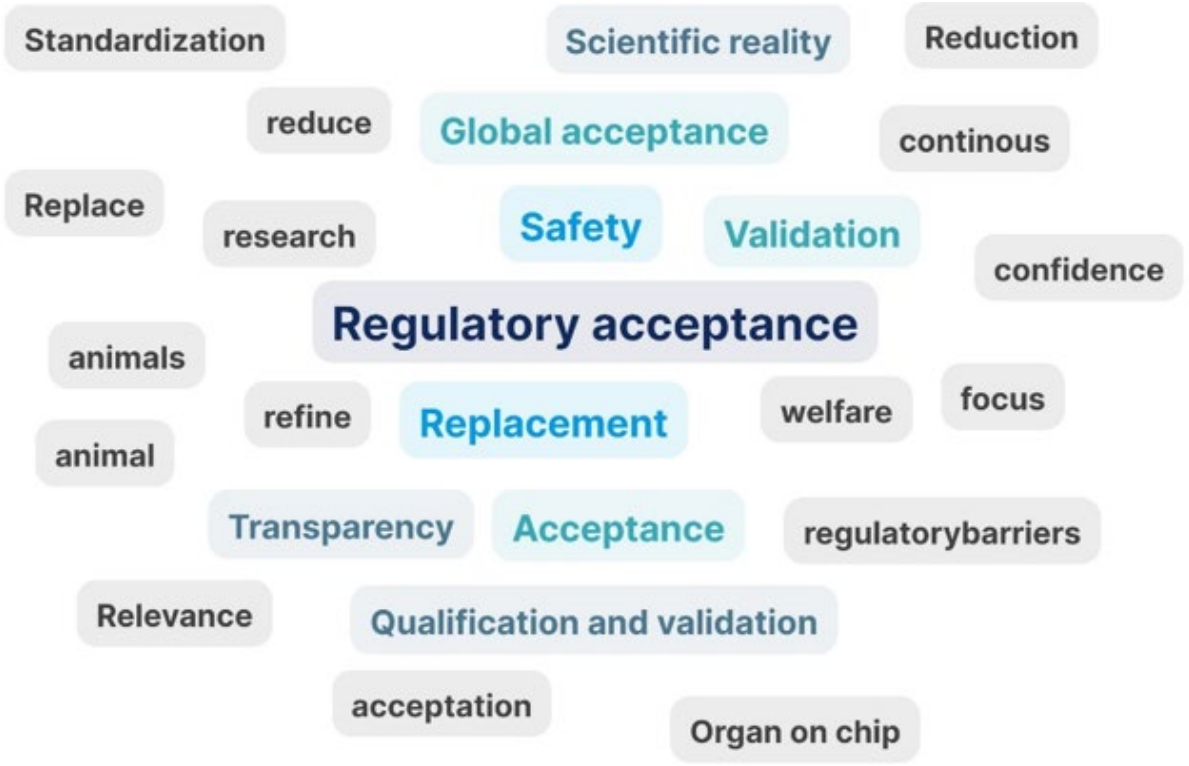
EMA
EUROPEAN MEDICINES AGENCY

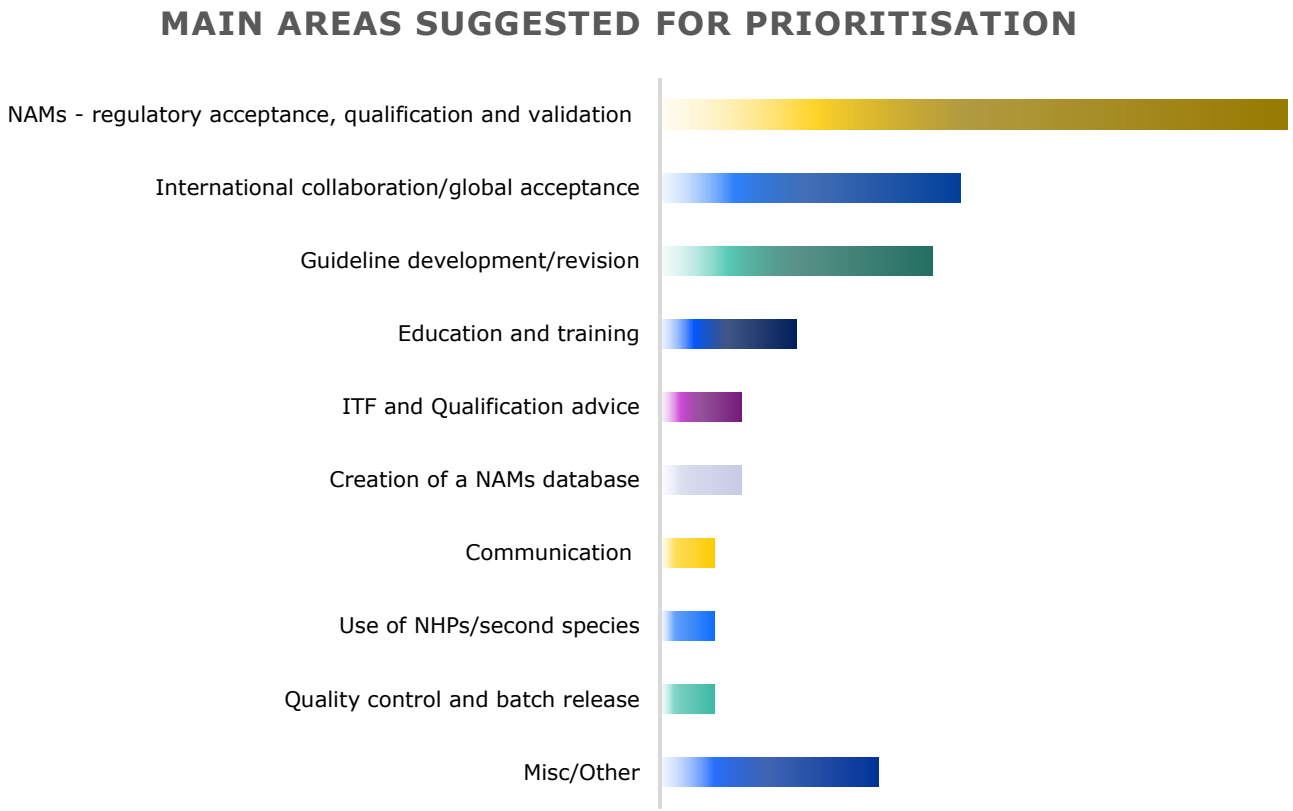
The International Medicines Regulators' Working Group on 3Rs

Supporting global harmonisation

Orla Moriarty, Translational Sciences Office EMA

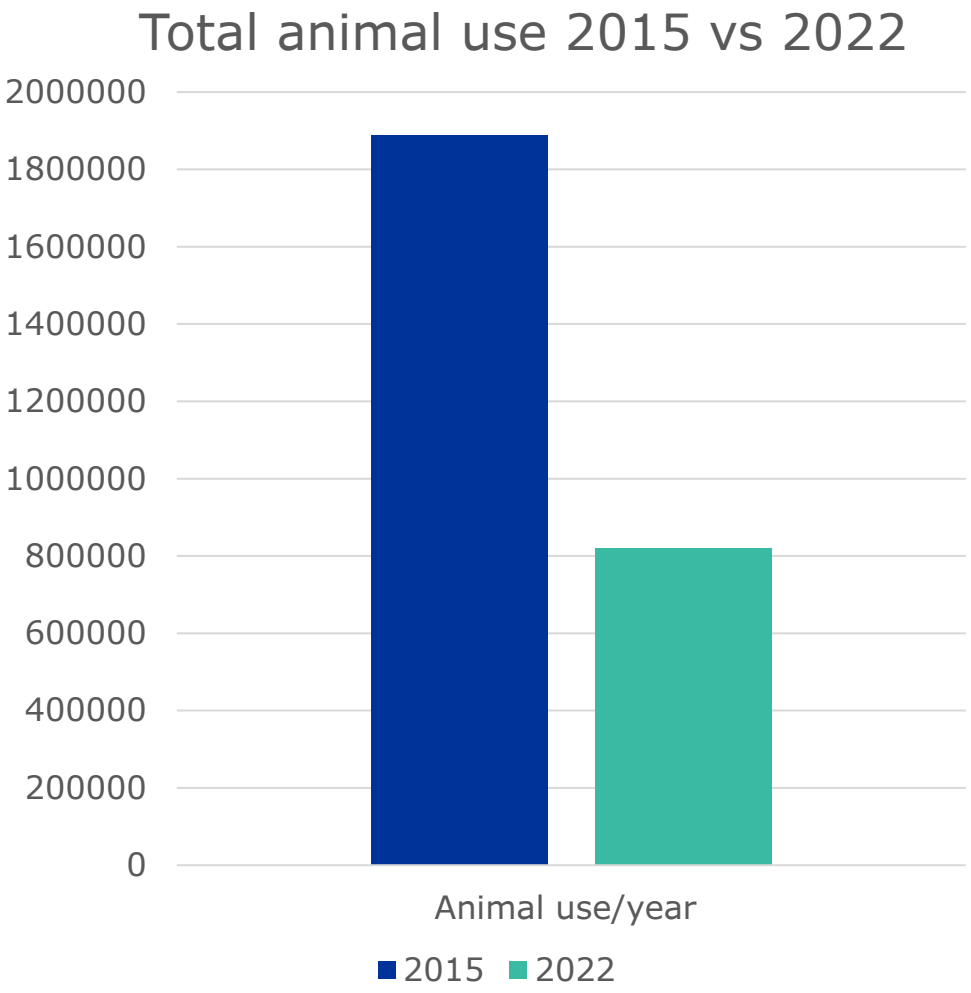
International collaboration – 3RsWP stakeholder feedback



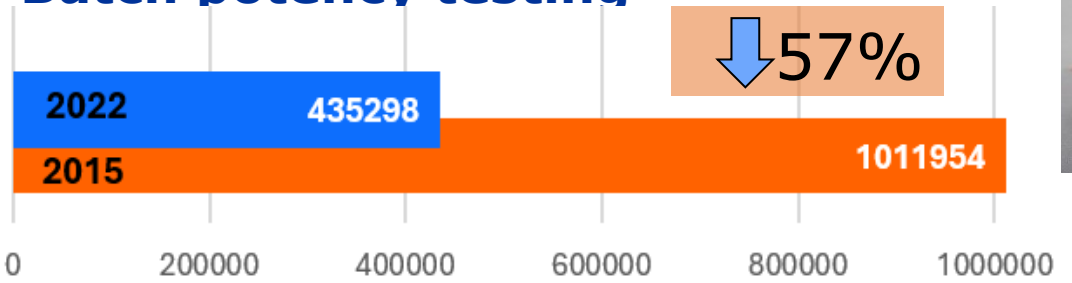


- Feedback received from stakeholders during annual meetings

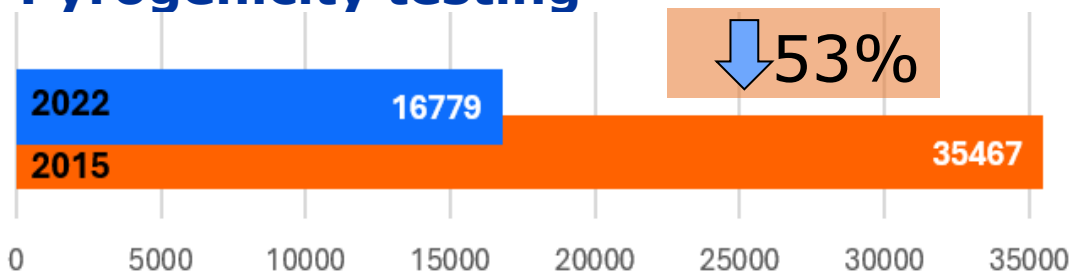
EU achievements to date



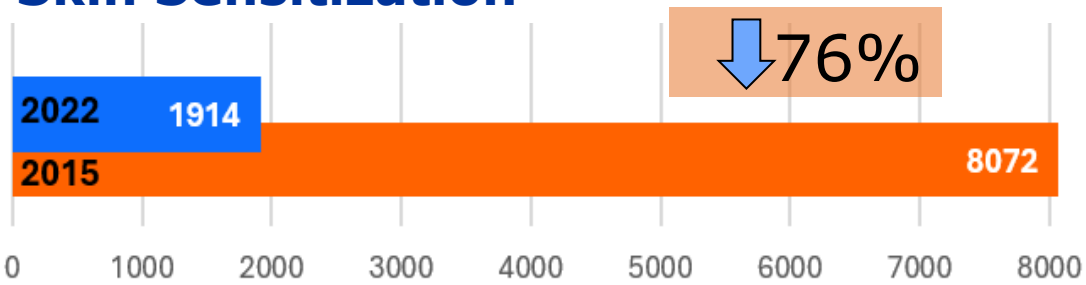
Batch potency testing



Pyrogenicity testing



Skin Sensitization



*regulatory use and routine production associated with human and veterinary medicines legislation, EU requirements – ALURES database

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International collaboration – why?

- Medicines development occurs on a **global** scale – Europe **cannot work in isolation**

- Continued reductions in animal use and promotion of the 3Rs requires global regulatory alignment to achieve harmonisation on:

    - Acceptance criteria for NAMs
    - Batch release requirements
    - Phasing out of obsolete tests

Establishment of the IMRWG3Rs

- Important to allow open dialogue between regulators while maintaining confidentiality

- Informal discussions with medicines regulatory agencies with confidentiality agreements with EMA

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Establishment of the IMRWG3Rs

Kick off discussions

Jan 2024

- State of the art in each region
- Agreement on establishment of group

ToR Drafting Group

Mar 2024

- EMA, Swissmedic, FDA & TGA

Agreement on ToR

June 2024

- Draft ToR agreed by group

Management Approval of ToR

Oct 2024

- Support from management of all participating regions

Publication of ToR

Jan 2025

- ToR available on EMA website

First IMRWG3Rs meeting

Jan 2025

- Recent updates from each region
- Agreement on standing agenda items and ways of work
- EMA to chair for 1st year with Swissmedic as cochair

IMRWG3Rs - Terms of Reference

Overarching Goal

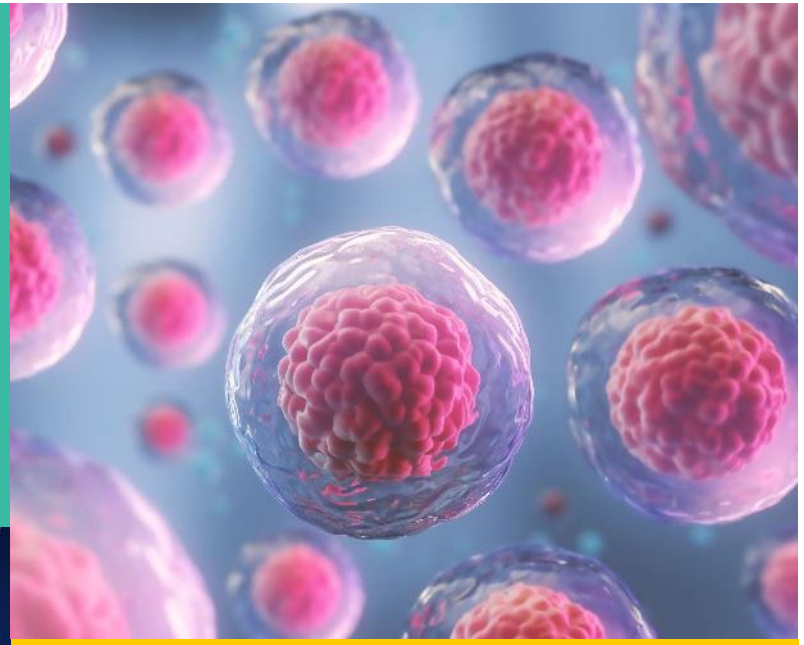
To foster a consistent global approach across regulatory jurisdictions to achieve internationally harmonised 3Rs (Replacement, Reduction, Refinement) recommendations and assist in the implementation of new alternative approaches for testing of human and veterinary medicinal products, wherever possible.

IMRWG3Rs - Terms of Reference

- More specifically, the group aims to facilitate:
  - Application of 3Rs in non-clinical testing
  - Agreement on acceptance criteria for NAMs within specific contexts of use
  - **Review of quality control and batch release requirements to encourage broader acceptance of the use of 3Rs-compliant methods where possible**
  - Support for the phasing out of obsolete tests
  - Development of a regulatory position paper on 3Rs which could be shared with other medicines regulatory authorities
  - Training and competence building through exchange of information on 3Rs-compliant methods
  - Sharing of information on 3Rs activities and developments in the participating regions, as well as opportunities for international stakeholder engagement

Meetings in 2025

- January
- Participation of IMRWG in 3RsWP meeting, April
- July
- November



Ways of working

- Updating on regional 3Rs developments
- Sharing of NAM case studies and data
- Harmonised guidance development
- Confidence building
- Aligned stakeholder engagement

Key discussions to date

- Qualification frameworks for NAMs
- Voluntary submission of NAM data to regulators
- ICH reflection paper on NAMs
- Sharing of information on events
- Global position statement on 3Rs

- At a meeting of its Executive Committee in 2025, ICMRA agreed to work on a statement on 3Rs

  "supporting work currently being done and encouraging regulators to be active in this area"

- ICMRA convened a drafting group which includes EMA and Swissmedic (chair and co-chair of IMRWG3Rs)

- Agreed by drafting group for all IMRWG regions to provide input and feedback

- Includes recommendations on reductions in animal use related to batch release testing (incl. pyrogenicity testing)

- Drafting at an advanced stage, will be submitted to the ICMRA plenary for approval

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SCIENCE MEDICINES HEALTH

Thank you

3Rs@ema.europa.eu

Follow us

Phasing Out Rabbit Pyrogen Tests: USP's Approach

Leslie Furr, MS
Principal Scientist, Microbiology Unit

Endotoxins and Pyrogens Tests

History

The beginning

- **1942** - RPT is added to USP XII
- **1955** - Added tests for transfusion and infusion assemblies

New technologies

- **1980** - USP <85> becomes official
- Replace RPT in monographs, where possible
- **2016-2017** - <151> & <161> revised to allow a validated, equivalent in vitro in place of in vivo rabbit pyrogen test, where appropriate
- **2019** Future of endotoxins and pyrogens workshop

Today

- **May 2024** USP hosts Modern methods for endotoxins and pyrogens workshop
- **Jan 2026** 2025-2030 Cycle Subcommittee re-formed
  - **Alternatives to Pyrogen Tests**
    - **Call for candidates Expert Panel**
    - **Call for data**

Rabbit Pyrogen Test <151>

13 remaining monographs referencing <151>

Monograph	Last Revised	Interferences
Ammonium Molybdate Injection	Prior to 2013	Ionic interference
Antithrombin III Human	01-Dec-2024	Protein binding/matrix effects
Trace Elements Injection	Prior to 2013	Ionic interference
Floxuridine	01-May-2020	Formulation-related; nucleosides/nucleotides
Floxuridine for Injection	01-May-2020	Formulation-related; nucleosides/nucleotides
Fluorescein Injection	01-May-2019	Colorimetric or optical interference
Indium In 111 Oxyquinoline Solution	Prior to 2013	Radioactive compounds
Oxacillin Injection	01-Dec-2021	β-lactam antibiotics
Polymyxin B Sulfate	01-May-2017	Polymyxins directly bind and neutralize endotoxins
Polymyxin B for Injection	01-Aug-2025	Polymyxins directly bind and neutralize endotoxins
Saline TS	Prior to 2013	Control
Sulfamethoxazole and Trimethoprim Injection	Prior to 2013	Colorimetric/pH-related interference
Verteporfin for Injection	01-May-2020	Colorimetric or optical interference

2025 – 2030 Endotoxins & Pyrogens Workplan

Why This Work Matters

Europe has already banned the Rabbit Pyrogen Test (RPT)

Global manufacturers may need to support both RPT and the Monocyte Activation Test (MAT)

Broad industry misalignment

Health authorities, companies, and regions are progressing at different speeds

Need for a modernized, harmonized approach to pyrogen testing

US Regulations

21 CFR 610.13 Purity

Mandates Rabbit Pyrogen Test (RPT) for injectable biologics

Creates a U.S. regulatory requirement for manufacturers to retain RPT unless exempted

Defines dose, procedure, and USP methods

Title 21 Food and Drugs

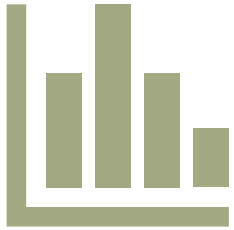
PART 610—GENERAL BIOLOGICAL PRODUCTS STANDARDS

21 CFR 610.13(b)

Test for pyrogenic substances. Each lot of final containers of any product intended for use by injection shall be tested for pyrogenic substances by intravenous injection into rabbits as provided in paragraphs (b) (1) and (2) of this section: Provided, That notwithstanding any other provision of Subchapter F of this chapter, the test for pyrogenic substances is not required for the following products: Products containing formed blood elements; Cryoprecipitate; Plasma; Source Plasma; Normal Horse Serum; bacterial, viral, and rickettsial vaccines and antigens; toxoids; toxins; allergenic extracts; venoms; diagnostic substances and trivalent organic arsenicals.

2025 – 2030 Endotoxins & Pyrogens Workplan

Alternatives to RPT: Next Steps



Immediate Action:
Call for Data

Alternative Pyrogen Tests

- MAT primary candidate alternative to RPT
- Issue a call for data to assess variability across product types

Recruit experts:
Call for candidates

Create an expert panel to review MAT as an alternative to pyrogen tests

- Evaluate other alternative tests and their potential inclusion
- Develop reference material to compliment documentary standards

2025 – 2030 Endotoxins & Pyrogens Workplan

Overview

Feb 2026

- Publish call for data

Mar 2026

- Begin recruiting for Expert Panel

*Spring 2027**

- Publish draft proposal(s)

*Spring 2028**

- Publish Chapter(s) in USP-NF

**Projected timing, subject to change*

2025 – 2030 Endotoxins & Pyrogens Workplan

Potential Outcomes

Test Method

- Below 1000 test method chapter
- Requirements and acceptance criteria

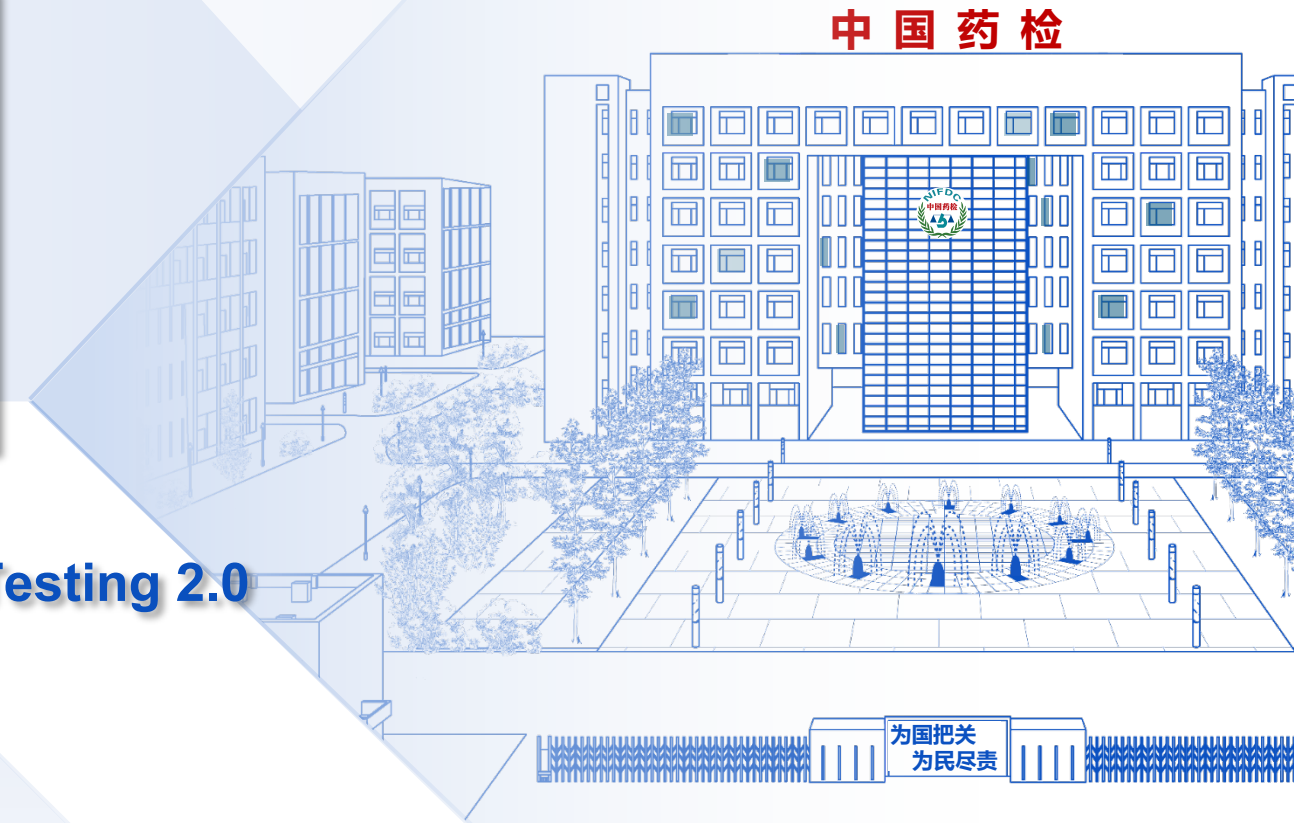
Guidance

- Above 1000 informational chapter
- Validation strategies for MAT
- Applicability across product classes
- Expectations for comparability to existing pyrogen methods

Reference Standards

- Growing need for non-endotoxin pyrogen (NEP) reference standards
- Development dependent on the chapter specific guidance:
  - Required analytes
  - Test sensitivity/fit-for-purpose
  - Validation strategy

Latest Progress of Pyrogen/Endotoxin Test in Chinese Pharmacopeia



EDQM-EPAA Hybrid Symposium: Pyrogen Testing 2.0

25/26 Feb 2026, Brussels, Belgium

Qing He, NIFDC

中国食品药品检定研究院

National Institutes for Food and Drug Control

Outline

1. A Brief Introduction of National Institutes for Food and Drug Control (NIFDC) and Chinese Pharmacopoeia (ChP)
2. Latest Progress and Requirement of Pyrogen/Endotoxin Test in ChP
3. Summary and Future Plan




National Institutes for Food and Drug Control (NIFDC)

- A subsidiary of National Medical Products Administration (NMPA)
- Center for Medical Device Standardization Administration of NMPA
- Chinese General Institute for Drug Inspection

1950's

2000's

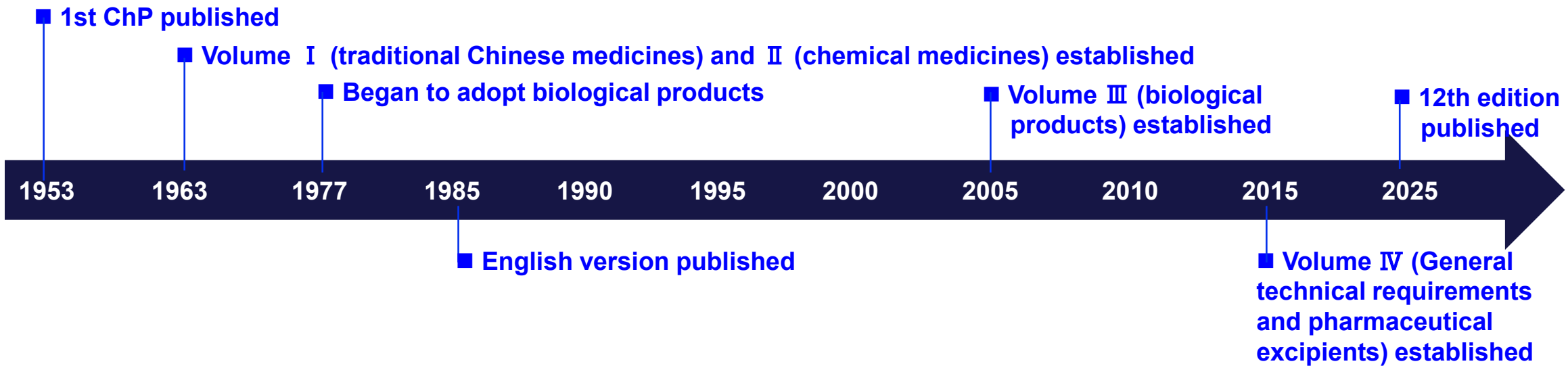
2016's

International Role and Cooperation of NIFDC

- **3 WHO Collaborating Centers**
  - **WHO Collaborating Center for Drug Quality Assurance**
  - **WHO Collaborating Center for Standardization and Evaluation of Biologicals**
  - **WHO Collaborating Center for Herbal Medicine**
- **Establish long-term cooperation mechanism with international authoritative counterparts**
- **Participate in the establishment and collaboration of WHO IS**

The role and development of Chinese pharmacopoeia (ChP)

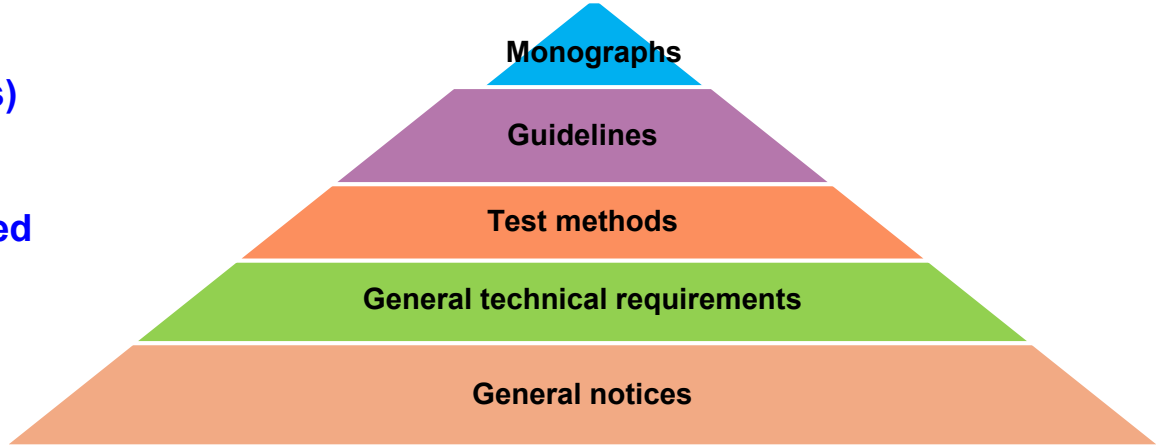
- The Chinese Pharmacopoeia (ChP) is the national standards to ensure drug quality and drug usage satety for the public.
- It is the statutory basis that must be strictly abided by in the development, manufacturing, inspection, operation, supply, administration and management of pharmaceutical product. It is the core of China's national drug standards and is also the most serious and authoritative drug standards.
- 12 editions of ChP have been published

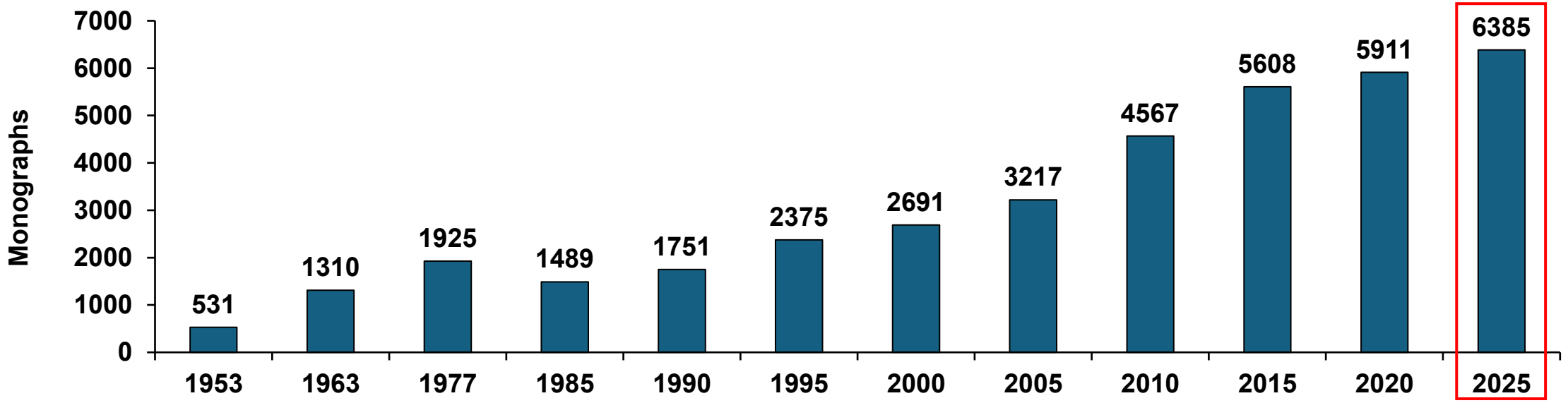


Overview of the 12th/2025 edition ChP

➢ 4 volumes

- Volume Ⅰ—Traditional Chinese Medicines (3069 monographs)
- Volume Ⅱ —Chemical Medicines (2776 monographs)
- Volume Ⅲ —Biological Products (153 monographs) and related general technical requirements (21)
- Volume Ⅳ — General technical requirements (389) and Pharmaceutical Excipients (387 monographs)





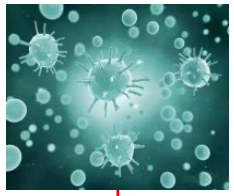
Development of pyrogen/endotoxin test in ChP: Moving towards to the 3Rs

- Rabbit pyrogen test (RPT)
  - has variations in responses
  - involves the use of animals *in vivo*, does not comply with the "3Rs" principle
  - expensive

1953

- Bacterial endotoxin test based on the amoebocyte lysate (LAL/TAL BET)
  - only detect gram-negative bacteria endotoxins
  - horseshoe crabs are the endangered species and the second-class protected animal in domestic

1990 (2nd supplement)



- Monocyte activation test (MAT)
  - based on the mechanism of fever reaction
  - involves the use of cells *in vitro*
  - can detect endotoxin and non-endotoxin pyrogens

2020

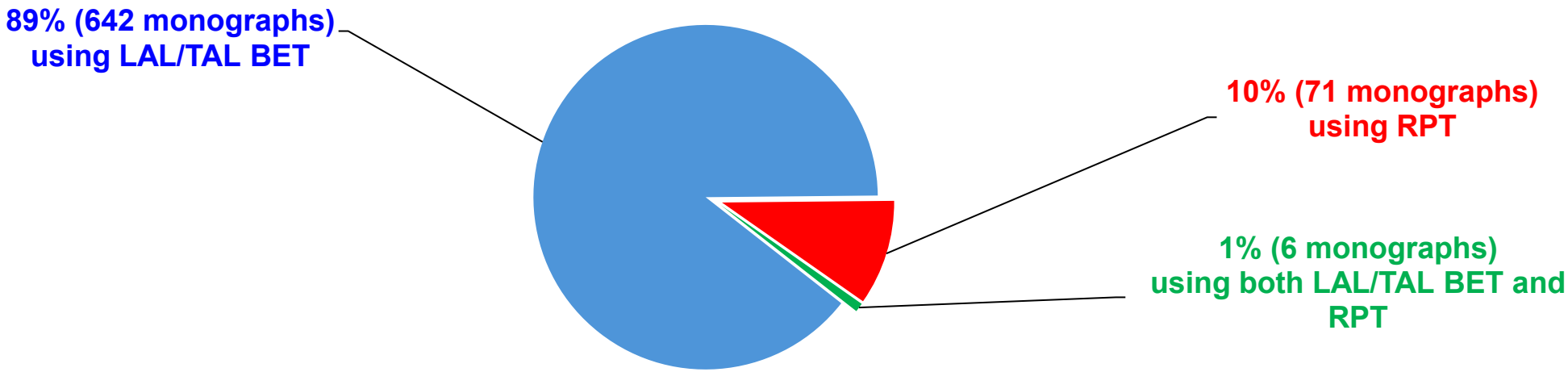
- BET based on the recombinant factor C (rFC BET)
  - based on the recombinant reagent, does not require the use of horseshoe crabs
  - better batch to batch consistency, specificity, and sustainability

- Report gene assay for pyrogen detection (RGA)
  - involves the use of transgenic cell line *in vitro*
  - uses the NF-κB as the pyrogenic marker

2020 (1st supplement)

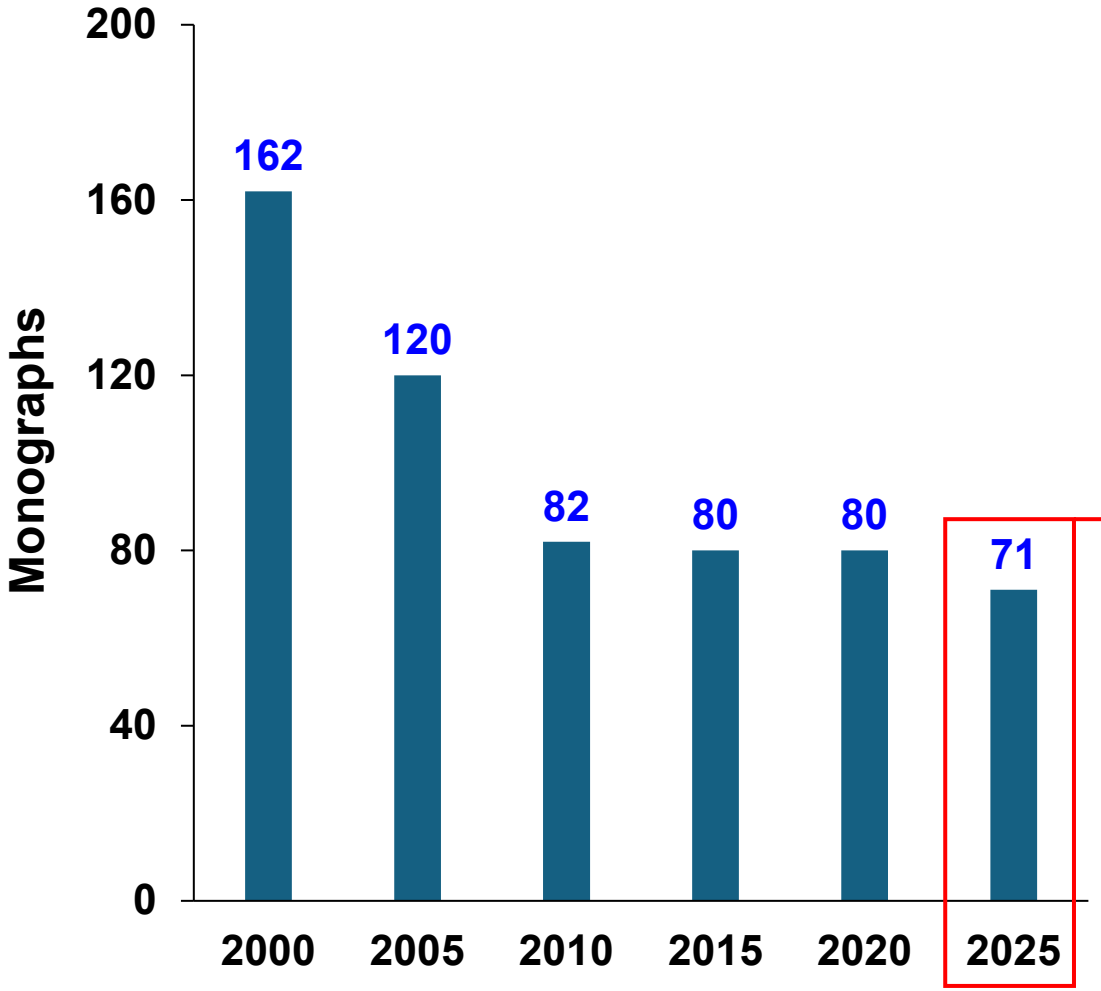
Overview of Pyrogen/Endotoxin Test in 2025 ChP

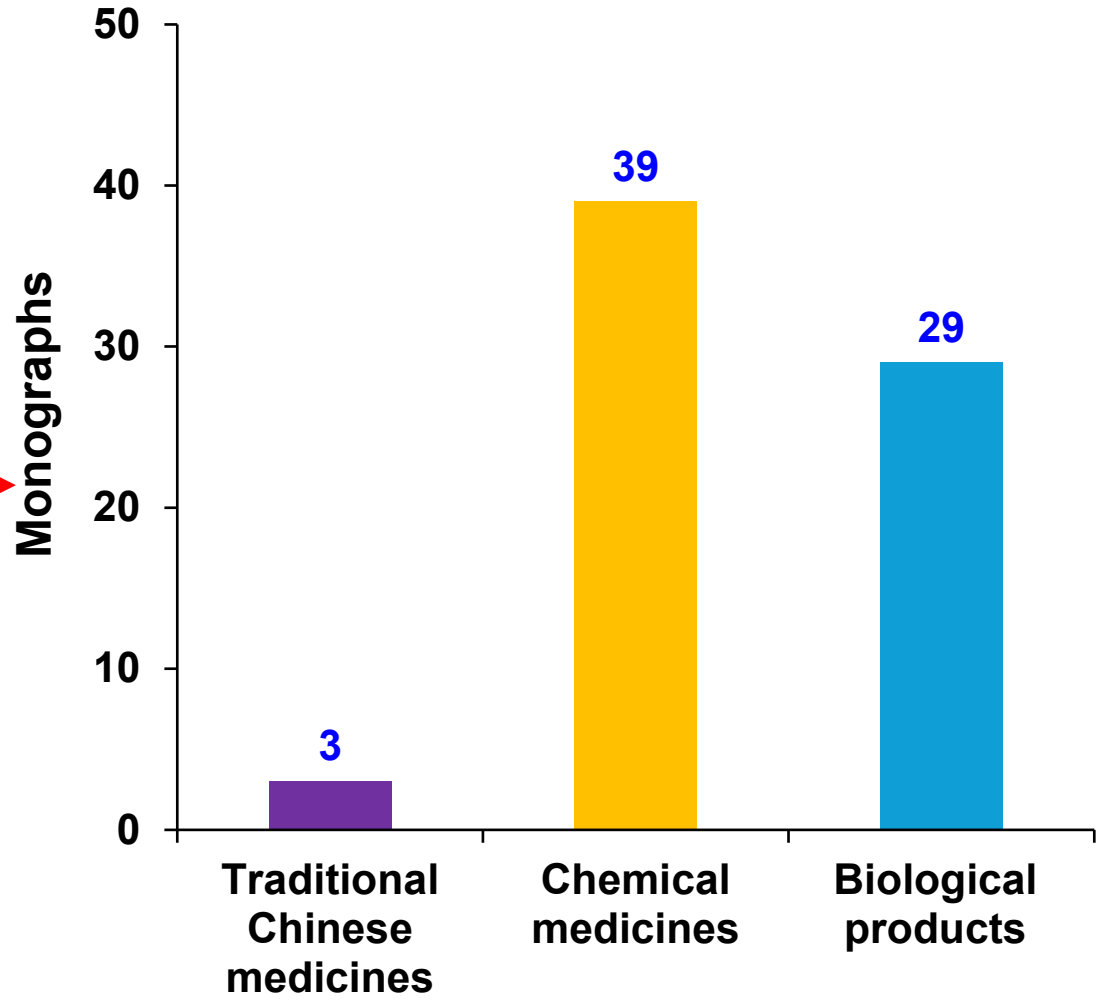
2025 ChP	Pyrogen	RPT	(General Principle 1142)	
		MAT	(Guideline 9301)	
		RGA	(General Principle 3309)	Supplementary method for pyrogen detection
	Endotoxin	LAL/TAL BET	(General Principle 1143)	
		rFC BET	(Guideline 9251)	Supplementary method for endotoxin detection



- No monographs have adopted the methods (MAT, RGA and rFC BET) for pyrogen/endotoxin detection

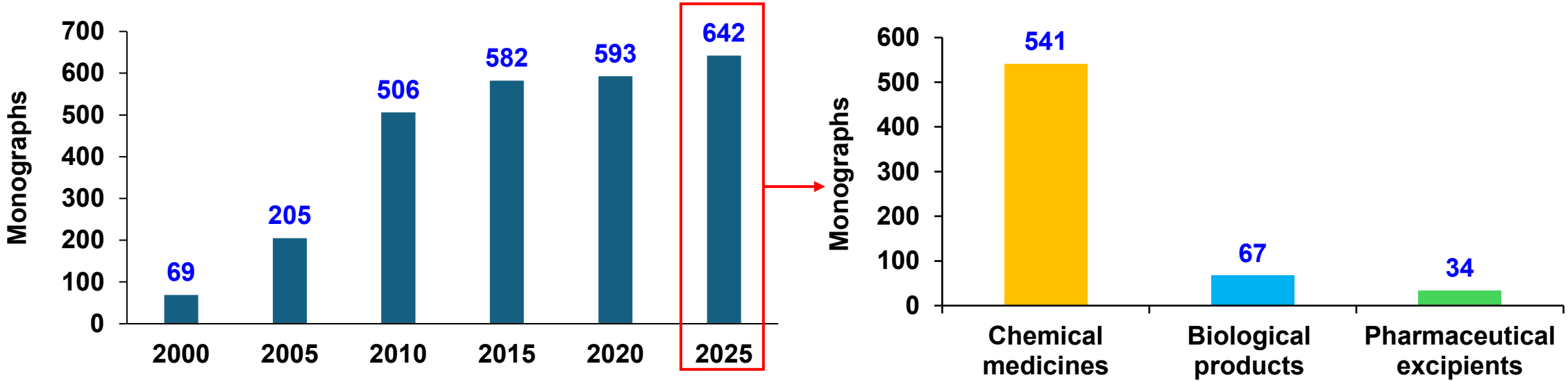
RPT in General Principle 1142 of 2025 ChP





LAL/TAL BET in General Principle 1143 of 2025 ChP

- LAL/TAL BET
  - Gel method
    - Limit assay (referee assay)
    - Semi-quantitative assay
  - Spectrophotometric method
    - Turbidimetric assay
      - Kinetic turbidimetric assay
      - Terminal turbidimetric assay
    - Chromogenic assay
      - Kinetic chromogenic assay
      - Terminal chromogenic assay



MAT in Guideline 9301 of 2025 ChP

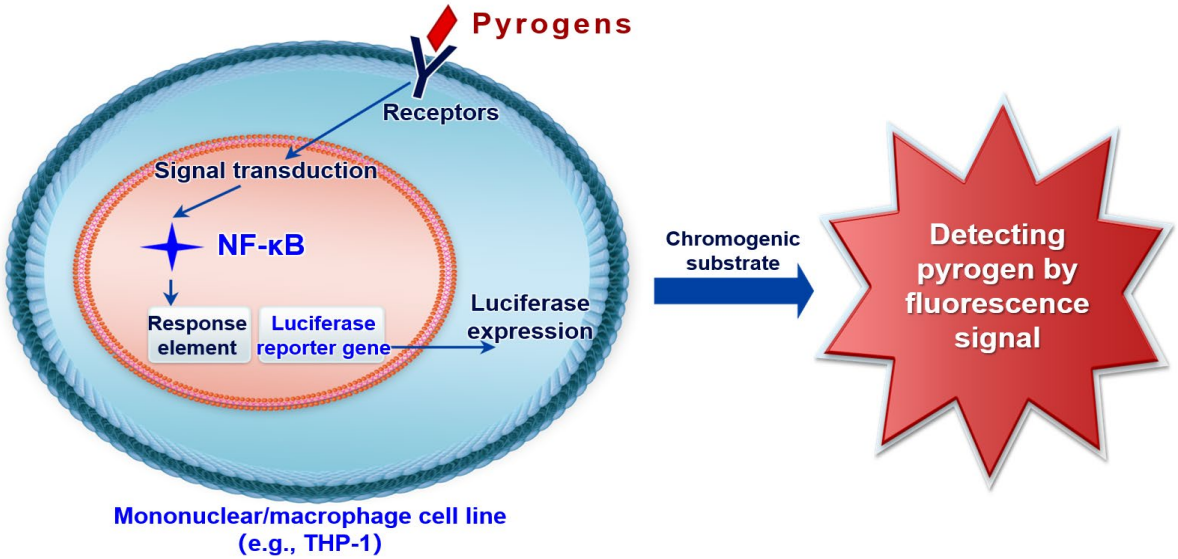
- **Included in the guideline 9301 for the application of safety tests for Injections: "the Intravenous injection could use the MAT after the risk analysis"**

Design of MAT	☐ quantitative test (corresponding to the method A of EP)
Version of MAT	☐ PBMC—IL-6
	☐ Fresh/cryopreserved human whole blood—IL-1β/IL-6
	☐ Mononuclear cell line HL60—IL-6

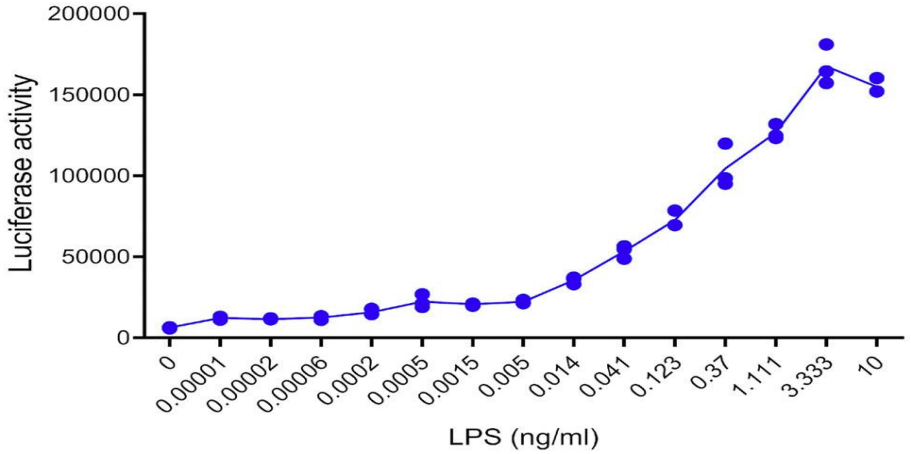
MATs	Within-lab reproducibility (%)	Inter-lab reproducibility (%)	Sensitivity (%)	Specificity (%)
PBMC—IL-6	86.7~100	78.5~96	90.1	92.3
Cryo pooled human whole blood—IL-1β	80.0~86.7	63.6~85.7	82.5	100
Cryo pooled human whole blood—IL-6	86.7~100	57.1~92.9	81.7	100

Chin J Pharm Anal, 2012, 32(10): 5-11. Innate Immun, 2018, 24(5):316-322.

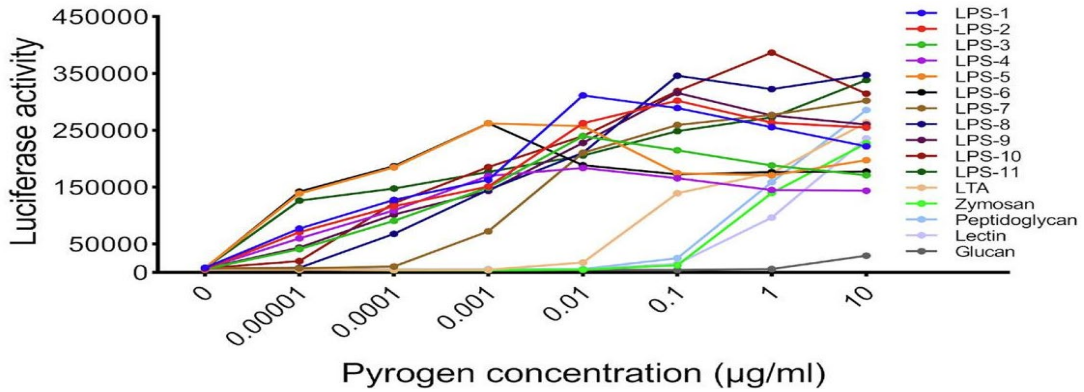
The Latest Progress—RGA in General Principle 3309 of 2025 ChP



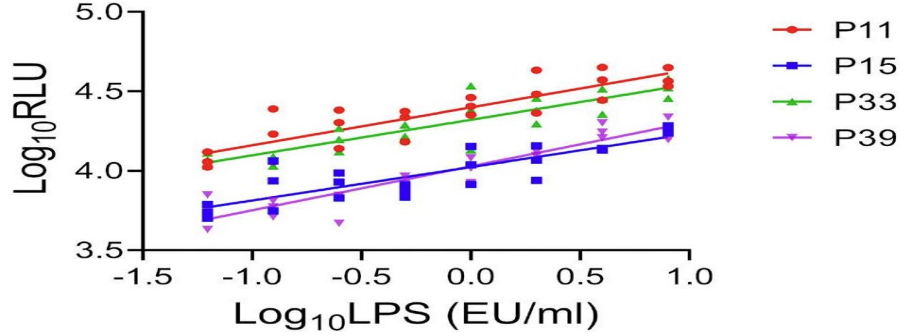
➢ significantly dose-effect relationship



➢ detect both endotoxins and nonendotoxins



➢ dose-effect responses at cell passages



Signal Transduct Target Ther. 2024;9(1):33.

Applicability of the test with different classes of biologicals

➢ Recovery of LPS spikes in biologicals

Drug		NF-κB response	
	Fold-dilution	Spike recovery (%)	Interference
Group A & C meningococcal polysaccharide vaccine	2000	61	no
Basiliximab	16	75	no
Rabies vaccine (Vero cells) for human use, freeze-dried	100	119	no
Japanese encephalitis vaccine (Vero cells), inactivated	100	111	no
Insulin aspart injection	160	63	no
Human albumin	2	79	no
Recombinant human erythropoietin injection (CHO Cell)	24	56	no

Signal Transduct Target Ther. 2024;9(1):33.

Validation of the test in different laboratories

➢ **Drugs involved in the validation experiment**

Drug	Endotoxin limit concentration (EU/ml)	Limit of endotoxin detection (EU/ml)	Maximum valid dilution
Group A & C meningococcal polysaccharide vaccine	1000	0.5	2000
Basiliximab	8	0.5	16
Rabies vaccine (Vero cells) for human use, freeze-dried	50	0.5	100
Recombinant human erythropoietin injection (CHO Cell)	12	0.5	24

➢ **Validation of the test in different laboratories**

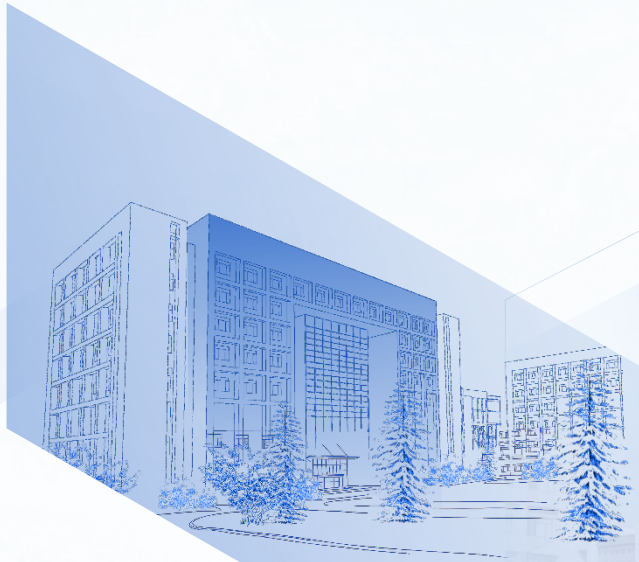
Test	Within-laboratory reproducibility (%)	Interlaboratory reproducibility (%)	Sensitivity (%)	Specificity (%)
THP-1/NF-κB	Lab. 1: 85 (51/60) Lab. 2: 80 (48/60) Lab. 3: 80 (36/45)	Lab. 1—Lab. 2: 83.3 (50/60) Lab. 1—Lab. 3: 95.6 (43/45) Lab. 2—Lab. 3: 86.7 (39/45)	89.9 (89/99)	90.9 (60/66)
Rabbit pyrogen test	/	/	57.9	88.3

Signal Transduct Target Ther. 2024;9(1):33.

Summary and Future Plan

	Stage 1: 2020-2025	Stage 2: 2025-2030	Stage 3: 2030-2035
Regulation	novel tests (MAT, RGA, rFC BET) as supplementary methods (not official)	corresponding general principles of the novel tests established (official)	novel tests provided to be alternative（flexible not mandated）
User	be familiar with the novel tests and the development trend	comparability and validation data accumulated	choose the tests based on validation and will
Tool	a few kits developed	more kits developed	more developed in-house and commercial kits available

Thank you for your attention



中国食品药品检定研究院

National Institutes for Food and Drug Control

25 Feb. 2026,
Pyrogen testing 2.0: Ethical, Evolving and Eco-friendly - Implementing safe, rapid, state-of-the-art and sustainable non-animal approaches worldwide

Recent Changes in the Landscape of Rabbit Pyrogen Testing and the Planned Listing of the Monocyte Activation Test in the General Information of the Japanese Pharmacopoeia

Katsuhiko HAYASHI, Ph.D.

Senior Research Scientist
Division of Microbiology
National Institute of Health Sciences, Japan,
under the Ministry of Health, Labour and Welfare (MHLW)

Disclaimer

The views and opinions expressed in this presentation are my own and do not necessarily reflect those of any affiliated organizations. The information shared is intended for informational purposes only and should not be interpreted as specific regulatory advice or guidance.

Thank you for your understanding.

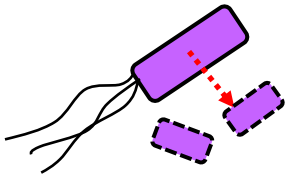
Disclosure of the Conflict of Interest (COI)

Katsuhiko HAYASHI has no financial conflicts of interest to disclose in relation to this presentation.

Pyrogens to be controlled

Endotoxins (Lipopolysaccharide, LPS)

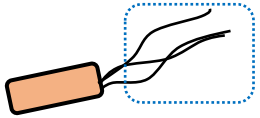
Cell wall of Gram negative bacteria



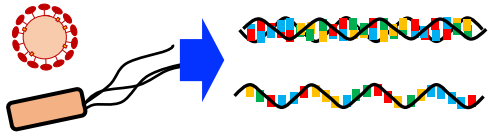
Non-Endotoxin Pyrogens (NEPs)

Cell wall of Gram positive bacteria

Bacterial Flagellins

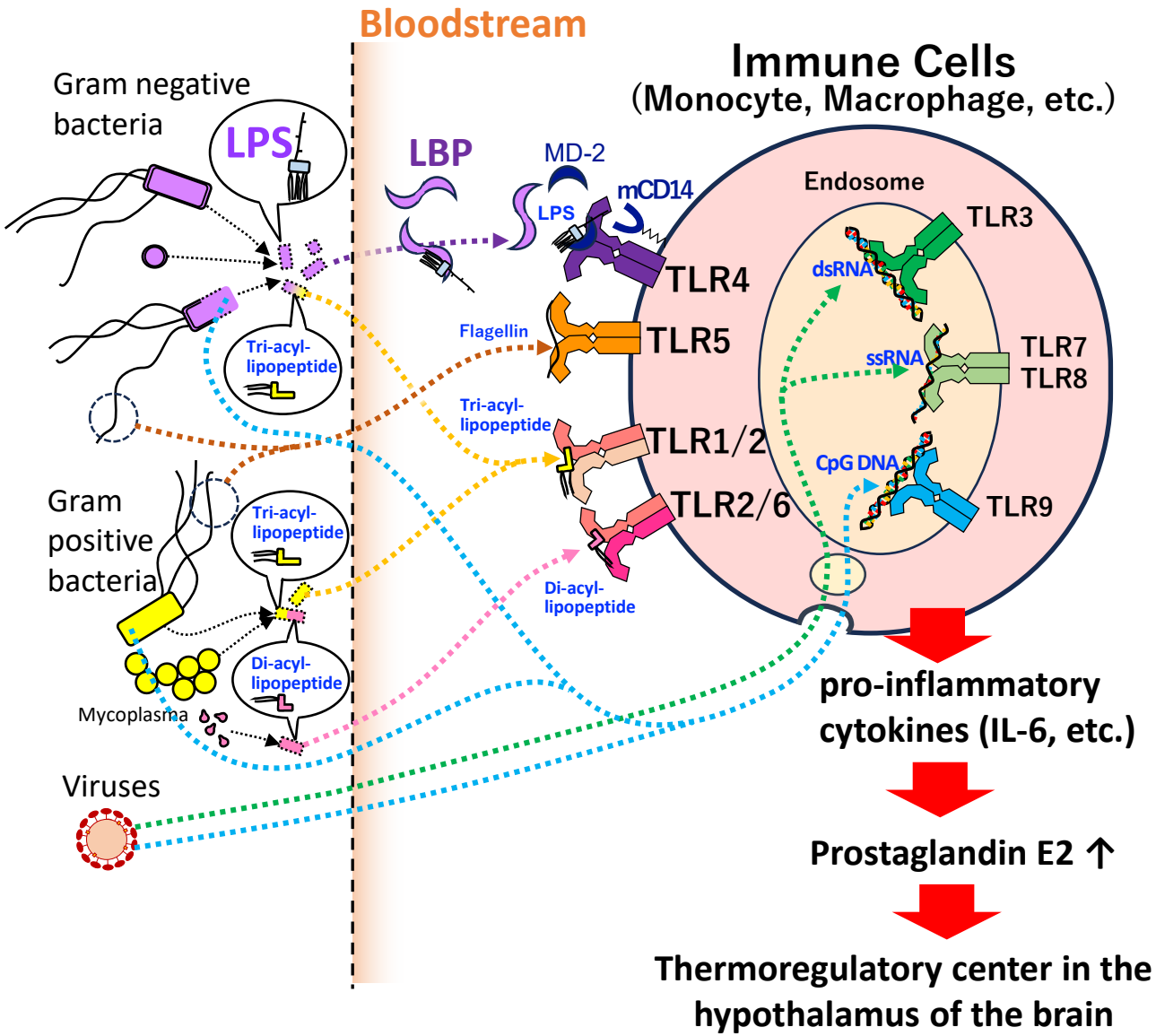


Viral/ Bacterial nucleic acid



Others

Chemicals, cytokines, etc.

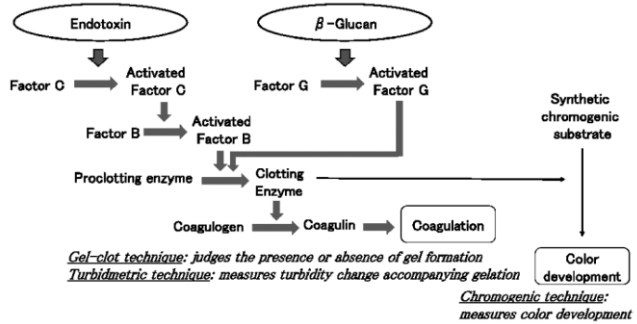


Methods to Detect Pyrogens in the Japanese Pharmacopoeia

BET (Bacterial Endotoxins Test)

JP18 General Tests, 4.01 Bacterial Endotoxins Test, since 1988

- Horseshoe crab amebocyte lysate that specifically reacts to LPS has been used.



rFC/rCR (Recombinant reagents for endotoxins evaluation)

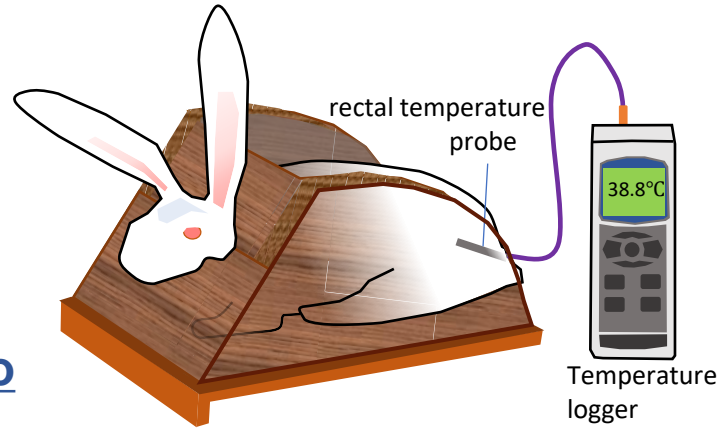
JP18 General Information, "Bacterial Endotoxins Test and Alternative Methods Using Recombinant Protein Reagents for Endotoxin Assay <*G4-4-180*>," since 2021

- For informational purposes regarding recombinant reagents, rFC and rCR.

RPT (Rabbit Pyrogen Test)

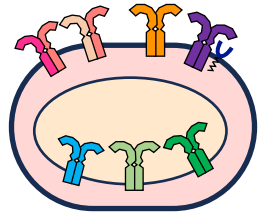
JP18 General Tests, 4.04 Pyrogen Test, since 1951

- Intravenous administration and rectal temperature measurement for 3 h.
- Both LPS and NEP can be detected in principle.
- **Under the General Rules for Preparations, injections meet the requirements of BET <*4.01*>. When the BET <*4.01*> is not applicable to aqueous vehicles, the RPT <*4.04*> may be applied instead.**



MAT (Monocyte Activation Test)

Upcoming JP19 General Information, Monocyte Activation Test <*G4-13-190*>, planned for inclusion in Apr. 2026



Current Regulation for Pharmaceuticals in Japan

Japanese Pharmacopoeia (JP)

General Notices
General Rules For Crude Drugs
General Rules For Preparations
General Tests
…
4. Biological Tests/Biochemical Tests/Microbial Tests
4.01 Bacterial Endotoxins Test
4.04 Pyrogen Test
…
Official Monographs
Crude Drugs and Related Drugs
Infrared Reference Spectra
Ultraviolet-Visible Reference Spectra
General Information

Minimum Requirements for Biological Products (MRBP)

General Rules
Official Monographs
General Tests
A. Test procedures
…
Bacterial Endotoxins Test → The BET in the JP shall apply mutatis mutandis.
…
Pyrogen Test → The same method as the JP is used, except for the injection dose.
…
B. Standards, Reference Preparations, Test Toxins and Units
C. Reagents, Test Solutions, etc.
D. Buffered Solutions and Culture Media

The MRBP specifies quality standards and test methods for biological products such as vaccines, toxoids, and blood products, while the JP specifies those for other pharmaceuticals.

Pharmaceuticals That Require the RPT in Japan

Japanese Pharmacopoeia

Sulfobromophthalein Sodium Injection; however, this product is not currently commercially available in Japan.

Minimum Requirements for Biological Products

12 items require the RPT (and the BET)

[Commercially available] 7 items

- **Human Antithrombin III, Freeze-dried Concentrated**
- **Purified Vi Polysaccharide Typhoid Vaccine**
- **Human activated protein C, freeze-dried concentrated**
- **Freeze-dried Gas Gangrene Antitoxin, Equine ***
- **Freeze-dried Diphtheria Antitoxin, Equine ***
- **Freeze-dried Habu Antivenom, Equine ***
- **Freeze-dried Mamushi Antivenom, Equine ***

[Currently no commercial product] 4 items

- Human Anti-D (Rho) Immunoglobulin
- Influenza vaccine†
- Adsorbed influenza virus vaccine (H5N1) †
- Adsorbed Cell Culture-derived Influenza virus vaccine (H5N1) †

* Because this product is manufactured in very few lots (approximately one lot every few years), comparison with the BET has not progressed.

† Other influenza HA vaccines are commercially available.

The RPT is now required for a few products in Japan.

25 items require either the RPT or the BET

- **Whole Blood**
- **Heat-treated Human Plasma Protein Fraction**
- **Human Serum Albumin**
- **Freeze-dried Human Fibrinogen**
- **Human Prothrombin Complex, Freeze-dried Concentrated**
- **Freeze-dried Human Blood-Coagulation Factor VIII**
- **Freeze-dried Human Blood Coagulation Factor IX Complex**
- **Freeze-dried Human Blood Coagulation Factor IX Concentrate**
- **Human Normal Immunoglobulin**
- **Freeze-dried Ion-exchange-resin Treated Human Normal Immuno-globulin**
- **Freeze-Dried Sulfonated Human Normal Immunoglobulin**
- **pH4-Treated Acidic Normal Human Immunoglobulin**
- **Polyethylene Glycol Treated Human Normal Immunoglobulin**
- **Freeze-dried Polyethylene Glycol Treated Human Normal Immunoglobulin**
- **Human Anti-HBs Immunoglobulin**
- **Freeze-dried Human Anti-HBs Immunoglobulin**
- **Polyethylene Glycol-treated Human Anti-HBs Immunoglobulin**
- **Human Anti-Tetanus Immunoglobulin**
- **Freeze-dried Human Anti-Tetanus Immunoglobulin**
- **Polyethylene Glycol Treated Human Anti-Tetanus Immunoglobulin**
- **Human Haptoglobin**
- **Influenza HA Vaccine**
- **Freeze-dried Human Anti-D (Rho) Immunoglobulin**
- Freeze-dried pH4-Treated Acidic Normal Human Immunoglobulin
- Freeze-dried pepsin-Treated Normal Human Immunoglobulin

Past Research to Replace the RPT with the BET for Blood Products

Research on the Application and Standardization of the Bacterial Endotoxins Test for Blood Products (in Japanese)
（血液製剤に対するエンドトキシン試験法の適用と基準化に関する研究）

Principal Investigator: Dr. Kazunari Yamaguchi, National Institute of Infectious Diseases (NIID)
MHLW Grants: 200735037A, 200735037B (2006–2007)

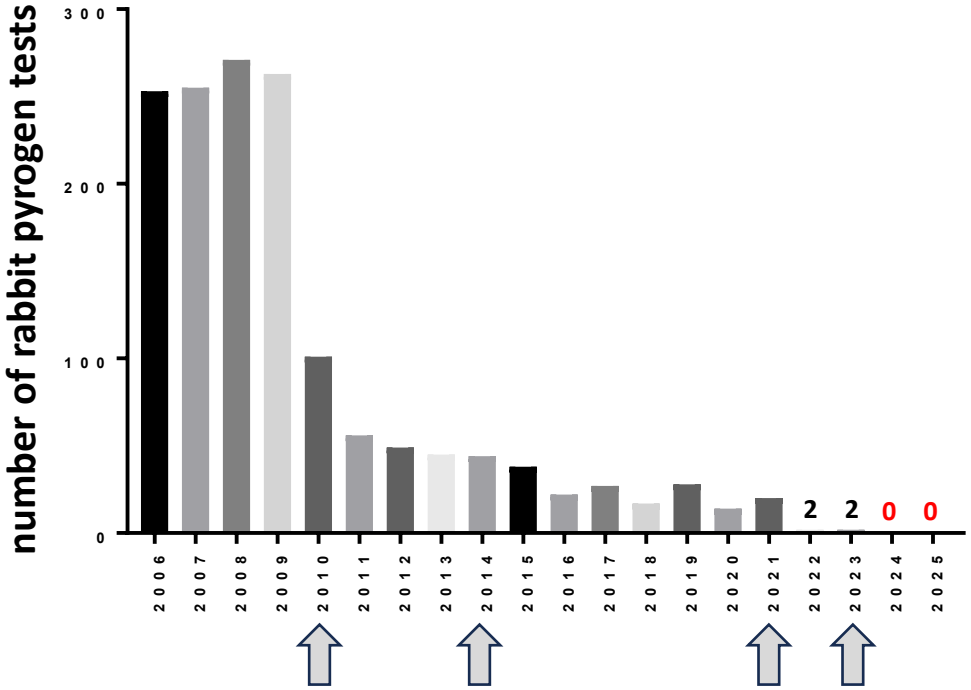
This study includes:
- Examination of the injection dose for the Rabbit Pyrogen Test
- Investigation of interference of blood products with the Bacterial Endotoxins Test
- Investigation of enhanced fever responses to bacterial endotoxins in blood products
- Evaluation of a pyrogen testing method using cytokine production from cultured human cells as an indicator
- Calculation of endotoxin limits and assessment of test method applicability

Some products enhanced pyrogenic responses in rabbits and human cells.
This was considered when setting endotoxin limits in the MRBP.

Phasing out of the RPT in National Testing for Biological Products

Shared by the National Institute of Infectious Diseases (NIID), Japan

Trend in the number of pyrogen tests over the past 20 years



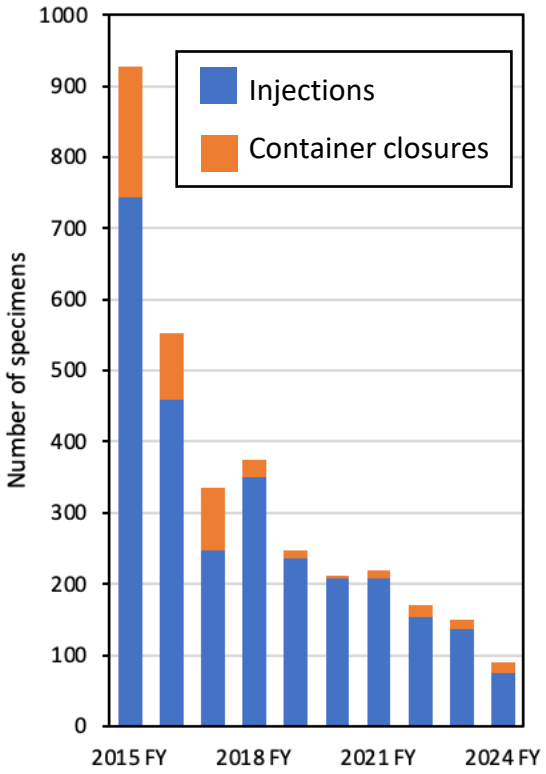
Selection of test types for lot release of biological products
Vaccines: Bacterial Endotoxins Test (BET)
Blood products: Rabbit Pyrogen Test (RPT) or BET
Antitoxins / antivenoms: RPT

Year	Event
2010	Major **8 blood products** shifted from RPT to BET (about 200 tests/year reduced).
2014	Discontinuation of RPT for **1 blood product** (Ig) (10 reduced per year).
2021	Discontinuation of RPT for **Antithrombin III** (20 reduced)
2023	Discontinuation of RPT for **Antitoxin and Antivenom** (0-5 reduced).
2024 ～	Japan has not conducted the RPT in national testing since 2024.

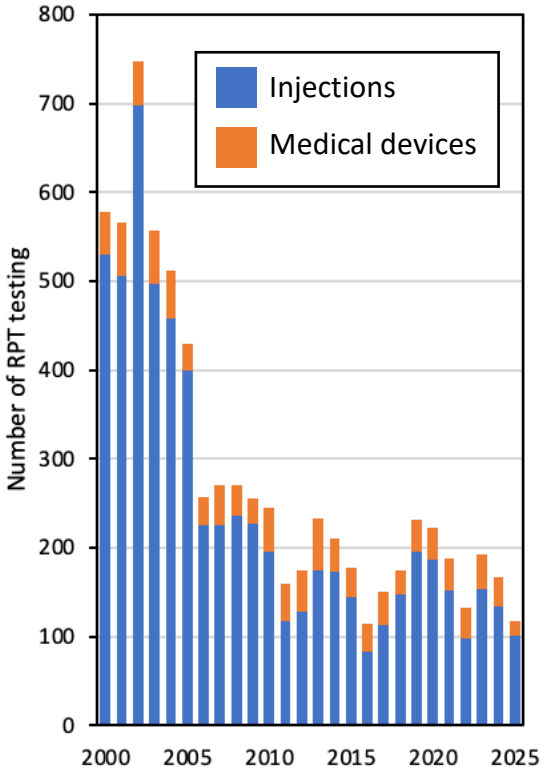
Japan has not conducted the RPT in national testing for biological products since 2024.

Decrease in the Number of RPTs Conducted in the Private Sector in Japan

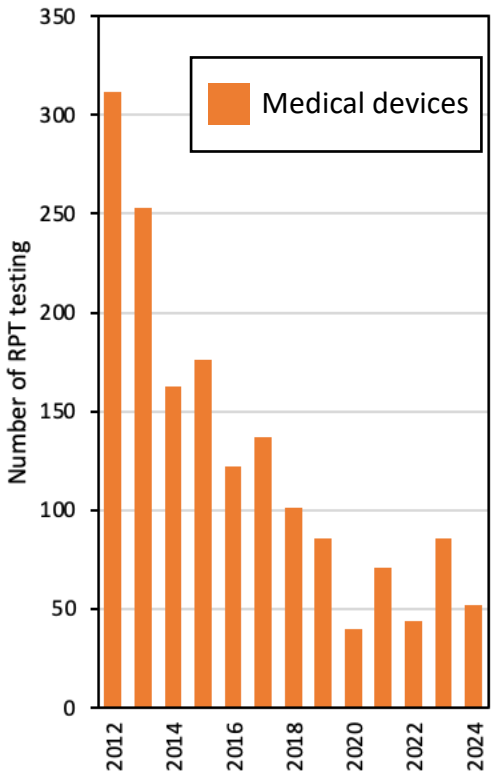
Company A



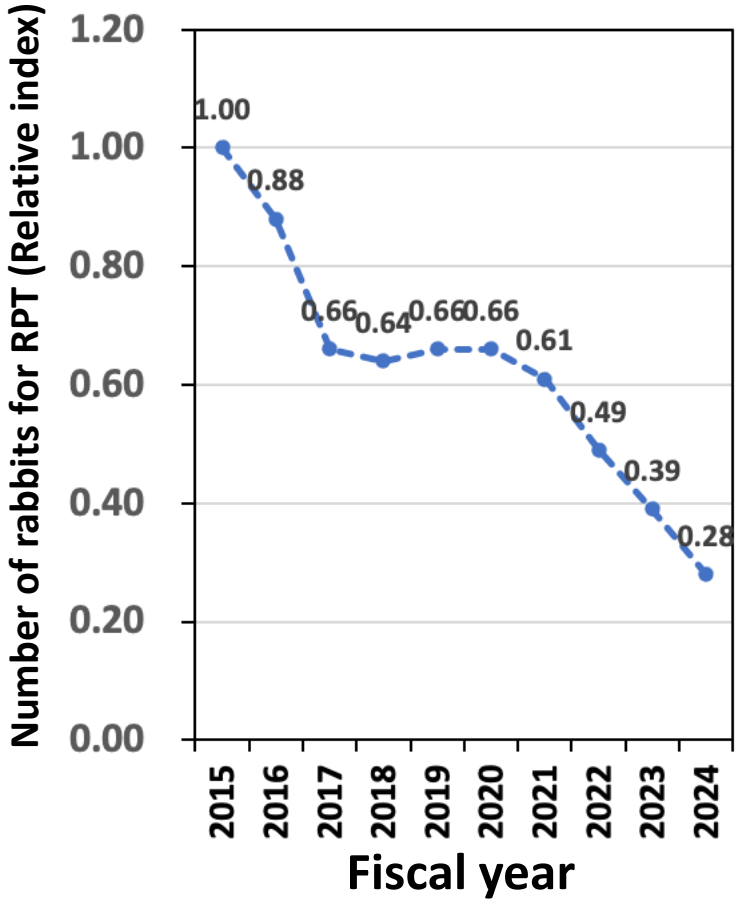
Company B



Company C



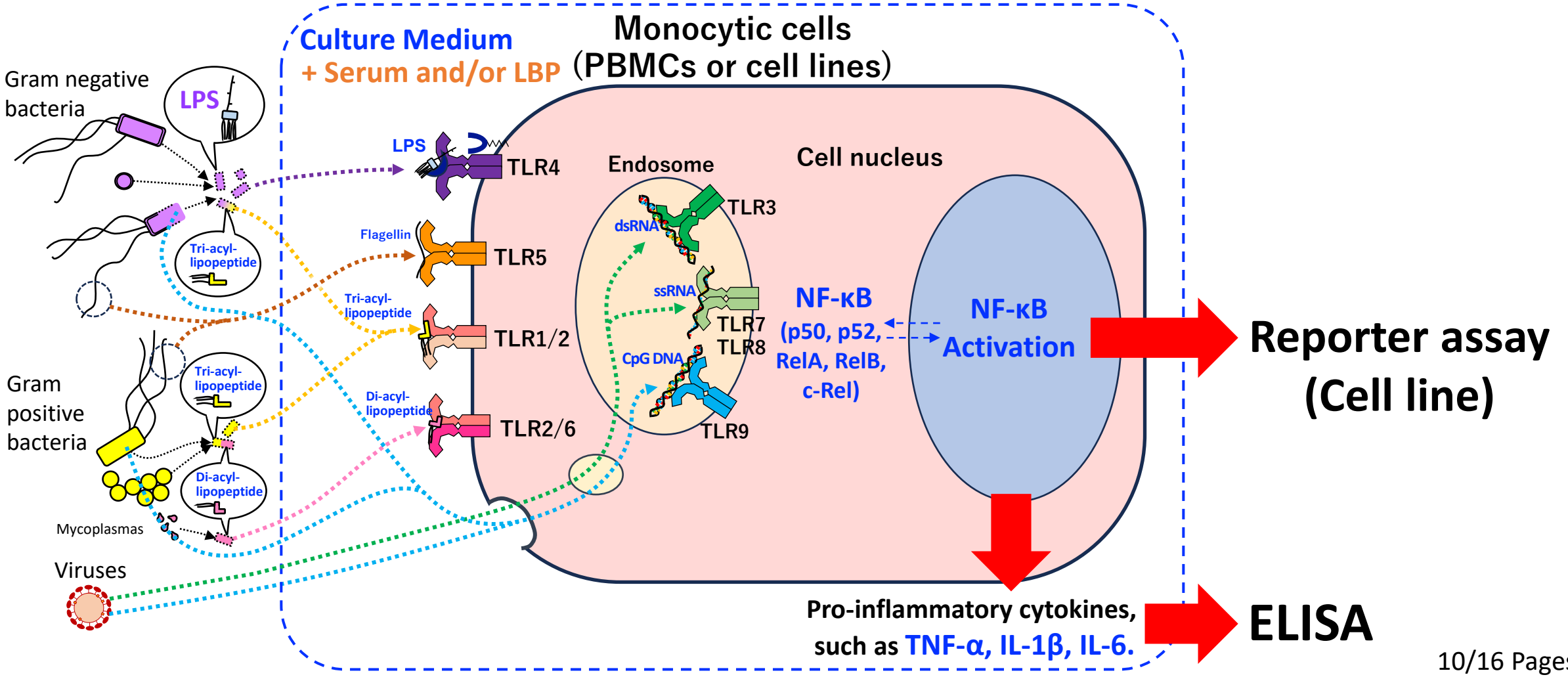
Number of rabbits used for the RPT (shared by a supplier)



- Transition from the RPT to the BET
- Discontinuation of pharmaceuticals that require the RPT

Monocyte Activation Test (MAT)

A method to detect pyrogens indirectly by measuring cytokines or signaling responses in whole blood, PBMCs, or monocytic cell lines.

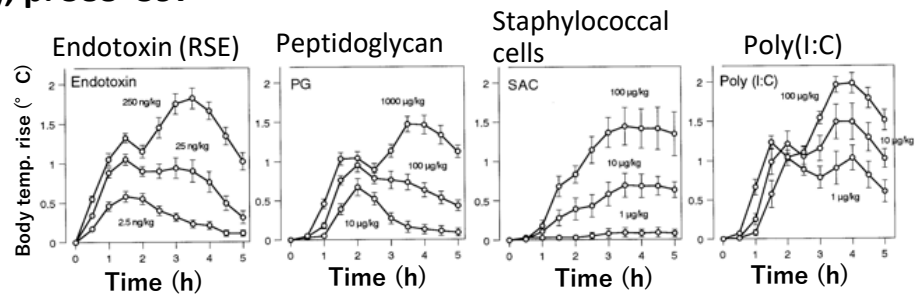


Past Research on the RPT and MAT in Japan

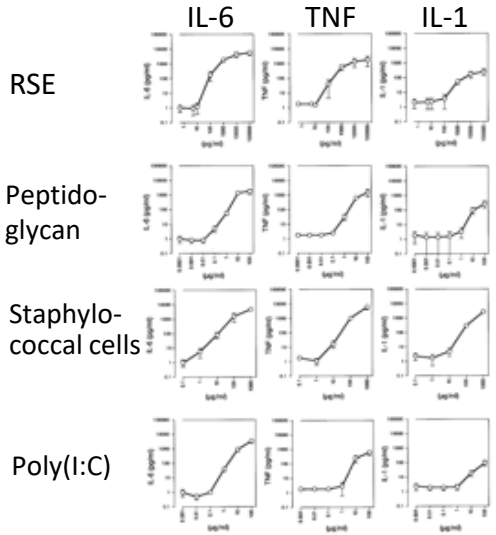
Evaluation of the In Vitro Pyrogen Test System Based on Proinflammatory Cytokine Release from Human Monocytes: Comparison with a Human Whole Blood Culture Test System and with the Rabbit Pyrogen Test

Y. Nakagawa et al, *Clinical and Diagnostic Laboratory Immunology* 2002, 9(3), p. 588–597

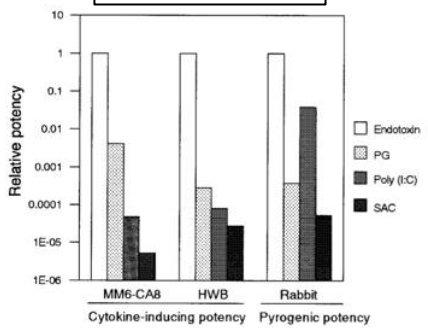
RPT



MAT by WB and MM6 cells



Evaluation



The RPT shows higher poly(I:C) sensitivity than MAT, but similar responses overall.

Research on the Application and Standardization of the Bacterial Endotoxins Test for Blood Products (in Japanese)

- Investigation of enhanced fever responses to bacterial endotoxins in blood products
- Evaluation of a pyrogen testing method using cytokine production from cultured human cells as an indicator

RPT

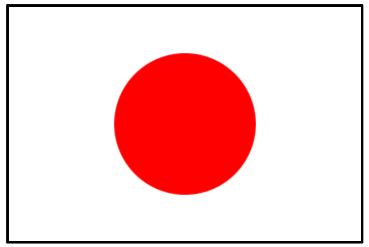
Human Blood Coagulation Factor VIII: 3.6-fold
Human Blood Coagulation Factor IX: 5.7-fold
Human Fibrinogen: 5.1-fold
Human Antithrombin III: 8.9-fold
Human Activated Protein C: 4.7-fold

in vitro assay (human cell line 28SC)

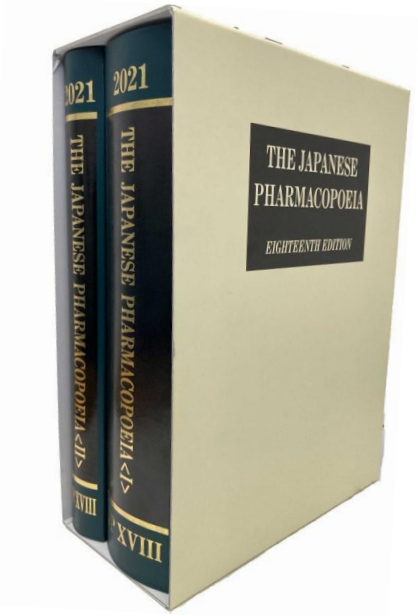
Human Haptoglobin: 1.35-fold
Human Antithrombin III: 3.3-fold
Human Activated protein C: 2.8-fold

The RPT and a 28SC cell-based *in vitro* assay showed enhanced pyrogenic effects of bacterial endotoxins in blood products.

The Monocyte Activation Test <*G4-13-190*> in the Upcoming JP19 General Information (planned for Apr 2026)



- The upcoming JP19 General Information does **NOT** describe the MAT as an alternative to RPT.
- The RPT can be replaced by alternative methods with better accuracy and precision, as described in JP General Notice 14.



Cells/ Preparatory testing

- Cells: Whole blood (WB), PBMCs, or monocytic cell lines, including reporter cell lines if justified.
- Preparatory testing: Evaluate the standard curve and LOD with RSE. Assess interference with RSE (50–200% recovery) and at least two NEPs (>50% recovery).

Methods

- Method 1: SEMI-QUANTITATIVE TEST and
  Method 2: REFERENCE LOT COMPARISON TEST
- No concrete methods are specified; validated methods can be used.

Validation of MAT Kits in Japan

The JaCVAM has been continuously evaluating cell line-based MAT kits.

Ministry of Health, Labour and Welfare, National Institute of Health Sciences
Japanese Center for the Validation of Alternative Methods

Why cell lines?

- **Limited access to fresh whole blood in Japan.** Blood for transfusion and blood products is collected exclusively by the Japanese Red Cross Society.
- To improve reproducibility.
- To ensure the safety of test personnel (reduced exposure to blood-borne pathogens).

MAT kits using cell lines under evaluation

- **PyroMAT™ (Merck)**
- **MyIc™MAT (MiCAN technologies)**
- **LumiMAT™ (FUJIFILM Wako)**

Evaluation of the RPT and MAT: Ongoing AMED Project in Japan

Study on the Evaluation of the Rabbit Pyrogen Test and Monocyte Activation Test

This study is being conducted at the NIHS.

We are collecting comparative data for the RPT and hPBMC-MAT to include the MAT as an RPT alternative.

- Evaluation of the LOD in the RPT and hPBMC-based MAT using JP RSE (LPS) and NEPs.
  - ➢ Tested NEPs could serve as MAT reference materials without the RPT.

- Transcriptome analysis in rabbit and human PBMCs.

Summary and Future Challenges to Be Addressed

- **The RPT is mostly phased out in Japan, with a few exceptions.**
  > National testing of the RPT has ended, but manufacturers still run the RPT.
  > The number of rabbits used for the RPT has been continuously decreasing.

- **Status of the RPT for biological products listed in the MRBP**
  > Alternative methods are not yet fully incorporated into the MRBP.
  > The RPT and BET in the MRBP are referenced to the JP. Updates are expected following completion of the JP revisions.

- **The MAT to be listed in the JP General Information**
  > The methods need validation.
  > Timing and contents of MAT for JP General Tests listing are still under discussion.

- **How to replace the RPT with the MAT?**
  > Do we need NEPs/LPS reference materials other than RSE?
  > Is simultaneous RPT/MAT testing necessary for transition?

Thank you for your attention.

INDIAN PHARMACOPOEIA COMMISSION

(Ministry of Health & Family Welfare, Government of India)
Sector 23, Raj Nagar, Ghaziabad 201002 (U.P.), India
E-mail: lab.ipc@gov.in; Website: www.ipc.gov.in

Pyrogen Testing in Indian Pharmacopoeia (IP) :Current Status and Way forward

(Dr Anil Kr Teotia)
Indian Pharmacopoeia Commission
Ghaziabad, India

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Disclaimer

This presentation is for **informational purposes only** and does not constitute professional, legal, or financial advice. The information provided is on an "as is" basis, and the presenter/organization makes no representations or warranties of any kind. Any reliance you place on the information is therefore strictly at your own.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Indian Pharmacopoeia Commission (IPC)

Indian Pharmacopoeia Commission is an autonomous institution under the Ministry of Health and Family Welfare, Govt. of India.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

IPC Mission and Vision

Mission

To promote public and animal health in India by bringing out authoritative and officially accepted standards for quality of drugs including APIs, excipients and dosage forms, used by healthcare professionals, patients and consumers

Vision

To promote the highest standards of drugs for use in humans and animals within practical limits of the technologies available for manufacture and analysis

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Rabbit Pyrogen Test (RPT) in IP

- Rabbit Pyrogen Test was introduced in 1955 in Indian Pharmacopoeia

- The worldwide trend of reduction or replacement of animal test has been taken note of and alternative procedures for Quality and Safety has been Introduced.

- Bacterial Endotoxin Test was introduced in IP 1996, 04th Edition, (Appendix No. 2.2.3). The test for Bacterial Endotoxin as a more suitable substitute for the test for Rabbit Pyrogen has been introduced for some monographs, it is proposed to gradually apply it to more items in the pharmacopoeia to the extent possible.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Replacement of Rabbit Pyrogen Test (RPT) in IP

- IPC has made visible progress on replacement of RPT with bacterial endotoxin test (LAL Test).
- Time to Time IPC has introduced amendments in several chemical monographs waiving the requirement of Pyrogen test which are replaced with BET.
- A chapter on Guidance on Bacterial Endotoxin Test (2.2.33) introduced in addendum 2024 to IP 2022.
- In most of the chemical monographs Rabbit Pyrogen test has been replaced with BET in IP 2026 (IP 2026 will be applicable from 1st July 2026) except few monographs.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Phasing out of Rabbit Pyrogen Test (RPT) in Biological Monographs

Vaccines and Immunosera for Human Use

- There are 77 monographs of Vaccines and Immunosera for Human Use in IP 2026. Out of 77, 25 monographs have both Rabbit Pyrogen Test and Bacterial Endotoxin Test. The statement is mentioned in Indian Pharmacopoeia as:

"Complies with the test for pyrogens or a validated test for Bacterial Endotoxin may be used instead of the test".

- 13 monographs of Vaccines and Immunosera for Human Use have Bacterial Endotoxin (BET) test requirement.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Phasing out of Rabbit Pyrogen Test (RPT) in Biological Monographs

- **Blood and blood related products:**
- There are 63 monographs of Blood and Blood related Products in IP 2026. Out of 63monographs, 11 monographs of Blood and Blood Related Products has both test Pyrogen or Bacterial Endotoxin (BET) with statement as:

"Complies with the test for pyrogen or, preferably and where justified and authorized with a validated in vitro test such as BET".

- 2 monographs have BET test requirement.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Phasing out of Rabbit Pyrogen Test (RPT) in Biological Monographs

❖ Biotechnology Derived Therapeutics Products

- There are 41 monographs of Biotechnology Derived Therapeutics Products in IP 2026. All have BET test requirement.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Monocyte Activation Test (MAT)

- IPC has introduced “2.2.25. Monocyte Activation Test” in IP 8th edition i.e., IP 2018.
- MAT is an alternative methodology to Rabbit Pyrogen Test.
- The Monocyte Activation Test (MAT) in India may growing over the next few years. However its adoption is driven by the pharmaceutical sector's need for reliable pyrogen testing, especially as a replacement for animal-based tests.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Way Forward

Recombinant Factor C (rFC) & Recombinant Cascade Reagent (rCR)

- Due to the global threat to the wildlife population of horseshoe crabs, bacterial endotoxin testing using Limulus Amebocyte Lysate (LAL) is being gradually replaced by recombinant reagents, such as rFC and rCR, as alternative non-animal-derived assays for the bacterial endotoxin test.

Pyrogen Testing in Indian Pharmacopoeia (IP): Current Status and Way Forward

Way Forward

- IPC is under discussion to include Recombinant Factor C (rFC) & Recombinant Cascade Reagent (rCR) for endotoxin testing in the IP.

- IPC EWG has proposed to include a short note on “Bacterial Endotoxin Testing using Recombinant Reagents” in IP for stakeholders which fulfil the requirement of alternative methods for bacterial endotoxin in future. However it is subjected to the approval of Scientific Body.

Thank You

USE OF IP & IPRS IS SOCIAL AND LEGAL OBLIGATION FOR "IP" PRODUCTS

국민 안심이 기준입니다
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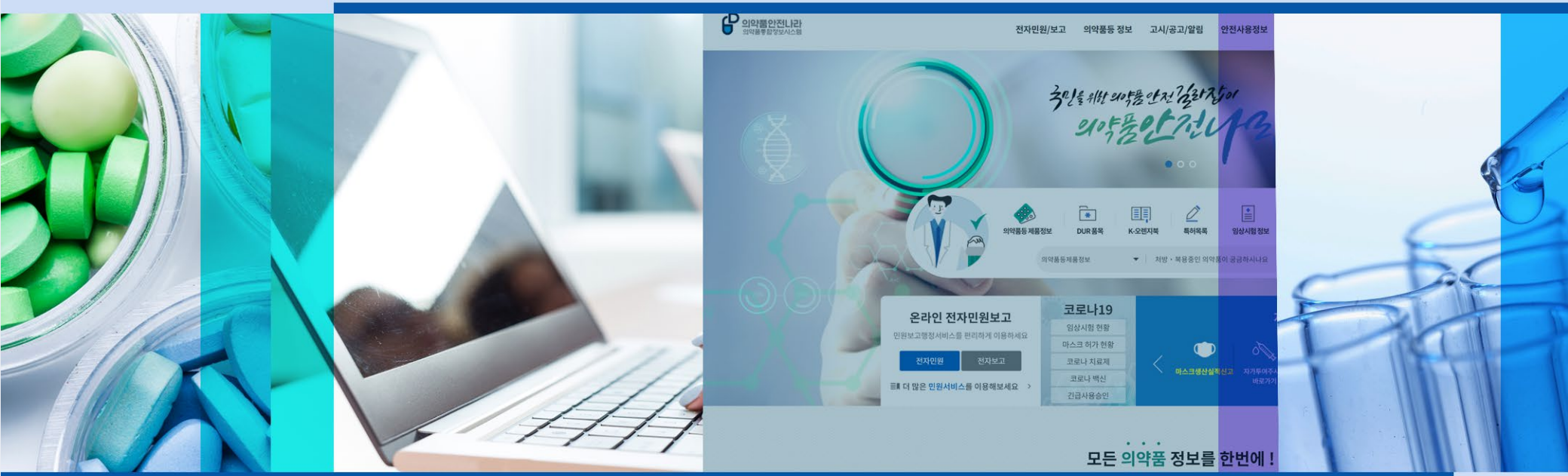

MFDS's Implementation Efforts for Alternatives to Animal Testing

Dr. Hokyung OH
Biologics Research Division

Ministry of Food and Drug Safety

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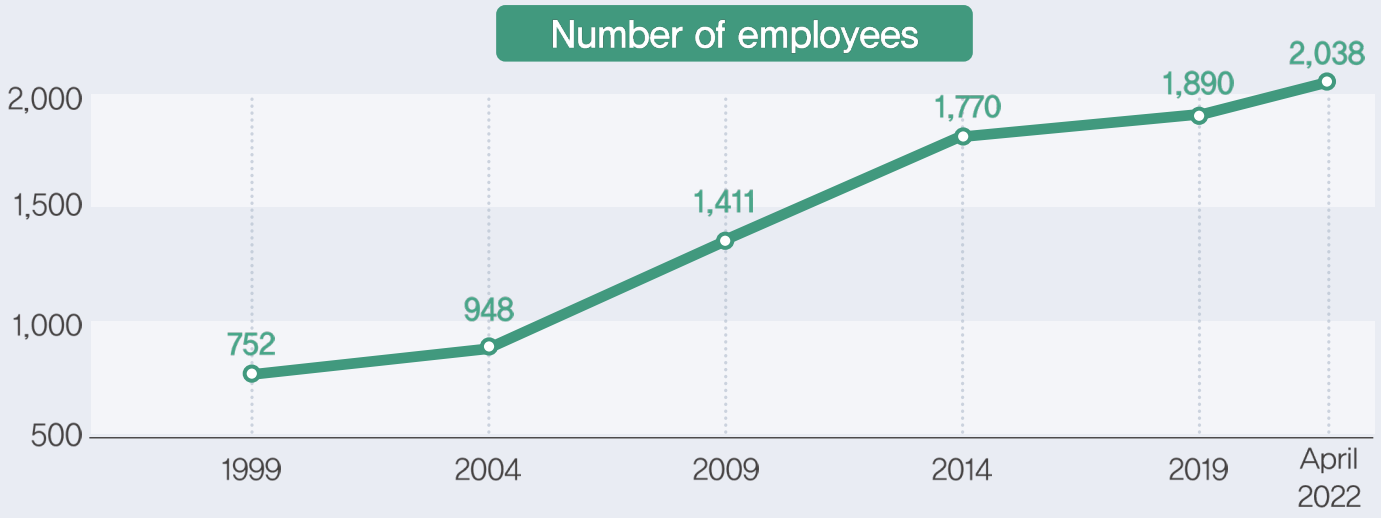
Introduction of the MFDS

The Ministry of Food and Drug Safety was established under the Prime Minister to deliver on its administrative duties to safeguard the safety of foods and drugs.

✓ Government Organization Act, Article 25: Ministry of Food and Drug Safety

- HQ: 1 Director General, 7 Bureaus, 2 Deputy Director Generals, 51 Divisions, 2 Teams
- Institutions under MFDS jurisdiction
  - National Institute of Food and Drug Safety Evaluation (NIFDS)
  - 6 Regional Offices: 2 centers, 17 Imported Food Inspection Centers
- Number of employees : Total 2,038* (as of 30 April, 2022)
  - HQ: 668
  - NIFDS: 439
  - Regional Offices: 896

* Excluding non-official employees



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Compendial Revision: Recent Developments

(Revision) Korean Pharmacopoeia

Recombinant factor C (rFC) (10/09/2023)

(Revision) Specifications and Test Methods for Biologicals

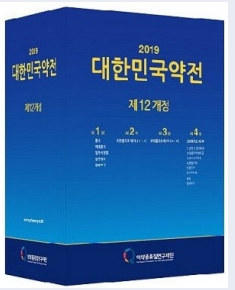
Agkistrodon (Salmusa/Mamushi) Antivenom (25/09/2024)

Diphtheria and Tetanus Potency Test (25/09/2024)

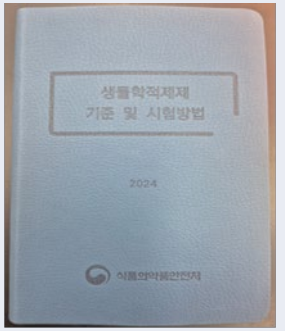
(Revision) Korean Pharmacopoeia

Monocyte Activation Test (27/03/2025)

Thus far, applications submitted by manufacturers to implement MAT for endotoxin testing have been approved. However, no such application has been made for pyrogen testing.



Korean Pharmacopoeia



Specifications and Test Methods for Biologicals

Compendial Revision: Way Forward

(Revision) Korean Pharmacopoeia

- Recombinant Cascade Reagent (rCR) (09/2026)

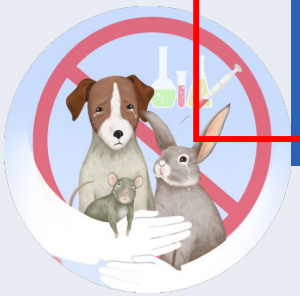
(Revision) Specifications and Test Methods for Biologicals

- 3Rs Concept, General Notices (12/2026)
- Specific Toxicity for Acellular Pertussis Vaccine (03/2027)
- Potency Assay (BINACLE) for *Clostridium botulinum* Toxin Type A (06/2027)

(Revision) Specifications and Test Methods for Biologicals

- Potency Assay (CBPA) for *Clostridium botulinum* Toxin Type A (12/2029)
- Potency Assay (Multiplex Immunoassay) for DTaP Vaccine (12/2029)

National Reference Reagents will be managed and distributed by the MFDS



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Research Project Overview

Name of Project

- Establishment and Validation of Non-Animal Alternatives to the National Lot Release Testing for Blood Products

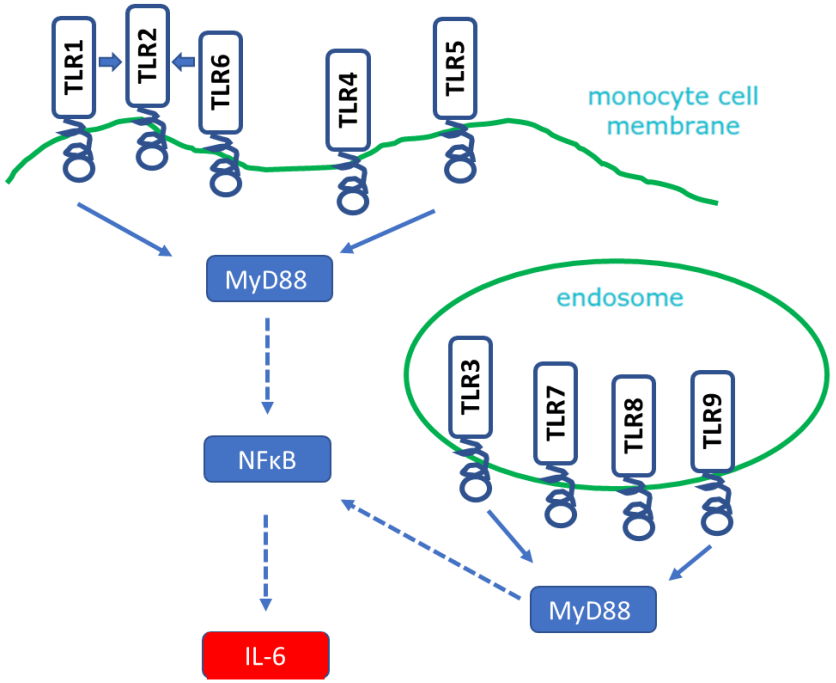
Period and Budget

- 08/2023 – 06/2025 (≈ 558,000 EUR)

MAT

- Cell lines used: PBMC, THP1, U937, TUR, HL-60
- • Endotoxin
  - Pam3CSK4: a synthetic tri-acylated lipopeptide
  - Heat-Killed *Staphylococcus aureus* (HKSA)
  - Peptidoglycan (PGN)
  - FSL-1: a synthetic di-acylated lipoprotein
  - Poly-IC: synthetic double-stranded RNA
  - Flagellin
  - Imiquimod: a synthetic immune response modifier
  - CL075: a thiazoloquinolone derivative
  - ODN200: synthetic class B CpG Oligodeoxynucleotide
  - Muramyl dipeptide (MDP)

Monocyte Activation Test



NEP	TLR
Pam3CSK4	TLR1/2
HKSA	TLR2
PGN	TLR2
FSL-1	TLR2/6
Poly-IC	TLR3
Flagellin	TLR5
Imiquimod	TLR7
CL075	TLR7/8
ODN2006	TLR9
MDP	NLR2

Endotoxin, Pam3CSK4 (a synthetic tri–acylated lipopeptide), HKSA (Heat–Killed *Staphylococcus aureus*), PGN (Peptidoglycan), FSL–1 (a synthetic di–acylated lipoprotein), Poly–IC (a synthetic double–stranded RNA), Flagellin, Imiquimod(a synthetic immune response modifier), CL075 (a thiazoloquinolone derivative), ODN2006 (synthetic class B CpG Oligodeoxynucleotide), MDP (Muramyl dipeptide)

Monocyte Activation Test

IL-6	THP1	HL60	U937	TUR	PBMC
Endotoxin	> 0.31 EU/mL 1-8 pg/ml	**> 0.01 EU/mL** **5-1300 pg/ml**	> 0.16 EU/mL 2-45 pg/ml	N.D	> 0.001 EU/mL 10-4500 pg/ml
Pam3CSK4	> 0.005 μg/mL 100-200 pg/ml	> 0.04 μg/mL 2-120 pg/ml	**> 0.01 μg/mL** **2-100 pg/ml**	N.D	> 0.05 μg/mL 10-2500 pg/ml
HKSA	N.D	**> 1.6 X 10^6 cells /mL** **200-800 pg/ml**	> 6.3 X 10^6 cells /mL 10-40 pg/ml	N.D	> 1.03 X 10^6 cells /mL 5-220 pg/ml
PGN	**> 0.31 μg/mL** **5-250 pg/ml**	**> 0.31 μg/mL** **5-200 pg/ml**	> 0.63 μg/mL 2-30 pg/ml	N.D	> 0.12 μg/mL 10-2200 pg/ml
FSL-1	> 0.01 μg/mL 50-450 pg/ml	> 0.01 μg/mL 25-160 pg/ml	**> 0.01 μg/mL** **5-250 pg/ml**	N.D	> 0.1 μg/mL 10-2000 pg/ml
Poly-IC	N.D	N.D	N.D	N.D	N.D
Flagellin	> 0.02 μg/mL 1-4 pg/ml	> 0.01 μg/mL 5-300 pg/ml	> 0.16 μg/mL 1-5 pg/ml	N.D	> 0.002 μg/mL 10-3500 pg/ml
Imiquimod	N.D	N.D	> 2.5 μg/mL 1-2 pg/ml	N.D	> 0.1 μg/mL 5-500 pg/ml
CL075	> 10 μg/mL 5-100 pg/ml	> 0.31 μg/mL 10-1500 pg/ml	> 0.31 μg/mL 5-700 pg/ml	N.D	> 0.1 μg/mL 10-3200 pg/ml
ODN2006	> 5 μg/mL 1-3 pg/ml	> 1.25 μg/mL 2-100 pg/ml	N.D	N.D	> 0.2 μg/mL 1-40 pg/ml
MDP	> 0.31 μg/mL 1-3 pg/ml	**> 0.01 EU/mL** **2-100 pg/ml**	N.D	N.D	> 0.15 μg/mL 5-800 pg/ml

Monocyte Activation Test

IL-1ß	THP1	HL60	U937	TUR
Endotoxin				
Pam3CSK4				
HKSA				
PGN				
FSL-1				
Poly-IC				
Flagellin				
Imiquimod				
CL075				
ODN2006				
MDP				

TNF-α	THP1	HL60	U937	TUR
Endotoxin				
Pam3CSK4				
HKSA				
PGN				
FSL-1				
Poly-IC				
Flagellin				
Imiquimod				
CL075				
ODN2006				
MDP				

High sensitivity, High reproducibility **Low sensitivity, Low reproducibility**

- ▶ Monocyte Cell Lines have low reproducibility in detecting pyrogenic contaminants due to inconsistent TLR expression.
- ▶ PBMC better reproduce obtained results.

Approval for Use of PBMC

How to request PBMC

The Enforcement Decree on Blood Management, Article 6 – Exceptions to the Disposal of Blood Unfit for Use sets forth that research institutions may be provided with blood for research purposes, provided that the blood is unfit for use. Such institutions include: medical institutions established pursuant to the Medical Service Act; pharmaceutical manufacturers established pursuant to the Pharmaceutical Affairs Act; Universities; and other types of research institutions. Such research institutions may request blood for research purposes by following the procedure listed below:

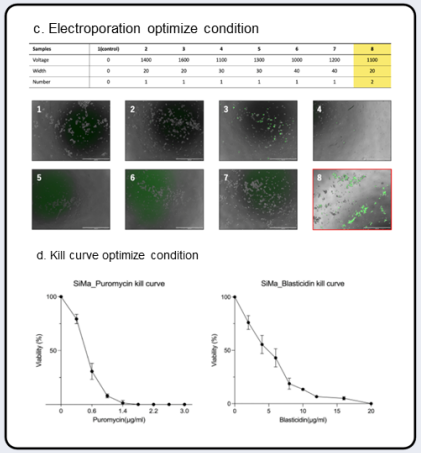
Required documents

- Request for blood for research purposes
- Research Plan, summarized
- Review request for the distribution of the blood
- Memorandum and confirmation regarding the blood
- Approval from the Institutional Review Board (IRB)
- Research plan submitted to the IRB

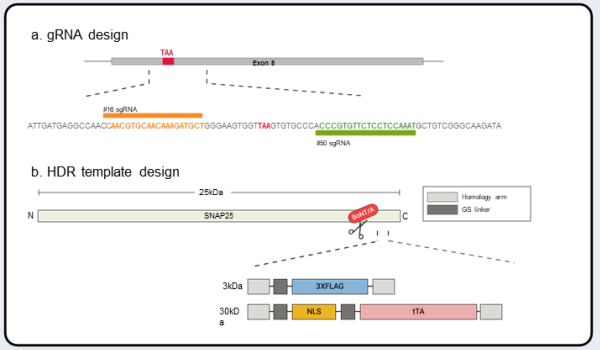
Optimized CRISPR Knock-in Monocyte Cell Line

Key Steps

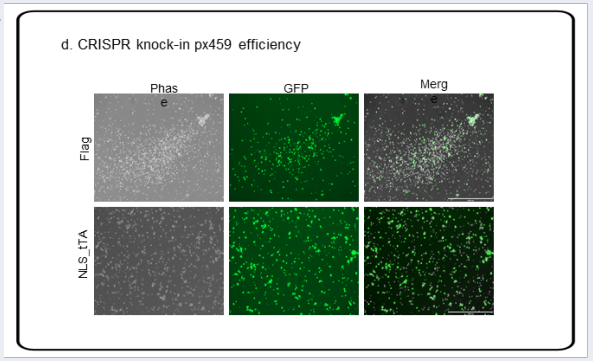
Electroporation condition & CRISPR Knock-in optimization

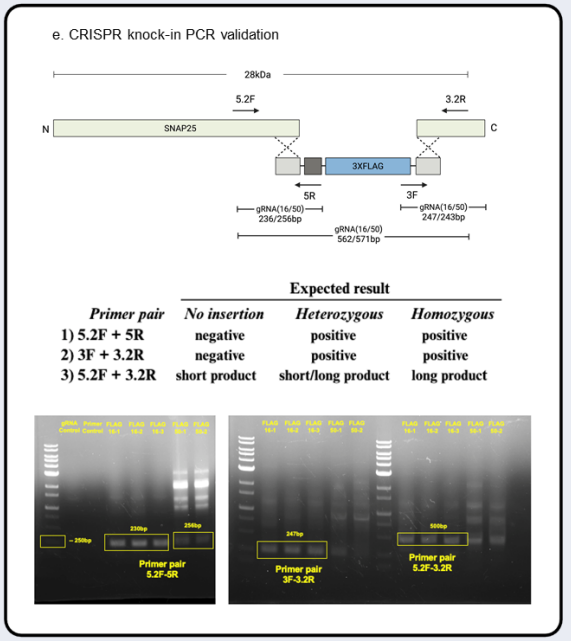


Cell line selection & Template design



CRISPR Knock-in & Validation





Primer pair	Expected result		
	No insertion	*Heterozygous*	*Homozygous*
1) 5.2F + 5R	negative	positive	positive
2) 3F + 3.2R	negative	positive	positive
3) 5.2F + 3.2R	short product	short/long product	long product

Summary

- **Revisions made by the MFDS to implement non-animal methods for biological products involve:**
  1. Recombinant factor C, Agkistrodon (Salmusa/Mamushi) antivenom (September 10, 2023)
  2. Diphtheria and tetanus potency test (September 25, 2024)
  3. Monocyte activation test (March 27, 2025)

- **Future revisions will involve:**
  1. Recombinant Cascade Reagent (rCR) (September 2026)
  2. Specific Toxicity for acellular pertussis vaccine (March 2027)
  3. Potency Assay (BINACLE) for *Clostridium botulinum* toxin Type A (June 2027)
  4. Potency Assay (CBPA) for *Clostridium botulinum* toxin Type A (December 2029)
  5. Potency Assay (multiplex immunoassay) for DTaP vaccine (December 2029)

- **Room for revision, found by research on MAT for blood products, remains:**
  1. Reference standards for non-endotoxin pyrogens are yet to be established.
  2. Monocyte cell lines have low reproducibility in detecting pyrogenic contaminants.
  3. While fresh PBMC has higher sensitivity than deep-frozen PBMC, mass supply of fresh PBMC is challenging.
  4. Considerations should be given to developing an optimized CRISPR knock-in monocyte cell line for MAT to ensure consistency in TLR expression.

Thank You

Ministry of Food and Drug Safety

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