European Directorate for the Quality of Medicines & HealthCare

Council of Europe

European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

Phasing out of the Rabbit Pyrogen Test (RPT)

Implementation in Europe: the regulatory perspective

Questions raised by stakeholders

Dr. Emmanuelle Charton, Head of Division B, European Pharmacopeia Department

EDQM, Council of Europe

Wednesday, 25 February 2026

FAQs - Suppression of the RPT from the Ph. Eur.

/... / General Chapters and Monographs

PYROGENICITY

- General chapter 5.1.13 entered into force on 1 July 2025. Does this new chapter apply to new products only?
- What methods should users employ now that the rabbit pyrogen test (RPT) is no longer an official Ph. Eur. test?
- What changes were made to general chapter 2.6.14. Bacterial endotoxins?
- Does the Ph. Eur. favour limulus amoebocyte lysate (LAL)-based methods over the recombinant factor C (rFC)-based method?
- I would like to use recombinant factor C (rFC), do I need to perform a validation?
- I want to use recombinant factor C (rFC) for my bacterial endotoxins test (BET): do I have to validate its equivalence against Methods A-F?
- What are the benefits of recombinant factor C (rFC)?
- What is the status of recombinant cascade reagents (rCR) in the Ph. Eur.?
- Does the revised version of general chapter 2.6.14. Bacterial endotoxins depart from the internationally harmonised version?

https://faq.edqm.eu/display/FAQS/PYROGENICITY

FAQs - Suppression of the RPT from the Ph. Eur.

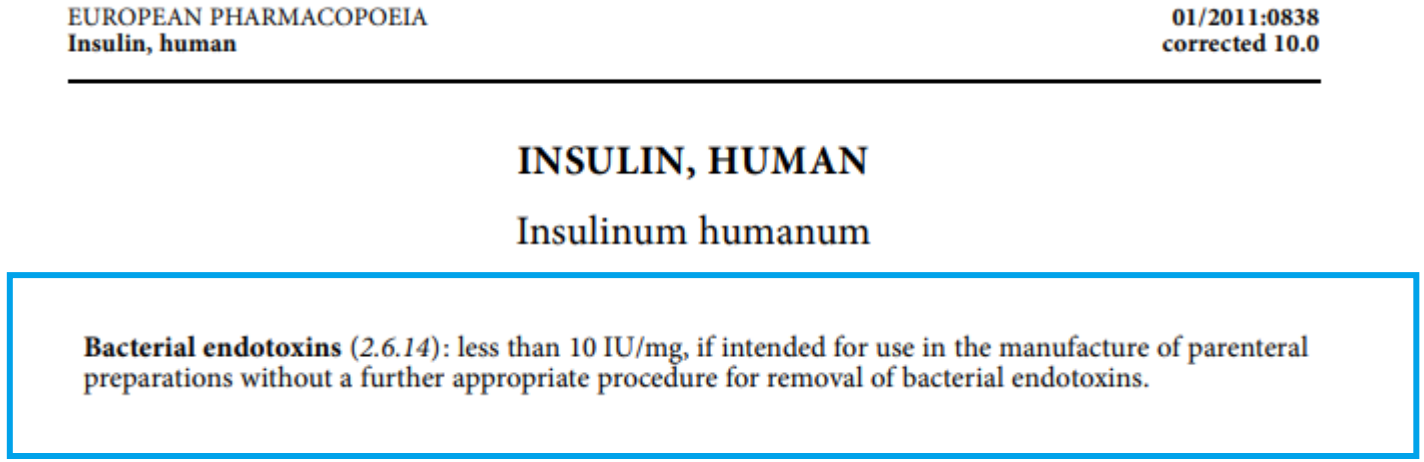
- **General chapter *5.1.13* entered into force on 1 July 2025. Does this new chapter apply to new products only?**
- No. General chapter *5.1.13. Pyrogenicity* is referenced in several monographs, including the general monographs *Substances for pharmaceutical use* (*2034*) and *Parenteral preparations* (*0520*), and applies to both new and existing products.

Follow-up question (not part of EDQM FAQs)

- **In the *Strategy for Removing or Replacing the Rabbit Pyrogen Test: New Pyrogenicity Strategy of the European Pharmacopoeia Commission* (published in September 2022) the following is stated: *"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."* The reply provided in the first FAQ seems to contradict the wording above. Could general chapter *5.1.13* be used to justify a retrospective assessment of pyrogenicity?**
- The FAQs should be considered complementary to the other documents published on this topic—such as *Strategy for Removing or Replacing the Rabbit Pyrogen Test: New Pyrogenicity Strategy of the European Pharmacopoeia Commission* (September 2022) - and not viewed separately from them. The additional considerations outlined in the *Strategy for Removing or Replacing the Rabbit Pyrogen Test: New Pyrogenicity Strategy of the European Pharmacopoeia Commission* remain valid.

Suppression of the RPT from the Ph. Eur. : retrospective assessment of pyrogenicity

Case study A: **a substance for pharmaceutical use covered by individual Ph. Eur. monograph**, marketing authorisation dossier of the substance **with BET** as specification attribute was approved by the competent authority; what is the impact of the new pyrogenicity strategy?



EUROPEAN PHARMACOPOEIA
Insulin, human

01/2011:0838
corrected 10.0

INSULIN, HUMAN

Insulinum humanum

Bacterial endotoxins (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

No retrospective assessment of pyrogenicity is required to determine whether the Bacterial Endotoxin Test (BET) is sufficient

228 individual monographs in this case

Suppression of the RPT from the Ph. Eur. : retrospective pyrogenicity assessment

Case study B: **a substance for pharmaceutical use not covered by individual Ph. Eur. monograph,** marketing authorisation dossier of the substance was approved by the competent authority; what is the impact of the new pyrogenicity strategy?

SUBSTANCES FOR PHARMACEUTICAL USE

Corpora ad usum pharmaceuticum

No longer in force

Bacterial endotoxins (*2.6.14*). The substance for pharmaceutical use complies with the test for bacterial endotoxins if it is labelled as a bacterial endotoxin-free grade or if it is intended for use in the manufacture of parenteral preparations or preparations for irrigation without a further appropriate procedure for the removal of bacterial endotoxins. The limit, when not indicated in the individual monograph, is determined in accordance with the recommendations of general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins.*

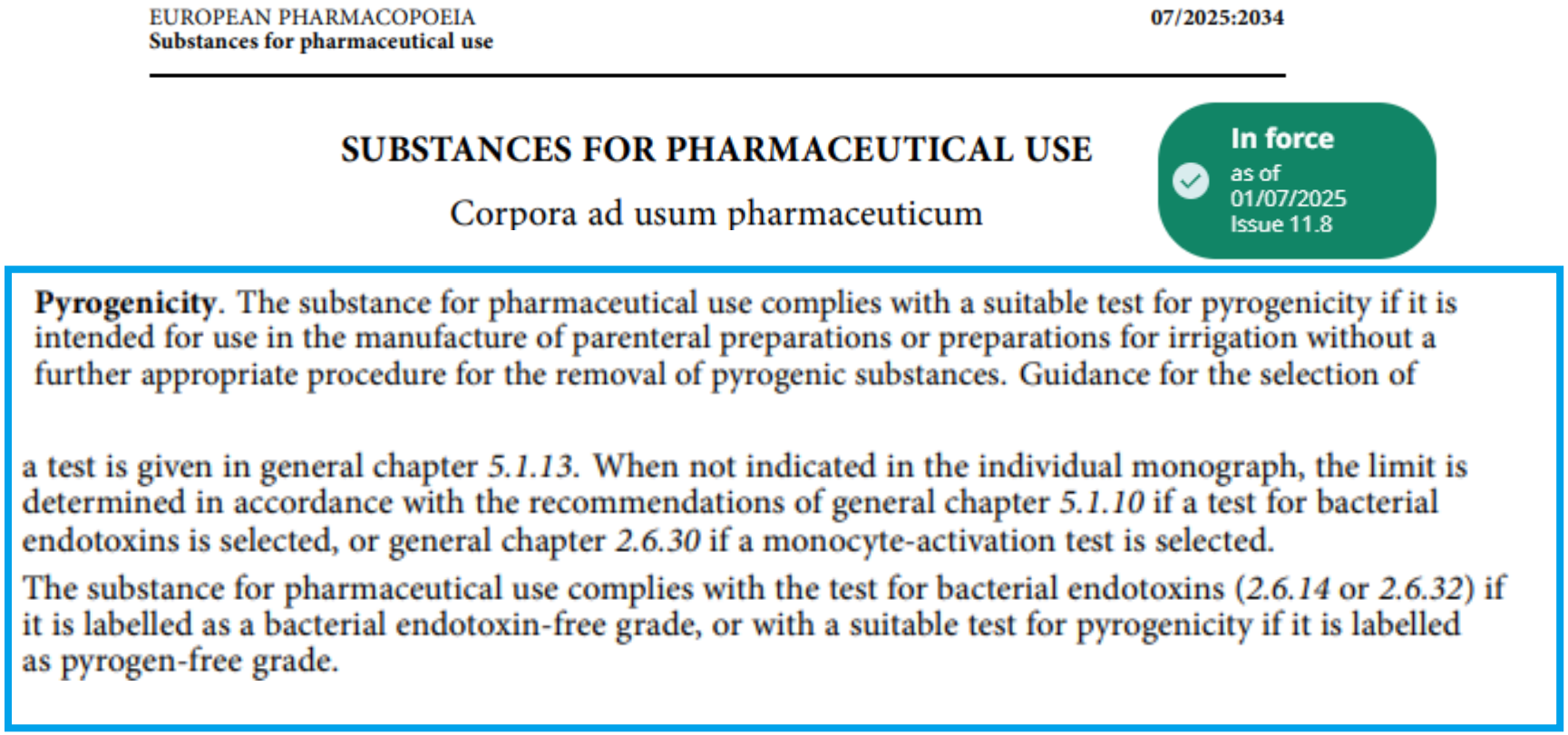
Pyrogens (*2.6.8*). If the test for pyrogens is justified rather than the test for bacterial endotoxins and if a pyrogen-free grade is offered, the substance for pharmaceutical use complies with the test for pyrogens. The limit and analytical procedure are stated in the individual monograph or approved by the competent authority. Based on appropriate test validation for bacterial endotoxins and pyrogens, the test for bacterial endotoxins may replace the test for pyrogens.

Case 1:
BET as specification attribute

Case 2:
a. Pyrogens as specification attribute
b. BET is used as pyrogenicity test and had already been approved

Suppression of the RPT from the Ph. Eur. : Retrospective pyrogenicity assessment

Case study B : a **substance for pharmaceutical use not covered by individual Ph. Eur. monograph,** marketing authorisation dossier of the substance was approved by the competent authority; what is the impact of the new pyrogenicity strategy?



EUROPEAN PHARMACOPOEIA
Substances for pharmaceutical use

07/2025:2034

SUBSTANCES FOR PHARMACEUTICAL USE

Corpora ad usum pharmaceuticum

In force
as of
01/07/2025
Issue 11.8

Pyrogenicity. The substance for pharmaceutical use complies with a suitable test for pyrogenicity if it is intended for use in the manufacture of parenteral preparations or preparations for irrigation without a further appropriate procedure for the removal of pyrogenic substances. Guidance for the selection of

a test is given in general chapter *5.1.13*. When not indicated in the individual monograph, the limit is determined in accordance with the recommendations of general chapter *5.1.10* if a test for bacterial endotoxins is selected, or general chapter *2.6.30* if a monocyte-activation test is selected.

The substance for pharmaceutical use complies with the test for bacterial endotoxins (*2.6.14* or *2.6.32*) if it is labelled as a bacterial endotoxin-free grade, or with a suitable test for pyrogenicity if it is labelled as pyrogen-free grade.

Case 1:
BET as specification attribute
No retrospective assessment is required to determine whether the BET is sufficient

Case 2:
a. Pyrogens as specification attribute
replacement is required
b. BET is used as pyrogenicity test and has already been approved
No retrospective assessment of pyrogenicity is required

Suppression of the RPT from the Ph. Eur. retrospective pyrogenicity assessment

Case study C: **a parenteral preparation not covered by individual Ph. Eur. monograph**, marketing authorisation dossier of the substance was approved by the competent authority; what is the impact of the new pyrogenicity strategy?

EUROPEAN PHARMACOPOEIA 07/2025:0520
Parenteral preparations

No longer in force

PARENTERAL PREPARATIONS

Parenteralia

Bacterial endotoxins - pyrogens. Parenteral preparations for human use, if applicable after reconstitution or dilution, comply with the test for bacterial endotoxins (*2.6.14*) or, where justified and authorised, with the test for pyrogens (*2.6.8*). Recommendations on the limits for bacterial endotoxins are given in general chapter *5.1.10*. The limit for intravitreal preparations is expressed per eye.

Where the label states that the preparation is free from bacterial endotoxins or that it is apyrogenic, the preparation complies with the test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*), respectively.

Parenteral preparations for veterinary use comply with the test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*) when the volume to be injected in a single dose is 15 mL or more and is equivalent to a dose of 0.2 mL or more per kilogram of body mass.

Case 1: BET as specification attribute

Case 2: Pyrogens as specification attribute

Suppression of the RPT from the Ph. Eur. : retrospective pyrogenicity assessment

Case study C: **a parenteral preparation not covered by individual Ph. Eur. monograph**, marketing authorisation dossier of the substance was approved by the competent authority; what is the impact of the new pyrogenicity strategy?

PARENTERAL PREPARATIONS

Parenteralia

Pyrogenicity. Parenteral preparations for human use, if applicable after reconstitution or dilution, comply with a suitable test for pyrogenicity. Guidance for the selection of a test is given in general chapter *5.1.13*. The limit, when not indicated in the individual monograph, is determined in accordance with the recommendations of general chapter *5.1.10* when a test for bacterial endotoxins is selected or general chapter *2.6.30* when a monocyte-activation test is selected. The limit for intravitreal preparations is expressed per eye.

Where the label states that the preparation is free from bacterial endotoxins, the preparation complies with the test for bacterial endotoxins (*2.6.14* or *2.6.32*). Where the label states that the preparation is apyrogenic, the preparation complies with the monocyte-activation test (*2.6.30*).

Parenteral preparations for veterinary use comply with a suitable test for pyrogenicity when the volume to be injected in a single dose is 15 mL or more and is equivalent to a dose of 0.2 mL or more per kilogram of body mass. Guidance for the selection of a test is given in general chapter *5.1.13*.

In force
as of 01/07/2025
Issue 11.8

Case 1: BET as specification attribute
No retrospective assessment is required to determine whether the BET is sufficient

Case 2: Pyrogens as specification attribute
A replacement is required

Conclusion case studies

For all these cases it is considered that there is no contradiction between the FAQ reply and the ***New Pyrogenicity Strategy of the European Pharmacopoeia Commission*** **(published in September 2022),** as compliance with Ph. Eur. would be maintained

FAQs - Suppression of the RPT from the Ph. Eur.

- **What methods should users employ now that the rabbit pyrogen test (RPT) is no longer an official Ph. Eur. test?**
- Recommended methods include:
  - Monocyte-activation test (MAT) (general chapter *2.6.30*) for broad pyrogen detection;
  - Bacterial endotoxins test (BET) using:
    - limulus amoebocyte lysate (LAL)-based methods (general chapter *2.6.14*);
    - recombinant factor C (rFC) (previously general chapter *2.6.32*, in general chapter *2.6.14*. as of Issue 13.1).

  According to general chapter *5.1.13. Pyrogenicity*, users must apply a risk-based approach if selecting the test for bacterial endotoxins as the sole method to assess pyrogenicity.

  Please also consult the EMA's Quality of medicines questions and answers: Part 1 European Pharmacopeia (Ph. Eur.) - Phasing out Rabbit Pyrogen Test

Thank you for your attention!

More information

www.edqm.eu

https://go.edqm.eu/Newsletter

Follow us on

edqm

@edqm_news

EDQMCouncilofEurope

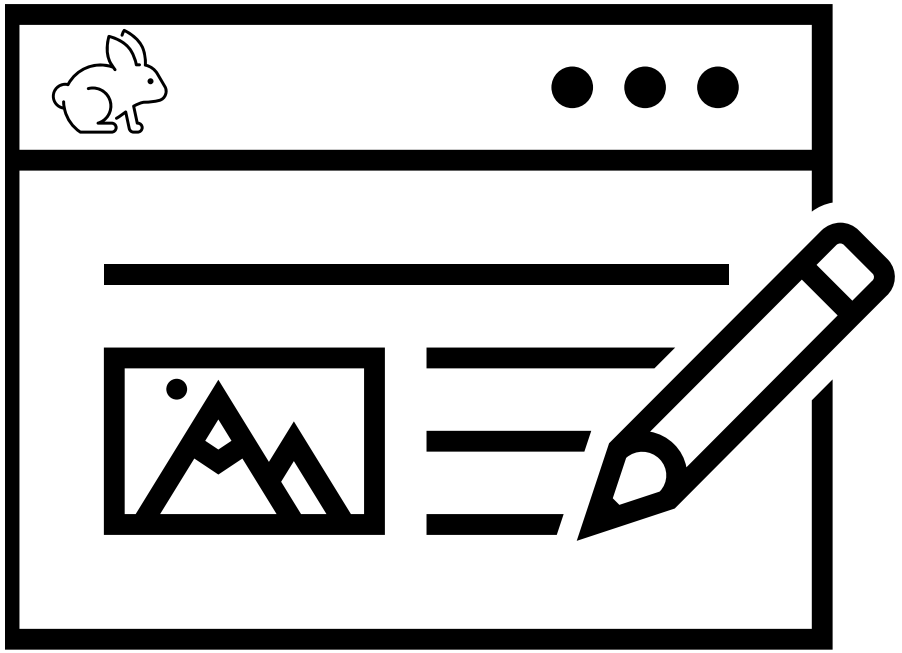
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

EMA
EUROPEAN MEDICINES AGENCY

Phasing out of the rabbit pyrogen test – Procedural aspects

How to update your dossier?



25th February 2026

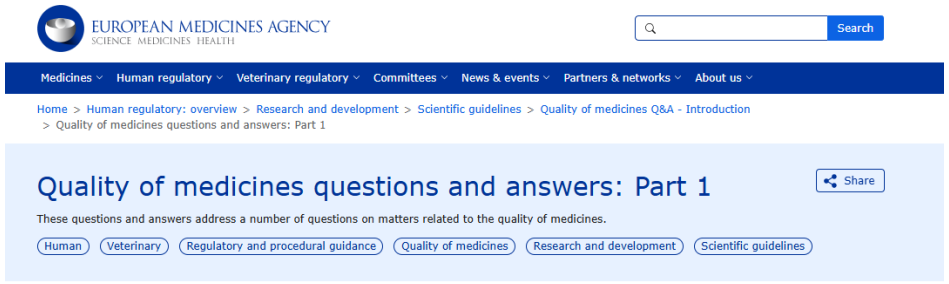
EDQM-EPAA Symposium: Pyrogen testing 2.0: Ethical, Evolving and Eco-friendly

Ph.Eur. phasing out of rabbit pyrogen testing

- Suppression of *2.6.8 Pyrogens (***1 January 2026***)*
- New general chapter: *5.1.13 Pyrogenicity,* referring to:
  - 2.6.30*. Monocyte-activation test*
  - 2.6.14*. Bacterial endotoxins*
  - 2.6.32*. Test for bacterial endotoxins using recombinant factor C*
- Possibility to rely only on endotoxin testing based on risk assessment
- The revised texts published in Supplement 11.8 of the Ph. Eur., with an **implementation date of 1 July 2025**.

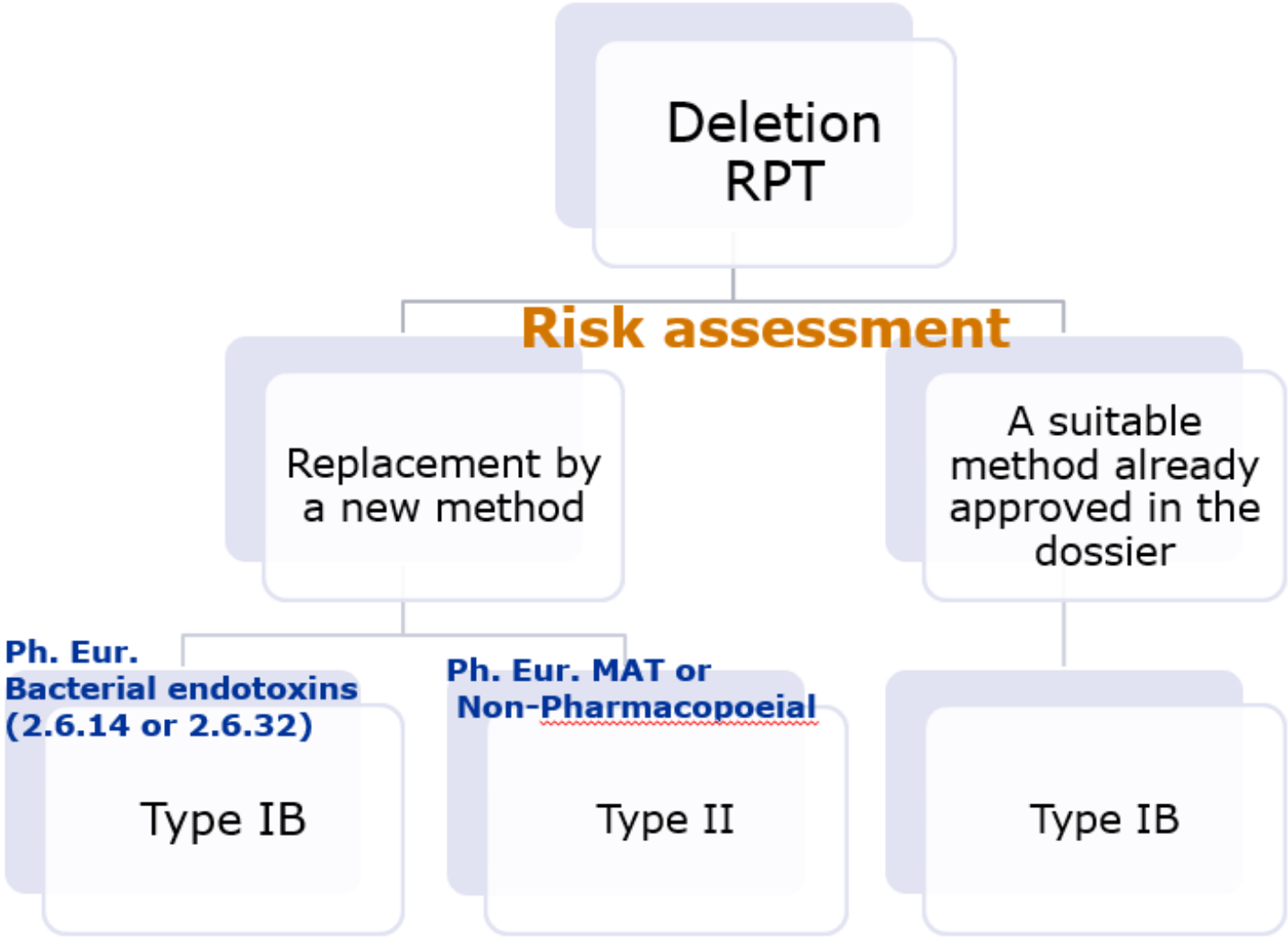
Guidance

- **Published guidance:**
  - Q&A (EMA Website)



[Quality of medicines questions and answers: Part 1 | European Medicines Agency (EMA)]

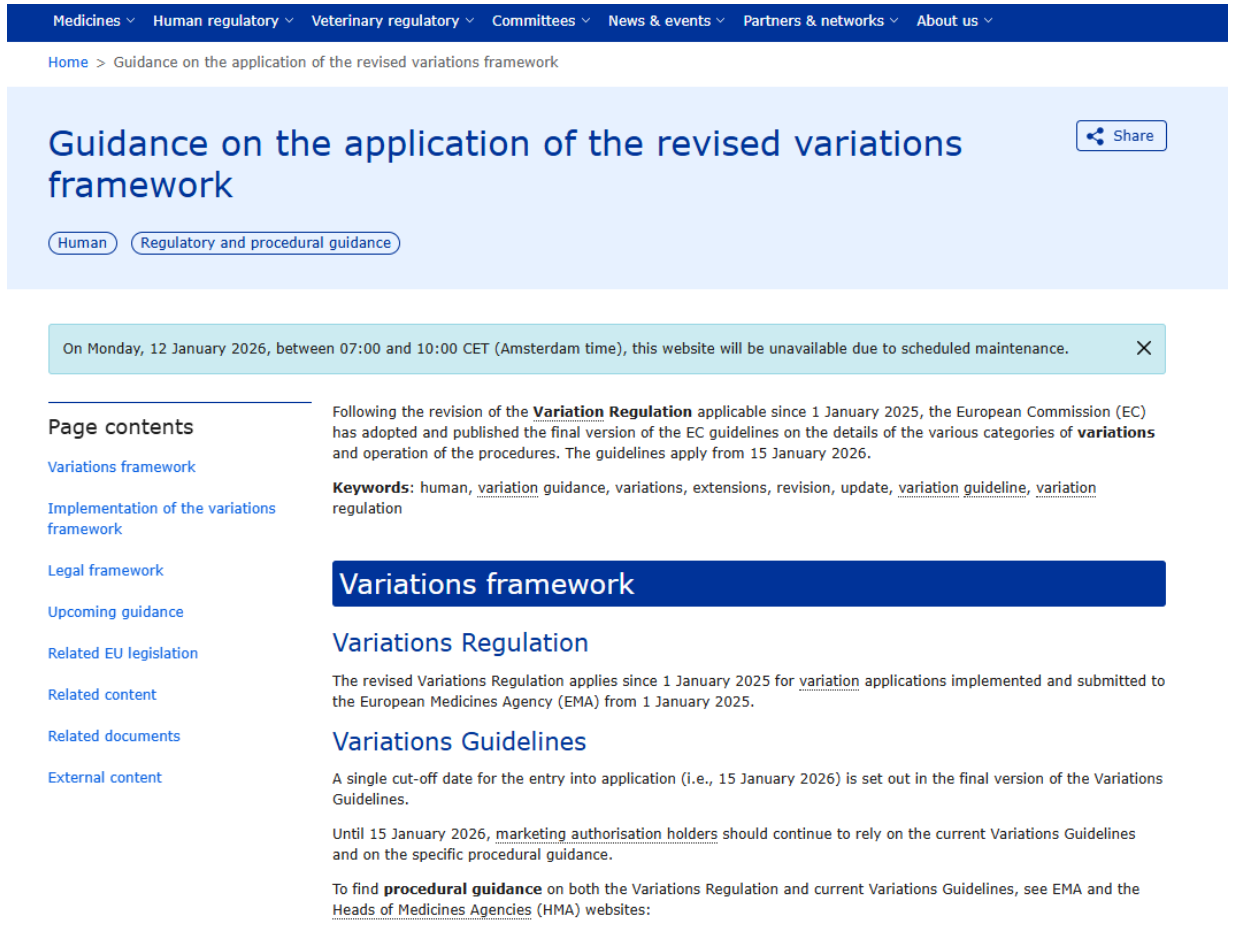
Guidance - Decision Tree



Guidance - Possible scopes
Variations framework

- Following the revision of the Variation Regulation applicable since 1 January 2025, the European Commission (EC) has adopted and published the **final version of the EC guidelines on the details of the various categories of variations** and operation of the procedures.
- The guidelines **apply from 15 January 2026**

- EUR-Lex - 52025XC05045 - EN - EUR-Lex
- Guidance on the application of the revised variations framework | European Medicines Agency (EMA)



Guidance - Possible scopes

- Q.I.b.1.z - Change in the specification attribute and/or acceptance criteria of an **active substance, starting material / intermediate / reagent** used in the manufacturing process of the active substance – Other changes
- Q.II.d.1.z - Change in the specification attribute and/or acceptance criteria of the **finished product** – Other changes
- Q.II.c.1.z - Change in the specification attribute and/or acceptance criteria of an **excipient** – Other changes

✓ …not exhaustive list

✓ “z” scopes to facilitate the submission - clearly identifying in the precise scope if it relates a replacement by a new method or a deletion without replacement (reliance on a suitable approved method).

Examples received

(B.II.c.2.c) - *Substantial change to or replacement of a biological/ immunological/ immunochemical test method or a method using a biological reagent* - **Addition of the compendial Monocyte Activation Test** (MAT, Ph. Eur. chapter 2.6.30) **to replace the Rabbit Pyrogen Testing (RPT) for an excipient** (adjuvant system)

Compendial excipient (revision of its monograph)

- The proposed acceptance criteria for MAT testing with its rationale are provided
- The proposed change covers the validation of a compendial MAT method:
    - validation parameters are tested (according to Ph. Eur. 2.6.30. method)
    - results of the validation are provided in the method validation report
- POSITIVE outcome: The validation met the acceptance criteria for all analysed parameters. The method validation results confirmed that MAT is a reliable and suitable method for determination of the pyrogenicity of the sample.

Examples received

(B.I.b.1.z) - *Change in the specification parameters and/or limits of an active substance, starting material / intermediate / reagent used in the manufacturing process of the active substance* – **Addition of the Ph.Eur. bacterial endotoxin test (LAL) to replace the Rabbit Pyrogen Testing (RPT) on an intermediate of the active substance**

- The change is based on regulatory requirements (compliant with European regulations), well-controlled manufacturing process robustness/consistency and historical manufacturing data. No rabbit Pyrogens test is required for this intermediate (only BET)
- POSITIVE outcome: the proposed change is in accordance with 3R principles, with current guideline recommendations and sufficient purification/eliminations steps in the manufacturing have been demonstrated. A significant number of manufacturing batches supports the claim of a well-controlled process.

- **REC -** In line with the 3Rs approach, the MAH should investigate the feasibility of replacing the Ph. Eur. LAL assay with Recombinant endotoxin testing and update EMA of the progress.

AskEMA example

- RPT and BET are approved - if endotoxins method is already approved in the AS specification as an additional test?
- if compliance with updated Ph.Eur. monograph (which removes the requirement for RPT) is implemented within 6 months?

Need to notify the authorities?

Yes!

Specifications - relying only on the endotoxin testing needs to be supported by a **risk assessment** (done by the variation type IB).

Conclusions

- Enabling the switch from animal testing to *in-vitro* alternatives is a priority for All, including Regulators

- Regulatory tools and guidance to enable the change

Run Away! | Cottontail rabbit escapes! | Eric Sonstroem | Flickr

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

Thank you

filipa.sameiro@ema.europa.eu

Follow us

25.02.2026

EMA 3RS WORKING PARTY SUPPORTING REGULATORY INTEGRATION OF SCIENTIFICALLY VALID 3RS TESTING APPROACHES

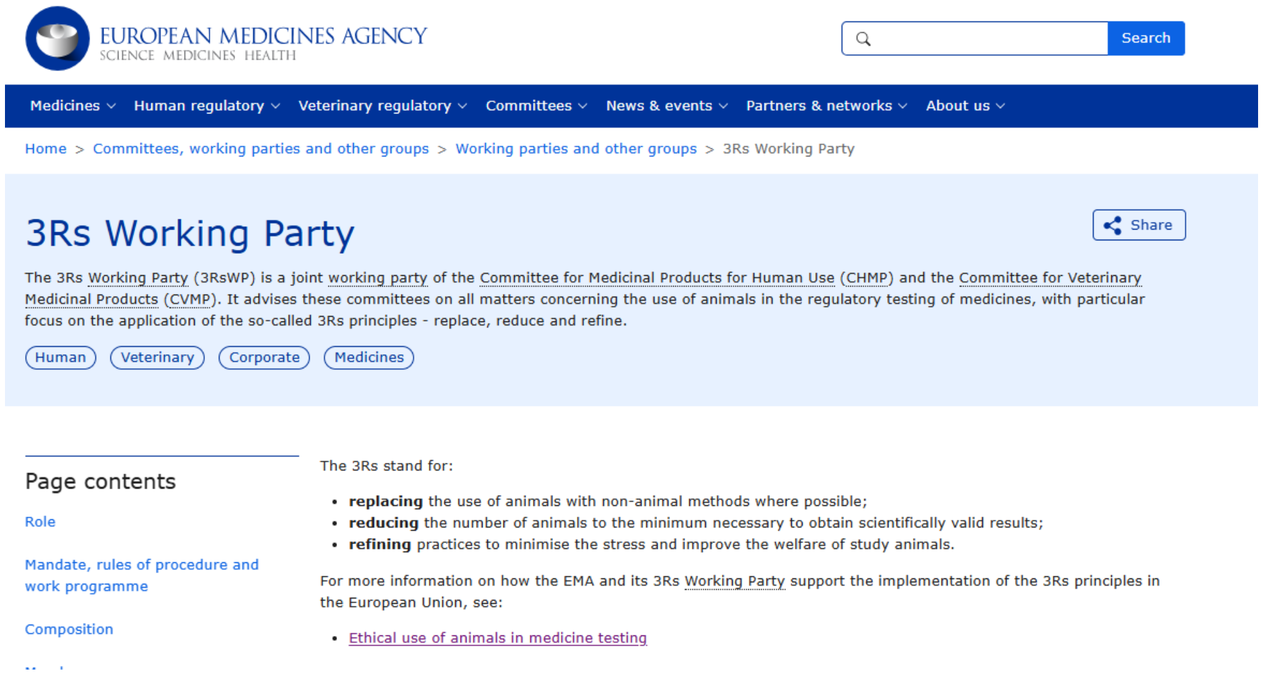
Sonja Beken
Coordinator Non-Clinical Evaluators, FAMHP
Chair 3Rs Working Party, EMA

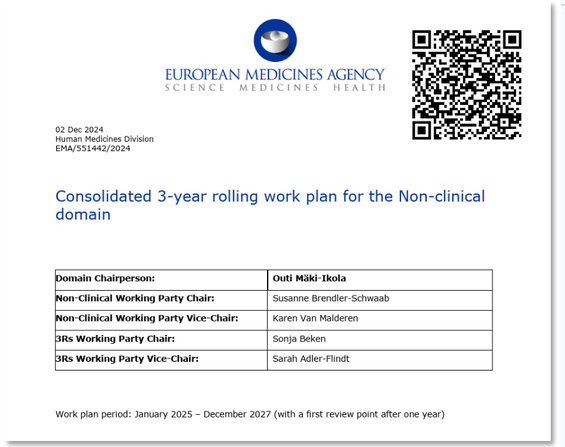
famhp

.be

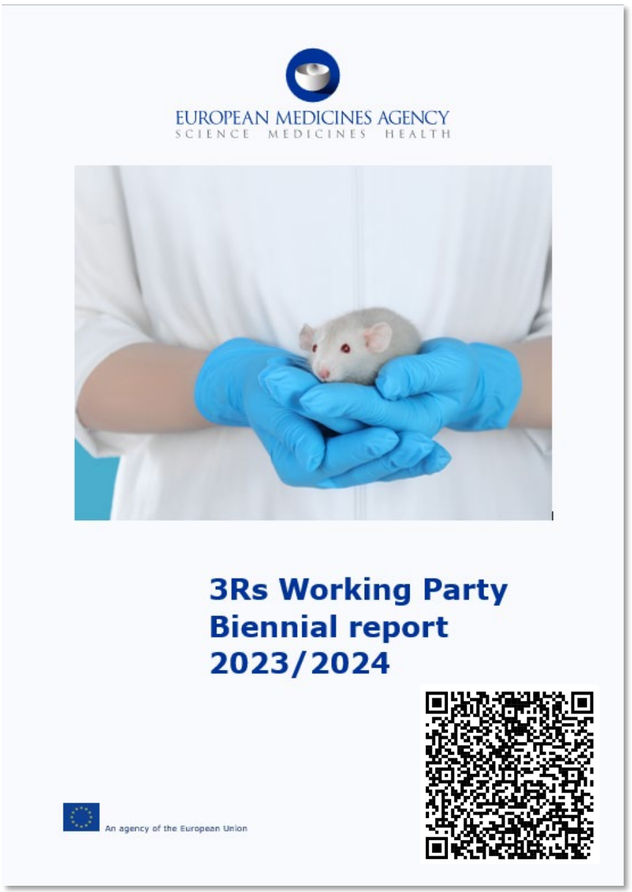
INTRODUCTION

EMA AND THE 3RS





Contact 3RsWP:
3Rs@ema.europa.eu



EMA 3RsWP webpage
https://www.ema.europa.eu/en/committees/working-parties-other-groups/chmp/3rs-working-party

EMA webpage on "Ethical Use of Animals"
https://www.ema.europa.eu/en/human-regulatory-overview/research-development/ethical-use-animals-medicine-testing

EMA webpage on "Regulatory acceptance of NAMs"
https://www.ema.europa.eu/en/human-regulatory-overview/research-development/ethical-use-animals-medicine-testing/regulatory-acceptance-new-approach-methodologies-nams-reduce-animal-use-testing

EMA COLLABORATES TO FOSTER THE 3RS

3RsWP

- Annual Stakeholder meeting
- Workplan consultation
- Reflection papers 3Rs opportunities – public consultation
- Webpage on regulatory acceptance
- ESEC to engage academia

Interaction Mechanisms

- ITF
- Scientific Advice
- Qualification
- Portfolio and Technology meetings
- Voluntary data submission

see EMA webpage:

EMA collaborative fora

- EPAA
- HESI
- IMRWG3Rs
- Scientific meetings and conferences
- EC Roadmap

European Commission

edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

efpia
animalhealth europe
medicines for europe
better access. better health.
AESGP
EUCROF
European CRO Federation
ACRO
ASSOCIATION OF CLINICAL RESEARCH ORGANIZATIONS
AVC
Association of Veterinary Consultants
EuropaBio
The European Association for Bioindustries
Vaccines Europe
An industry for healthy lives
European Animal Research Association
EUCOPE
European Confederation of Pharmaceutical Entrepreneurs AISB
Access VETMED
epaa
The European Partnership for Alternative Approaches to Animal Testing
efsa
EUROPEAN FOOD SAFETY AUTHORITY
ECHA
EUROPEAN CHEMICALS AGENCY
OECD
PETA SCIENCE CONSORTIUM INTERNATIONAL e.V.
Advancing 21st Century Toxicology
NC 3Rs
National Centre for the Replacement Refinement & Reduction of Animals in Research
ECEAE
THE EUROPEAN COALITION TO END ANIMAL EXPERIMENTS
Cruelty Free INTERNATIONAL
EUROGROUP FOR ANIMALS
Humane World for Animals
ASPIS
EUROOCS
innovative health initiative

3RWPS ANNUAL STAKEHOLDER MEETINGS

3RsWP BRT Operational Expert Group :

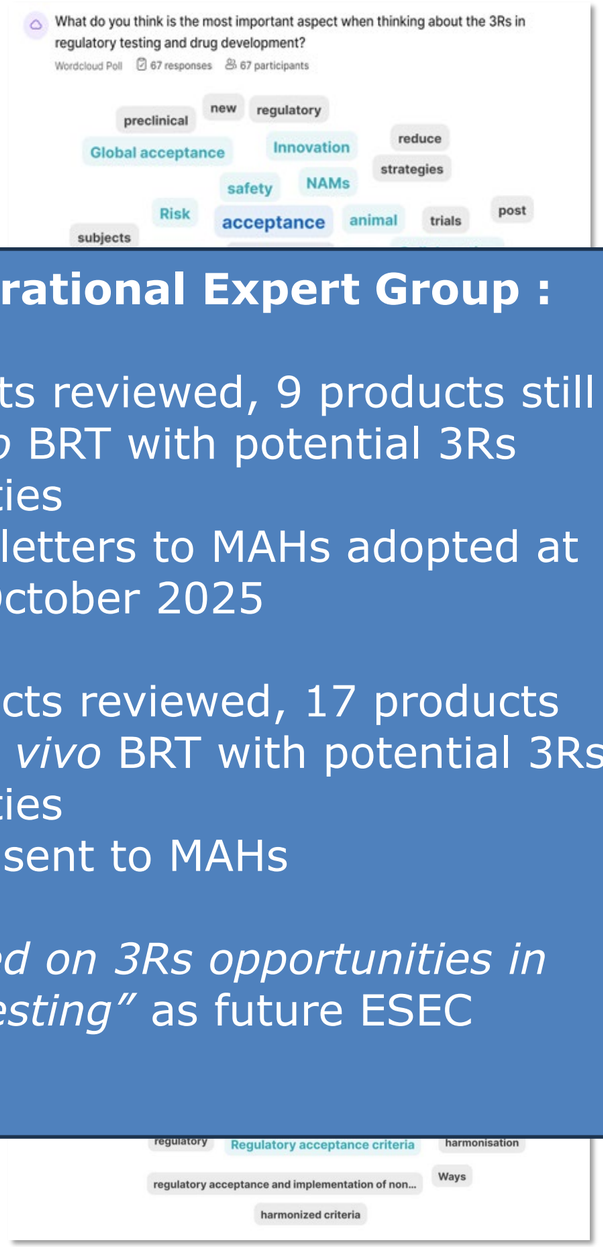
VMPs:

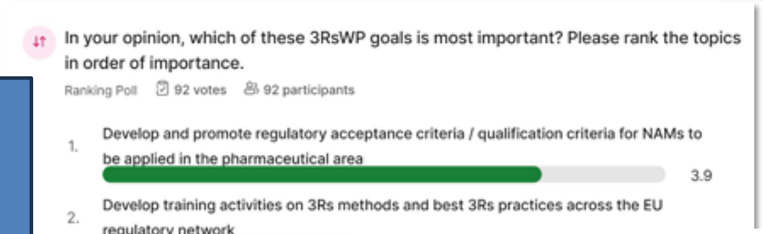
- 67 products reviewed, 9 products still use *in vivo* BRT with potential 3Rs opportunities
- Response letters to MAHs adopted at CVMP in October 2025

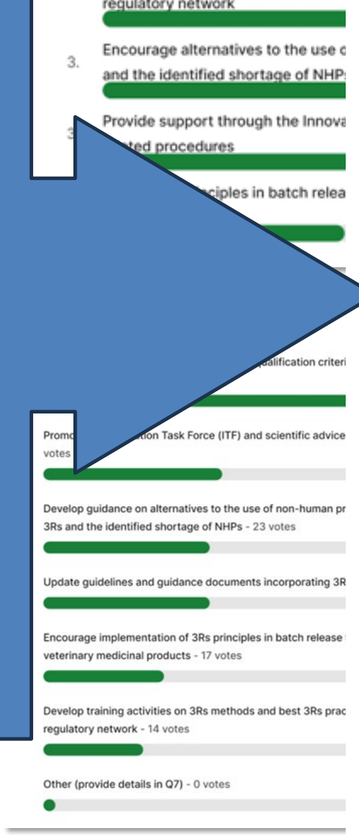
HMPs:

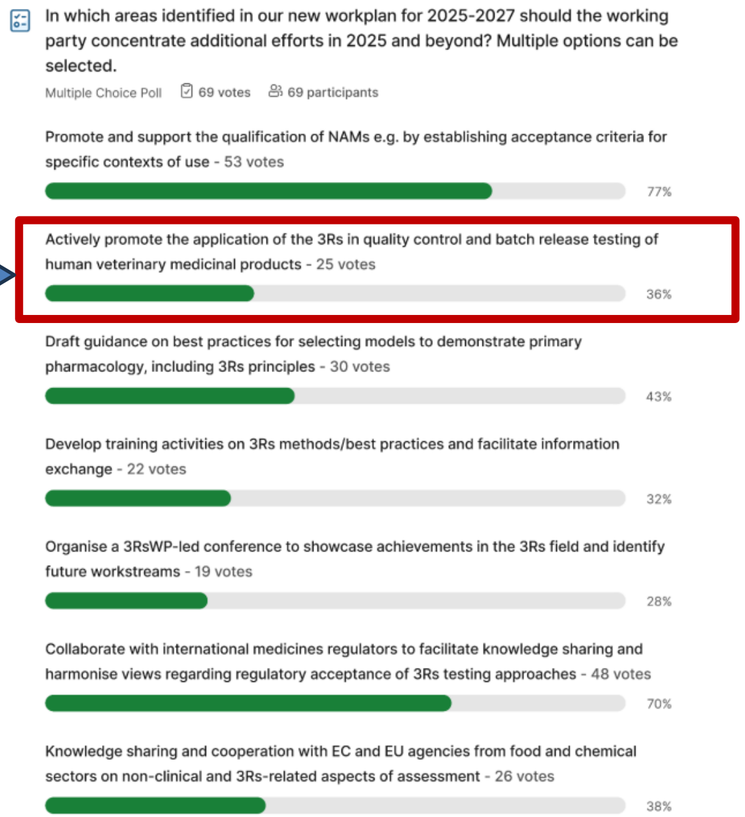
- 281 products reviewed, 17 products still use *in vivo* BRT with potential 3Rs opportunities
- Letters to sent to MAHs

"Lessons learned on 3Rs opportunities in batch release testing" as future ESEC webinar







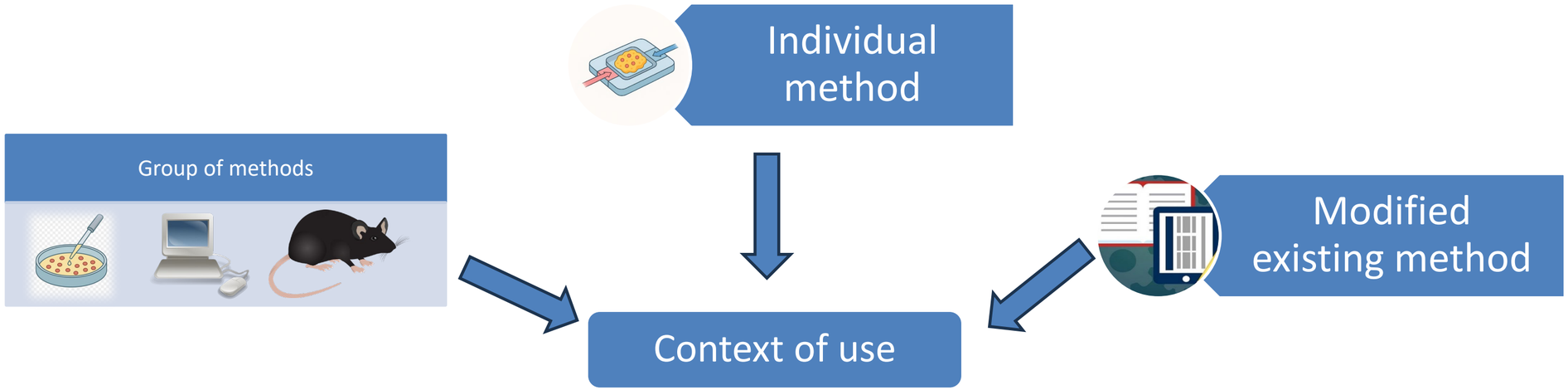


REGULATORY ACCEPTANCE OF 3Rs TESTING APPROACHES

NEW APPROACH METHODOLOGIES OR NAMs

WHAT IN A 3Rs NAME?

Novel Approach Methods or NAMs *are innovative test methods or testing approaches aimed to progressively replace, reduce, and refine (3Rs) traditional animal studies. These include in vitro, in silico, in chemico, ex vivo, refined in vivo and other advanced biology-based approaches and structured combinations of these (e.g. Weight of Evidence approaches). NAMs are designed to generate data that provide an equivalent or improved translation (as compared to current test methods) to support human or target species-relevant regulatory decision making.*



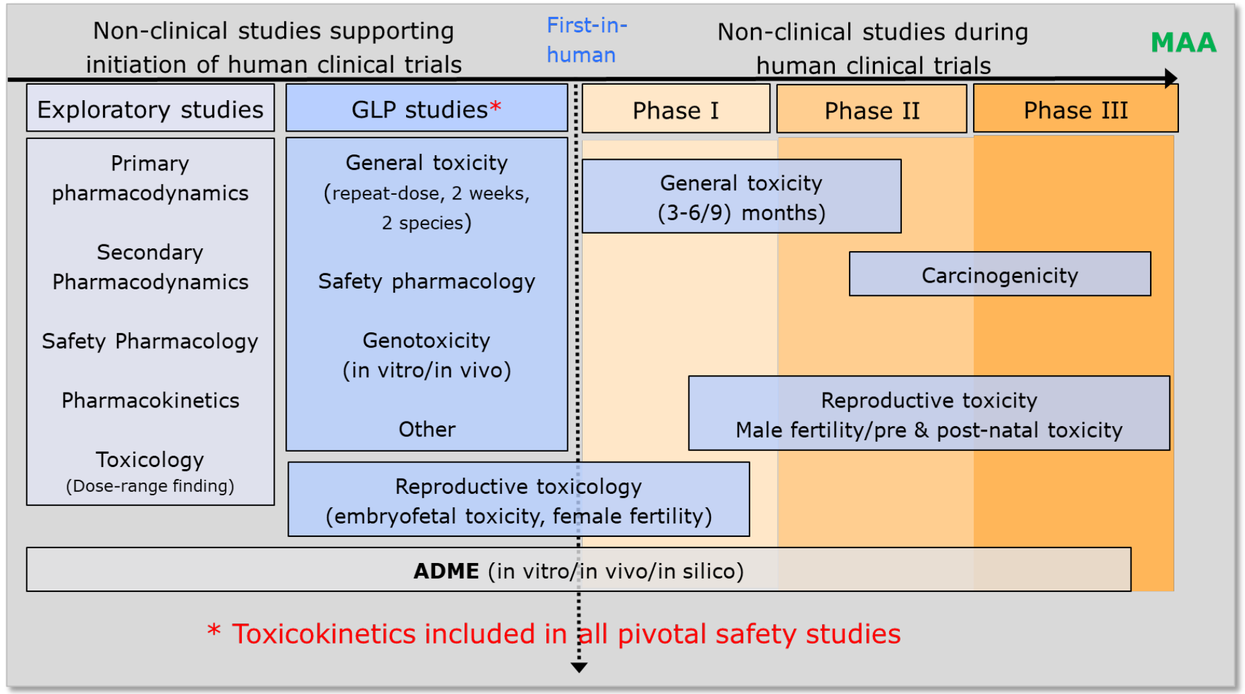
WHAT IS REGULATORY ACCEPTANCE

Regulatory acceptance refers to
the official recognition by a regulatory authority (such as the EMA) that a method, tool, or dataset is scientifically valid and suitable for use in regulatory decision-making

Context-of-Use

A CoU defines the specific intended application of a NAM in the development or regulatory assessment of medicinal products, and the conditions under which it can inform regulatory decision making.

CONTEXTS OF USE



Context of use	Disease area	Key tissue model	End user
Disease mechanisms	Cancer	Tumor models	Biomedical researchers Clinicians Pharmaceutical industry
	Neurodegenerative diseases	Brain, BBB, neurons, retina	
	Cardiometabolic disorders	Heart, lung, liver, pancreas, vessels, adipose	
	Autoimmune diseases	Immune system, gut, pancreas, neurons, skin	
	Fibrosis	Connective tissues, lung, liver, kidney	
Drug efficacy	Cancer	All types	Industry: pharmaceutical, cosmetics Biomedical researchers
	Neurodegenerative diseases	Brain, BBB, neurons	
	Cardiometabolic disorders	Heart, lung, liver, pancreas, vessels	
	Autoimmune diseases	Immune system, gut	
	Fibrosis	Connective tissues, lung, liver, kidney	
Drug toxicity	All types	ADME pathway (liver, kidney), barrier systems (gut, lung, BBB), heart, brain, immune system	Industry: pharmaceutical, cosmetics Biomedical researchers
Personalized medicine: – Patient stratification (adverse effects, dynamics/resistance, identification of vulnerable population) – Companion diagnostics (responders, disease progression)	Cancer	All types	Pharmaceutical industry Hospitals/clinicians
	Rare diseases	All types	
	Systemic diseases	Multi-organs	
	Autoimmune diseases	Immune system, gut	

Workshop Report

Building Blocks for a European Organ-on-Chip Roadmap

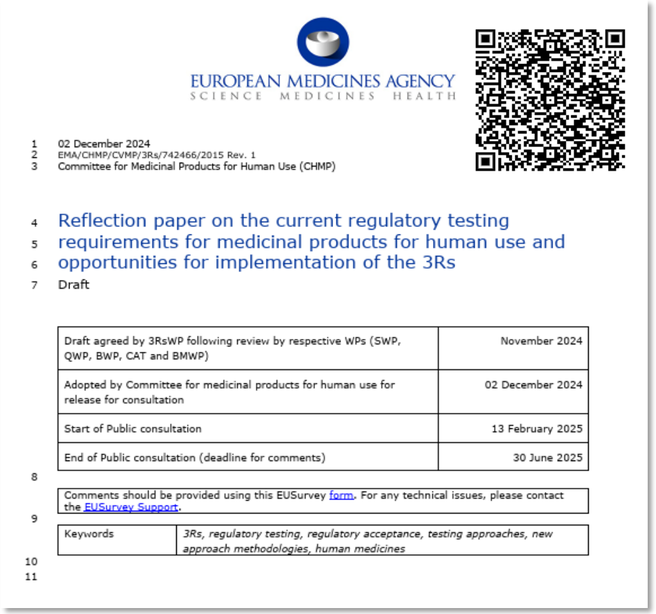
doi:10.14573/altex.1905221

Regulatory acceptance criteria are driven by the COU

Regulatory use of NAMs is diverse:

- *Integration in Weight-of–Evidence approaches (e.g. ICH S1B(R1))*
- *Part of integrated testing strategies (Q&A ICH S7B/E14)*
- *Very limited 1-to-1 replacement*

CONTEXT OF USE



EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

02 December 2024
EMA/CHMP/CVMP/3Rs/742466/2015 Rev. 1
Committee for Medicinal Products for Human Use (CHMP)

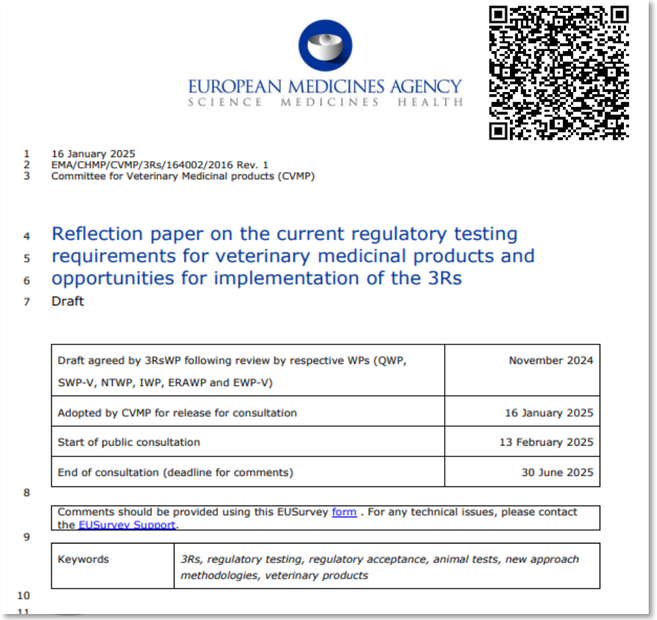
Reflection paper on the current regulatory testing requirements for medicinal products for human use and opportunities for implementation of the 3Rs

Draft

Draft agreed by 3RsWP following review by respective WPs (SWP, QWP, BWP, CAT and BMWP)	November 2024
Adopted by Committee for medicinal products for human use for release for consultation	02 December 2024
Start of Public consultation	13 February 2025
End of Public consultation (deadline for comments)	30 June 2025

Comments should be provided using this EUSurvey form. For any technical issues, please contact the EUSurvey Support.

Keywords	*3Rs, regulatory testing, regulatory acceptance, testing approaches, new approach methodologies, human medicines*



EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

16 January 2025
EMA/CHMP/CVMP/3Rs/164002/2016 Rev. 1
Committee for Veterinary Medicinal products (CVMP)

Reflection paper on the current regulatory testing requirements for veterinary medicinal products and opportunities for implementation of the 3Rs

Draft

Draft agreed by 3RsWP following review by respective WPs (QWP, SWP-V, NTWP, IWP, ERAWP and EWP-V)	November 2024
Adopted by CVMP for release for consultation	16 January 2025
Start of public consultation	13 February 2025
End of consultation (deadline for comments)	30 June 2025

Comments should be provided using this EUSurvey form . For any technical issues, please contact the EUSurvey Support.

Keywords	*3Rs, regulatory testing, regulatory acceptance, animal tests, new approach methodologies, veterinary products*

3.4. CHMP Biologics Working Party and European Pharmacopoeia (Ph. Eur.)

Overview of animal testing requirements for biological medicinal products (Biologics Working Party (BWP) - CHMP)

Topic	Regulatory provision	Animal testing requirements	Implemented 3Rs opportunities	Newly identified opportunities for 3Rs implementation
Pyrogenicity testing of biologicals including vaccines and animal sera for human use	Ph. Eur. chapter 2.6.8 Pyrogens *Note: this chapter will be suppressed from the Ph.Eur. by 1 January 2026* Ph. Eur. chapter 5.1.13 *Note: chapter 5.1.13 is to be implemented 1 July 2025* Ph.Eur. chapter 5.2.11 Carrier proteins for the production of conjugated polysaccharide vaccines for human use. General monograph 'Vaccines for human use' (0153) Ph. Eur. monograph on 3-O-Desacyl-4'-monophosphoryl lipid A (MPL) (2537), and	RPT previously required at various stages of development and as final lot release test.	In June 2021, the European Pharmacopoeia (Ph. Eur.) Commission took the decision to completely replace the RPT 2.6.8 in the Ph. Eur. with MAT (2.6.30) or BET (2.6.14/2.6.32) within approximately 5 years. Subsequently, in June 2024, the Ph. Eur. Commission adopted revised text for 57 monographs, where the RPT has been deleted with an implementation date of 1 July 2025. Accordingly, the requirements to carry out the RPT in monographs for vaccines for human use have been deleted. As a result, the new requirement for pyrogenicity in the revised general monograph 'Vaccines	
	specific monographs on individual vaccines and animal immunosera for human use: Animal immunosera for human use (0084) HepB (1056) Hib (1219) DTaP-Hib (1932) DTaP-IPV-Hib (2065) Hib-MenC (2067) Men PS vaccine (0250) MenC conjugate vaccine (2112)		new requirement in the general monograph 0153 refers to the general chapter 5.1.13 (to be implemented on 1 July 2025), which provides guidance for the selection and implementation of a suitable test for pyrogenicity: MAT (as described in 2.6.30) or BET (as described in 2.6.14/2.6.32). Likewise, the general monograph 'Animal immunosera for human use' (0084) has been revised to replace the requirement to carry out the RPT by a new requirement for pyrogenicity, referring to the general chapter 5.1.13.	

Pyrogenicity Testing

DEMONSTRATION OF SCIENTIFIC VALIDITY

- Defined test methodology (protocol, endpoints)
- Relevance within a particular context of use (including accuracy)
- Context of use (including limitations)
- Reliability/robustness
- Voluntary submission of data (safe harbour)

What was measured, and how was that measurement achieved?

What does that measurement mean? And when can I use it?

Do I get the same results each time?

A powerful oportunity to align regulatory learning

THE COU-DEPENDENT APPROACH
NOT A ONE-FITS-ALL SCHEME

Aspect	NAMs for pivotal safety or quality	NAMs for Efficacy
Risk Tolerance	Very low: human safety and qualifty control requires high certainty	High: Part of larger evidence base, including clinical efficacy data
Evidence Nature	Highly documented, rigorous demonstration of scientific validity and translatability	Best scientific practice/merit. Supports evaluation of efficacy in clinic → often supplementary
Regulatory Expectation	ICH guidelines, PhEUR monographs, product specific (validation) procedures, qualification procedures	Flexible, state-of-the-art should be used
Decision Focus	Risk profiling and mitigation → conservative, qualified methods Quality control → conservative, product-specific validation	Understanding MoA and demonstrating value for clinical exploration → exploratory approaches welcomed

HOW TO ACHIEVE REGULATORY ACCEPTANCE?

Case-by-Case Acceptance in product submissions

- NAMs used for regulatory decision making (e.g., Clinical Trial Applications, Marketing Authorization Application)
- Evaluation based on demonstration of scientific validity/merit and relevance to the medicinal product and target indication

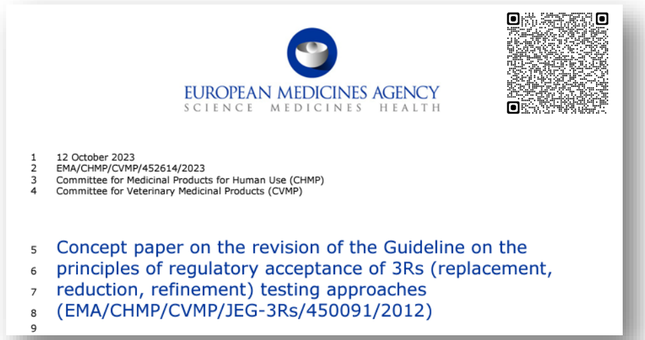

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

15 December 2016
EMA/CHMP/CVMP/JEG-3Rs/450091/2012
Committee for Medicinal Products for Human Use (CHMP)
Committee for Medicinal Products for Veterinary Use (CVMP)

Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches

EMA Qualification for broad NAM use

- A formal EMA process to assess & endorse a novel method for a specific context of use in the development of a medicinal product
- Provides regulatory endorsement for broader application

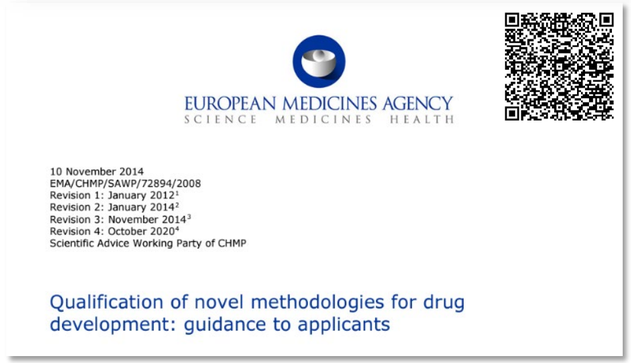

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

12 October 2023
EMA/CHMP/CVMP/452614/2023
Committee for Medicinal Products for Human Use (CHMP)
Committee for Veterinary Medicinal Products (CVMP)

Concept paper on the revision of the Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches (EMA/CHMP/CVMP/JEG-3Rs/450091/2012)

Integration into Guidelines or PhEUR

- NAMs can be incorporated into official guidance (e.g., ICH, EMA, EDQM)
- Examples: ICH M7 allows use of (Q)SAR models for mutagenicity assessment of impurities; PhEUR adopts the MAT and BET as replacement for RPT


EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

10 November 2014
EMA/CHMP/SAWP/72894/2008
Revision 1: January 2012[1]
Revision 2: January 2014[2]
Revision 3: November 2014[3]
Revision 4: October 2020[4]
Scientific Advice Working Party of CHMP

Qualification of novel methodologies for drug development: guidance to applicants

EMA PROCEDURES SUPPORTING REGULATORY ACCEPTANCE OF NAMS

EMA INTERACTION TO FOSTER REGULATORY ACCEPTANCE OF NAMs

Innovation Task Force

- Early Dialogue
- Informal exchange
- Regulatory, technical & scientific topic
- Free of charge
- Vet & Human

Portfolio and technology meetings

- Pharma companies with large medicinal product portfolios
- Issues impacting portfolio progression
- Anticipate scientific & regulatory needs
- Identify innovative technologies

Scientific Advice

- Product Specific or Broad Pipeline
- Formal scientific guidance
- Study design / Methodology
- Vet & Human separate
- Reduced Fees (e.g. SME and academia)

Qualification of a Novel Methodology

- Innovative methods medicines R&D
- Acceptability of a methodology in a specific context of use
- Vet: through SA
- Reduced Fees (e.g. SME and academia)

Safe harbour

- Voluntary data submission
- Independent evaluation
- Builds regulatory confidence & experience
- Support the drafting of CoU based qualification criteria

CONCLUDING REMARKS

Context of Use drives expectations

- Defines how, when, and for what purpose NAM data can be used
- Determines the level of evidence required
- No “one-size-fits-all” approach

Early dialogue is critical

- Early engagement with regulators reduces uncertainty
- Builds shared understanding of scientific validity and relevance
- Fosters integration of NAM data into regulatory decision-making

Scientific validity builds regulatory confidence

- Context of Use
- Clear methodology and endpoints
- Demonstrated relevance and reliability
- Transparent understanding of limitations

Voluntary Data Sharing

- Enables learning through experience
- Supports trust-building between stakeholders
- Helps shape future regulatory testing paradigms fully aligned with the 3Rs

CONTACT

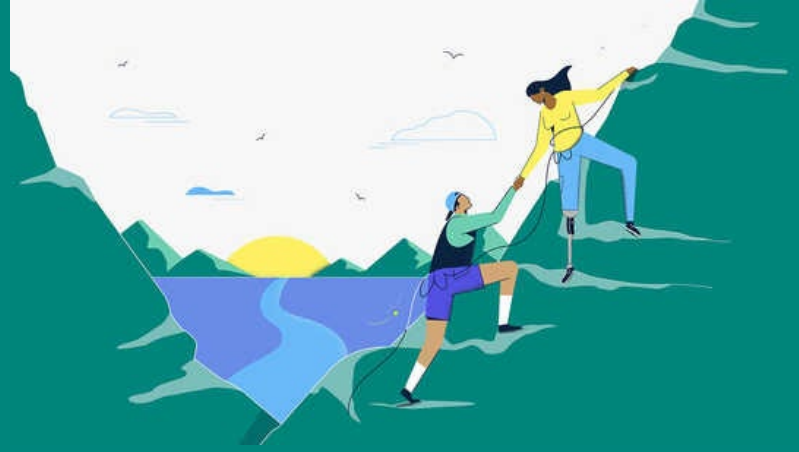
Federal Agency for Medicines and Health Products - FAMHP

Avenue Galilée/Galileelaan 5/03
1210 BRUSSELS
tel. + 32 2 528 40 00

welcome@fagg-afmps.be
www.famhp.be

Entity of Non-Clinical Evaluators
Sonja.beken@fagg-afmps.be

Follow the FAMHP on



Validated, QC-compatible pyrogen testing for vaccines: industry deployment of a reporter -cell monocyte activation test (MAT)

Sijia Yi

MRL, Analytical Research & Development, Cell based Sciences, Merck & Co., Inc., West Point, PA, USA

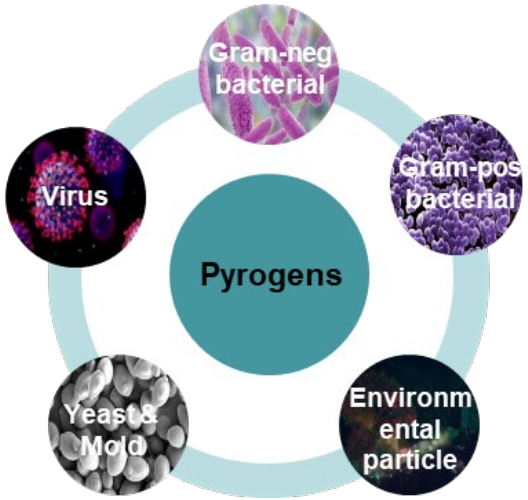
2026 **Joint EDQM -EPAA Hybrid Symposium**

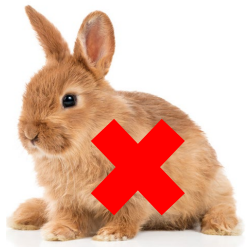
25-26 February 2026, Brussels, Belgium

MSD

Why phase out RPT: industry and business drivers

- **Regulatory momentum** : The Rabbit Pyrogen Test (RPT) is being phased out. Ph. Eur. has removed RPT and adopted the Monocyte Activation Test (MAT) for pyrogen testing, with implementation starting in **2026**.
- **Risk and limitations** : RPT shows high variability and notable high failure/re-testing rate, impacting reliability and timelines.
- **Strategic alignment with 3Rs** : Transitioning to MAT supports corporate sustainability goals, quality modernization, and ongoing efforts to reduce, refine, and replace animal testing in pharmaceutical development and quality control.





RPT (in vivo)

Eliminating in EU by 2026

MAT (in vitro)

MSD

MSD RPT to MAT transition: history and milestones

Feasibility with PBMC:
- High variability
- Cell sourcing constraints

Cell system re-screen:
- Evaluated in house and commercial human monocytic reporter cell lines
- Identified THP1 NFkB reporter cell lines
- Positive scientific feedback from EU OMCLs

MSD pipeline programs using RPT:
- ✓ Biologics: ≥4 programs
- ✓ Vaccines: ≥4 programs

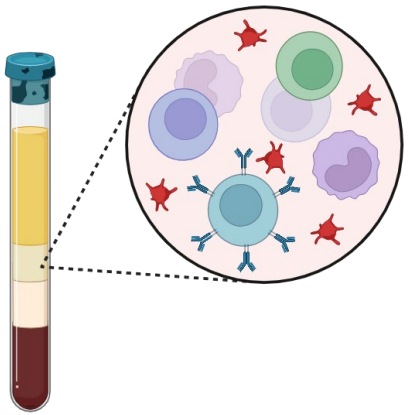
Re-development with monocytic cell lines (MonoMac6 cells):
- Assay redeveloped
- Failed validation
- Supply issues

Validation and implementation with reporter cells
- Product specific method developed
- Successfully validated in GxP lab
- Implementation in QC lab

MSD

MAT cell system selection: PBMC vs Reporter cells

PBMC or WBC

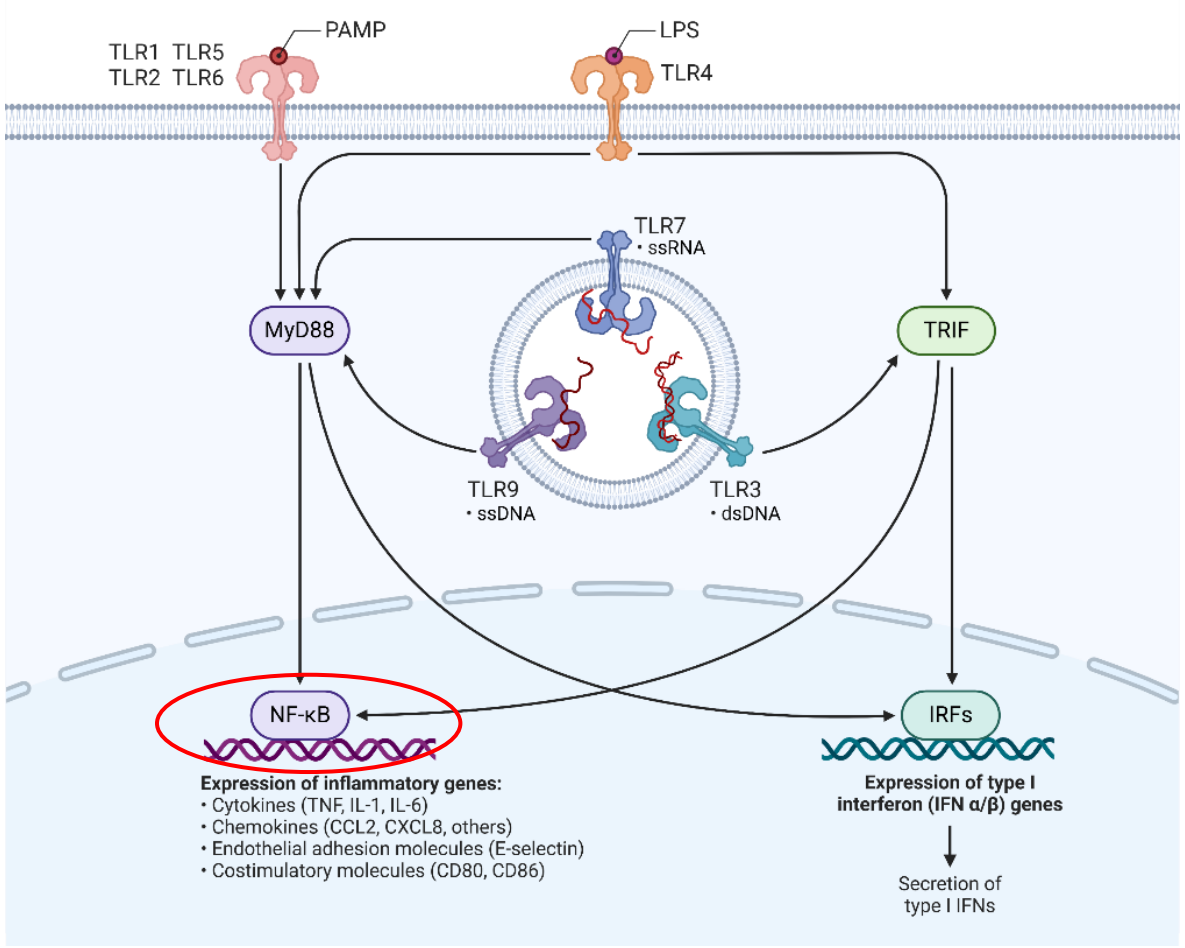


AP-1

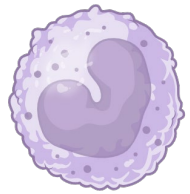
NFkB

IRF

Endogenous Pyrogens:
TNFα, IL-1β, IL6, IL-8



Human monocytic NFkB reporter cell lines



- U937-NFkB-Nluc (in-house)
- THP1-Lucia-NFkB (InvivoGen)
- TLR Bioassay cells (Promega)

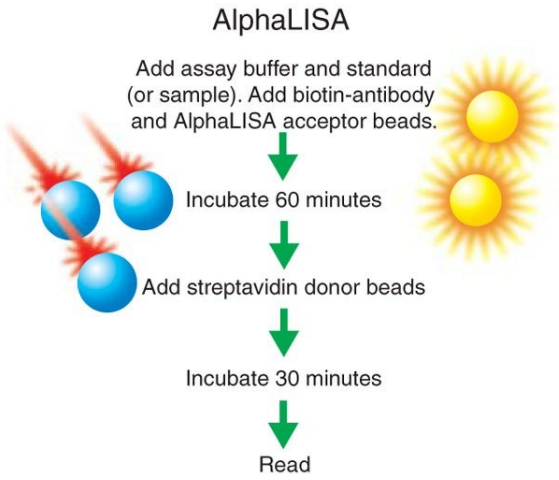
Streamlined MAT workflow using human reporter cell lines

Human primary cells
- **PBMC or WBC**

Monocytic cell lines
- MM6

Incubate with test substance

Cytokine detection by ELISA or AlphaLISA



- ❑ High variability
- ❑ Inconsistency
- ❑ Several steps

Reporter cell line
- TLR Bioassay cells

Seed cells and incubate overnight

Incubate with test substance

Add luciferase substrate
Shaking for ~10min

Read

- ✓ Low lot-to-lot variability
- ✓ Good consistency/robustness
- ✓ Easy

MSD

Establishing THP1 reporter cells as the MAT test system

Requirements from EP 2.6.30: TLR2, TLR4, and TLR5

TLR agonist	Name	PBMCs		THP1-Lucia-NFkB		TLR Bioassay cells		RPT sensitivity
		EC50[1]	LoD[2]	EC50[1]	LoD[2]	EC50[1]	LoD[2]	
TLR4	USP-RSE (LPS) (EU/ml)	0.050	0.010	6.286	0.02	1.867	0.032	→ 5-10 EU/mL/kg
TLR1/2	Pam3CSK4 (ng/ml)	3.689	0.063	27.83	<0.457	2.945	<0.001	
TLR2	HKLM (cells/ml)	2.84E+06	5.68E+04	1.50E+08	1.50E+06	5.94E+07	<6.86E+05	
TLR2	LTA-SA (ng/ml)	103.2	3.416	325.8	20.448	148.2	1.8	→ 20 µg/mL/kg
TLR2/6	FSL-1 (ng/ml)	0.1618	0.010	1.352	0.007	2.048	0.0027	
TLR3	Poly(I:C) HMW (ng/ml)	4342	567.103	NC	NC	NC	NC	
TLR3	Poly(I:C) LMW (ng/ml)	NC	>5555	NC	NC	NC	NC	
TLR5	FLA-ST ultrapure (ng/ml)	1.328	0.085	53.42	0.117	37.810	<1.372	
TLR7	Imiquimod (ng/ml)	6460	321.404	NC	NC	NC	NC	
TLR7/8	R848 (ng/ml)	206	4.670	3442	334.416	1.87E+04	1.50E+03	
TLR9	ODN2006 (µM)	70.64	32.771	NC	NC	NC	NC	

- THP-1 reporter cell lines responded strongly to multiple TLRs, especially TLR1,2,4,5, and 6. Based on performance and RTU model, **TLR Bioassay cells** were selected as the MAT cell source in MSD.
- **MAT** showed **superior sensitivity** to both endotoxin and non-endotoxin pyrogens compared with RPT.

Note: 1. The EC50 was determined by 4PL model.

2. The LoD was defined as the lowest concentration giving a signal greater than the blank (~8 negative controls) + 3 s tandard deviations. The concentrations for wells were used in the calculations mentioned above.

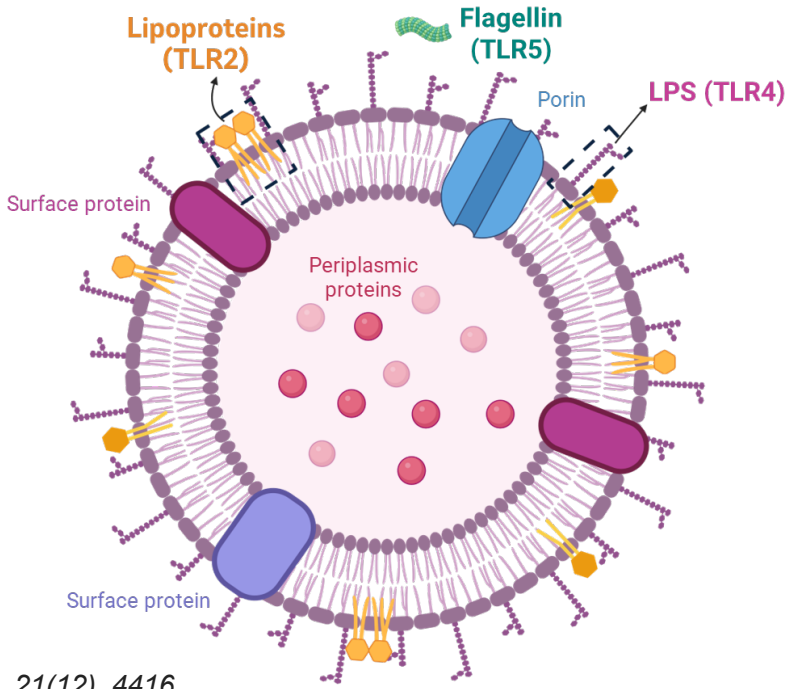
NC, not calculable (EC50/LoD could not be determined because a dose response curve was not obtained).

MSD

Product -specific MAT development example: OMPC

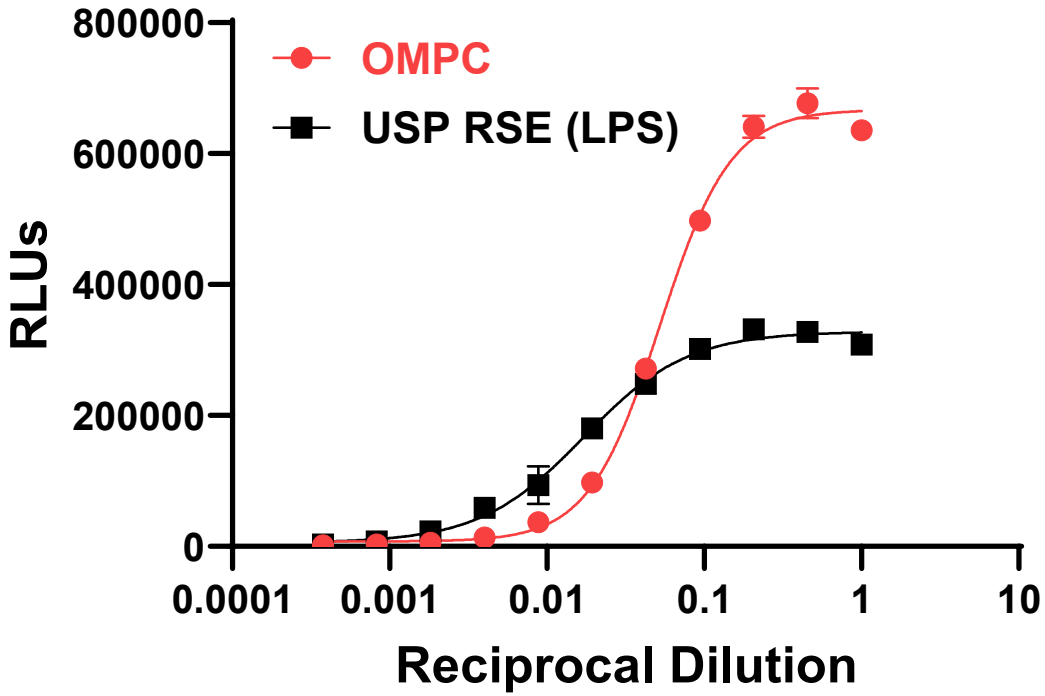
- **Outer membrane protein complex (OMPC)** is extracted and purified from N. meningitidis (gram-negative bacterium) and used as a carrier for various vaccines.

OMPC activates **TLR4, TLR2,** and **TLR5** (<10%)



Outer membrane protein complex (OMPC)

Int. J. Mol. Sci. 2020, 21(12), 4416



- **Reference lot comparison method** in MAT is considered as a more suitable method for **OMPC**, which is inherently pyrogenic.
- **Validated reference lot** for OMPC is necessary due to the presence of NEP and the OMPC dose response curve is **NOT parallel** with USP Reference Standard Endotoxin (USP RSE).

MSD

MAT method validation strategy for OMPC

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES quantitat. limit		ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

(1) in cases where reproducibility (see glossary) has been performed, intermediate precision is not needed

(2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(3) may be needed in some cases

- Based on ICH Q2(R1) and EP 2.6.30 & 2.6.40, reference lot comparison method is validated as a **quantitative content method**.
  The following validation performance characteristics should be tested:
  - Specificity
  - Linearity
  - Precision
    - Repeatability
    - Intermediate Precision
  - Accuracy
  - Range
  - Robustness

MSD

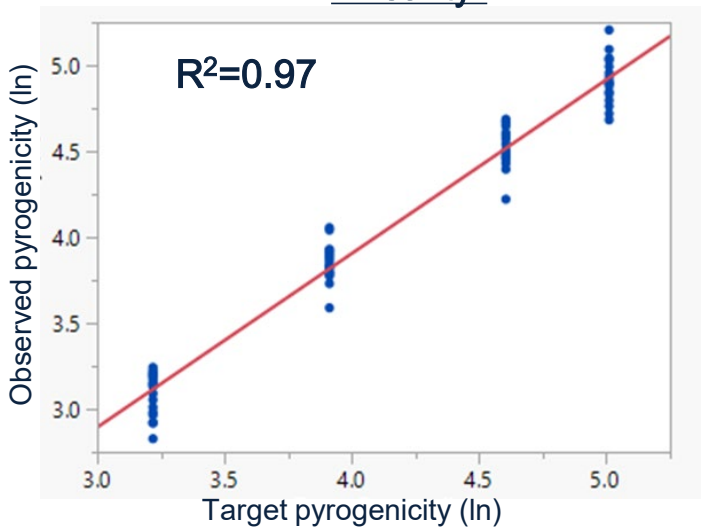
MAT method robustness for OMPC

- The critical method parameters and their effects on assay performance due to deliberate variations in critical method parameters were evaluated by a Design of Experiments (**DoE**) study.
  - Cell seeding density
  - Cell seeding time
  - Sample treatment time
  - Substrate incubation time
- The study demonstrated robust assay performance in terms of **linearity, accuracy, and precision**, indicating that the MAT assay is **robust** for testing OMPC samples.

Accuracy/relative bias

Target (%)	N	Geomean (%)	% Relative Bias	90% Lower CL of Relative Bias (%)	90% Upper CL of Relative Bias (%)
25	20	22.1	-11.7	-15.6	-7.5
50	20	46.9	-6.2	-9.9	-2.2
100	20	91.6	-8.4	-12.3	-4.3
150	20	136.3	-9.2	-13.5	-4.6

Linearity



Precision

Target%	Random Effect	%RSD
25	Analyst	14.5
	Day[Analyst]	0
	Residual	5.9
	Total	**15.7**
50	Analyst	8.2
	Day[Analyst]	2.8
	Residual	8.5
	Total	**12.1**
100	Analyst	10.8
	Day[Analyst]	0
	Residual	8.2
	Total	**13.6**
150	Analyst	1.8
	Day[Analyst]	0
	Residual	12.5
	Total	**12.7**
Combined	Analyst	9.6
	Day[Analyst]	0.8
	Residual	9.3
	Total	**13.4**

1. Day[Analyst] represents the day-to-day variability by an analyst.
2. Residual represents repeatability, the within day variability or plate-to-plate variability.
3. $\%RSD = \sqrt{\exp(Variance) - 1} \times 100$

MSD

MAT QC robustness for OMPC

- The assay robustness, reproducibility, and suitability for QC use were evaluated by a Design of Experiments (**DoE**) study.
  - Ready-to-use (RTU) cell lots
  - Assay plate types
  - FBS vendors used in assay media
  - Instruments (plate readers)
  - Analysts (from development and QC labs)
- Acceptance criterion: total assay variability ≤ 30% RSD.
- Result: observed total variability for OMPC samples **12.4%** and **9.6%** RSD across all tested factors.
- Therefore, the assay is regarded as **robust** and **suitable for routine QC use** .

MAT assay variability estimates for the OMPC samples

Sample	Random Effect	Variance Component Estimate	%RSD
Sample-1	Random Block	0	0.0
	RTU Cell Lot	0.0046	6.8
	Assay Plate Type	0.0043	6.5
	FBS	0.0018	4.3
	Residual	0.0045	6.7
	Total	0.0152	12.4
Sample-2	Random Block	0.0020	4.4
	RTU Cell Lot	0.0021	4.5
	Assay Plate Type	0.0000	0.0
	FBS	0.0000	0.0
	Residual	0.0052	7.2
	Total	0.0092	9.6

Successful validation for OMPC using reporter cell -based MAT

- All acceptance criteria were met, confirming successful validation
- Data support end-to-end analytical performance and consistent routine GxP lab operation

Performance Characteristic	Acceptance Criteria	Results	Pass/Fail
Specificity	Report results.	No interference to assay results from matrix	Pass
Linearity	Coefficient of Determination (R^2): ≥ 0.95	ln(Observed RP) = -0.047 + 1.006 ln(Target RP) $R^2 = 0.99$	Pass
Precision - Repeatability	Percent (%) Relative Standard Deviation (RSD) per level: $\leq 30\%$	Mock OMPC Samples: %RSD $\leq 6.8\%$	Pass
Precision - Intermediate Precision	% RSD per level: $\leq 30\%$	Mock OMPC Samples: %RSD $\leq 20.3\%$ OMPC Samples: %RSD $\leq 11.7\%$	Pass
Accuracy - Relative	% Relative Bias: $\pm 30\%$	Relative bias: -8.0% to 4.1%	Pass
Range	Acceptable accuracy, linearity and precision.	25% - 300% relative pyrogenicity	Pass

- Reporter cell-based MAT:
  - **<5%** initial failure rate with root causes identified
  - **0%** failure rate on repeat runs
- MM6 cell-based MAT:
  - **~36%** initial failure rate
  - **~54%** failure rate on repeat runs

MSD

Conclusion and next steps

- **Promising test system** : selection of human monocytic reporter cell line enables detection of a broad range of pyrogens and performs effectively in the MAT.
- **Streamlined workflow for rapid implementation** : reporter cell –based MAT is simple, reliable, consistent and robust; ready -to-use (RTU) cells facilitate validation, transfer, and routine QC.
- **Hurdles and next steps:**
  - **Challenge**: Setting “pass/fail” specs for licensed products with inherent pyrogenicity given limited batches and difficulties of bridging with RPT .
  - **Next steps** : MSD is developing a specification strategy to ensure safety and lot -to-lot consistency for inherently pyrogenic vaccines.

Reference: Yi et. al., *Vaccines* **2025**, *13*(10), 1009; **https://doi.org/10.3390/vaccines13101009**

MSD

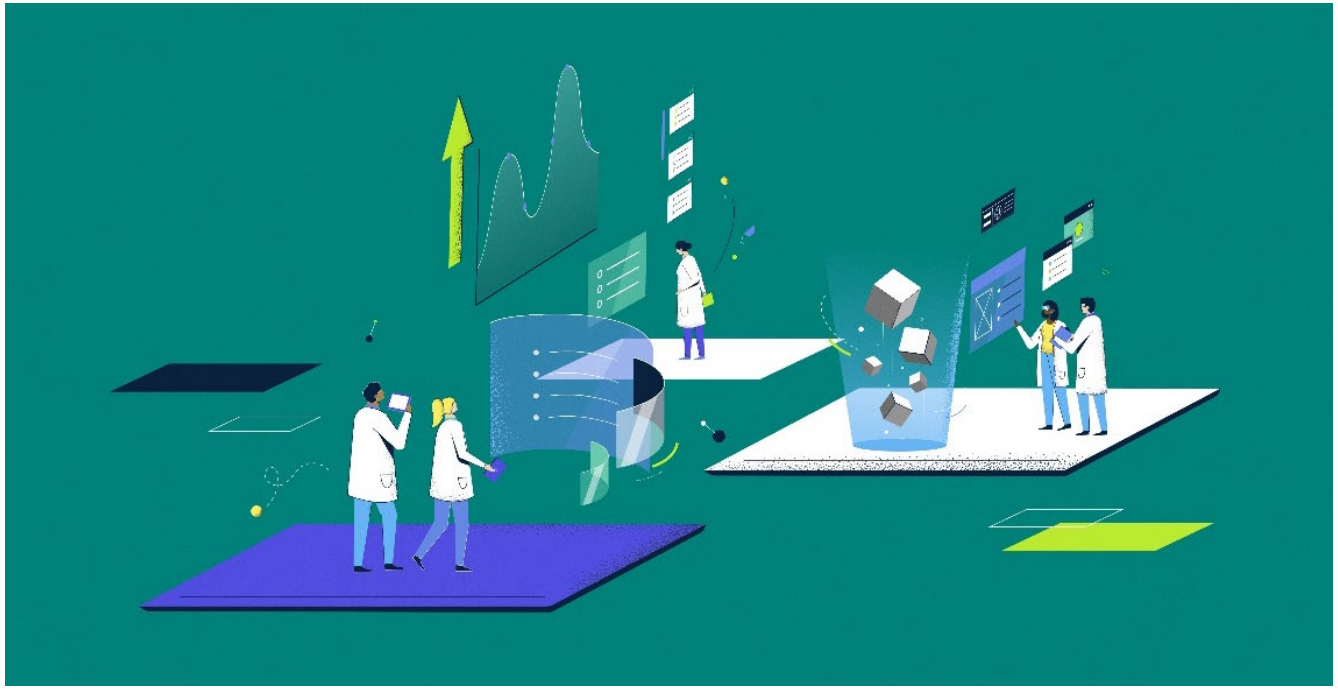
Acknowledgements

Jenny Xu
Melissa Whiteman
Stephen Wu
Shara Dellatore
Daniel DiStefano
Frank Celeste
Christopher J Wang
Daisy Sun
Asra Mirza
Robert Bailer
Kevin Gurney

Liping Song
Radhakrishna Tirumalai
Amy Eckert
Kartik Narayan
Joseph Anthony Micucci
Suzanne W Matlock
Joey Marie Ingram
J Paul Detweiler

Jason Karasinski
Jennifer Quinn
Denis Streltsov
Bhupendra Singh
Elizabeth Anne Thoryk
Kishore Joshi

April Fike
Stephanie Overholt
Dominic Tulio
Kevin Ludwig
Stephanie Rangel
Tori Stachura
Ashley Nicole Plawa
Donald Conover
Christopher Brennan



Merck & Co., Inc.
Tel: +1215-652-4546
E-mail: sijia.yi@merck.com
Address: 770 Sumneytown Pike, West Point, PA, USA, 19486

MSD

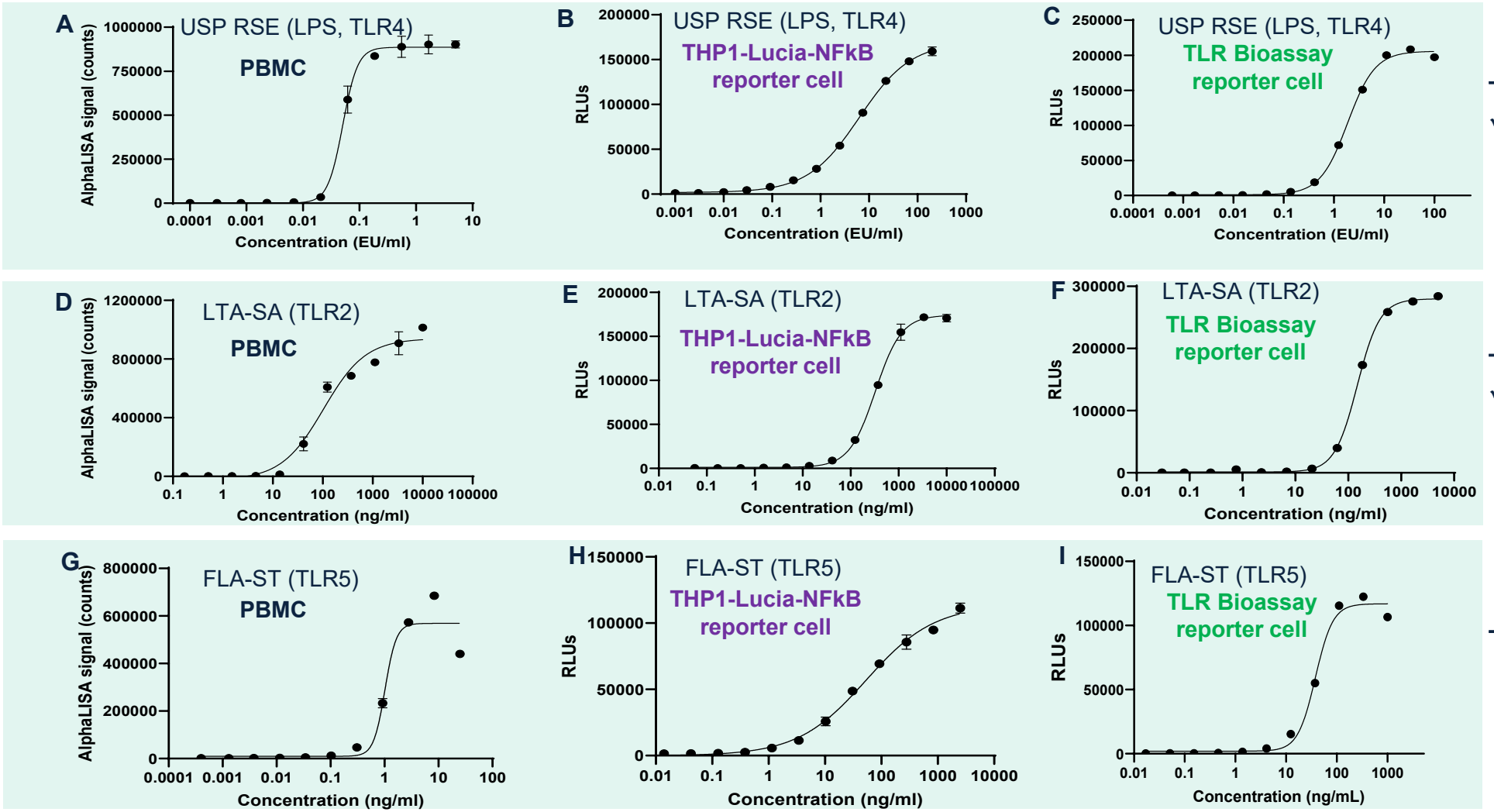
Back-up slides

Selection of human monocytic reporter cell lines as MAT test system

Under Ph. Eur. 2.6.30, section 5 -6, human monocytic cell lines are appropriate when meeting the requirements:

- Validity of reference standard curve
  - Endotoxin (method 1) or product-specific reference lot (method 2)
  - Dose response curve with a good fit between data points and regression model
    - $R^2 \geq 0.975$
- "The test system should ensure that **at least TLR4 and 2 other TLR ligands** that reflect the most likely contaminant(s) of the preparation tested are detected."
  - **TLR4**: LPS
  - **TLR2**: Lipoteichoic acid or peptidoglycan
  - **TLR5**: Flagellin.

Detection of diverse Endotoxin and NEP



TLR4
✓ Lipopolysaccharide (LPS)

TLR2
✓ Lipoteichoic acid (LTA-SA)

TLR5
✓ Flagellin (FLA-ST)

Requirements from EP 2.6.30: TLR2, TLR4, and TLR5

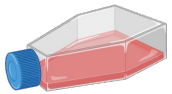
USP RSE: US Pharmacopeia Reference Standard Endotoxin

MAT assay workflow for OMPC

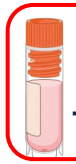
1 Cell seeding

Plate cells on 96-well plate and allow grow overnight

Continuous cell

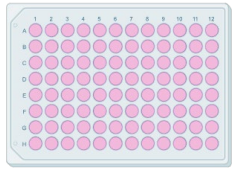


or



"Ready-to-use" (RTU)

-80 °C

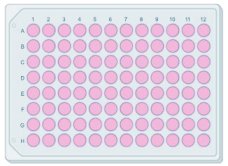


O/N

37 °C

2 Treatment

Add 2.2-fold serial dilutions of RS, TA, and SSS

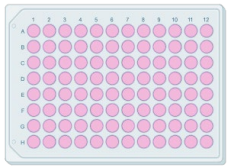


4-24 h

37 °C

3 Luciferase substrate addition

Add Bio-Glo-NL reagent



10 min

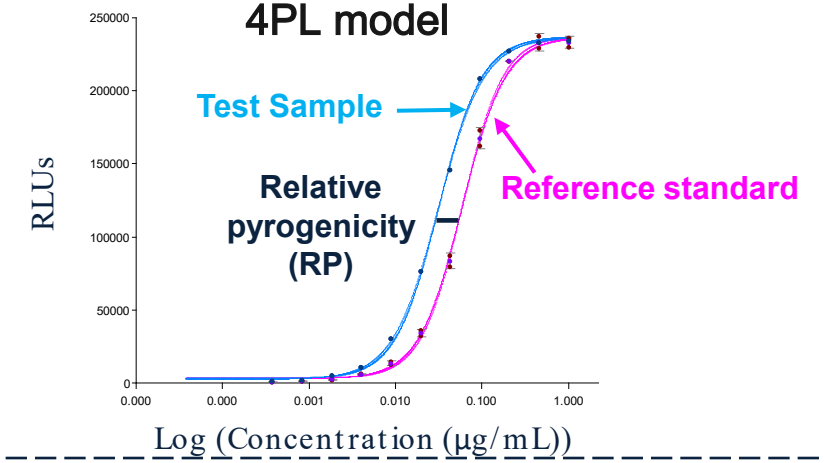
Room Temperature

4 Detection, analysis & report

Read at luminescence unit (RLU)

Data analysis and report in Cytation:

- ✓ Integrated assay acceptance criteria
- ✓ Reportable relative pyrogenicity values from multiple plates
- ✓ Final report outcome: "Pass/Fail"

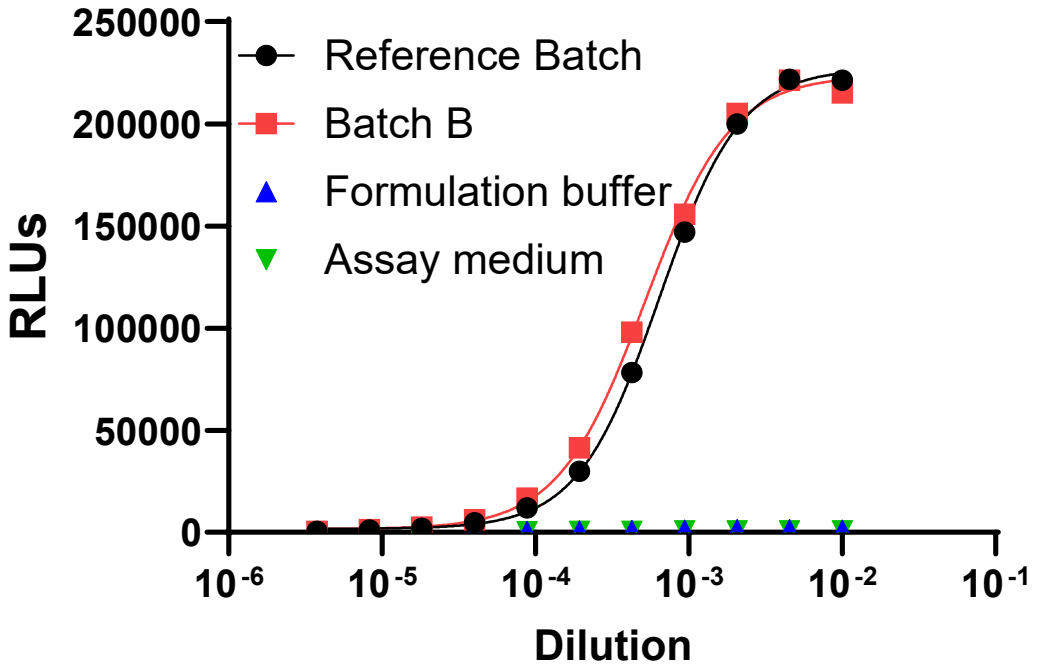


MSD

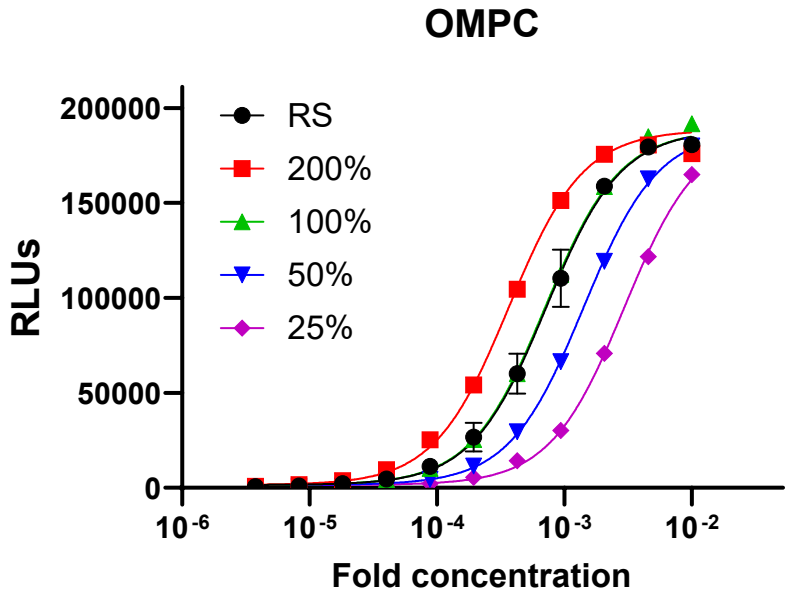
MAT assay performance characteristics: specificity

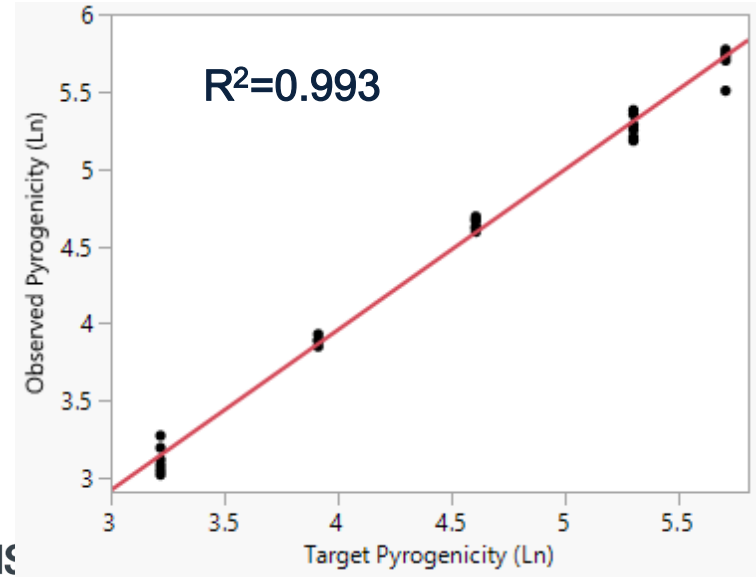
The MAT method is specific to the analyte (OMPC) and exhibit no interference from other components in the matrix.

- Specific: OMPC Batch B (a different batch from reference batch)
- Non-specific: formulation buffer



MAT assay performance characteristics in pre -validation





- Sample at 5 levels (25% to 300% of nominal test concentration (NTC)) were tested
- Precision at different levels
  - Repeatability
  - Intermediate precision
- Relative accuracy: the relative bias between the measured % relative pyrogenicity and the target %relative pyrogenicity
- 2 analysts, 6-10 runs for each level

Level	Intermediate Precision (%RSD)	Repeatability (%RSD)	Relative Bias (%)
L1 (25%)	8.5	8.1	-10.3
L2 (50%)	3.3	3.0	-1.2
L3 (100%)	3.3	3.3	4.6
L4 (200%)	7.7	7.7	-1.8
L5 (300%)	9.8	9.8	-0.4
Overall	6.9	6.9	-2.0

Advancing sustainable pyrogen testing: Global progress in RPT replacement with non-animal approaches

Emmanuelle Coppens, Global Analytical Expert, Global Analytical Sciences, Sanofi, Marcy l'Etoile

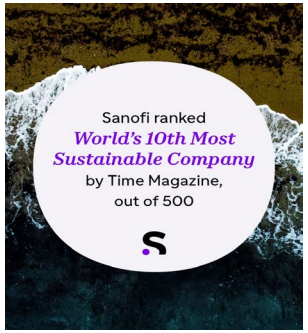
February 25, 2026

Sanofi's Integrated Research and Testing Strategy (IRTS) as a sustainability fundamental

Lays out Sanofi guidelines to affirm **rigorous & state-of-the-art science**

- to **select** the best available, feasible, and translatable **models**
- to address **scientific questions**
- to adhere to **regulatory requirements**,

With the aim to **stop using live animals** whenever possible



Improving animal use by preclinical package, study rationale and design

+

Phasing in NAMs (New Approach Methodologies)

+

Replacing by qualification, validation, acceptance

+

Waiving and challenging obsolete tests

=

Objective of 50% global reduction of animal use:
- **Between 2020 and 2030**
- **Internal and external use**

sanofi

Sanofi's strategy for non-animal-based quality control has several benefits

- **Regulatory Compliance (EU Directive 2010/63, European Pharmacopoeia, other local regulations)**

3Rs — THE PRINCIPLES OF HUMANE EXPERIMENTAL TECHNIQUE — RUSSELL & BURCH

Reduction	Refinement
• **Minimize the number** of animals per experiment	• **Minimize suffering** and improve animal **welfare**

- Replacing challenge potency tests by **serological methods**
- Using **single-dilution** method design instead of multi-dilution design
- Use of **humane endpoint** for lethal or invasive assay

Replacement

- **Avoid** or **replace** the use of animals

Replacing *in vivo* assays : Developing and implementing ***in vitro* alternatives** using **animal free reagents**

The 4th R — edqm

Removal

- **Removing/ not performing unjustified tests** : redundant, unnecessary

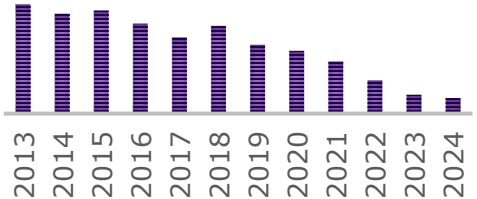
* European Directorate for the Quality of Medicines

- **Analytical Performance**

automation

RELEVANCE (state of the art technology and scientific value)
RELIABILITY (less variability, invalid and false Out Of Specifications)
REDUCED CYCLE TIME (faster time to market and improved availability for patients)

- **85% decrease of animal use for quality control between 2013 & 2024**



sanofi

Sanofi's strategy : aiming at improved Quality Control with scientifically relevant non-animal based analytical testing

Ongoing

Test in **animals only for preclinical** package

Development of tetanus residual toxicity *in vitro* functional assay (BinACle) + removal of irreversibility test

Innovative **state of the art *in vitro/in silico* technologies** (eg High Throughput Sequencing) applied for **clinical batch testing** and **product characterization**

New Products

Established Products

Rabbit Pyrogen test replacement by *in vitro* functional assays (MAT/BET)

Licensure without *in vivo* QC testing

In vitro potency development for DTaP (Diphtheria, Tetanus & acellular Pertussis) vaccines

Achieved

Legacy or redundant safety tests removal eg Abnormal toxicity test (ATT) & specific toxicity tests

In vitro potency for viral and polysaccharide vaccines

Cell based assays for toxins (Diphtheria, Pertussis)

Full in vitro Adventitious agents testing

Specific animal based testing requirements (National/Regional Pharmacopoeias, Importation)

Which test methods are described in Pharmacopoeias

	Rabbit Pyrogen Test (RPT) ~~Ph. Eur. 2.6.8~~ / USP <151> / JP 4.04 / Russian Ph GPM 1.2.4.0005.15 / ChP <1142> / Indian Ph 2.2.8 : Not harmonized	Bacterial Endotoxin Tests (BET) *LAL/TAL*: Ph. Eur. 2.6.14 / USP <85> / JP 4.01: harmonized (ICH 2001)/ ChP 1143 *rFC*: Ph. Eur. 2.6.32 / ChP guideline 9251 / / EAEU 2.1.6.12 / South Korea Ph/ rFC/rCR: JP guideline JP G4-4-180 / USP <86>	Monocyte Activation Test (MAT) Ph. Eur. 2.6.30 revised and 2.6.40 IP 22 2.2.25 ChP supplement 2020 3309 "Reporter Gene Assay" ChP 2025 guideline <9301> Ph Korea guideline 2024 Draft J Ph guideline 2024 Brazil Ph 5.5.2.7.1
Principle	Body temperature elevation post IV injection	Hemolymph clotting in contact with endotoxins / or recombinant reagent	Mimics the first step of fever mechanism – uses human cells
Method	Limit Assay (0.5 IU/mL/kg)	Detection or Quantitative Assay shown to be sensitive to 0.005 IU/mL	**Ph. Eur. method 1: Limit Assay (semi-quantitative)** **Ph. Eur. method 2: Reference lot comparison**
Goal	Safety Test Product/Process Consistency	Safety Test Product/Process Consistency	Safety Test Product/Process Consistency
Advantages	Compendial method (US, ~~Eur~~ and JP but not harmonized) Sensitive to all rabbit pyrogens	Compendial method harmonized for LAL based BET (US, EU, JP) Sensitive and fast	Compendial method (EU) Sensitive to all pyrogens
Drawbacks	*In vivo* Not harmonized through Pharmacopeias Variable Not representative of human biology Injection route Dilution of the product (vaccine) **Deleted in Ph. Eur. (effective Jan 2026)**	*Ex vivo (*horseshoe crab is endangered (*Tachypleus tridentatus*) or vulnerable (*Limulus polyphemus*) « Only sensitive to endotoxins from Gram negative bacteria » **rBET compendial only in Ph. Eur. (rFC)**	*In vitro* - **Based on human cells** **Compendial method for Ph. Eur. since 2010**

***MAT & rBET* are generally not compendial methods outside Europe**

Overview of current situation for pyrogenicity testing

Pyrogenicity Testing:

Choice of test according to a risk assessment

Rabbit pyrogen test phasing out :

- **Few remaining RPT replacements with BET** are under worldwide submission or approval for legacy products with an endotoxin risk only
- **Ongoing validation of MAT** for inherently pyrogenic vaccines in order to replace the RPT

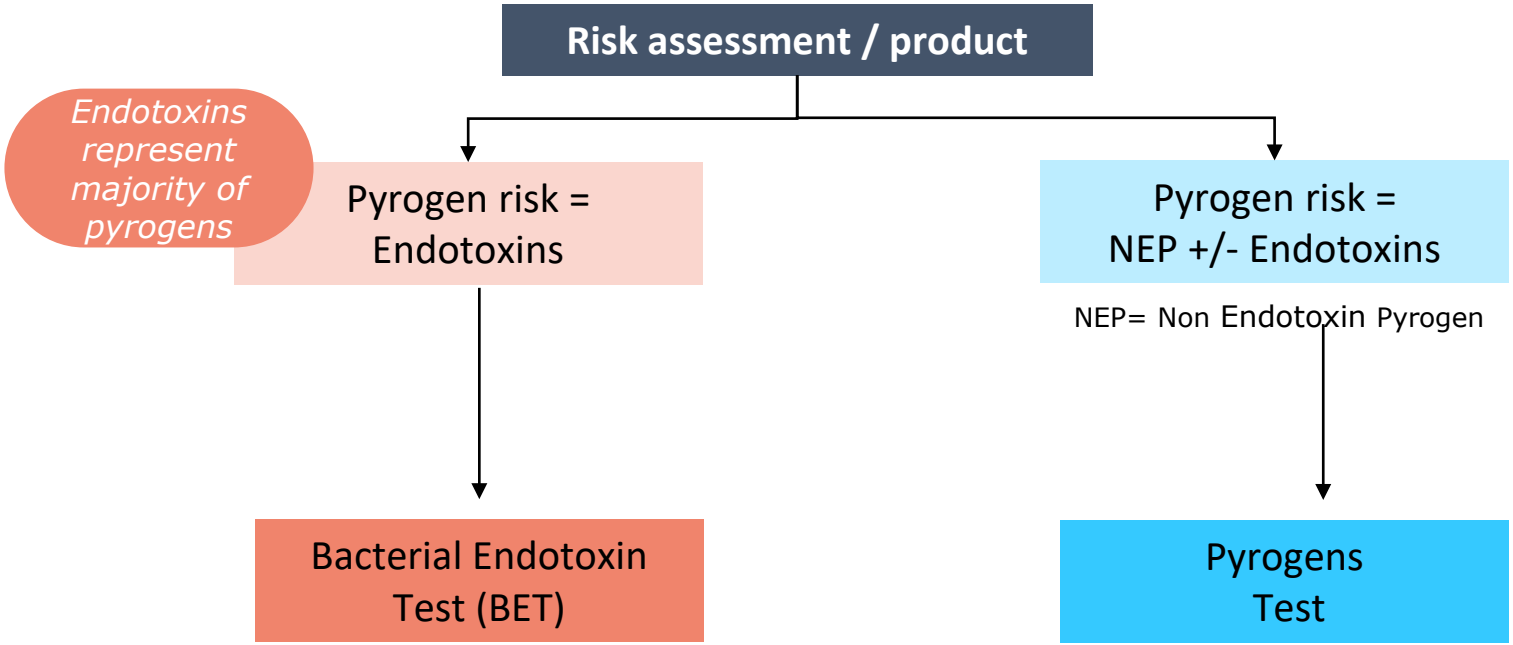
Replacement of horseshoe crab blood lysate :

- **Achieved for pharmaceutical water for some sites/ongoing for others**
- **Ongoing** for products

RPT: Rabbit Pyrogen Test
MAT: Monocyte Activation Test
BET : Bacterial Endotoxin Test

Pyrogens – which test methods for their detection

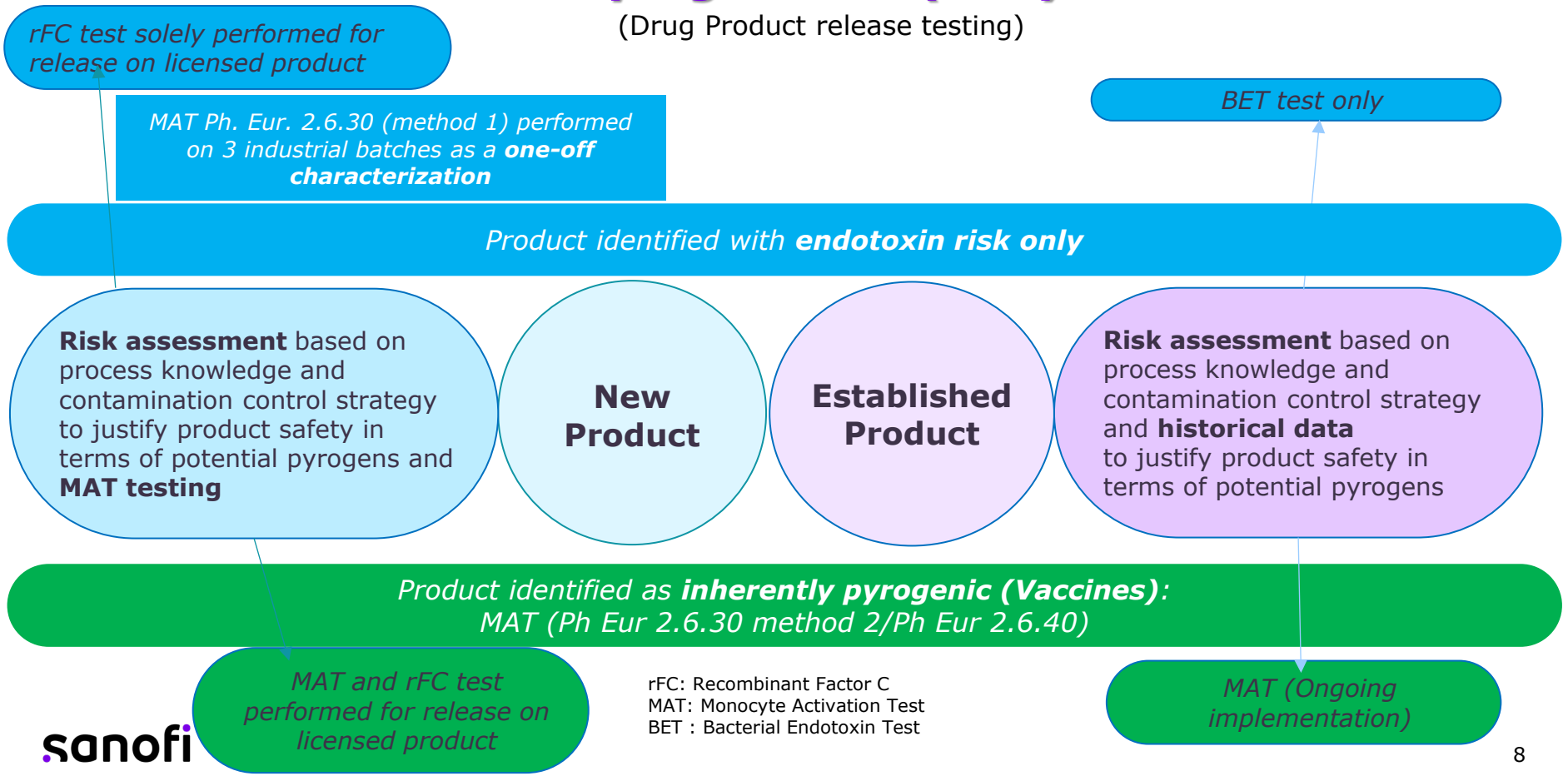
A risk assessment is performed to identify (new product) or confirm (established product) which is the candidate method for pyrogens detection depending on the nature of pyrogens



As per Ph. Eur. 5.1.13 Pyrogenicity (Guideline)

Pyrogenicity Testing Strategy - Phasing out of Rabbit Pyrogen Test (RPT)

(Drug Product release testing)



rFC: Recombinant Factor C
MAT: Monocyte Activation Test
BET : Bacterial Endotoxin Test

sanofi

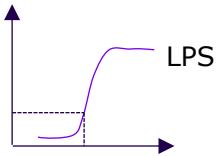
2 MAT Methods used in sanofi

Methods of MAT described in **Ph. Eur. 2.6.30** (method 1 and 2)

Method 1 : Limit Test / Semi-quantitative Test

- Comparison of the preparation being examined with endotoxin standard
- **Result: pass/fail (Endotoxin Equivalent/ml must be lower than the "Endotoxin Limit Concentration"**)

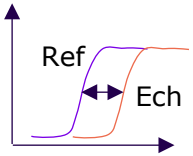
  - **→ used in Sanofi for product characterization when no NEPs are expected**



Method 2 : Reference Lot Comparison Test (Vaccines)

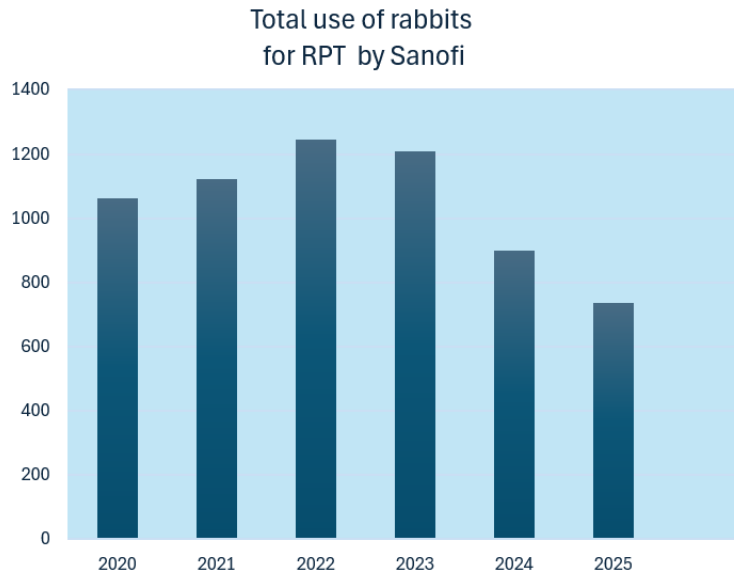
- Comparison of the preparation being examined with a validated reference lot of that preparation
- **Result: Pyrogen Unit /mL or ratio (must be below a defined limit)**

  - **→ used in Sanofi when the pyrogen content (NEPs and/or endotoxin) of the vaccine to be tested is inherently high** (method according to **Ph. Eur. 2.6.40** « MAT for vaccines containing inherently pyrogenic components »)

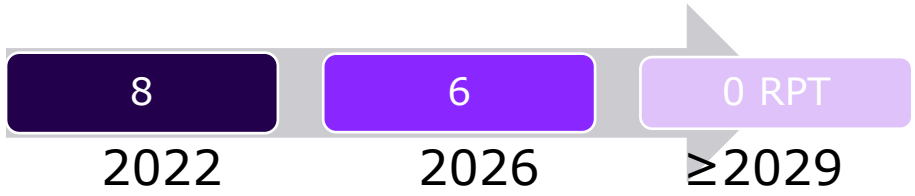


Current situation - RPT release testing on established products

Evolution of rabbits used (2020-2025)



Evolution of number of products tested with RPT (2022-?)



- Products with endotoxin risk only :
  - 4 still released with RPT (ongoing or planned submissions)
- Inherently pyrogenic vaccines:
  - 2 products with ongoing MAT implementation

RPTs still performed in EU in 2026, planned outside EU after 2026

Case studies - Switch to BET as sole method to assess pyrogenicity on established products

- **Challenge from some Health Authorities outside Europe** :
  - Several Q&As with strong rationale, risk assessment & supportive data (RPT, BET) were needed for approval
  - 2 cases of submission withdrawal (non-compliance to local Pharmacopoeia, insufficient proof of contamination control through process)

- **Request from EMA** to replace Type IA variation by a **Type IB** one (in alignment with EMA Q&As update 27 MAY 2025)

Case studies - Use of MAT & RPT for new products

- **Products with endotoxin risk only** :
  - **MAT** performed on all new products as **characterization test** on DP (Drug Product) on 3 industrial batches
  - **RPT** performed on a **case by case** basis according to specific regulatory requirements **additionnally to MAT**

- **Products inherently pyrogenic (vaccines)** :
  - **MAT & RPT performed in parallel** in early clinical phases (RPT as release test) to meet all regulatory requirements
  - **Openness** from European Health Authorities to use **MAT for release** on phase III batches (if sufficient available data allowing to set acceptance criteria)

Remaining challenges and next steps for alternative pyrogenicity testing

Challenges

- **RPT needs to be maintained beyond 2026**
- All **pharmacopeias not aligned** on the topic
- Obtaining **acceptance of MAT outside EU vs obligation to phase out RPT in EU**
- MAT & rBET are still seen as **alternative methods outside EU** → potential requirement for **comparability data** between MAT & RPT and rFC & LAL **and need for full validation package**
- Setting of **acceptance criteria**
- Technical challenge with **some matrices**

Remaining challenges and next steps for alternative pyrogenicity testing

Next steps

- Continue to **advocate for compendial harmonization**
- For commercialized products :
  - Perform **MAT validation, acceptance criteria setting and submission** for inherently pyrogenic commercialized products (Ph. Eur. method 2)
  - Finalize **rFC roadmap across the company** (implementation in QC & regulatory pathway)
- For new products :
  Generate **characterization data with MAT** (Ph. Eur. method 1)
  Obtain **licensure** with **rFC as release test**

- Fully implement **double sourcing for MAT critical reagents** (PBMCs & Detection kits)
- Assess **serum free** cell culture media for PBMCs
- Assess **rapid MAT solutions** eg reporter gene approach
- Start to **evaluate** available **rCR (recombinant Cascade Reagent)** kits
- Continue to semi automate and look into full **automation**

sanofi

Acknowledgments (Sanofi) : T. Bonnevay, H. Chausse, O. Chipier, M. Couriera, D. Damjanovic, K. Drollée, N. Dudoignon, C. Fernandez, S. Oudin, T. Petters, A. Porsche, S. Richard, P. Riou, P. Sachs, C. Southey-Pillig, K. Thibault-Duprey, D. Trelat & all sanofians who contributed

Thank You

This work was funded by Sanofi.
Emmanuelle Coppens is a Sanofi employee and may hold shares and/or stock options in the company.

sanofi

Emmanuelle.coppens@sanofi.com

Waive and Replace the Rabbit Pyrogen Test in Lifecycle Vaccine Release

Shahjahan SHAID
GSK

Disclosure

Shahjahan SHAID is an employee of the GSK group of companies.

This work was sponsored by GlaxoSmithKline Biologicals SA.

GSK is committed to the replacement, reduction and refinement of animal studies (3Rs). Non-animal models and alternative technologies are part of our strategy and employed where possible. When animals are required, application of robust study design principles and peer review minimises animal use, reduces harm and improves benefit in studies.

Agenda

- Why is GSK relying on Animal Testing and the commitment to 3R?
- Achievement and Future 3R challenge on the Rabbit Pyrogen Test
- Take home message

Why is GSK relying on animal testing and the commitment to 3Rs

A small, vital and crucial part of the business

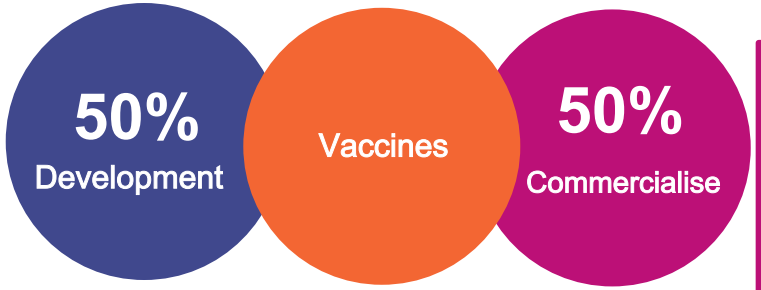
Why is GSK relying on Animal Testing

Animal Assays are required by Compendia for lifecycle vaccines

Laboratory animals used in Vaccines

R&D:

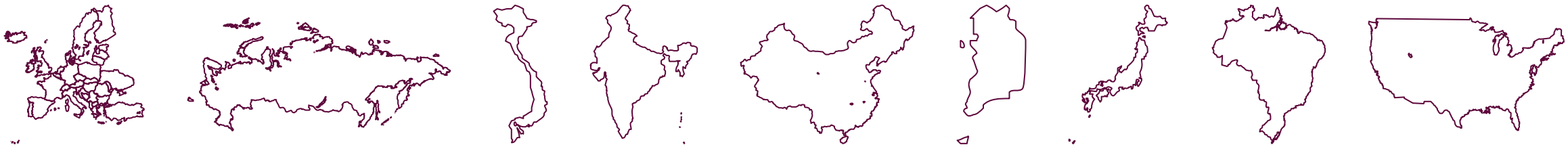
- Basic research on disease processes
- Use of models of diseases to test candidate
- Preclinical safety, efficacy, stability testings



QC:

- Animal Testing in QC mainly for Release of Vaccines in lifecycle products
- Production and control development (process and testing validation, detoxification, inactivation, etc. …)

Animal assays are required by WHO and regional Pharmacopeias which are often **not aligned** e.g. EDQM has prioritized a substitution where scientifically possible.



The substitution in a biologics license application **might therefore be rejected** by certain countries and can take years due to number of countries involved for each product.

Impact of in vivo assays in lifecycle vaccines

Animal Assays have a disproportionately high impact on the release compared to other tests

Release

Stability

Process Changes

Potency

- Serological
- Challenge

Safety / Purity

- General safety tests
- **Rabbit Pyrogen Test**
- Safety (detoxification, extraneous agent, etc.)

Identification

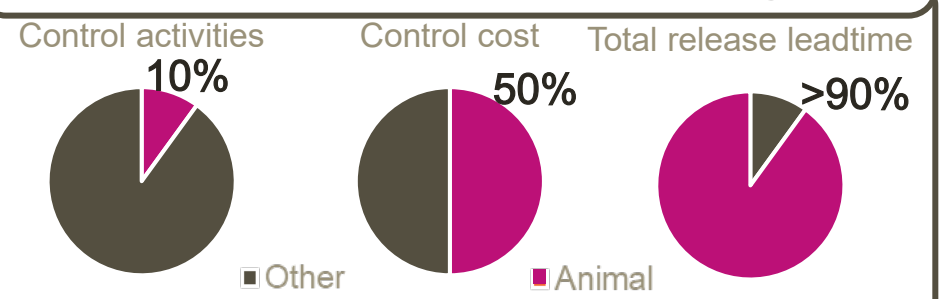
- Vaccine strain
- Antigen

Impact

Cycle time in QC testing

> 100 days

Animals used in QC testing



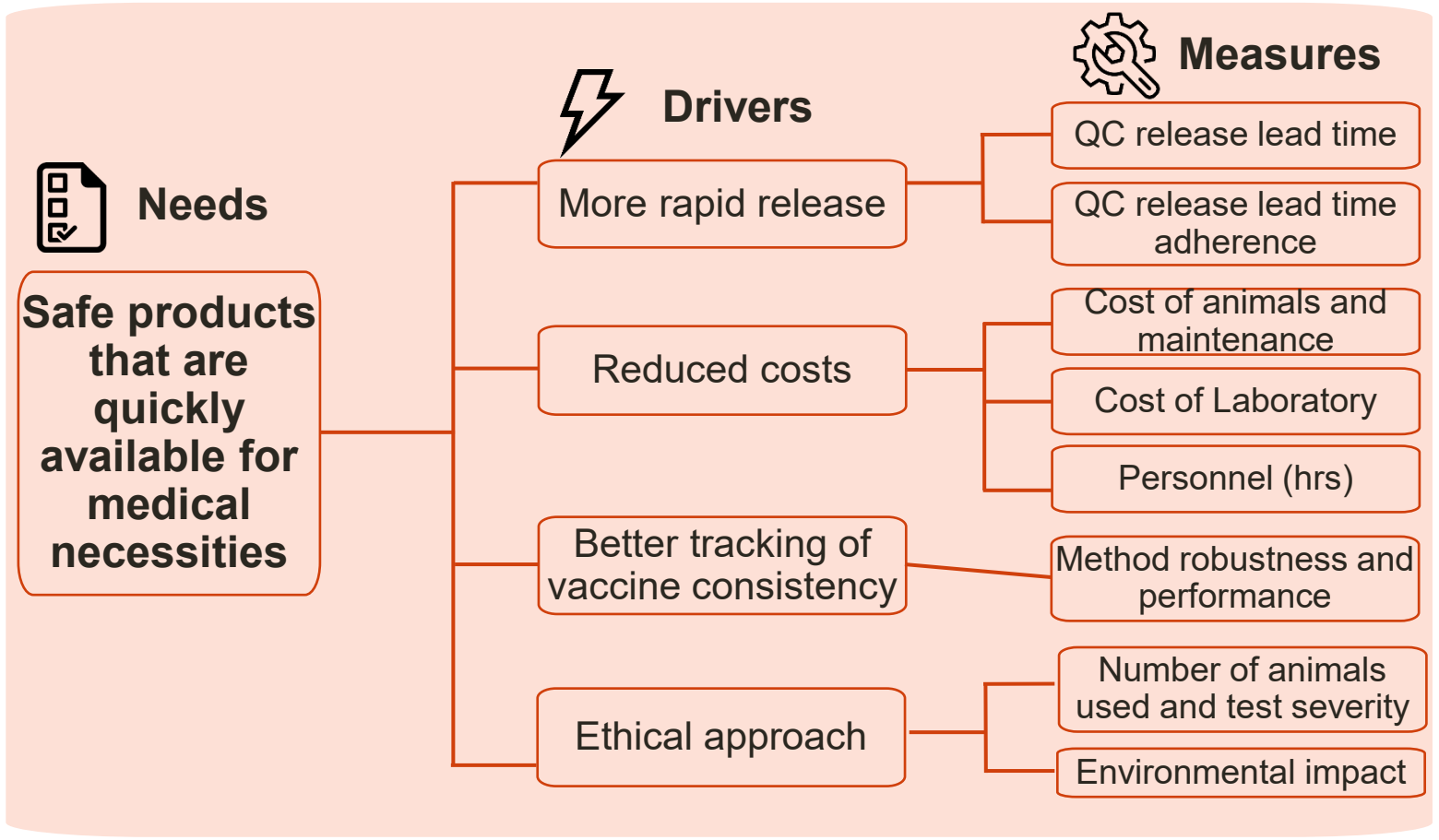
Supply impact by in vivo intrinsic variability (repeats)

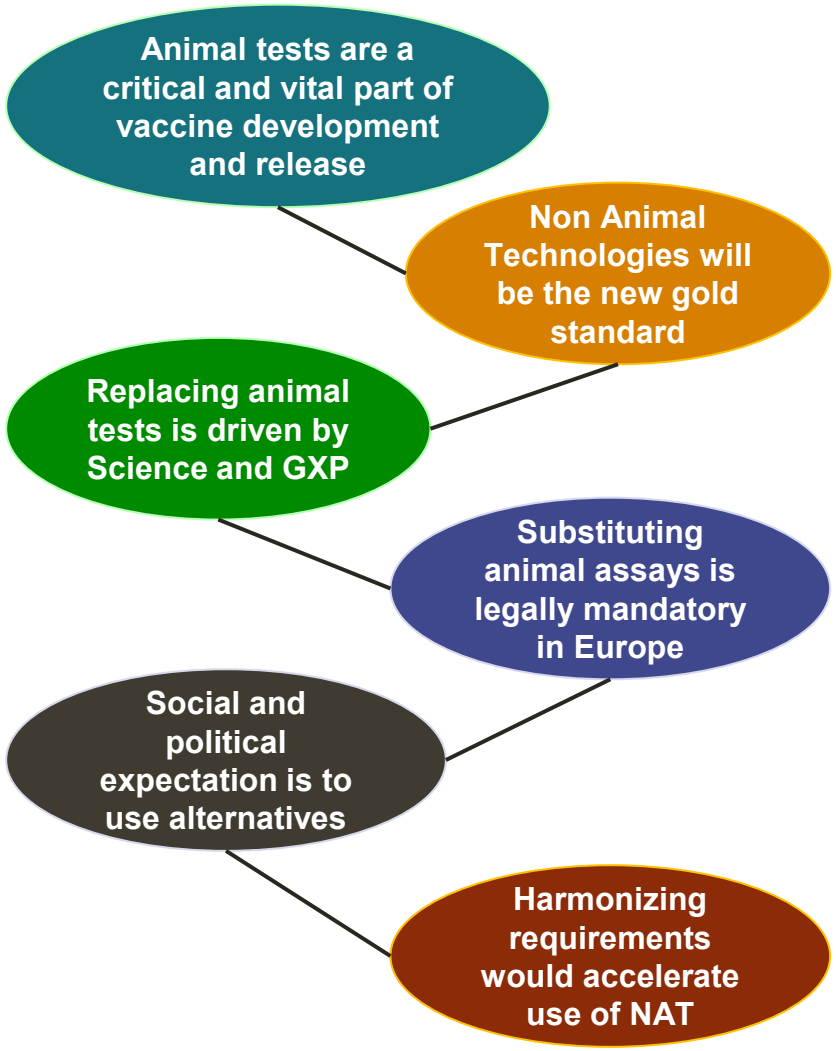
Second testing by NCL

- Animal numbers
- Lead time
- Costs

Harmonizing requirements would accelerate use of NAT

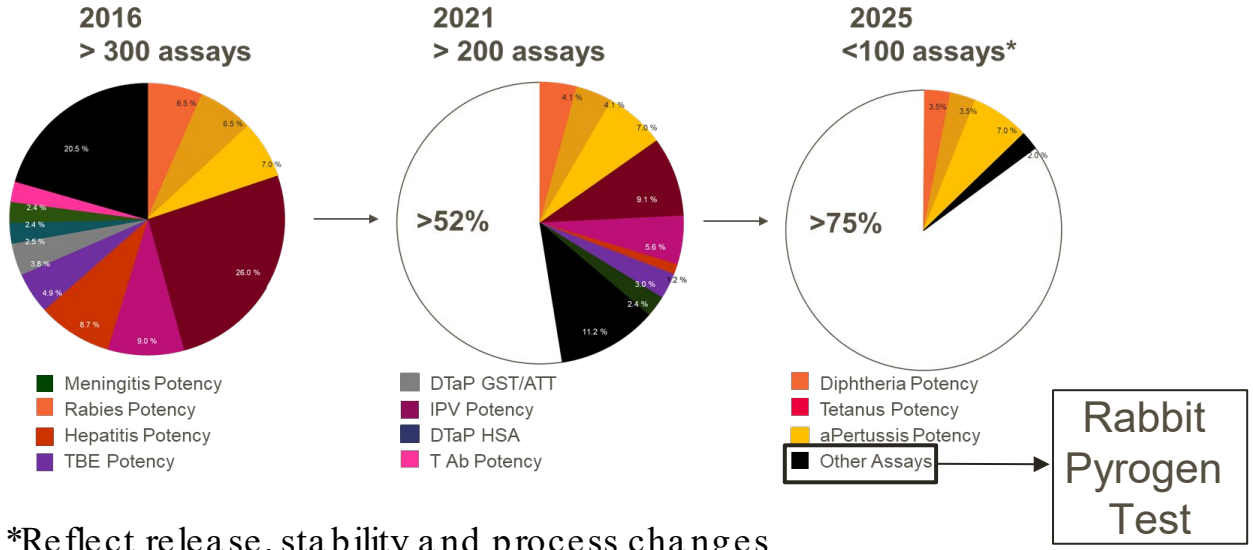
Replacing animal tests is a win win for patients and manufacturers



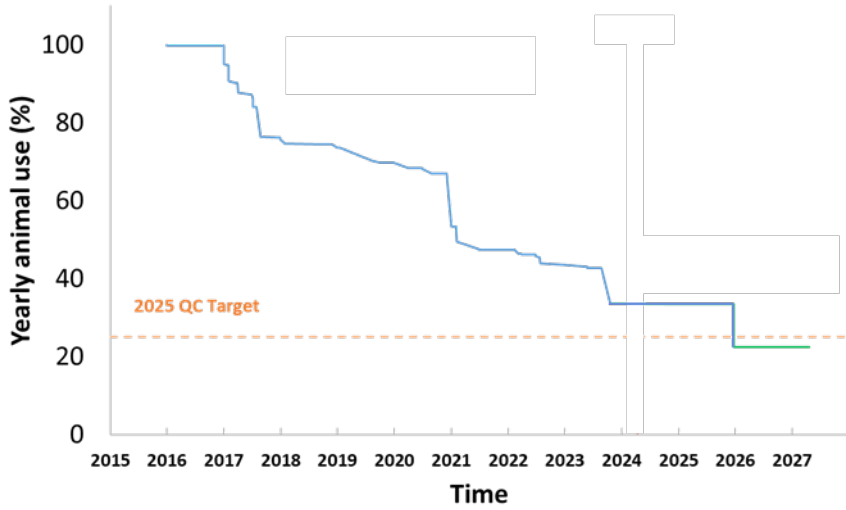


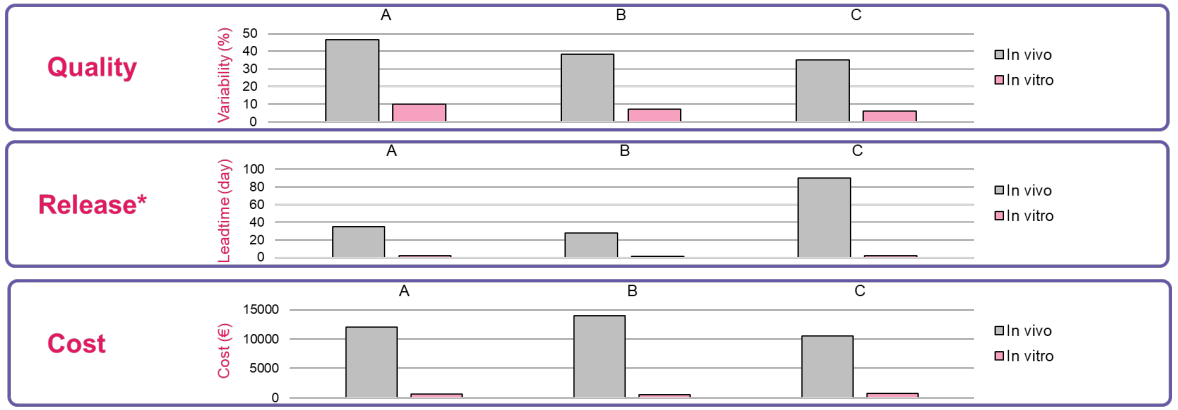
3R & GSK: Prioritize replacement to reduce animal use by 75% from 2016-2025 in QC

In vitro replacement does not jeopardize quality. Faster release; less repeats; reduced cost



*Reflect release, stability and process changes





*Difference before and after is shown

Achievement and Future 3R challenge on the Rabbit Pyrogen Test

1. Remove and rely on controls at other steps

2. Substitute by the BET test as a release

3. Replace by the MAT test as a release

GSK

Pyrogen detection is crucial and therefore mandatory to ensure patient safety

Several techniques are available allowing animal-free detection of pyrogens

Exogenous pyrogens

Non-endotoxin pyrogens

- Components from Gram-positive bacteria
- Yeast and mould
- Virus
- Particles of the environment

Endotoxins

Components from Gram-negative bacteria (LPS)

Pyrogen detection

MAT: Ph. Eur. 2.6.30

- Recognizes pyrogens with a high sensitivity.
- Established in the PEP
- Mentioned in the Ch.P and USP

RPT: Ph. Eur. 2.6.8, removal by 2026

- Measure of the **rabbit** body temperature after injection of the product.
- Former gold standard.

BET Guideline: Ph. Eur. 5.1.10

Guidelines for using the test for bacterial endotoxins (and MAT)

rFC: Ph. Eur. 2.6.32

- Non-animal-derived reagent that can detect endotoxins.
- Accepted as equivalent in the E.P.

LAL: Ph. Eur. 2.6.14

- 1st commercially available endotoxin method
- Best established and harmonized alternative to the RPT
- Broad acceptance from authorities around the world

Endotoxin detection

Different Assay design of MAT

Primary Cells

Blood from healthy donors

PBMCs* isolation, qualification and cryopreservation

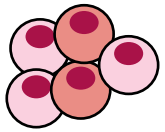
Stimulation of PBMCs with RSE** and Vaccine

Most common Readout:
IL*** detection on PBMCs supernatants by a sandwich ELISA

Data Analysis

Secondary Cells

Cell line derived macrophages i.e.THP1, NOMO-1



Permanent Cell line already qualified

Stimulation of PBMCs with RSE** and Vaccine

Several Readout:
IL by a sandwich ELISA,
IL by Fluorescence
Reporter gene by Fluorescence
Reporter gene by PCR

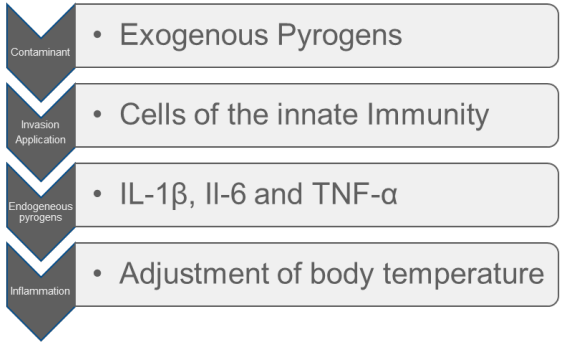
Data Analysis

**PBMCs= Peripheral Blood Mononuclear Cells* ***RSE = Reference Standard Endotoxin* ****IL = Interleukin i.e. IL-6, IL-1, TNF-a*

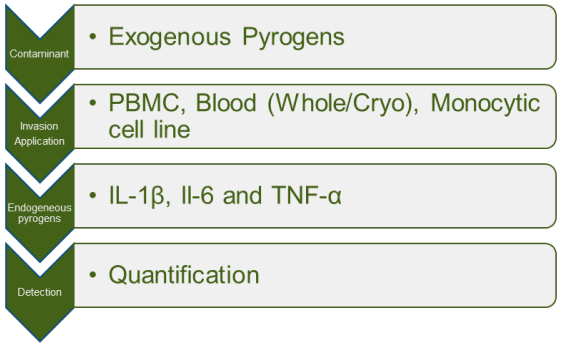
Comparison of method key characteristics of RPT and MAT

RPT is not required due to availability of Endotoxin tests and MAT

RPT



MAT



	RPT	MAT
Specificity	All rabbit pyrogens	All human pyrogens
Reagent origin	Direct animal use	Human-derived, Cell line
Availability	Since 1950s, broadly	Since 2010s, growing
Test type	Qualitative	Quantitative
Endpoint	Body temperature	Cytokine, ELISA
Duration	24h, lead time > 1week	~8h (lead time ~2days)

Endotoxin / LPS	TLR 4 has 72% homology with human on amino acid level
Single stranded RNA	TLR 7 and TLR 8 absent
DNA	TLR 9 broader recognition pattern than human

(1) doi: 10.3389/fimmu.2014.00316, (2) https://doi.org/10.1007/s00251-009-0381-z (3) https://doi.org/10.1016/j.cimid.2012.03.008

Legal requirement in different jurisdictions

Today the pyrogen methods are not aligned between the Compendia's. The trend continues. What will be the "common ground" ?

	Non-endotoxin pyrogens			Endotoxin pyrogens		
	RPT	MAT	Other methods	LAL	rFC	rLAL
E.P	Not foreseen by Jan 2026	Solemn method 2026	Not foreseen	Available	Available Equivalent method	To be determined
WHO	Available	Proposed*	Not foreseen	Foreseen	Proposed*	Proposed*
USP	Available	Discussed**	Not foreseen	Available	Available	Available
China	Available	Available	In discussion	Available	Available	To be determined
Korea	Available	Available	In discussion	Available	Available	To be determined
Japan	Available	Available	Not foreseen	Available	Alternative method	Alternative method
India	Available	Available	Not foreseen	Available	Alternative method	To be determined
Brazil	Available	Available	Not foreseen	Available	Expected	To be determined

* TRS has been published in Nov 2025

** FDA guidance on using MAT, USP Micro expert team has called for data, Europhorum

GSK

Rabbit Pyrogen Test will be retired in the European Pharmacopoeia by 2026

Substitution by Non-animal technologies is mandatory in the EU


DIRECTIVES

DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

of 22 September 2010

on the protection of animals used for scientific purposes

Will be deleted from the E.P. by 2026 (incl 59 references).

According to the EU directive 2010/63 the use of non-animal technologies instead of animal tests is mandatory.

RPT will be an alternative method not described in the E.P.
Leaving the manufacturers with MAT, RFC, TAL, LAL

What are the possibilities to ensure safety and ethics without the Rabbit Pyrogen Test in QC:

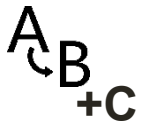
1. Remove and rely on controls at other steps | **2. Substitute by the BET test as a release** | **3. Replace by the MAT test as a release**

Substitute the Rabbit Pyrogen Test (RPT) with a risk-based approach

Avoid test duplication and rely on the most impactful test

1. Remove and rely on controls at other steps

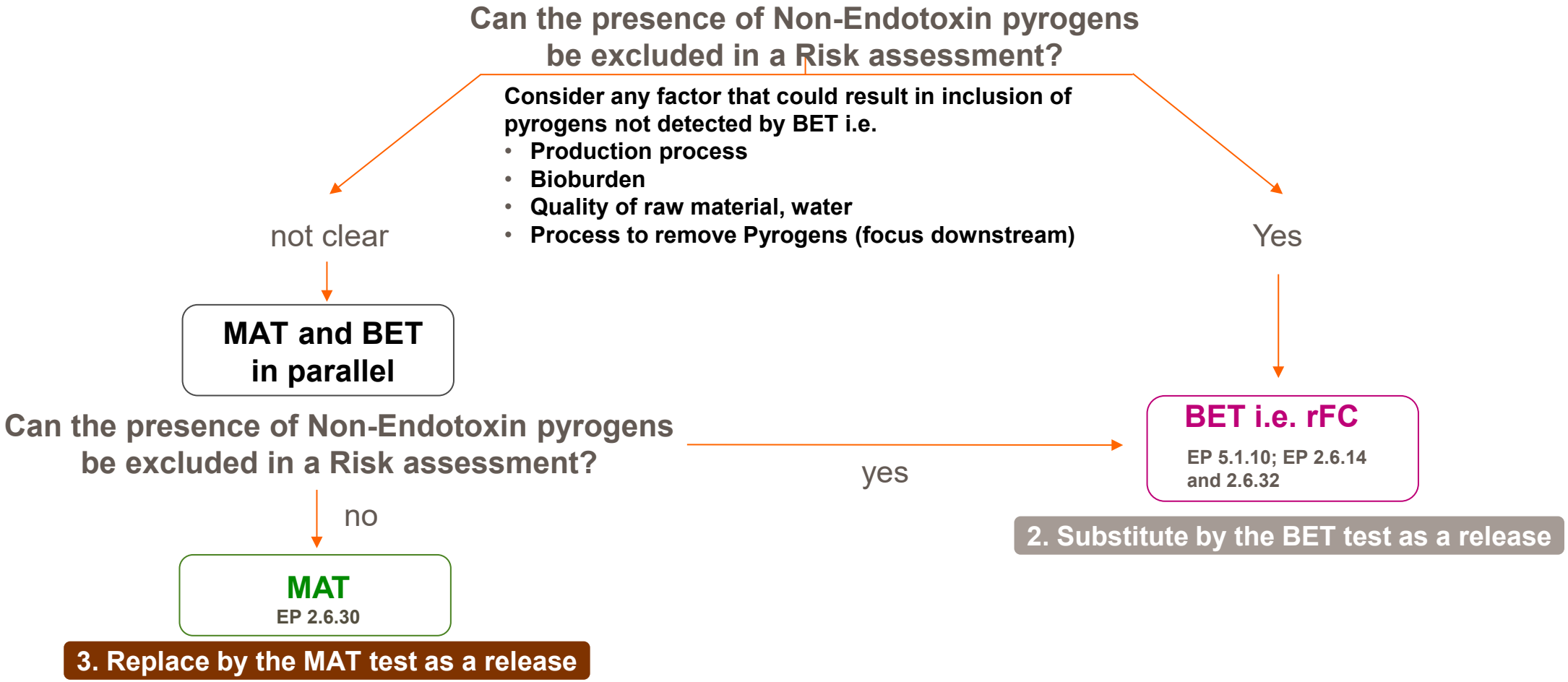
Lifecycle based on the **consistency approach**: Paradigm shift moving away from the current focus on testing **each batch as unique** with high reliance on *in vivo* models, to an **integrated in-process and final product quality monitoring** program including all available data allowing a shift to non-animal methods.
The consistency approach is based upon the principle that the **quality of a biologic is the result of the strict application of a quality system and consistent production**. Subsequent **batches are determined to be similar to clinically evaluated batches** and therefore acceptable for release through the in-process testing that comprises this quality system.



Incoming Material e.g. Antibiotics	Pyrogenicity is reduced in the manufacturing process and tested at intermediate/ Drug substance or Drug product level
	Pyrogenicity is analyzed by the Supplier and part of the Certificate of Analysis
	Pyrogenicity is evaluated at later manufacturing steps

Substitution or replacement decision depends on a risk-based approach

*EP 5.1.13 describes the selection of the most adequate method**



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

Substitute the Rabbit Pyrogen Test (RPT) with a risk-based approach

The manufacturing level and overall test schedule of the product needs to be considered as well

2. Substitute by the BET test as a release

The choice of the **most appropriate** pyrogen detection strategy in **QC testing should depend on a risk-based approach** without the use of RPT. This approach should take into consideration the **lifecycle, manufacturing process, QC testing** and if applicable the **intrinsic pyrogenic characteristics of the product**.
The current GMP for human vaccines allow to assess, test and control how limited the risk is of a possible pyrogen contamination. Modern pharmaceutical manufacturers have thorough **validated controls, specifications and limits** embedded in the also **validated manufacturing process** as demanded by the **GMP rules**. Contaminants are therefore appropriately controlled.
The **absence of lipopolysaccharides** in materials or products combined with the ruling out of the presence of non-endotoxin pyrogens **is a strong indicator to justify the absence of exogenous pyrogens**.

Final Container

There is no intrinsic pyrogenicity in the product or matrix interference with the rFC/LAL/TAL test. Pyrogenicity is evaluated as a combination of the rFC/LAL/TAL and further tests e.g. bioburden sterility, environenmental monitoring

Meningoccoccal A, C, W135 Vaccine

Replace the Rabbit Pyrogen Test (RPT) with an equivalent Method

MAT recognizes pyrogens by mimicking the fever response to pyrogens in humans

3. Replace by the MAT test as a release

MAT recognizes pyrogens with a high sensitivity using **human derived monocytes** to mimic the fever response to pyrogens in vivo. Depending on the **Interleukin** (IL-6) response variability a semi quantitative or **full quantitative assay** for not inherently pyrogenic substances and a full quantitative assay for inherently pyrogenic substances are described.
Besides the technical abilities to detect all human pyrogens the MAT also allows a **validation according to today's ICH guidelines**.

The MAT is established in QC and R&D to ensure a sustainable retirement of the RPT in GSK

Final Container

Endotoxin Test is not applied and instead MAT is the method of choice due to

the intrinsic pyrogenicity compositon of the vaccine and

matrix interference with the LAL/rFC test.

Meningococcal B Vaccine

Take Home Message

Several strategies and methods are in place to stop or replace use of RPT

GSK
2016 > 20 RPT
2021 < 10 RPT
2025 1 RPT

1. Remove and rely on controls at other steps
2. Substitute by the BET test as a release
3. Replace by the MAT test as a release

gsk.com