of endotoxin/kg rabbit body mass as the pyrogenic dose, taking into account the injection volume. In the monographs on *Haemodialysis, solutions for (0128)*, *Haemofiltration and haemodiafiltration, solutions for (0861)*, and *Peritoneal dialysis, solutions for (0862)*, a limit for pyrogenicity expressed in endotoxin equivalents per mL has been introduced. This limit was obtained from the previous limit in the BET.

2.9 **Containers** (Section 3 of the Ph. Eur.): The two general chapters that prescribed the RPT (*3.3.4. Sterile plastic containers for human blood and blood components and 3.3.7. Sets for the transfusion of blood and blood components*) have been revised in a similar fashion as the monographs on blood products (*see section 2.5 above*), i.e. the requirement for "Pyrogens" has been replaced with a new requirement for "Pyrogenicity", referring to new chapter *5.1.13. Pyrogenicity*.

2.10 **Radiopharmaceuticals**: The general monograph *Radiopharmaceutical preparations (0125)* has been revised in accordance with the changes made to other general monographs that prescribed the RPT (requirement for "Bacterial endotoxins – Pyrogens" replaced with a new requirement for "Pyrogenicity", referring to new general chapter *5.1.13. Pyrogenicity*. In addition, the MAT has replaced the RPT as a test that may be prescribed when the nature of the radiopharmaceutical preparation results in interference in the BET and it is not possible to eliminate the interfering factors.

A list of the impacted texts and the corresponding Ph. Eur. groups of experts/working parties is provided in the Appendix.

3. Revision of general chapters 2.6.30 and 5.1.10

As previously mentioned, general chapters *2.6.30* and *5.1.10* have also been revised further to the elaboration of new general chapter *5.1.13. Pyrogenicity* as certain parts of these two texts had been moved to new chapter *5.1.13*.

In addition, it should be noted that a separate general revision of *2.6.30* (major technical revision of the chapter) has been carried out to ensure that the text reflects the experience accrued with the MAT since 2010 and to fix certain issues reported by users. Major resulting changes include a new description of sensitivity, revised validity criteria for the endotoxin standard curve, the merging of Methods A and B into a single semi-quantitative test and revised assay acceptance criteria. In view of the planned suppression of the RPT from the Ph. Eur., revision of the MAT chapter is critical to keep the chapter up to date and support all users. Revised chapter *2.6.30* was released for public consultation in *Pharmeuropa* 34.2 (April 2022). The public consultation for all the other texts included in this project is planned for *Pharmeuropa* 35.1 (January 2023).

4. Additional considerations

Importantly, the introduction of new Ph. Eur. general chapter *5.1.13* and the revisions of the above-mentioned Ph. Eur. texts do not call into question strategies involving the BET that are already used by manufacturers to control the pyrogenicity of their products and have been authorised by

the competent authority, nor is it intended to prompt a retrospective assessment of pyrogenicity. A statement to underline this has been included in the explanatory notes accompanying new general chapter *5.1.13* and all the Ph. Eur. texts revised as part of this project.

Timelines

This 5-year project officially started in June 2021, with the endorsement by the Ph. Eur. Commission of a way towards the deletion of the RPT from the Ph. Eur. It is considered that a further 4 years will be necessary to complete the suppression of the RPT from the Ph. Eur. The table below gives a *tentative* schedule for publication in *Pharmeuropa* and the envisaged implementation dates for all the Ph. Eur. texts impacted.

Assuming that all the elaborations and revisions take place according to schedule, by July 2025 all references to *2.6.8* will have been removed from these texts, although the chapter itself will still be included in the Ph. Eur. Deletion of the chapter will then ensue (by 1 July 2026).

Text	Publication in Pharmeuropa	Envisaged implementation date
New general chapter *5.1.13*	Jan 2023 (Pharmeuropa 35.1)	July 2025
Revision of general chapter 5.1.10	Jan 2023 (Pharmeuropa 35.1)	July 2025
Revision of general chapter 2.6.30	Apr 2022 (Pharmeuropa 34.2)	July 2024
Revision of general monograph 2034	Jan 2023 (Pharmeuropa 35.1)	July 2025
Revision of dosage form monograph 0520	Jan 2023 (Pharmeuropa 35.1)	July 2025
Revision of other Ph. Eur. texts referencing the RPT	Jan 2023 (Pharmeuropa 35.1)	July 2025
Suppression of general chapter 2.6.8		July 2026

Appendix

Text number	Title	Type of text	Group of experts / Working Party
TEXTS REFERRING TO GENERAL CHAPTER *2.6.8. PYROGENS*			
2034	*Substances for pharmaceutical use*	General monograph	BET (Bacterial Endotoxin Test)
1290	*Amikacin sulfate*	Individual monograph	7 (Antibiotics)
0709	*Chloramphenicol sodium succinate*	Individual monograph	7 (Antibiotics)
0319	*Colistimethate sodium*	Individual monograph	7 (Antibiotics)
0663	*Dicloxacillin sodium*	Individual monograph	7 (Antibiotics)
0668	*Flucloxacillin sodium*	Individual monograph	7 (Antibiotics)
0033	*Kanamycin acid sulfate*	Individual monograph	7 (Antibiotics)
0032	*Kanamycin monosulfate*	Individual monograph	7 (Antibiotics)
0203	*Polymyxin B sulfate*	Individual monograph	7 (Antibiotics)
1296	*Calcium levulinate dihydrate*	Individual monograph	10D (Organic chemistry – synthetic and semi-synthetic products)
0412	*Sodium citrate*	Individual monograph	9 (Inorganic Chemistry)
0177	*Glucose*	Individual monograph	CRB (Carbohydrates)
0178	*Glucose monohydrate*	Individual monograph	CRB (Carbohydrates)
2811	*Intravesical preparations*	Dosage form monograph	12 (Dosage forms and pharmaceutical technical procedures)
0520	*Parenteral preparations*	Dosage form monograph	12 (Dosage forms and pharmaceutical technical procedures)
1116	*Preparations for irrigation*	Dosage form monograph	12 (Dosage forms and pharmaceutical technical procedures)
1264	*Solutions for organ preservation*	Individual monograph	12 (Dosage forms and pharmaceutical technical procedures)
0125	*Radiopharmaceutical preparations*	General monograph	14 (Radiopharmaceutical Preparations)
0084	*Immunosera for human use, animal*	General monograph	15 (Human Vaccines and Sera)
2062	*Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)
1932	*Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)
1933	*Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)

Text number	Title	Type of text	Group of experts / Working Party
2067	*Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) andhaemophilus type b conjugate vaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)
2065	*Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugatevaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)
2066	*Diphtheria, tetanus, pertussis (whole cell), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)
2622	*Haemophilus type b and meningococcal group C conjugate vaccine*	Individual monograph	15 (Human Vaccines and Sera)
1219	*Haemophilus type b conjugate vaccine*	Individual monograph	15 (Human Vaccines and Sera)
1056	*Hepatitis B vaccine (rDNA)*	Individual monograph	15 (Human Vaccines and Sera)
3066	*Meningococcal group A, C, W135 and Y conjugate vaccine*	Individual monograph	15 (Human Vaccines and Sera)
2112	*Meningococcal group C conjugate vaccine*	Individual monograph	15 (Human Vaccines and Sera)
0250	*Meningococcal polysaccharide vaccine*	Individual monograph	15 (Human Vaccines and Sera)
2150	*Pneumococcal polysaccharide conjugate vaccine (adsorbed)*	Individual monograph	15 (Human Vaccines and Sera)
0966	*Pneumococcal polysaccharide vaccine*	Individual monograph	15 (Human Vaccines and Sera)
0216	*Rabies vaccine for human use prepared in cell cultures*	Individual monograph	15 (Human Vaccines and Sera)
1375	*Tick-borne encephalitis vaccine (inactivated)*	Individual monograph	15 (Human Vaccines and Sera)
2537	*3-O-Desacyl-4'-monophosphoryl lipid A*	Individual monograph	15 (Human Vaccines and Sera)
5.2.11.	*Carrier proteins for the production of conjugated polysaccharide vaccines for human use*	General chapter	15 (Human Vaccines and Sera)
3.3.4.	*Sterile plastic containers for human blood and blood components*	General chapter	16 (Plastic materials, plastic containers and closures)
3.3.7.	*Sets for the transfusion of blood and blood components*	General chapter	16 (Plastic materials, plastic containers and closures)
0024	*Human fibrinogen*	Individual monograph	6B (Human Plasma and Plasma Products)
0209	*Anticoagulant and preservative solutions for human blood*	Individual monograph (MP)	6B (Human Plasma and Plasma Products)

Text number	Title	Type of text	Group of experts / Working Party
0255	*Human albumin solution*	Individual monograph	6B (Human Plasma and Plasma Products)
0275	*Human coagulation factor VIII*	Individual monograph	6B (Human Plasma and Plasma Products)
0338	*Human normal immunoglobulin for intramuscular administration*	Individual monograph	6B (Human Plasma and Plasma Products)
0554	*Human prothrombin complex*	Individual monograph	6B (Human Plasma and Plasma Products)
0878	*Human antithrombin III concentrate*	Individual monograph	6B (Human Plasma and Plasma Products)
0918	*Human normal immunoglobulin for intravenous administration*	Individual monograph	6B (Human Plasma and Plasma Products)
1223	*Human coagulation factor IX*	Individual monograph	6B (Human Plasma and Plasma Products)
1224	*Human coagulation factor VII*	Individual monograph	6B (Human Plasma and Plasma Products)
1644	*Human coagulation factor XI*	Individual monograph	6B (Human Plasma and Plasma Products)
1646	*Human plasma (pooled and treated for virus inactivation)*	Individual monograph	6B (Human Plasma and Plasma Products)
1928	*Anti-T lymphocyte immunoglobulin for human use, animal*	Individual monograph	6B (Human Plasma and Plasma Products)
2298	*Human von Willebrand factor*	Individual monograph	6B (Human Plasma and Plasma Products)
2387	*Human α-1-proteinase inhibitor*	Individual monograph	6B (Human Plasma and Plasma Products)
2788	*Human normal immunoglobulin for subcutaneous administration*	Individual monograph	6B (Human Plasma and Plasma Products)
2818	*Human C1-esterase inhibitor*	Individual monograph	6B (Human Plasma and Plasma Products)
0128	*Haemodialysis, solutions for*	Individual monograph	DIA (Dialysis)
0861	*Haemofiltration and haemodia-filtration, solutions for*	Individual monograph	DIA (Dialysis)
0862	*Peritoneal dialysis, solutions for*	Individual monograph	DIA (Dialysis)
2.6.30	*Monocyte-activation test*	General chapter	BET (Bacterial Endotoxin Test)
NEW TEXT			
5.1.13	*Pyrogenicity*	General chapter	BET (Bacterial Endotoxin Test)
OTHER TEXTS IMPACTED			
5.1.10	*Guidelines for using the test for bacterial endotoxins*	General chapter	BET (Bacterial Endotoxin Test)
0153	*Vaccines for human use*	General monograph	15 (Human Vaccines and Sera)
2.6.8	*Pyrogens*	General chapter	BET (Bacterial Endotoxin Test)