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EDQM-EPAA Hybrid Symposium

"Pyrogen testing 2.0: Ethical, Evolving and Eco-friendly



Optimizing MAT through an Integrated Approach: Rigorous Validation and Smart Automation



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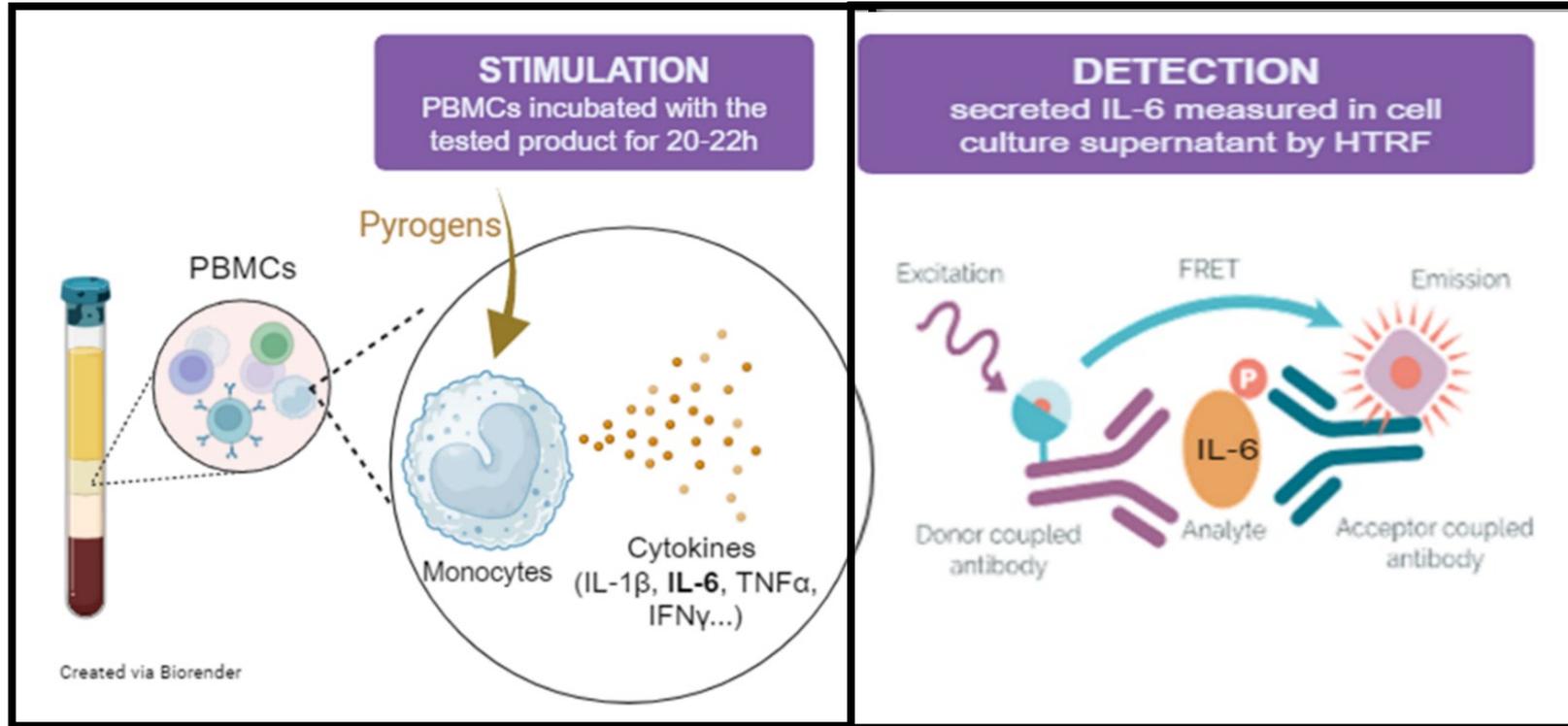
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MAT

Validation Strategy
across Sanofi

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Monocyte Activation Test Principle



Sanofi MAT design

- Human PBMCs
 - ↳ Pool of 4 donors
- 20-22h stimulation
- IL-6 detection in cell supernatant as the most suitable read-out
 - ↳ ELISA or HTRF assay
- PLA analysis
- Pyrogenic titer calculated vs a LPS reference (method 1) or a reference lot (method 2-2.6.40)

PYROGENS – Risk assessment & Analytical associated methods

Risk assessment / product – process

NEP= Non Endotoxin Pyrogen

PPQ = Product Performance Qualification

Pyrogen risk =
Endotoxins

Pyrogen risk =
NEP +/- Endotoxins

Characterization
Test

MAT
Method 1
Demonstration of the absence
of NEP on PPQ batches

R&D
Method
QUALIFICATION

Release
Test

BET

BET

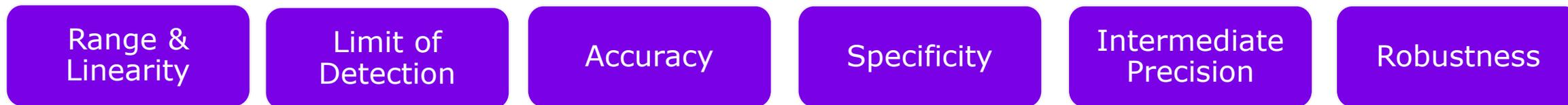
QC
Method
VALIDATION

MAT
Method 2-2.6.40
Consistency approach

MAT for Demonstration of the absence of NEP Characterization Test

According to Ph. Eur. 2.6.30, USP<1225>, USP<1223>, ICH Q2

Generic validation of the MAT format regarding LPS and NEPs:



Product-specific validation in 3 batches:



Compliance with Product specification (ECL)

- **Preparatory interference test**
 - Free of interferences in case of 50-200% recovery of spiked endotoxin
- **NEP Recovery Experiments**
 - Use historic batches with NEP contamination
 - or
 - ≥ 2 NEPs need to be detectable (e.g. Flagellin, LTA)
 - ≥ 1 NEP spiked in the sample
- **Interference with detection system**
 - $\leq 20\%$ in presence or absence of the sample with the read-out

MAT for Product Consistency assessment Release Test

According to Ph. Eur. 2.6.30, 2.6.40 USP<1033>ICH Q2R2

Pre-requisites: Method Qualification & Robustness study (R&D environment)

Specificity

Linearity

Intermediate
Precision

Accuracy

Theoretical
LOD

**No acceptance
criteria**

Product-specific validation:

Table 1 – Validation performance characteristics based on measured quality attributes

Measured Quality Attribute Analytical Procedure Performance Characteristics to be Demonstrated (2)	IDENTITY	IMPURITY (PURITY) Other quantitative measurements (1)		ASSAY Content or potency
		Quantitative Test	Limit Test	Other quantitative measurements (1)
Specificity (3) Specificity Test	+	+	+	+
Range				
Response (Calibration Model)	-	+	-	+
Lower Range Limit	-	QL [†]	DL	-
Accuracy (4) Accuracy Test	-	+	-	+
Precision (4) Repeatability Test	-	+	-	+
Intermediate Precision Test	-	+ (5)	-	+ (5)

— signifies that this performance characteristic is not normally evaluated

+ signifies that this performance characteristic is normally evaluated

[†] in some complex cases DL may also be evaluated

QL: Quantitation limit DL: Detection limit

ICH Q2R2

Matrix interference study (2.6.40) & Specificity

Test performance

Range

-Accuracy and Precision on the reportable range
-definition of range

Accuracy at each level (x5)

Precision at each level of the working range (X3)

USP<1033>

Linearity of the reportable range

Internal reference batch

- Product stability justification
- Control Chart Slope –EC50

Positive Control

Purified Endotoxin

- Control Chart

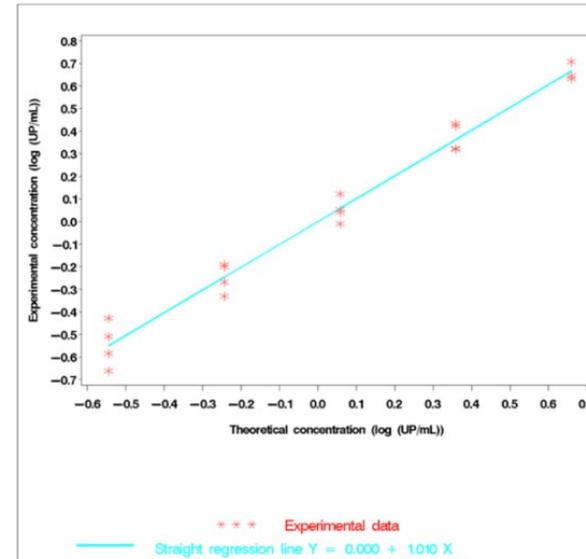
Double sourcing for critical reagents

- PBMCs
- HTRF IL6-kit

MAT applied to different vaccines having intrinsic pyrogenic compounds is a precise and accurate analytical method

Characteristics	Results
Signal at LOQ	Mean of blanks signal + 10 x std deviation of blanks: 618
Specificity	Comparison of mean signal obtained for blanks and LPS: Mean HTRF ratio of blanks: 467 Mean HTRF ratio of LPS: 9838
Linearity	$P_{\text{regression}} < 0.0001$ $Y = 0.000 + 1.010 X$ Where X = theoretical expected concentration (log(PU/mL)) and Y = measured concentration (log(PU/mL)) $R^2 = 0.9801$ Linearity range: [0.286 – 4.570] PU/mL
Accuracy	Level -2 (0.286 PU/mL): 102 % Level -1 (0.571 PU/mL): 100 % Level 0 (1.143 PU/mL): 99 % Level +1 (2.285 PU/mL): 104 % Level +2 (4.570 PU/mL): 101 %
Precision	Overall mean: 0.058 log(PU/mL) ; arithmetic form: 1.143 PU/mL Relative standard deviations of repeatability and intermediate precision are respectively: 6.3% and 12% 95% confidence interval of intermediate precision for 1 run with 1 measurement: $\pm 0.111 \text{ log(PU/mL)}$ that is x/: 1.29 in arithmetic form

Figure 2: Linearity graph – F test

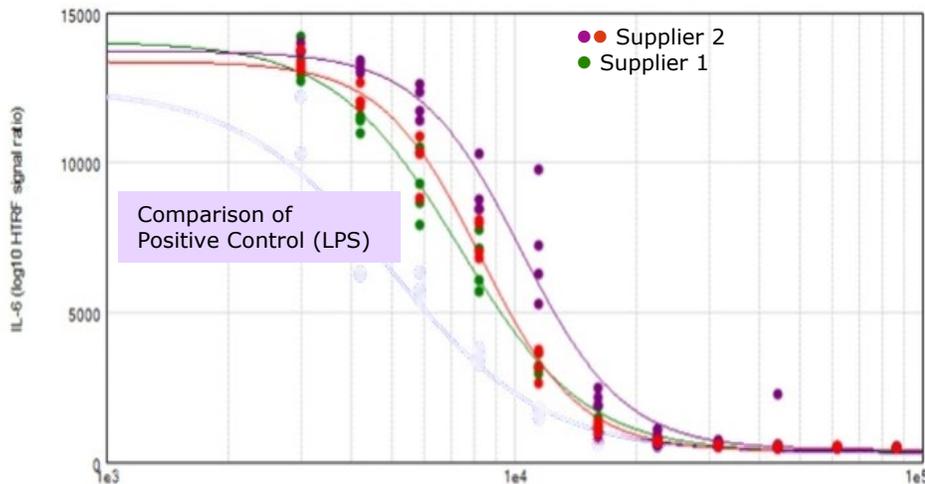


Assay qualification completed for different products
Go for validation as Release Test

Double sourcing for critical reagents PBMCs

Two Suppliers fulfilling our URS for PBMCs pools preparation and characterization

- CoA provided by the supplier for each pool of PBMCs according to 2.6.30
- Control at receipt by Sanofi on purified pyrogens & specific vaccines



Overlay of the reference and positive control for both PBMCs sources

Both PBMCs sources included in qualification studies



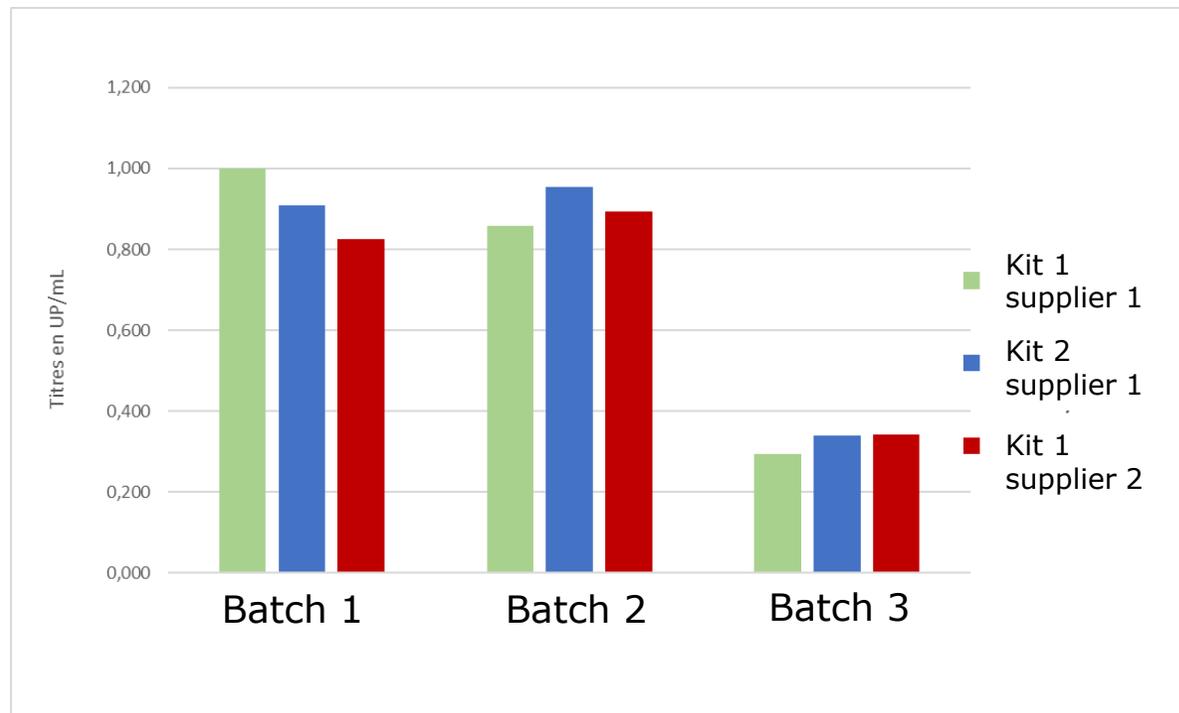
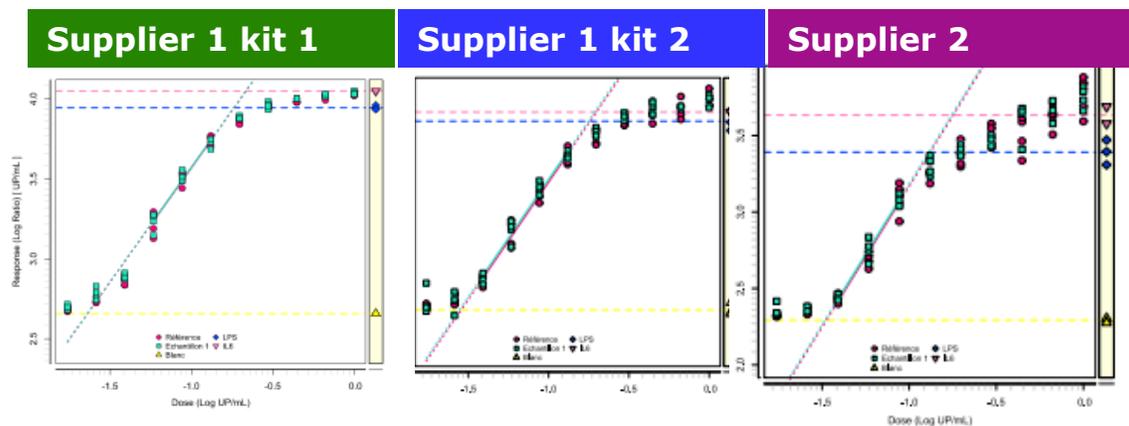
All qualification criteria met with double-sourced PBMCs

Double sourcing for critical reagents

IL-6 HTRF kits

- Same cell culture supernatants evaluated with 3 different kits

Evaluation of IMPACT ON PYROGENIC TITERS



Differences on raw data (signal magnitude)



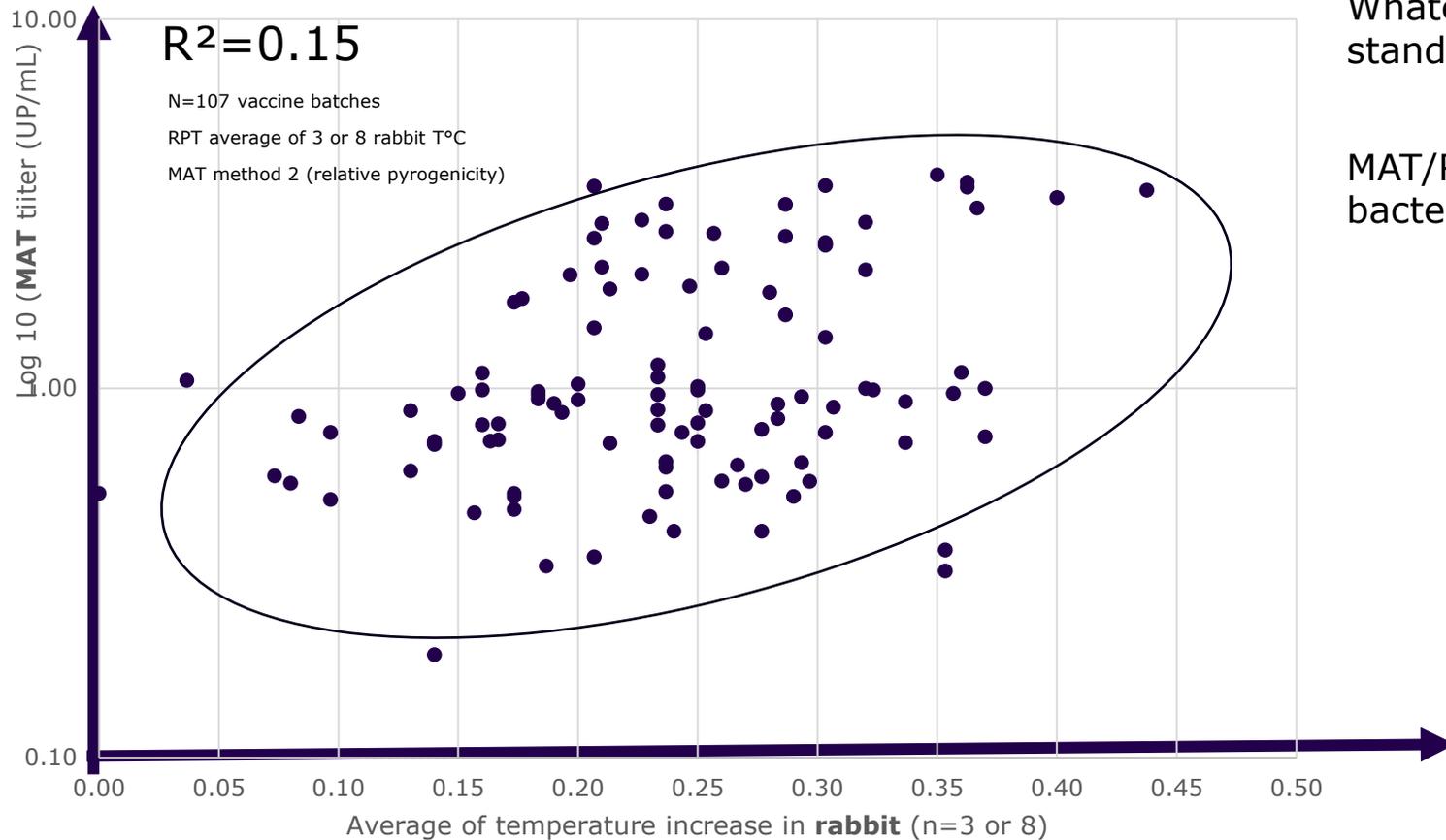
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But No impact on the calculation of the relative pyrogenic titer = Same Pyrogenic titer whatever the Kit

MAT as a quantitative approach to pyrogen testing to replace RPT

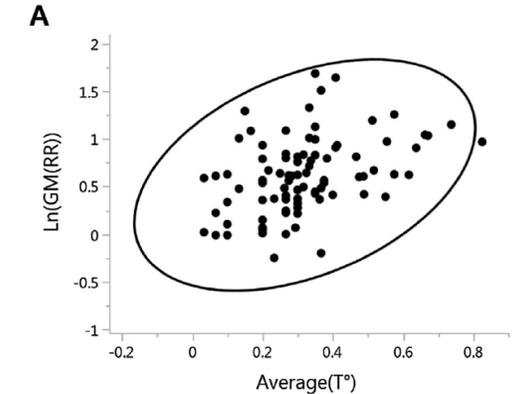
Comparison MAT vs RPT for Bacterial based-vaccine inherently pyrogenic

MAT
Method 2



Whatever the MAT results, the RPT results are within the standard RPT variability

MAT/RPT comparison as previously described for another bacterial based-vaccine (Bexsero)



<https://doi.org/10.1016/j.vaccine.2018.10.082>

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MAT is more discriminant than RPT and acceptance criterion definition is critical

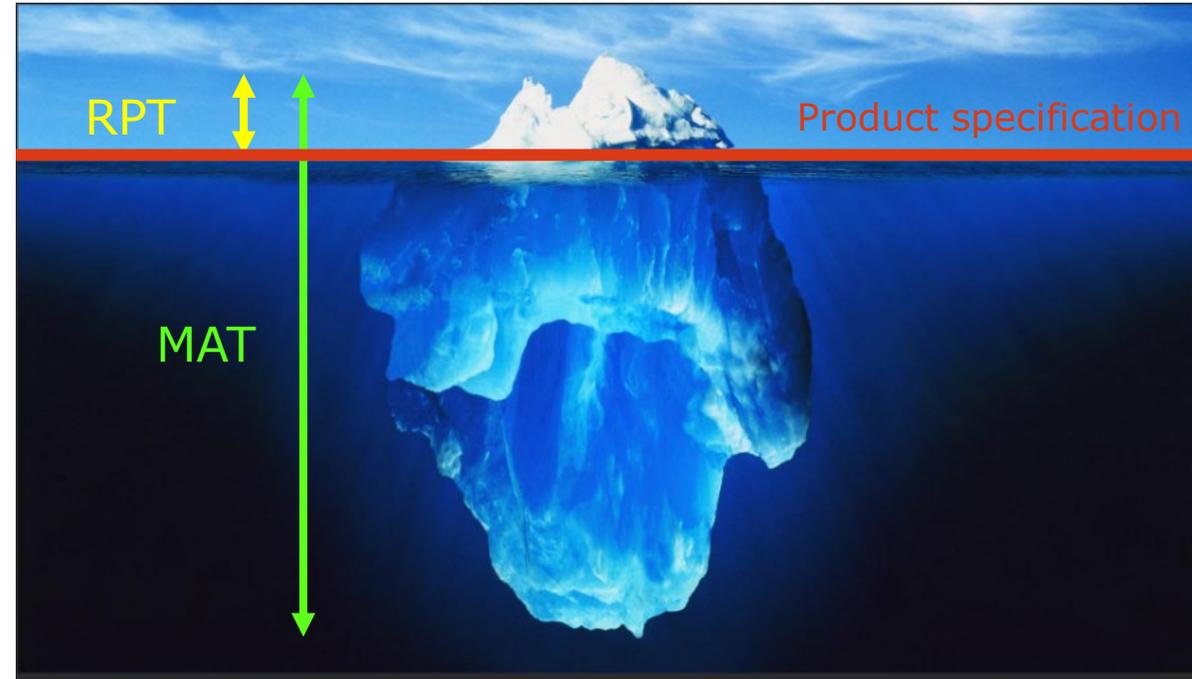
Key messages

For non pyrogenic product

- MAT method 1 = Characterization Test
- MAT performance can be documented
- MAT is applicable to various matrices

For inherently pyrogenic product

- MAT method 2-2.6.40: Consistency & Quantitative approach = Release Test
- MAT performance can be documented: reproducible, robust, and critical reagents management.
- MAT is a valuable and fit-for-purpose alternative method more discriminant than RPT



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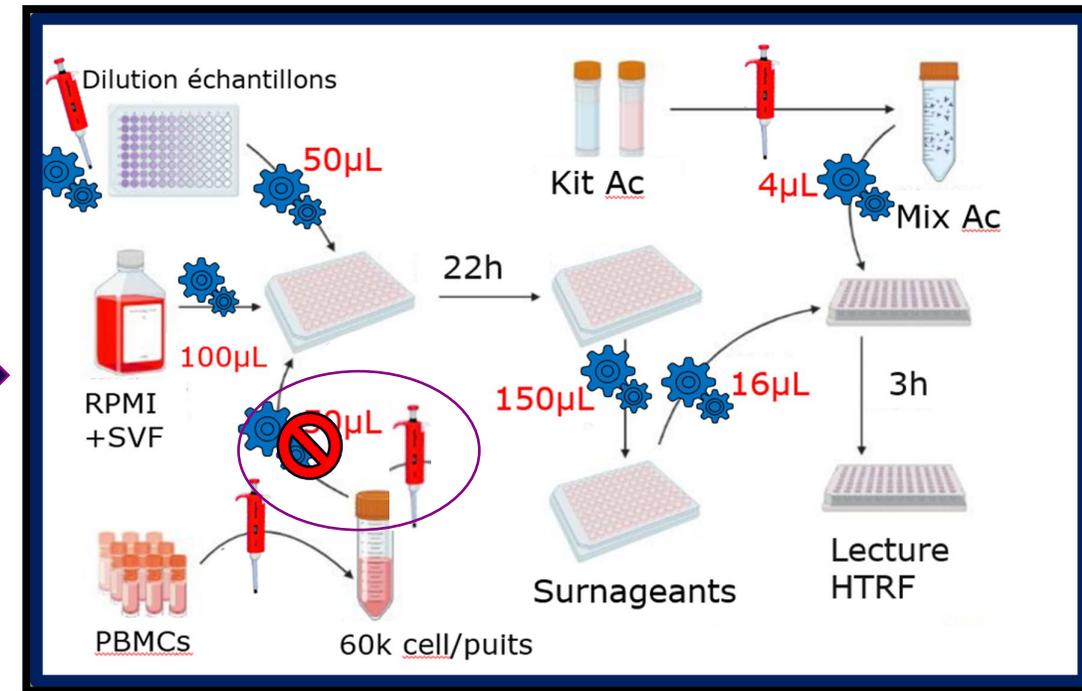
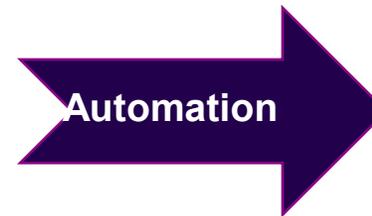
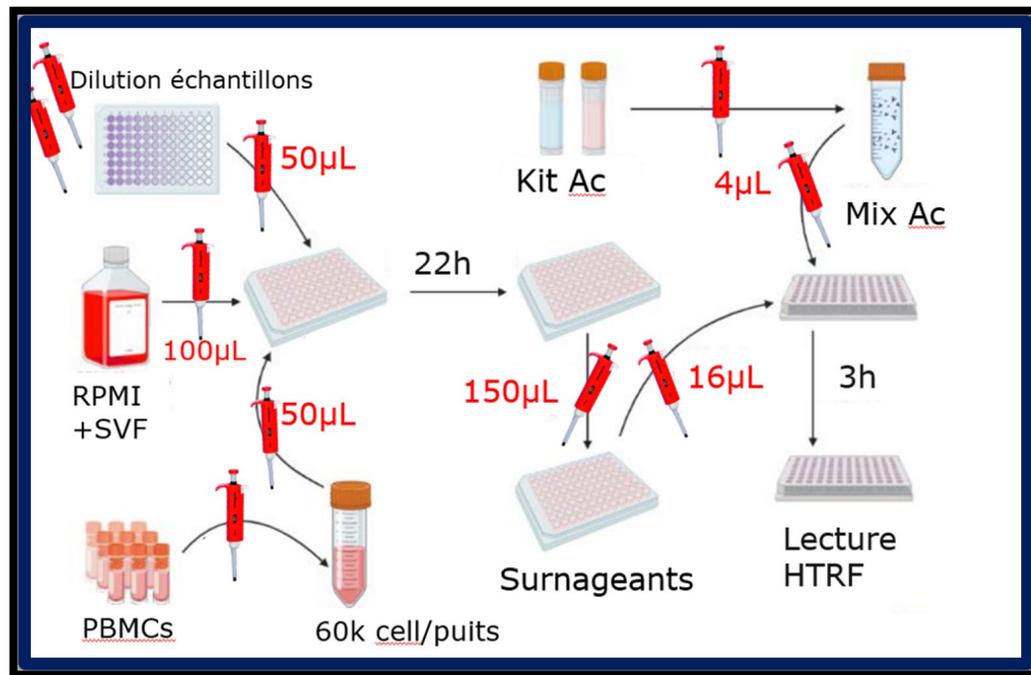
Smart Automation



MAT : Transforming Challenges into Automation Opportunities

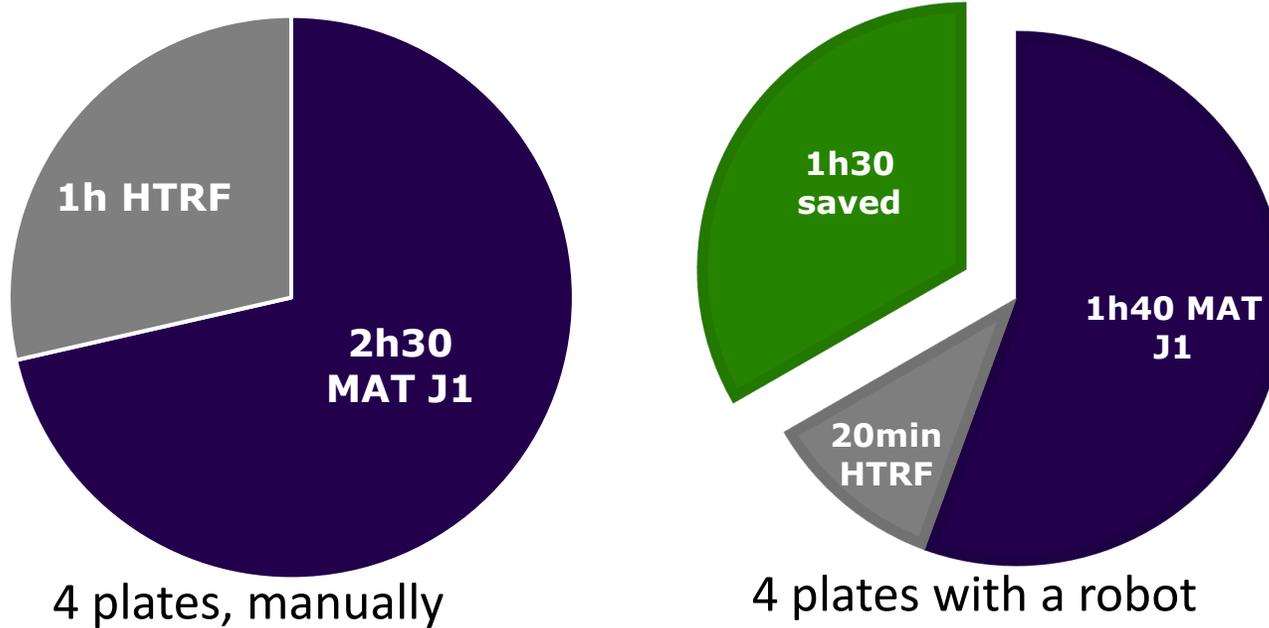
- Many pipetting steps over 2 days
- Long and Tedious handling
 - 1 plate = 1 sample (quadruplicate)
- Risk of error
- Musculoskeletal disorder for technologist

- A minimum of 4 plates / run
- Save operator time
- Significantly reduce pipetting steps
- At least same reproducibility than manual testing
- Independant program from day 1 and day 2



Comparison of Manual versus Automated MAT

Focus on operator time

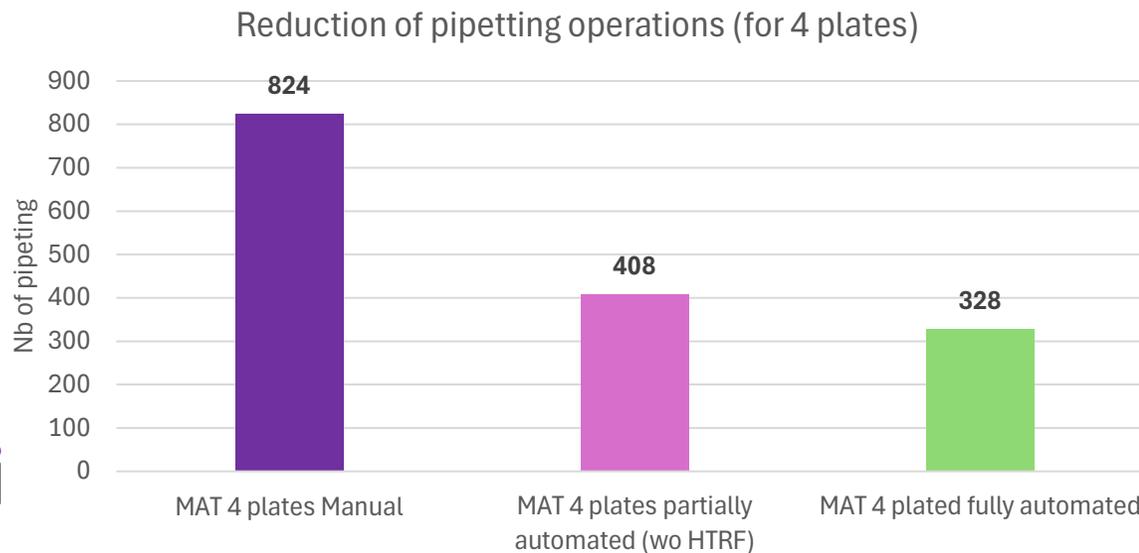


Focus on Assay performance

Fidélité MAT - QU-WIN-0020517			
Precision study			
N	GMean	Repeatability GCV(%) (95% one-sided upper confidence limit)	Intermediate precision GCV(%) (95% one-sided upper confidence limit)
12	2.1383	9.09 (16.06)	10.17 (23.15)

- **1h30 saved for operator → dedicated to more added value activities (analysis, interpretation)**
- **Automation free technologists from repetitive pipetting: manual pipetting steps dramatically reduced by 2.5-fold**
- **No impact of the automation on assay performance**

Focus on pipetting



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Conclusions



MAT is broadly deployed by Sanofi

MAT PBMC/IL-6

- MAT method 1 as Characterization Test on the final batches (PPQ)
 - Part of Product & Process pyrogen risk assessment
 - Applicable to several matrices
- MAT method 2-2.6.40 as Release Test
 - Consistency & Quantitative approach
 - Full validation strategy in place
 - First implementation for QC release expected between 2027-2028

Perspectives

Automation

- MAT based on PBMC is robust enough to be automated
- Drastic reduction of repeated pipetting tasks and technologist time

Still working on more QC friendly approaches MAT2.0 with fully characterized cell lines

- Pre-requisites: rapid, stable, sustainable, robust, sensitive to all pyrogens

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Aknowlegments

Sanofi R&D vaccines (Marcy l'Etoile, Toronto)

Delphine Trelat
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Héloïse Chausse
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Sanofi Bioanalytics Frankfurt

Parysatis Sachs
Dirk Usener
Maureen Spohn
Heike Wurtinger
Finja Schneider

This work was funded by Sanofi.

The author is a Sanofi employee and may hold shares and/or stock options in the company.

QC requirements for MAT method validation and qualification of PBMCs or cell lines

Liliana Alleri

Pyrogen testing 2.0: Ethical, Evolving and Eco-friendly
February 26th, 2026

- ❖ Donor qualification and validation strategy for:
 - ❖ MAT assay applied to intrinsically pyrogenic product (Method 2): the example of Bexsero*
 - ❖ MAT assay for all other products (Method 1): instances of a semi-quantitative method
- ❖ Critical parameters for selecting and implementing cell lines for QC testing

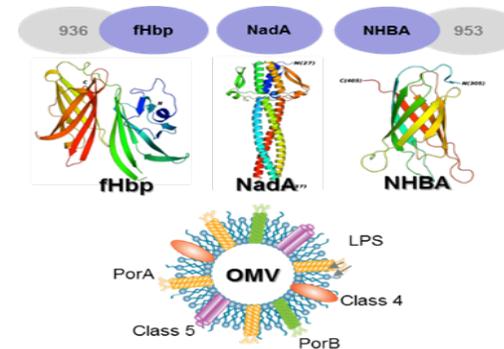
** Bexsero is a trademark owned by the GSK group of companies.*

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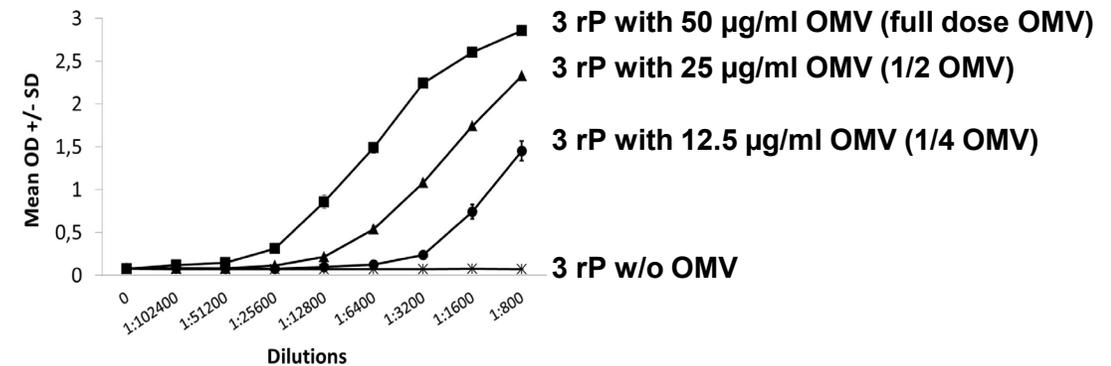
* *Bexsero is a trademark owned by the GSK group of companies.*

The Bexsero vaccine elicits cytokines production in the cells

- ❖ Bexsero vaccine is constituted by three recombinant protein antigens and the Outer Membrane Vesicles (OMV) from serogroup B *N. meningitidis* adsorbed onto aluminum hydroxide.
- ❖ OMV component stimulates PBMC to produce IL-6, in a concentration-dependent manner.
- ❖ The MAT measures the overall pyrogenic response of the Bexsero batches



Bexsero components



IL-6 production in hPBMC stimulated with ad hoc Bexsero formulations

Reference: "Monocyte-activation test to reliably measure the pyrogenic content of a vaccine: an in vitro pyrogen test to overcome in vivo limitations" S. Valentini, G. Santoro, F. Baffetta, et al. *Vaccine*, 37 (2019), pp. 3754-3760

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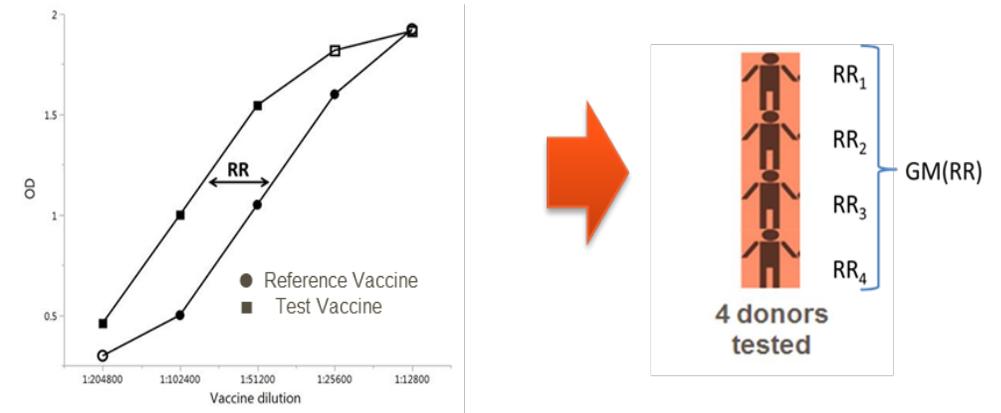
A 'Reference lot comparison test' using a Bexsero lot as Reference is applied for QC release of lots

Donor qualification step

- PBMCs' donors are stimulated with a full dose-response curve of Bexsero vaccine
- The linear part of the vaccine response curve for each donor is identified and used in the test

Final Format

- Relative Response (RR) versus a qualified Bexsero reference standard batch using a Parallel Line Assay
- The reportable result for MAT is the Geometric Mean (GM) of RRs for the tested donors. Number of donors can be selected based on the desired assay variability



Reference: "Monocyte-activation test to reliably measure the pyrogenic content of a vaccine: an *in vitro* pyrogen test to overcome *in vivo* limitations"
S. Valentini, G. Santoro, F. Baffetta, et al. *Vaccine*, 37 (2019), pp. 3754-3760

Method Validation for inherently pyrogenic products conducted according to Pharmacopoeial requirements for MAT and biological assay

Validation strategy aligned with requirements of USP <1032> and <1033>, ICH Q2(R1) and Ph. Eur. chapters 2.6.30, 2.6.40 and 5.3.

- The use of the method for QC testing of Bexsero was approved in 60 different countries
 - The test replaces both LAL and RPT tests on Bexsero DP for EU/RoW while it replaces RPT only for US
- Studies for method development and validation were conducted following Pharmacopoeia Guidelines

Assay development

- Parallelism assessment between Product and RSE
- Donor qualification set up with a Product Reference lot
- Evaluation of:
 - Mathematical Model,
 - plate layout and final assay format
 - potential interference of the product matrix in MAT detection system
 - assay robustness

Product-specific validation

- Accuracy of the measured Relative Response over the range of interest
- Intermediate precision
- Standard Linearity and Sample linearity
- Assay robustness

A concordance study MAT-RPT demonstrated a superior sensitivity of the MAT assay in detecting pyrogenicity in Bexsero lots

Study conducted to fulfill authority submission requirements

- Comparative pyrogenicity testing of Bexsero lots with elevated OMV levels versus the target formulation
- Objective: Determine whether batches containing out-of-specification (increased) OMV amounts have a higher likelihood of failing the MAT compared with the RPT.

Lot	MAT			RPT*		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Lot 1	FAIL	FAIL	FAIL	PASS	PASS	FAIL
Lot 2	FAIL	FAIL	FAIL	PASS	FAIL	FAIL
Lot 3	FAIL	FAIL	FAIL	PASS	FAIL	PASS
Lot 4	FAIL	FAIL	FAIL	FAIL	PASS	PASS

**Animal studies were ethically reviewed by the local AWB and carried out at a GSK facility in accordance with European legislation (EU DIR 63/2010), and the GSK Policies on the Care, Welfare and Treatment of Animals*

❖ Donor qualification and validation strategy for:

- ❖ MAT assay applied to intrinsically pyrogenic product (Method 2): the example of Bexsero*
- ❖ MAT assay for all other products (Method 1): instances of semi-quantitative methods

❖ Critical parameters for selecting and implementing cell lines for QC testing

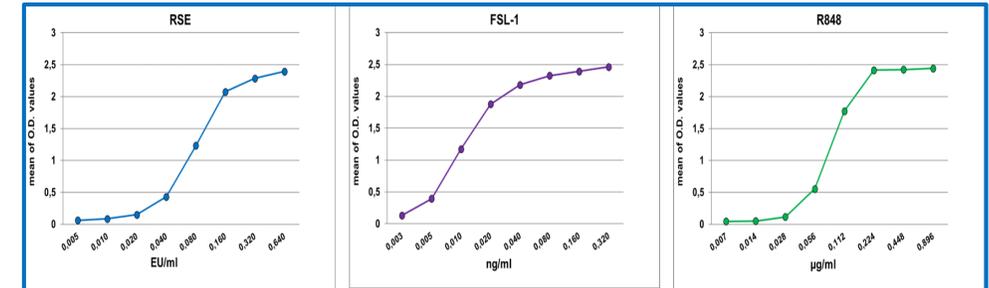
* Bexsero is a trademark owned by the GSK group of companies.

Method 1 is applied for products for QC release of lots

Application of the method to non-intrinsically pyrogenic products or products exhibiting dose-response curves broadly parallel to the RSE

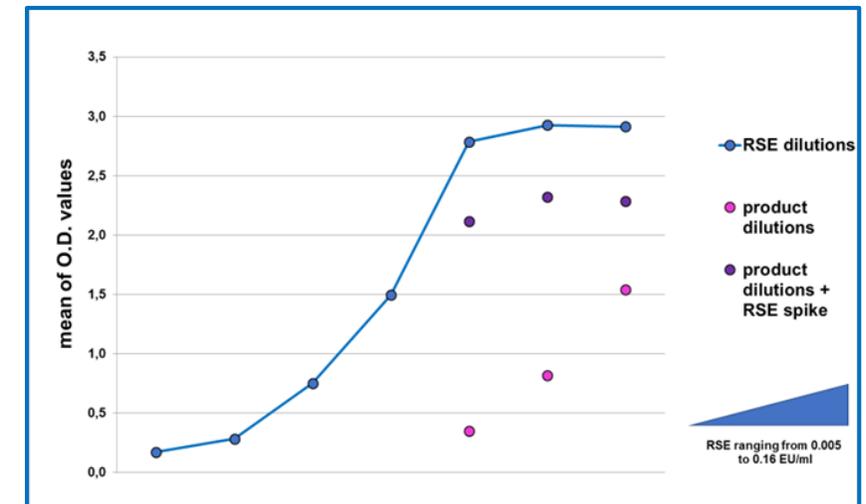
Donor qualification step

- PBMCs' donors are stimulated with a full dose-response curve of RSE and two NEPs (i.e. FSL-1 and R848)



Final Format

- A 4-PL model is applied to the RSE full dose response curve (8 data points)
- 3 product dilutions are tested in quadruplicate, pure and spiked with RSE (concentration corresponding to ED50 of dose RSE curve):
 - optimum dilution* (Sol.A), 1/2 Sol.A (Sol. B), MVD
- Validity criteria are applied to RSE dose response curve
- 50-200% criterion is applied to each product dilution



*Optimum dilution = first dilution of the product for which the endotoxin recovery (in the test for interfering factor) is ~~not~~ **met** within the validity range 50-200%

Method Validation for non-inherently pyrogenic products conducted according to Pharmacopoeial requirements for MAT and biological assay

Validation strategy aligned with requirements of USP <1225>, ICH Q2(R1) and Ph. Eur. 2.6.30

- The use of the method for QC testing of GSK products was approved in 2025 (US, EU, 80% RoW)
- Studies for method development and validation were conducted following Pharmacopoeias Guidelines

Assay development

- Donor qualification and sensitivity assessment for RSE and two NEPs:
 - FSL-1* synthetic diacylated lipoprotein derived from Mycoplasma salivarium, agonist for TLR2/TLR6 heterodimer.
 - R848* a small molecular weight imidazoquinoline compound, agonist for TLR7/8
- CLC and MVD determination
- Test for interfering factors on product matrix with both endotoxin and NEP spikes to identify the optimal dilution
- Evaluation of:
 - potential interference of the product matrix in MAT detection system
 - assay robustness

Product-specific validation

- Tests for interfering factors:
 - At least 3 product lots tested
 - For each test batch of product, the recovery percentage for both RSE and at least 1-non endotoxin pyrogen, has to fall within the range of 50-200% for all the analyzed dilutions for the pool of donors used in the test.
- Test to assess endotoxin-like pyrogens detectability (if needed):
 - Artificially contaminated samples (with contaminant potentially present in the product) are prepared in parallel with uncontaminated samples and tested.

Outcome of Method 1 Validation: product dilutions are free of interference

- Pre-validation activities assess all product dilutions for interference with the RSE and two designated NEPs to identify suitable product dilution ranges and potential matrix effects.
- Validation activities conducted using three independent product lots at the optimal dilution, 1/2 MVD, and the MVD to confirm assay specificity on the identified product dilutions

Example from a product-specific validation

RSE recovery (%)				FSL-1 recovery (%)			
	Dilution #1	Dilution #2 (1/2 MVD)	Dilution #3 MVD		Dilution #1	Dilution #2 (1/2 MVD)	Dilution #3 MVD
Lot#1	139%	59%	92%	Lot#1	125%	180%	114%
Lot#2	104%	143%	89%	Lot#2	120%	103%	118%
Lot#3	109%	112%	91%	Lot#3	110%	167%	127%

Outcome of method validation: product dilutions enable to detect pyrogenicity in the product (if present)

- Test included in product-specific validation when product contamination during manufacturing is possible (e.g., products derived from endotoxin-like pyrogens).
- Test is conducted by:
 - ✓ Preparing artificially contaminated samples in parallel with uncontaminated controls and test both by MAT.
 - ✓ Estimating Endotoxin Equivalents relative to the RSE dose–response curve. Contaminated samples must show higher Endotoxin Equivalent values than the matched uncontaminated samples.
- Example: Two contaminated samples (S1, S2) were assessed by MAT; both showed higher Endotoxin Equivalents than their uncontaminated counterparts.

Example from a product-specific validation

Sample	Endotoxin Equivalents
Lot #1	259 EE
Lot #2	117 EE
Lot S#1	18130 EE
Lot S#2	5850 EE

MAT submission experience: regulatory outcomes in EU, US and RoW

Method 2

- ❖ The method was submitted to all HAs in 2017 as a proposed replacement for both the RPT and LAL assays for Bexsero release.
- ❖ The change was accepted by EU and RoW authorities.
- ❖ The US regulator required a concordance study to demonstrate that MAT was more sensitive than RPT. After GSK provided this evidence, MAT was accepted as a replacement for RPT in the US (replacement of LAL was not accepted)

Method 1

- ❖ A variation was submitted in 2024 for other GSK products. Approvals were subsequently obtained in the EU, US, and about 80% of RoW jurisdictions.
- ❖ The validation data package was judged robust, even though the Ph. Eur. revised Chapter 2.6.30 during the submission process; those changes had an impact on the dossier but did not prevent approvals.
- ❖ No further requests for animal studies were made by all regulators.

- ❖ Donor qualification and validation strategy for:
 - ❖ MAT assay applied to intrinsically pyrogenic product (Method 2): the example of Bexsero*
 - ❖ MAT assay for all other products (Method 1): instances of semi-quantitative method
- ❖ Critical parameters for selecting and implementing cell lines for QC testing

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From a PBMCs-based to a cell lines-based MAT assay: balancing standardization with biological relevance

Background

- MAT method 2 is accepted worldwide for Bexsero, 80% of RoW regulators have approved MAT method 1 use for other GSK products.
- No information currently available on the regulatory acceptance for commercial MAT kits

PBMCs: Preserving Biological Complexity

- Better reflection of in vivo human immune heterogeneity and higher sensitivity to NEPs signaling pathway
- Captures complex cell–cell interactions and rare responders
- Critical for translational relevance in safety assessment

Cell Lines: Controlled Biological Contexts

- Increased assay standardization and reproducibility
- Reduced donor-to-donor variability
- Easier automation and QC
- Easier method transfer
- Fewer ethical constraints

From a PBMCs-based to a cell lines-based MAT assay: balancing standardization with biological relevance

Key attributes to consider for assessing the comparability of cell lines to PBMCs

- Demonstration of comparable method performance is required to support their use in a QC environment. Requirements for their suitability:
 1. Non product specific validation:
 - Comparable sensitivity to the RSE
 - Broad detection of relevant pyrogen. Testing of a broad spectrum of pyrogens is needed to demonstrate their detectability
 2. Product-specific validation:
 - Similar product interference profiles should be demonstrated to support MAT comparability, despite differences in the pyrogen-elicited pathways.
 - For non-intrinsically pyrogenic products: demonstrate equivalent pyrogen detection limits (EE/mL) in artificially contaminated (spiked) samples.
 - For intrinsically pyrogenic products: demonstrate equivalent relative accuracy and intermediate precision using ad-hoc formulated samples at multiple target potencies across the validated range of the current PBMC-based MAT.

Conclusions and considerations

Conclusions

- The Monocyte Activation Test (MAT) has been validated and globally adopted as an ethical, evolving, and eco-friendly alternative to traditional in vivo pyrogen testing methods.
- MAT Method 2 has replaced both Rabbit Pyrogen Test (RPT) and Limulus Amebocyte Lysate (LAL) for Bexsero in EU/RoW, and RPT in the US, demonstrating superior sensitivity in detecting pyrogenicity compared to RPT.
- MAT Method 1 has been applied for semi-quantitative testing of other GSK products. Its validation, aligned with key regulatory standards (USP <1225>, ICH Q2(R1), and Ph. Eur. 2.6.30), facilitated approvals across major markets, including the EU, US, and ~80% of RoW jurisdictions.

Future considerations:

- PBMC-based MAT provides critical biological complexity and translational relevance, essential for accurate pyrogen detection. Transitioning to cell-line-based MAT could offer significant advantages in standardization, automation, and reduced ethical constraints.
- To ensure patient safety, regulatory approval and maintain confidence, robust validation strategies are essential to demonstrate comparable sensitivity and detection capabilities to the currently accepted PBMC-based MAT.



Thank you

Sara Valentini
Giovanna Santoro
Maria Teresa Savino
Sara Chellini
Chiara Capitani
Badiaa Bouzya
Shahjahan Shaid
Stefania Berti
Anna Coslovi
Benedetta Romi

Thank you for your attention
QUESTIONS?

GSK



Validation and Implementation of a Monocyte Activation Test as a Replacement for the Rabbit Pyrogen Test

EDQM/EPAA Conference “Pyrogen testing 2.0: Ethical and Eco-friendly”

25-26 February 2026, European Commission, AlberBorschette Conference Center, Brussels, Belgium

Dr. Sven Deutschmann
Network QC
Quality and Compliance for Roche Pharma Technical Op

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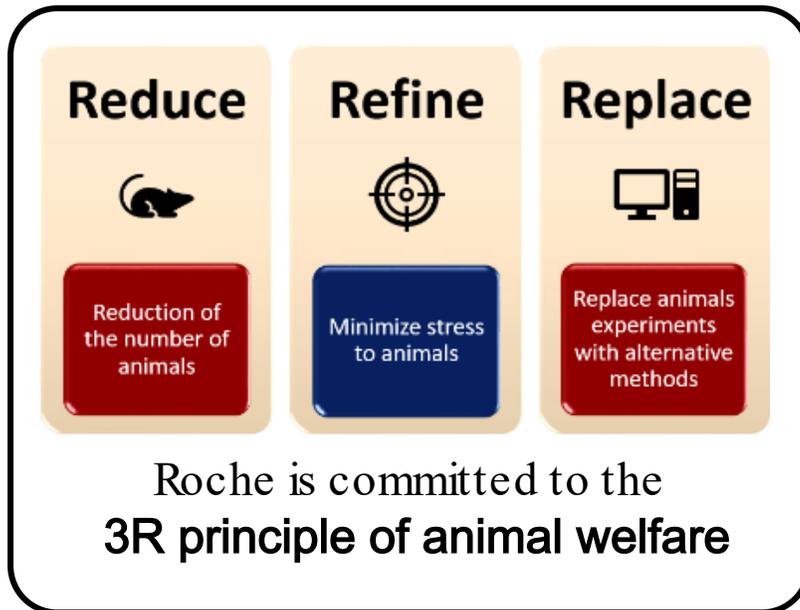


Introduction

MAT Validation and Implementation

Roche's 3R Commitment

Roche's 3R-Commitments



- **Purpose:** The goal is to avoid animal experiments altogether (Replacement), to limit the number of animals (Reduction) and their suffering (Refinement) in tests to an absolute minimum "3R-Principle".
- **Long-Term Outcomes:** Avoidance of animal testing (Replacement) to limit the number of animals (Reduction) and their suffering (Refinement) in tests to an absolute minimum.
- **Scope:** *In vivo* assays within the Global QC Network of Quality and Compliance for Roche Pharma Technical Operations.
 - Virus Detection Assays for Cell Bank Release and PHCCF-IPC-Testing
 - Replacement of the LAL-based BET with the rFC-based BET
 - Replacement of the RPT with the MAT
- **Out of Scope:** *In vivo* assays outside Global QC Network of Quality and Compliance for Roche Pharma Technical Operations.

MAT Validation and Implementation

Replacement of the Rabbit Pyrogen Test with the Monocyte Activation Test (1)

Vision: Replacement of the Rabbit Pyrogen Test with the MAT

Problem statement The Rabbit Pyrogen test (RPT) is required in the US as per 21 CFR 610.72(b) *Test for pyrogenic substances* *Each lot of final containers of any product intended for use by injection shall be tested for pyrogenic substance by intravenous injection into rabbits.*”



In vivo Assay: The RPT is a one-time characterization test, which is performed on three registration batches (usually PPQs).



21 CFR 610.09(a) Equivalent methods and processes: *“Modification of any particular test method ... shall be permitted only under the following conditions: The applicant presents evidence [...] demonstrating that the modification will provide assurances of the safety, purity, potency, and effectiveness of the biological product equal to or greater than the assurances provided by the method or process specified in the general standards or additional standards for the biological product.”*

➤ In former times, Roche performed for each BLA a comparison study between the RPT and the BET.

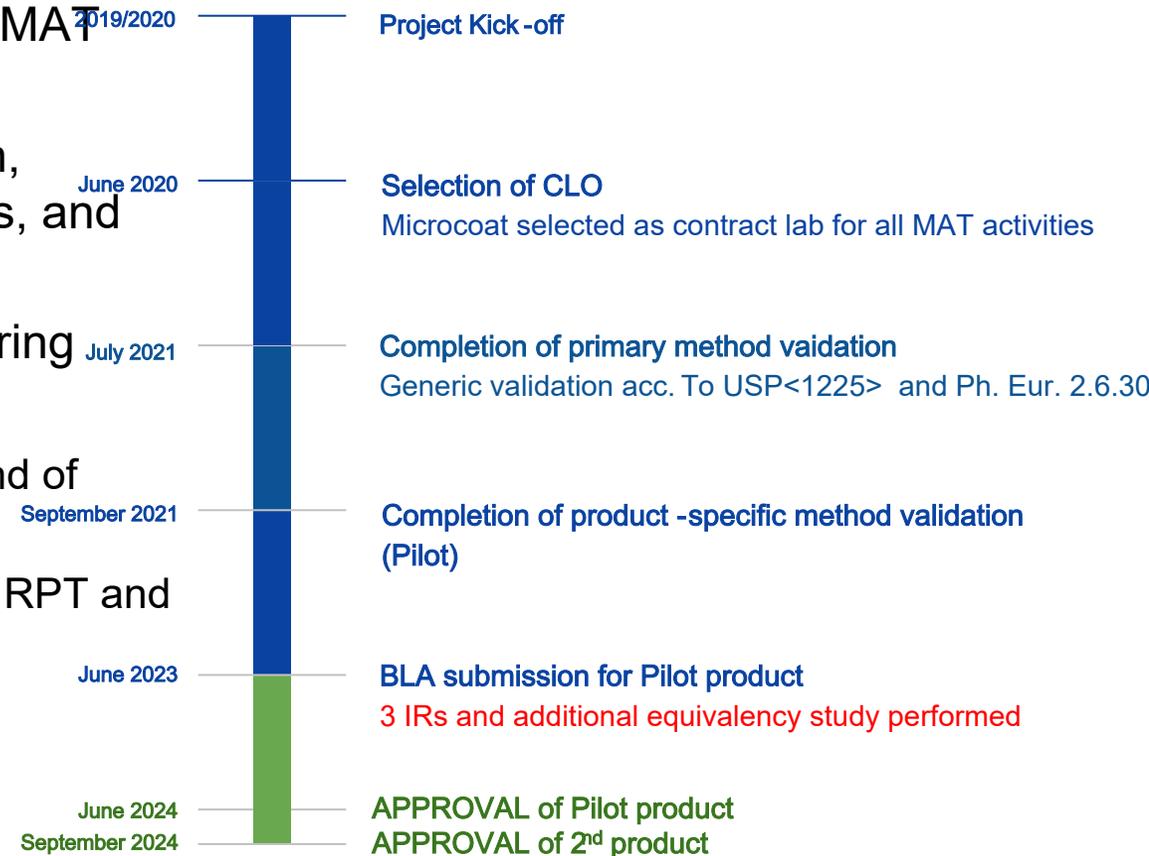
In vitro assay: After suitable validation of the Monocyte Activation Test (MAT) is a suitable *in vitro* method to detect pyrogens. Hence, the RPT can be replaced with the MAT for the one-time characterization test required for filing (note: 2 commercial products successfully filed in the USA).

MAT Validation and Implementation

Replacement of the Rabbit Pyrogen Test with the Monocyte Activation Test (2)

Project Timeline & Overview

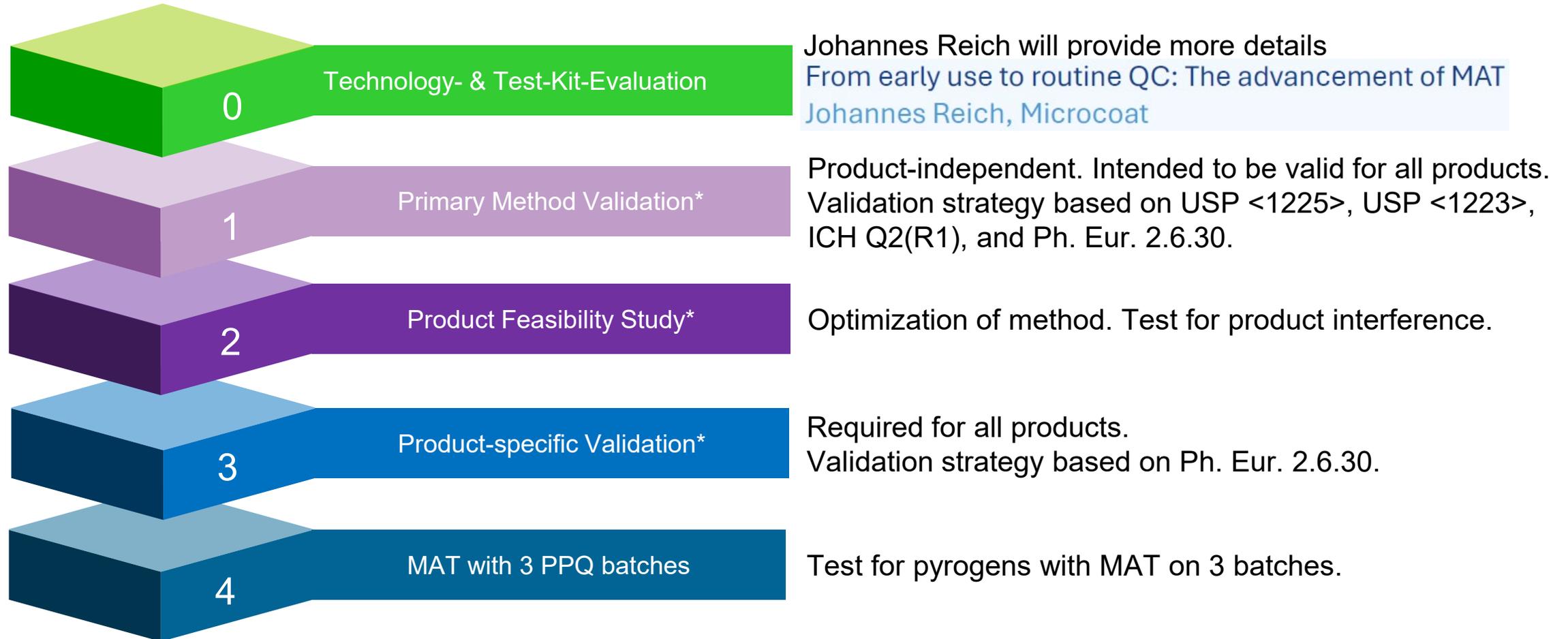
- Selection of a CLO (Microcoat, Bernried, Germany) for all MAT activities
- Parameter of the generic method validation: Limit of detection, Correctness (Accuracy), Specificity, Precision, Robustness, and Range & Linearity
- Parameter of the product-specific validation: Test for interfering factors & Interference with readout
- 2 Pilot products: Submission of 1st Pilot product in June 2023, and of 2nd Pilot product in September 2023
- IR: FDA requested data to demonstrate the equivalence between RPT and MAT
- FDA approvals in June 2024 (1st Pilot product) and September 2024 (2nd Pilot product)



Validation and Implementation Strategy

MAT Validation and Implementation

Overview



* For more information see: Burgmaier, L.; van den Berg, J.; Gajewi, M.; Röder, R.; Reich, J.; Deutschmann, S.M. (2025), *Validation of the Monocyte Activation Test Demonstrating Equivalence to the Rabbit Pyrogen Test*. *Int. J. Mol. Sci.* 2025, 26, 11136. <https://doi.org/10.3390/ijms262211136>

MAT Validation and Implementation

Primary Method Validation: Overview

Validation Strategy and Overview

- Validation strategy based on requirements from USP <1225>, USP <1223>, ICH Q2(R1), and Ph. Eur. 2.6.30 (for semi-quantitative tests)
- Primary method validation intended to be valid for all products

- Parameters validated:

Range and Linearity	Limit of Detection	Correctness (Accuracy)	Specificity	Precision	Robustness	Comparability
---------------------	--------------------	------------------------	-------------	-----------	------------	---------------

- Pyrogens used:

Pyrogens	Description
Endotoxin Standard RSE	Standard dilutions and spiking of samples, binds to TLR4
Flagellin (<i>B. subtilis</i>)	Basic component of bacterial flagella, binds to TLR5 and TLR11
Lipoteichonic acid (LTA) (<i>S. aureus</i>)	Major component of the cell wall of gram positive bacteria, binds to TLR2
Peptidoglycan (PGN BS) (<i>B. subtilis</i>)	Peptidoglycan forms a mesh like layer as part of the cell wall of bacteria, binds to TLR2
Pam3CSK4	Pam3CSK4 (Pam3CysSerLys4) is a synthetic triacylated lipopeptide and binds to TLR1 and TLR2

MAT Validation and Implementation

Primary Method Validation: Range / Linearity & Limit of Detection

Range and Linearity

Limit of Detection

Correctness (Accuracy)

Specificity

Precision

Robustness

Comparability

1

Range and Linearity: *“Linearity is the ability of the method to obtain test results that are proportional to the concentration of the analyte within a given range”*

- Preparation of standard curve with four RSE concentrations (range from 0.05 EU/mL to 0.4 EU/mL)
→ Acceptance criteria passed (based on regression of standard curve)

2

3

Limit of Detection: *“LOD is expressed as the lowest endotoxin concentration which can be reliably detected. In the MAT, the LOD has to be above the cut-off value”*

- Measurement of blank samples and determination of the cut-off value (2 assays with 2 lots)
→ Acceptance criteria passed (Signal of LOQ > Cut-off)

4

MAT Validation and Implementation

Primary Method Validation: Accuracy & Specificity

Range and Linearity

Limit of Detection

Correctness (Accuracy)

Specificity

Precision

Robustness

Comparability

1

Correctness (Accuracy): “Closeness of a measured pyrogenicity to the expected pyrogenicity (adapted definition from ICH Q2)”

- Comparison of defined pyrogen spike with pyrogen standard dilutions
- Pyrogens used: 5 (RSE, LTA, Flagellin, Pam3CSK4, PGN-BS)
- Acceptance criteria passed (spike recovery between 50 – 200%)

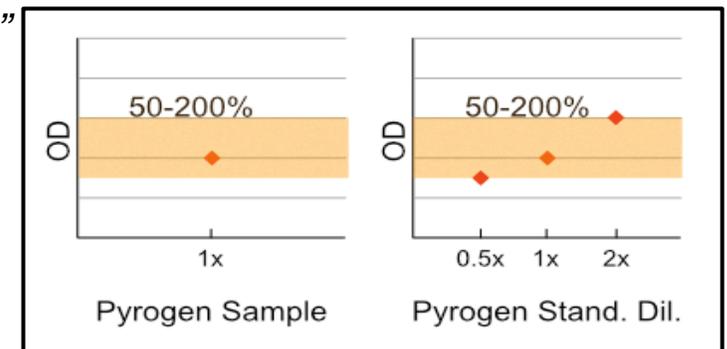
2

3

Specificity: “Ability of the method to unequivocally assess pyrogen contamination”

- Various pyrogens must be detectable with the MAT
- Pyrogens used: 5 (RSE, LTA, Flagellin, Pam3CSK4, PGN-BS)
- Acceptance criteria passed (spike recovery between 50 – 200%)

4



Note: Figures for illustration purpose only

MAT Validation and Implementation

Primary Method Validation: Precision

Range and
Linearity

Limit of
Detection

Correctness
(Accuracy)

Specificity

Precision

Robustness

Comparability

1

Intra-assay precision: *“Replicates of a single sample obtained under the same conditions analyzed in a single assay”*

- Pyrogen samples in three dilutions were measured multiple times within assay
- Pyrogens used: 5 (RSE, LTA, Flagellin, Pam3CSK4, PGN-BS)
→ Acceptance criteria passed (based on %CV for each pyrogen)

2

3

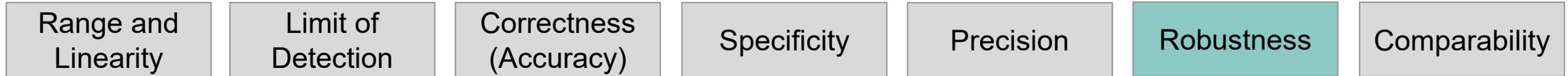
Inter-assay precision: *“Measurements with changes in conditions such as different operators, days, or equipment”*

- Pyrogen samples in three dilutions were measured multiple times within different assays
- Pyrogens used: 4 (RSE, LTA, Flagellin, PGN-BS)
→ Acceptance criteria passed (based on %CV for each pyrogen)

4

MAT Validation and Implementation

Primary Method Validation: Robustness (1)



1

Robustness: *“Capacity to remain unaffected by small but deliberate variations in the method.”*

2

Lot-to-Lot-Comparability of the HaemoMAT® Kit

→ No difference between HaemoMAT® Kit Lots

Robustness of the IL-6 measurement over time

→ Only passed for timepoints up to 50 min between addition of stop solution and measurement of ELISA

3

Robustness of thawing of PBMCs

→ Only passed for pre-incubation time up to 30 min

4

Robustness of stimulation time of PBMCs

→ Passed for PBMC stimulation times between 17h and 24h

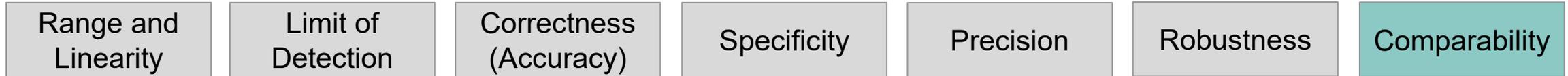
Robustness of thawing of the cell culture plate

→ Passed only for up to one freeze-thaw cycle

* Parameters selected based on risk assessment

MAT Validation and Implementation

Primary Method Validation: Comparability (1)



Comparability - Comparability / Equivalence of the MAT with RPT:

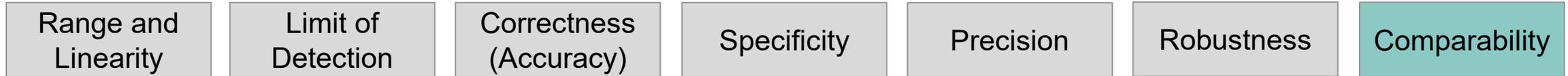
- “... equal to or greater than the assurances provided by the method or process specified in the general standards or additional standards for the biological product.” (21 CFR 610.09(a))
- “... provided that they enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official procedures were used.” (Eur. General Notices 1.1.2.5 Alternative analytical procedure)

Feedback from FDA:

- „... data demonstrating that the ~~XMAT~~ kit is better or equivalent in detecting non endotoxin pyrogens (NEP) to RPT using a panel of NEP. This information may be provided by the vendor.”
 - Comparability Assessment for the **specific** test-kit or method in use is needed
 - In accordance with the requirements specified in other Guidance Documents, this data can/should be generated by the vendor of the test kit
- „We are asking for **one-time, non-product specific study**, that compares the proposed ~~XMAT~~ method with the RPT using a panel of non endotoxin pyrogens (NEP). As indicated before, this study may be conducted by the vendor **supported by data in the literature** .“
 - A study supported by data in the literature was designed, which required NO further head-to-head comparison studies between MAT and RPT.

MAT Validation and Implementation

Primary Method Validation: Comparability (2)



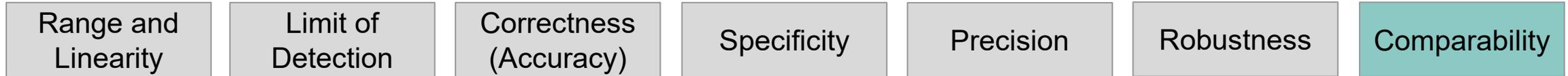
Comparability - Study Design and Results (1):

- **Literature search:** Peer-reviewed publications reporting head-to-head-comparison experiments (MAT vs. RPT) and results
- **NEP sources:** Check the availability of the NEPs used in the publications. Some NEPs could still be ordered from the same manufacturers under the same catalogue number.
- **Qualification of NEPs:** The NEPs were solubilized, diluted as described in the respective publication, and tested for contamination with endotoxins. Some NEPs were contaminated with endotoxins, which reduced the number of usable Pyrogens:

Pyrogens (species)	RSE	LTA (<i>S. aureus</i>)	PGN (<i>S. aureus</i>)	LTA (<i>S. aureus</i>)	PGN (<i>B. subtilis</i>)
Vendor	EDQM	Sigma-Aldrich	Sigma-Aldrich	Microcoat	Microcoat
Endotoxin Content in NEP preparation	N/A	0.46 EU/mL	0.08 EU/mL	< 0.05 EU/mL (not detectable)	< 0.05 EU/mL (not detectable)

MAT Validation and Implementation

Primary Method Validation: Comparability (3)



Comparability - Study Design and Results (2):

- **MAT experiments:** With the different NEPs, MAT was performed using the RPT-threshold concentration described in the literature, and the sensitivity of the MAT was determined.
- **Results:**

Pyrogen	Vendor	RPT-threshod from literature	MAT-result using RPT-threshold concentration from literature
RSE	EDQM	0.18 EU/mL	Positive
LTA	Sigma-Aldrich	0.35 µg/mL	Positive
PGN	Sigma-Aldrich	0.61 µg/mL	Positive
LTA	Microcoat	N/A	Positive (0.35 µg/mL)
PGN	Microcoat	N/A	Positive (0.61 µg/mL)

MAT Validation and Implementation

Product Feasibility Study (1)

- 
- Determine the maximum valid dilution: $MVD = \frac{CLC \times C}{sensitivity}$
 - Choose product dilutions at MVD and below (0.5 MVD & 0.25 MVD)
 - Test interference
 - with endotoxins and NEPs
 - with the detection system
 - Several batches should be evaluated

MAT Validation and Implementation

Product Feasibility Study (2)

- Determine the maximum valid dilution (MVD) $\frac{CLC \times C}{sensitivity}$
- Choose product dilutions at MVD and below

▶ Endotoxin Limit of product = 167.5 EU/mL

$$\frac{167.5 \text{ EU/mL} \times 1}{0.125 \text{ EU/mL}} = 1340 = \text{MVD}$$

Dilution	1:335 (0.25 x MVD)	1:670 (0.5 x MVD)	1:1340 (1 x MVD)
Unspiked Sample / [OD]	0.104	0.126	0.118
Endotoxin Spike – Unspiked Sample / [OD]	0.27	0.35	0.38
LTA Spike – Unspiked Sample / [OD]	1.8	0.93	0.73
Flagellin Spike – Unspiked Sample / [OD]	0.42	0.32	0.19
Status	Valid	Valid	Valid

OD – OD(BLK)	0.5 x Spike (= 50 % Recovery)	2 x Spike (= 100 % Recovery)
Endotoxin	0.061	1.076
LTA	0.171	1.884
Flagellin	0.074	0.88

MAT Validation and Implementation

Product specific validation

- Validation strategy based on requirements Ph. Eur. 2.6.30
- Product-specific validation required for all products
- Parameters validated:

1
2
3
4

Test for interfering factors
(Endotoxins and NEPs)

Batch to batch comparison of the
product

Interference with the readout

MAT Validation and Implementation

Product specific validation (1)

Test for interfering factors
(Endotoxins and NEPs)

Batch to batch comparison of the
product

Interference with the readout

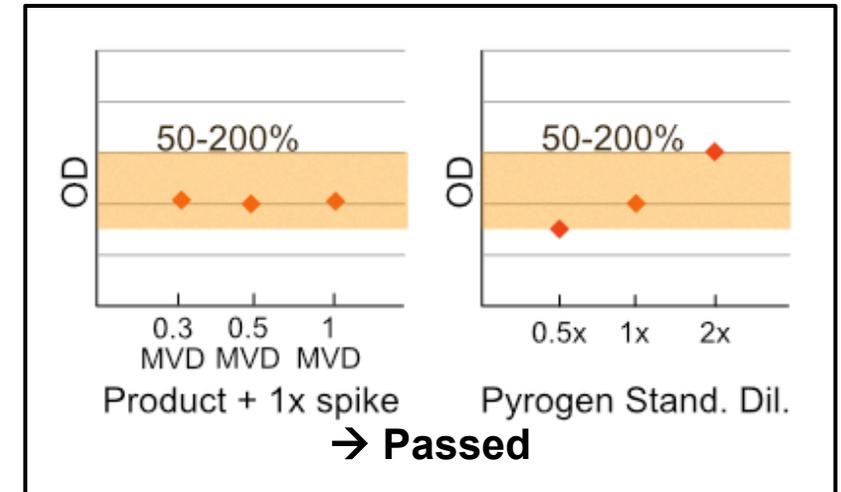
“According to Ph. Eur. 2.6.30, it has to be verified, that the preparation being examined does not contain factors interfering with the Monocyte-Activation Test.”

Assay procedure:

- Product was diluted to 0.3x MVD, 0.5x MVD and 1x MVD and spiked
- Pyrogens used: 3 (RSE, LTA, Flagellin)
- Product batches: 3

Acceptance criteria:

- Spike recovery of pyrogens must be between 50-200% of the corresponding pyrogen concentration of the standard dilution



Note: Figures for illustration purpose only

MAT Validation and Implementation

Product specific validation (2)

Test for interfering factors
(Endotoxins and NEPs)

Batch to batch comparison of the
product

Interference with the readout

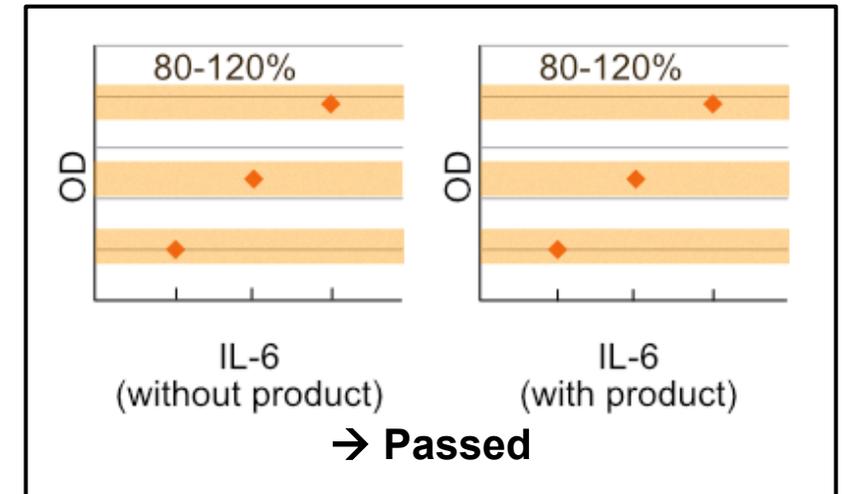
“According to Ph. Eur. 2.6.30, it has to be verified, that the preparation being examined does not contain factors interfering with the Monocyte-Activation Test.”

Assay procedure:

- IL-6 standard dilution series was prepared and measured with product (at 0.3x MVD) and without product
- Pyrogens used: none
- Product batches: 1

Acceptance criteria:

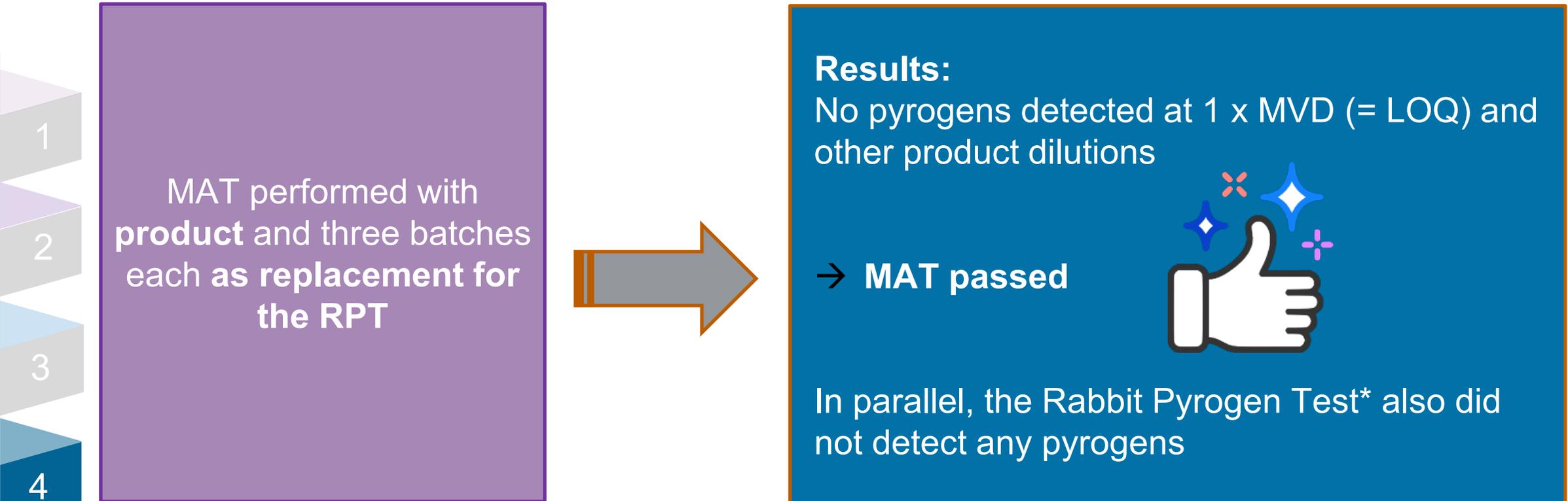
- The OD of the standard dilutions with and without product must be between 80-120%



Note: Figures for illustration purpose only

MAT Validation and Implementation

MAT with three PPQ batches



* Note: RPT was waived after two Pilot Products

MAT Validation and Implementation

Current Status

Summary

Products	Status
MAB # 1	FDA approved
MAB # 2	FDA approved
MAB # 3	Successful Product Specific Method Validation and PPQ-Testing, Ready for Submission
MAB # 4	Feasibility Tests failed due to readout interference --> Rabbit Pyrogen Test-Results for US-Filing
MAB # 5	Feasibility Study passed
MAB # 6	Feasibility Study failed --> Rabbit Pyrogen Test-Results for US-Filing
MAB # 7 - #9	Testing in preparation for PPQ-Campaigns in 2026
si-RNA-Product	Successful validation and test for pyrogenicity of a batch manufactured at a CDMO with bioburden excursion (Investigation Program)

- Selection of appropriate kit, method, readout, etc. is essential
- It may be necessary to have established different test kits/methods as products can be differently compatible with test kits/methods (as expected)

MAT Validation and Implementation Summary

Conclusion:

- MAT successfully validated for several products
- MAT reliably detects endotoxins and a range of non-endotoxin pyrogens (if compatible with the product)
- It may be necessary to have established different test kits/methods as products can be differently compatible with test kits/methods
- After completion of generic validation only moderate effort for follow-up products (interference testing & product-specific validation)
- MAT accepted by the FDA as a replacement for the RPT (CFR-requirement for Biologics)
- Filing in the U.S. requires increased effort, as MAT is still considered an alternative assay to RPT in the U.S. (and other regions)

Doing now what patients need next



From early use to routine QC: The advancement of MAT

Microcoat Biotechnologie GmbH

Dr. Johannes Reich

Bruxelles, 26-Feb-2026, Joint EDQM-EPAA Symposium "Pyrogen Testing 2.0"

Agenda

- 1 – Monocyte Activation Test (MAT) – Pyrogenicity Test
- 2 – Monocyte Activation Test (MAT) – Verification / Validation
- 3 – Case studies:
 - 1) Excerpt from verification
 - 2) Replicates and CV
 - 3) PPC Recovery >200%
 - 4) PPC Recovery <50%
 - 5) Inherent pyrogenicity
- 5 – Summary

5.1.13. PYROGENICITY

Medicinal products administered parenterally may cause side-effects due to potential contamination by pyrogenic substances (microbial contaminants, process-related impurities) or due to inherent pro-inflammatory activity, including pyrogenicity, ranging from fever to life-threatening shock-like symptoms. The aim of pyrogenicity testing is to limit to an acceptable level the risk of a febrile reaction in the patient due to the administration of the product concerned.

5. General texts

Ref: EUROPEAN PHARMACOPOEIA 11.8, 5.1.13. Pyrogenicity

Bacterial Endotoxin Test (BET)
(+ Risk Assessment)

Monocyte Activation Test (MAT)

- BET only detects endotoxin
- MAT detects endotoxin + non-endotoxin-pyrogens
- Detected activity may differ between MAT and BET
- Different reaction mechanism BET vs. MAT: Factor C vs. TLR

Monocyte Activation Test (MAT) – Verification / Validation

Requirements:

- a) According to Ph. Eur. chapter 2.6.30, only a product-specific verification is required
- b) Full method validation in addition to the product-specific verification, if no equivalent pharmacopoeia reference available:

- Range and Linearity
- Limit of Detection for endotoxin and representative NEPs
- Accuracy
- Specificity
- Precision Robustness
- **MAT/RPT comparison**



Pyrogen	Exposure Dose Leading to Fever in Rabbits	Corresponding Concentration in the MAT ^a	NEP Concentrations Tested Using the HaemoMAT [®]			
			Conc. 1	Conc. 2	Conc. 3	Conc. 4
Reference Standard Endotoxin [18]	Administered: 10 EU/kg rabbit bodyweight Normalized: 0.18 mg/mL rabbit blood ^a	≤0.18 EU/mL (corresponds to ≤0.0000216 mg/mL)	0.1 EU/mL ^b	0.05 EU/mL ^c	0.025 EU/mL ^c	0.0125 EU/mL ^c
LTA [18,19]	Administered: 20 mg/kg rabbit bodyweight Normalized: 0.35 mg/mL rabbit blood ^a	≤0.35 mg/mL	0.350 mg/mL ^b	0.030 mg/mL ^d	0.015 mg/mL ^d	0.0075 mg/mL ^d
	Administered: 75 mg/kg rabbit bodyweight Normalized: 1.315 mg/mL rabbit blood ^a					
PGN [18]	Administered: 35 mg/kg rabbit bodyweight Normalized: 0.61 mg/mL rabbit blood ^a	≤0.61 mg/mL	0.610 mg/mL ^b	0.122 mg/mL ^d	0.061 mg/mL ^d	0.0305 mg/mL ^d
Zymosan [16]	Administered: 14 mg/kg rabbit bodyweight Normalized: 0.25 mg/mL rabbit blood ^a	≤0.25 mg/mL	0.250 mg/mL ^b	0.125 mg/mL ^d	0.0625 mg/mL ^d	0.0313 mg/mL ^d

Monocyte Activation Test – Example 1

Valid Positive Product Controls (PPC) during interference testing

- Endotoxin Limit of product = 5 EU/mL

$$\frac{5 \text{ EU/mL} \times 1}{0.125 \text{ EU/mL}} = 40 = \text{MVD}$$

→ Choose product dilutions at MVD and below

Dilution	1:10 (0.25 x MVD)	1:20 (0.5 x MVD)	1:40 (1 x MVD)
Unspiked sample [EE/mL]	< 1.25	< 2.50	< 5.00
PPC Recovery [%]			
Endotoxin Spike	84	86	90
LTA Spike	83	77	81
Flagellin Spike	111	102	94
Status	Valid	Valid	Valid



Monocyte Activation Test – Example 2 (1/2)

Challenges during real life sample testing – Replicates

7-1-1. Test procedure

Using the validated test method, prepare the solutions shown in Table 2.6.30.-1 and culture **4 replicates** of each solution with the qualified cells (whole blood preparation, PBMC or monocytic cell line).

Ref: EUROPEAN PHARMACOPOEIA, 2.6.30. Monocyte Activation Test

CV Criteria?

Monocyte Activation Test – Example 2 (2/2)

Challenges during real life sample testing – Replicates and CV

Case 1: No coefficient of variation (CV):

	OD 450 nm – 570 nm				
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	1.956	0.485	0.650	86
#2	0.163	0.177	0.189	0.397	48

 OK?

Monocyte Activation Test – Example 2 (2/2)

Challenges during real life sample testing – Replicates and CV

Case 1: No coefficient of variation (CV):

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	1.956	0.485	0.650	86
#2	0.163	0.177	0.189	0.397	48



OK?

Case 2: CV-criteria (eg.: 35%) + Outlier Test

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	1.956	0.485	0.650	86
#2	0.163	0.177	0.189	0.397	48



OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	*1.956*	0.485	0.650	31
#2	0.163	0.177	0.189	*0.397*	7

Monocyte Activation Test – Example 2 (2/2)

Challenges during real life sample testing – Replicates and CV

Case 1: No coefficient of variation (CV):

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	1.956	0.485	0.650	86
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➔ OK?

Case 2: CV-criteria (eg.: 35%) + Outlier Test

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	1.956	0.485	0.650	86
#2	0.163	0.177	0.189	0.397	48

Outlier Test

➔

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#1	0.345	*1.956*	0.485	0.650	31
#2	0.163	0.177	0.189	*0.397*	7

Case 3: CV-criteria (eg.: 35%)

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#3	0.125	0.093	0.179	0.062	44
#4	1.031	1.523	0.656	0.554	47

Re-Test

➔

OD 450 nm – 570 nm					
Sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4	CV [%]
#3	0.096	0.089	0.117	0.135	19
#4	1.039	1.273	1.482	1.022	18

Monocyte Activation Test – Example 3

Challenges during real life sample testing – PPCs Recovery > 200%

Spike recovery must be within 50-200 per cent. However, if there is synergism between the PRR ligand and the test solution, it is sufficient for spike recovery to be greater than 50 per cent.

Ref: EUROPEAN PHARMACOPOEIA, 2.6.30. Monocyte Activation Test

Dilution	1:5 (0.25 x MVD)	1:10 (0.5 x MVD)	1:20 (1 x MVD)
Unspiked sample [EE/mL]	< 1.25	< 2.50	< 5.00
PPC Recovery [%]			
Endotoxin Spike	132	101	85
LTA Spike	>200	>200	>200
Flagellin Spike	89	102	75
Status	Valid	Valid	Valid



Monocyte Activation Test – Example 4 (1/3)

Challenges during real life sample testing – PPCs Recovery <50%

- Endotoxin Limit of product = 6 EU/mL

$$\frac{6 \text{ EU/mL} \times 1}{0.125 \text{ EU/mL}} = 48 = \text{MVD}$$

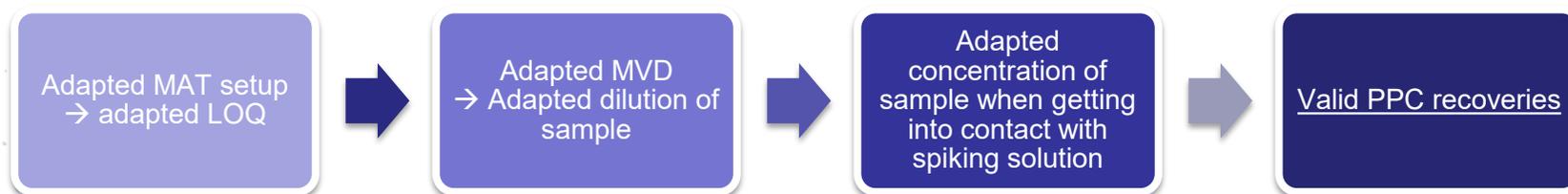
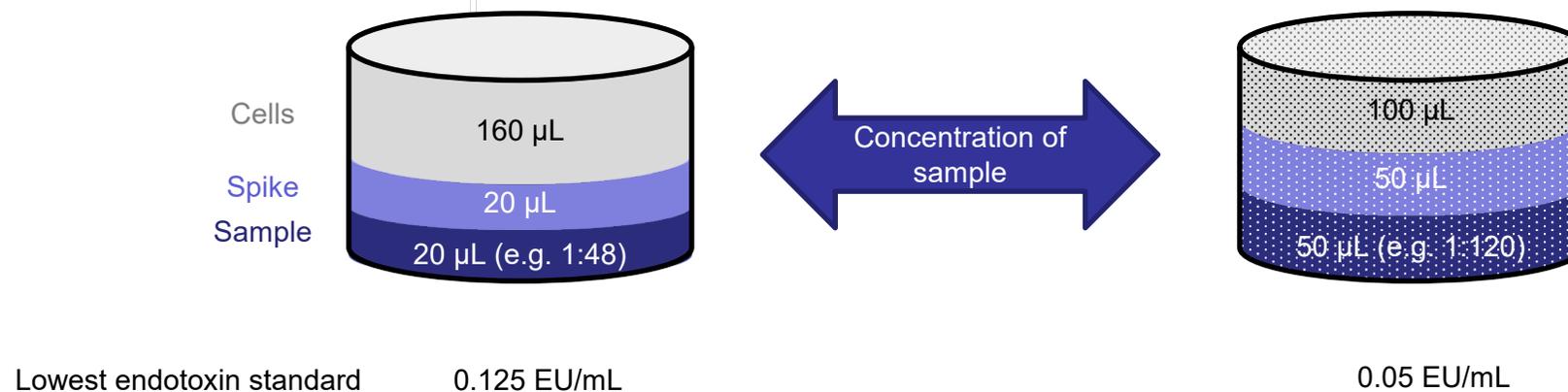
→ Choose product dilutions at MVD and below

Dilution	1:12 (0.25 x MVD)	1:24 (0.5 x MVD)	1:48 (1 x MVD)
Endotoxin PPC [%]	41	75	80
LTA PPC [%]	34	39	48
Flagellin PPC [%]	28	51	65
Status	Invalid	Invalid	Invalid

Monocyte Activation Test – Example 4 (2/3)

Challenges during real life sample testing – PPCs Recovery <50%

➤ Adaptation of MAT setup:



Final concentration of the sample in the well stays the same

Monocyte Activation Test – Example 4 (3/3)

Challenges during real life sample testing – PPCs Recovery <50%

- Endotoxin Limit of product = 6 EU/mL

$$\frac{6 \text{ EU/mL} \times 1}{0.04 \text{ EU/mL}} = 150 = \text{MVD}$$

→ Choose product dilutions at MVD and below

Dilution	1:37.5 (0.25 x MVD)	1:75 (0.5 x MVD)	1:150 (1 x MVD)
Endotoxin PPC [%]	101	165	144
LTA PPC [%]	68	71	89
Flagellin PPC [%]	88	66	67
Status	Valid	Valid	Valid



Monocyte Activation Test – Example 5

Challenges during real life sample testing – Inherent pyrogenicity

Pyrogenicity is an integral part of medicinal product (eg.: Vaccine)



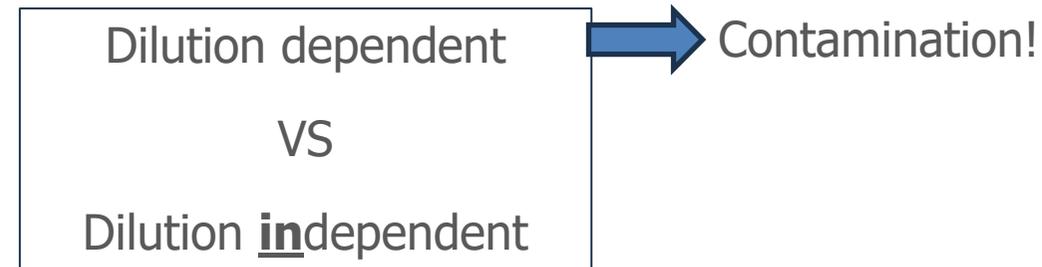
2. METHODS

2-1. INFORMATION REGARDING THE CHOICE OF METHODS

Method 1 may not be appropriate if the product has high inherent pro-inflammatory activity (spike recovery exceeding 200 per cent). **In this case Method 2 is recommended.**

Ref: EUROPEAN PHARMACOPOEIA 11.8,
2.6.30. Monocyte Activation Test

Unexpected pyrogenicity of medicinal product (eg.: Therapeutic protein)



Interference?

Monocyte Activation Test – Example 5

Challenges during real life sample testing – Inherent pyrogenicity

→ Elevated background signal

		Method 1			
		measured values [EE/mL]	EE/mL x dilution	PPC [%]	Status
untreated	96	0.452	43.4	28	Invalid
	192	0.433	83.1	31	Invalid
	384	0.351	135	39	Invalid
	768	0.229	176	43	Invalid

5min 80° C



		Method 1			
		measured values [EE/mL]	EE/mL x dilution	PPC [%]	Status
inactivated	96	<0.125	<12	225	Invalid
	192	<0.125	<24	169	Valid
	384	<0.125	<48	107	Valid
	768	<0.125	<96	94	Valid



Control experiment: Endotoxin and Flagellin hotspike

		Hotspike	
		Recovery Endotoxin [%]	Recovery Flagellin [%]
inactivated	96	115	80
	192	90	93
	384	121	144
	768	108	96



Summary and evolution of the MAT

10 Years Ago

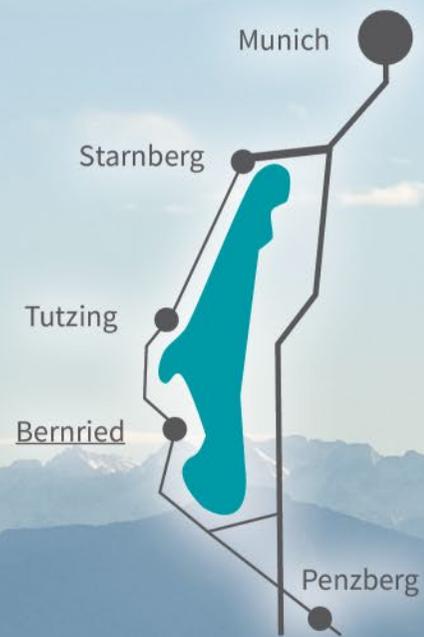
- Unreliable cell logistics
- High effort, non-standardized methods
- Limited experience and training

3 Years Ago

- Discussion on MAT applicability
- Multiple vendors available
- Stable cell logistics
- Growing expertise

Today

- MAT required to demonstrate absence of NEPs and to replace RPT
- Strategic discussion on when to implement MAT
- Procedures in place to overcome interference
- Trend toward outsourcing





MAT validation strategy assessment

Barbora Ladinová, State Institute For Drug Control, Czechia and BWP (EMA) member

02 March 2026

Introduction

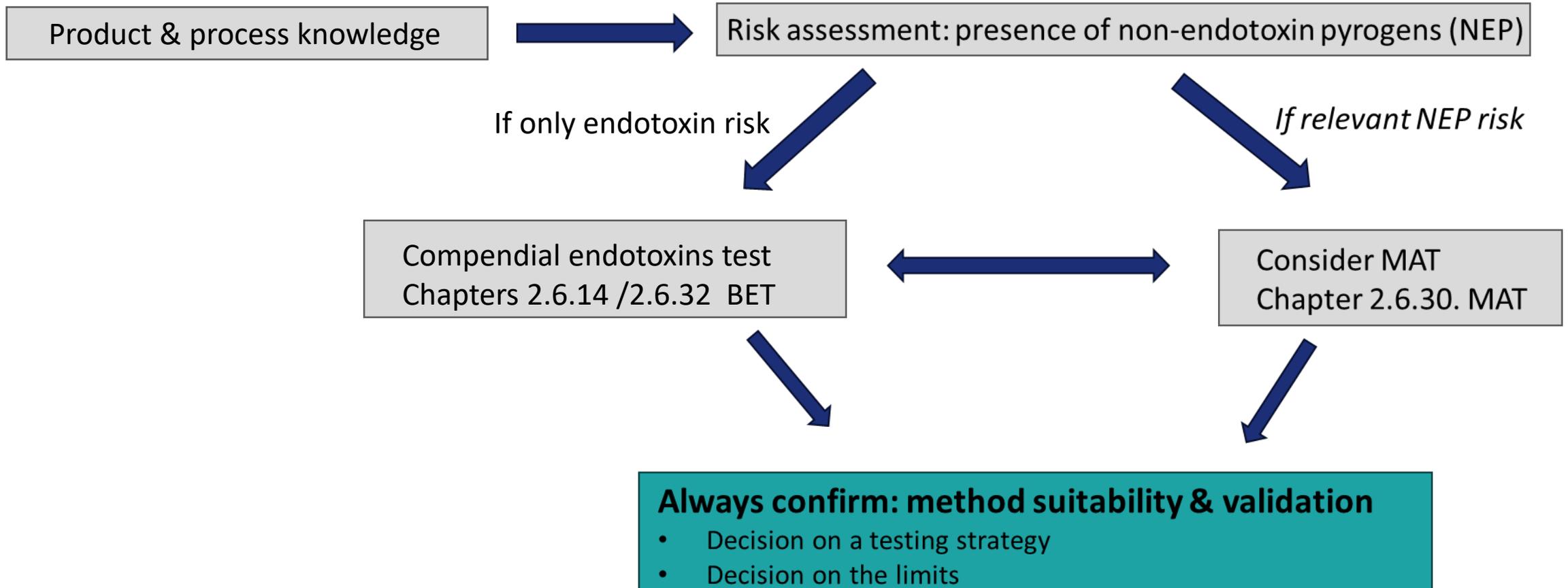
- **Pyrogens**
 - Endotoxins (LPS, Gram-negative bacteria)
 - Non-endotoxin pyrogens (Gram+, fungi, viruses, cell debris, process impurities)
- ➔ Historically reliant on animal-derived methods (**LAL, RPT**)
 - Not aligned with the EMA/EDQM 3Rs initiative promoting reduction of animal use
- **Monocyte-Activation Test (MAT)**
 - Human-relevant, animal-free in vitro assay detecting the full spectrum of pyrogens; preferred regulatory replacement for the Rabbit Pyrogen Test



Evolution of Pyrogen & MAT in Europe

- **1940:** First use of the Rabbit Pyrogen Test for safety testing of parenteral medicines
- **1950 -1960:** RPT adopted globally as the regulatory “gold standard” for pyrogen testing
- **1971:** Rabbit Pyrogen Test included in the European Pharmacopoeia (1st edition, vol II)
- **1990:** Human monocyte cytokine response identified as pyrogen mechanism
- **2000 - 2005:** Interlaboratory validation & robustness studies
- **2010:** Directive 2010/63/EU significant milestone by introducing legal provisions for scientific animal protection in the EU
- **2010:** MAT introduced in European Pharmacopoeia (Chapter 2.6.30)
- **2012 - 2016:** Adoption for biologics & vaccines supported by European Medicines Agency
- **2016 - 2020:** Progressive Phase-Out of RPT
 - ✓ EMA/EDQM 3Rs initiative accelerates transition away from animal-based methods.
 - ✓ Risk-based approaches increasingly used to justify MAT or no testing for NEPs.
- **2024:** Formal adoption of general chapter 5.1.13 Pyrogenicity
- **2026:** Rabbit Pyrogen Test (chapter 2.6.8) is no longer present in any general chapter or monograph

Pyrogen testing strategy according to Ph. Eur. 5.1.13 – a risk-based approach



Low risk of non-endotoxin pyrogens (NEP): when MAT is not scientifically required

- **Product-related factors**
 - chemically defined or highly purified substance
 - simple molecular structure ...
- **Raw materials & excipients**
 - synthetic or compendial grade materials
 - animal-free origin ... (animal derived origin risk assessment is expected)
- **Manufacturing process**
 - closed system manufacturing
 - sterile filtration (0.22 µm)
 - validated endotoxin removal...
- First cases: RPT has been removed based on risk assessment only, without MAT introduction: Guideline on the replacement of rabbit pyrogen testing by an alternative test for plasma derived medicinal products [guideline-replacement-rabbit-pyrogen-testing-alternative-test-plasma-derived-medicinal-products_en.pdf](#)

Elevated or uncertain risk of non-endotoxin pyrogens (NEP): when MAT becomes appropriate

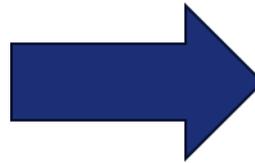
- **Product-related factors**
 - complex biological products ...
- **Raw materials & excipients**
 - biological or natural origin materials
 - serum, cell culture components ...
- **Manufacturing process**
 - cell-based production
 - higher bioburden risk ...
- **Microbiological uncertainty**
 - Gram-positive bacteria...
- **Etc...**

MAT suitability: technical limitations and validation challenges

- **Matrix-related interference**
 - excipient interference
 - adjuvants or inherent immunostimulation (cytokine release)
 - lipid nanoparticles (LNPs), saponins, CpG oligonucleotides
 - high viscosity, lipidic matrices, protein aggregates ...
- **Cytotoxicity or cell incompatibility**
 - monocyte cytotoxicity
 - altered cellular metabolism
 - suppressed or exaggerated cytokine response
 - invalid or non-interpretable read-out ...

EU regulatory view: RPT as a last resort: exceptional use only

- ✓ **Risk remains clinically relevant**
 - broader pyrogen risk cannot be excluded
 - endotoxin-only testing not sufficient
- ✓ **Only when MAT is not technically suitable**
 - non-resolvable matrix interference
 - inherent immunostimulation
 - monocyte cytotoxicity
 - inability to demonstrate suitability or validation



- **Case-by-case assessment**
- **Strong data package required**
- **Documented risk assessment**



**EXCEPTION TO
THE RULES**

Global regulatory landscape: phasing-out of Rabbit Pyrogen Test

- **United States (U.S. Food and Drug Administration / CFR)**
 - Rabbit Pyrogen Test still referenced in CFR
 - New products expected to comply
 - **Alternatives** to RPT, including MAT, are acknowledged by FDA and encouraged when validated
- **International Council for Harmonisation (ICH) / WHO**
 - ICH Q9 / risk-based principles apply
 - WHO guidance supports alternatives and 3Rs

Regulatory position on MAT and animal testing - BWP Position

- BWP Interested Parties Meeting October 2025 with EFPIA and VE (Enabling Global Implementation of Changes to Pyrogenicity and Endotoxin Testing)
- MAT remains the preferred standard
- No intention to expand the burden faced by Industry (i.e. no desire to introduce the need for MAT testing for situations of low risk of NEP and no RPT test would have previously been performed/required)
- Animal testing should not persist due to legacy requirements
- Regulatory decisions must be science-based, not region-dependent



Conclusion – EU regulatory perspective and BWP view

Can a ‘paper-based’ risk assessment replace MAT when endotoxin and NEP risk are low?

- BWP confirms that paper-based risk assessments are acceptable.
- If the risk of endotoxins and NEPs is assessed as low, no MAT testing is required.
- There is no intention to increase the burden on industry or introduce MAT where it was not previously needed.

Are non-endotoxin pyrogens (NEPs) a relevant risk for biological medicinal products?

- BWP acknowledges that most products do not present a NEP risk.
- A small number of products may present a genuine NEP risk.
- MAT testing is expected only when a real NEP risk is identified.

If NEP risk exists, can existing RPT data generated for the FDA be accepted by EMA to avoid double testing?

- BWP expressed concern about accepting RPT data, as it contradicts Europe’s move away from animal testing. Therefore, RPT is generally not accepted as an alternative to MAT.
- Exceptionally, RPT data may be accepted only when MAT cannot be used for compelling scientific or technical reasons.
- Such cases are expected to be rare and exceptional.



THANK YOU FOR YOUR ATTENTION

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Health Canada perspective on the Monocyte Activation Test: Insights and Experiences

Cynthia Allen, PhD
Senior Scientific Evaluator
Vaccine Quality Division
Biologic and Radiopharmaceutical Drugs Directorate
Health Canada

“My comments and point of view are an informal communication and represent my own best judgement. These comments do not bind or obligate Health Canada.”

Under the authority of the **Food and Drugs Act (FDA)**

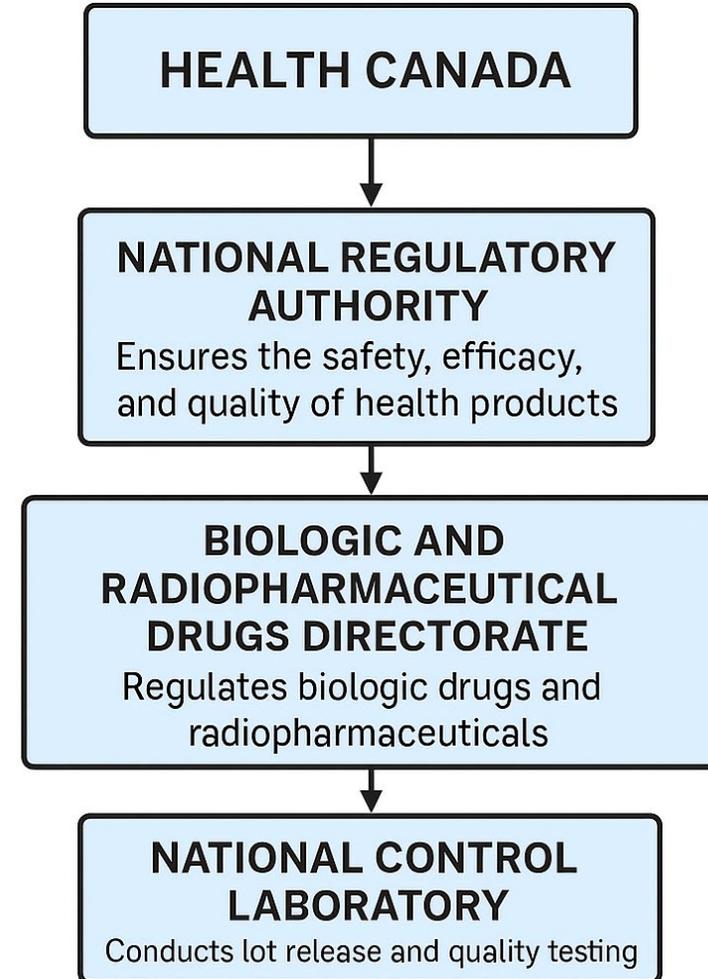
- Schedule D of the FDA is the legal classification of biologic drugs to which Division 4 applies
- **Food and Drug Regulations (FDR) – Part C**
 - Division 4 in Part C defines regulations that are specific to biologic drugs

National Regulatory Authority

- Comprehensive Regulatory Oversight
- Market Authorization and Surveillance
- Enforcement and Scientific Evaluation
- Global Harmonization and Collaboration

National Control Laboratory

- Independent Quality Control Testing
- Global Regulatory Compliance
- Support for Safety and Manufacturing



Health Canada does not have an explicit policy or guidance document dedicated specifically to the MAT/BET

Quality expectations for pyrogen control rely on ICH aligned principles.
(ICH Q6A/Q6B, ICH Q9)

Canada recognizes pharmacopeial standards such as:

- Ph. Eur. monograph 5.1.13
- Ph. Eur. 2.6.30
- Ph. Eur. 2.6.40
- USP <151>

WHO Technical Report Series

- Guidelines on the replacement or removal of animal tests for the quality control of biological products

Health Canada guidance documents:

- Guidance document on submission information for biologic drugs (Division 4, Schedule D)
- Post-Notice of Compliance (NOC) Changes: Quality Document
- Guidance for Sponsors Lot Release Program for Schedule D (Biologic) Drugs

Biologic and Radiopharmaceutical Drugs Directorate (BRDD) ensures product quality, safety and efficacy and committed to using risk-based evaluations, scientific evidence and alignment with international scientific standards. Regulatory mandates strongly support scientifically robust, validated and human-biologically relevant assays for control of biologics quality.

Monocyte Activation Test is NOT automatically accepted (just like any test)

- Product specific data are required
- Demonstration that the assay is fit for purpose

Regulatory Considerations

- Example of Quality Risk Management (QRM) factors:
 - Identification and stratification of pyrogen-related risks
 - Justification of control strategy for endotoxin and non-endotoxin pyrogen risks
 - Validation and qualification summaries supporting method suitability
 - Lifecycle approach to controlling endotoxin and non-endotoxin pyrogens
 - Scientific justification for selecting MAT over BET testing
 - Alignment of pyrogen controls with release specifications and in-process controls (IPC)
- BRDD minimum expectations:
 - Science-based justification for method selection including rationale and evidence that endotoxin testing alone is insufficient
 - Scientific validation demonstrating MAT suitability for specific product
 - Product-specific feasibility – e.g., matrix interference studies, appropriate controls
 - Trending and lifecycle management demonstrating ongoing method suitability

Risk Assessment for Pyrogens in Biologics

1. Hazard Identification

- Endotoxin
- Non-Endotoxin pyrogens
 - Biologics manufactured using cell lines, fermentation, recombinant systems, or human/animal-derived materials are at **increased risk** of NEPs

2. Exposure Assessment

- Biologic Product Factors
 - Route of administration, Dose & Volume, Patient population
- Manufacturing Process Factors
 - Potential pyrogen introduction points (cell culture/fermentation, harvest, purification, formulation, fill/finish)
 - Environmental & personnel contamination can also introduce pyrogens

3. Risk Characterization

High	Moderate	Low
<ul style="list-style-type: none"> • Intravenously or intrathecally administered • Large volume parenteral • Biologics using microbial expression systems • Process with insufficient clearance steps • Historical evidence of endotoxin excursion 	<ul style="list-style-type: none"> • Biologics manufactured using mammalian cell culture • Subcutaneous or intramuscular administered low volume 	<ul style="list-style-type: none"> • Purified recombinant proteins • Clearance steps • Environmental controls • Low bioburden processes

4. Risk Control and Mitigation

- Control Strategies
 - Design level controls – use of low endotoxin raw materials, excipients, WFI systems, use of single use equipment
 - Process Controls
 - Microbial control throughout upstream & downstream manufacturing
 - Purification steps for pyrogen clearance
 - Ensure depyrogenation conditions where applicable (e.g., 250°C dry heat)
 - Robust cleaning & sanitization
- Analytical Controls
 - Endotoxin Testing
 - LAL/TAL assay (Kinetic chromogenic or turbidimetric).
 - Validated for interference (inhibition/enhancement testing), and detection limits aligned with maximum allowable endotoxin levels
 - Non-Endotoxin Pyrogen Testing
 - Monocyte Activation Test (MAT) detecting broad pyrogenic responses via cytokine release
 - Useful for biologics containing complex matrices, products where LAL is insufficient or gives false positives/negatives
- Environmental and Equipment Controls

5. Monitoring and Trending

- Establish routine trending of endotoxin results (e.g., control charts)
- Investigate deviations such as:
 - endotoxin test failures
 - elevated bioburden
 - environmental monitoring excursions
 - cleaning failures
- Annual product quality review (APQR/PQR) assessment

6. Residual Risk Evaluation

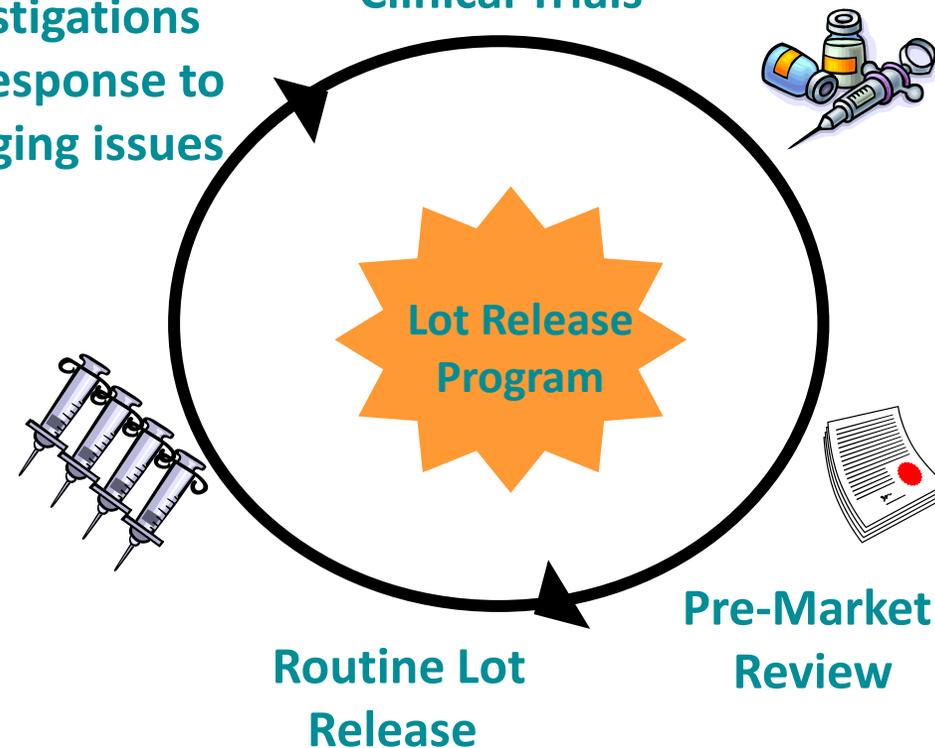
- After implementing controls, residual risk should be assessed by:
 - Verification of validated clearance factors
 - Endotoxin and MAT testing for each batch (**as applicable**)
 - Review of historical trend data
 - Assessment of robustness of microbial control programs
- Residual risk is typically **low** when:
 - All raw materials meet endotoxin specs
 - Clearance steps are validated
 - Environmental monitoring is stable
 - LAL/MAT testing meets acceptance criteria

Health Canada's Lot Release Lifecycle Approach

- Every lot of a Schedule D biologic drug must undergo Lot Release before sale in Canada
- Products are placed in one of four Evaluation Groups requiring different levels of regulatory oversight
- Group assignment is based on product risk factors including the nature of the product, indication, manufacturer's production and testing history, BRDD's testing experience, post-market experience
- The Lot Release program is a risk-based approach which allows BRDD to allocate resources based on priorities – risk is periodically reassessed and movement through the evaluation groups may be bi-directional.

Support for investigations and response to emerging issues

Clinical Trials



Monocyte Activation Testing in Health Canada Lot Release Labs

BRDD has conducted the MAT however it is NOT routinely performed

During the regulatory review process, BRDD assesses the suitability of the MAT for measuring pyrogenic activity of various vaccine products

This could include a product specific verification, evaluating repeatability, intermediate precision, linearity, sensitivity, specificity

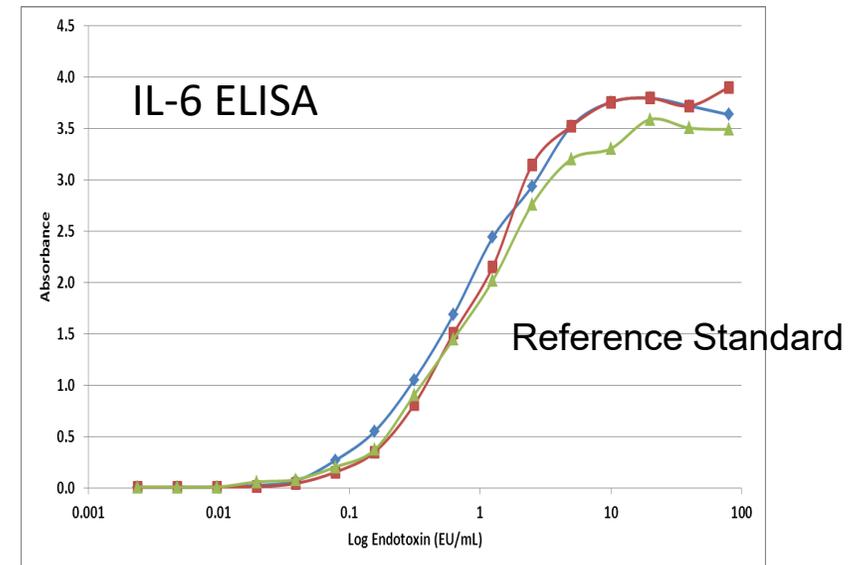
Important to note: a function of the MAT is to ensure lot-to-lot consistency of the pyrogenic activity, particularly in inherently pyrogenic products

In alignment with our lot release guidance, pyrogen testing by Health Canada is not an absolute requirement

Monocyte Activation Test at BRDD

- Verification of Mono-Mac-6 (MM6) cells
- Use of immortalized monocytic cell line over whole blood /primary human peripheral blood mononuclear cells (PBMCs)
 - Higher reproducibility – eliminates inter-donor variability
 - Greater stability – tolerant to handling stress
 - Ready-to-Use – MM6 based MAT kits are available
 - Time Saving
 - Continuous & reliable supply chain
 - Validated Cell Source – qualification of a working cell bank
 - Adequate sensitivity for endotoxins

Preliminary test to establish the appropriate cell passage number/days in culture for most consistent MAT results.

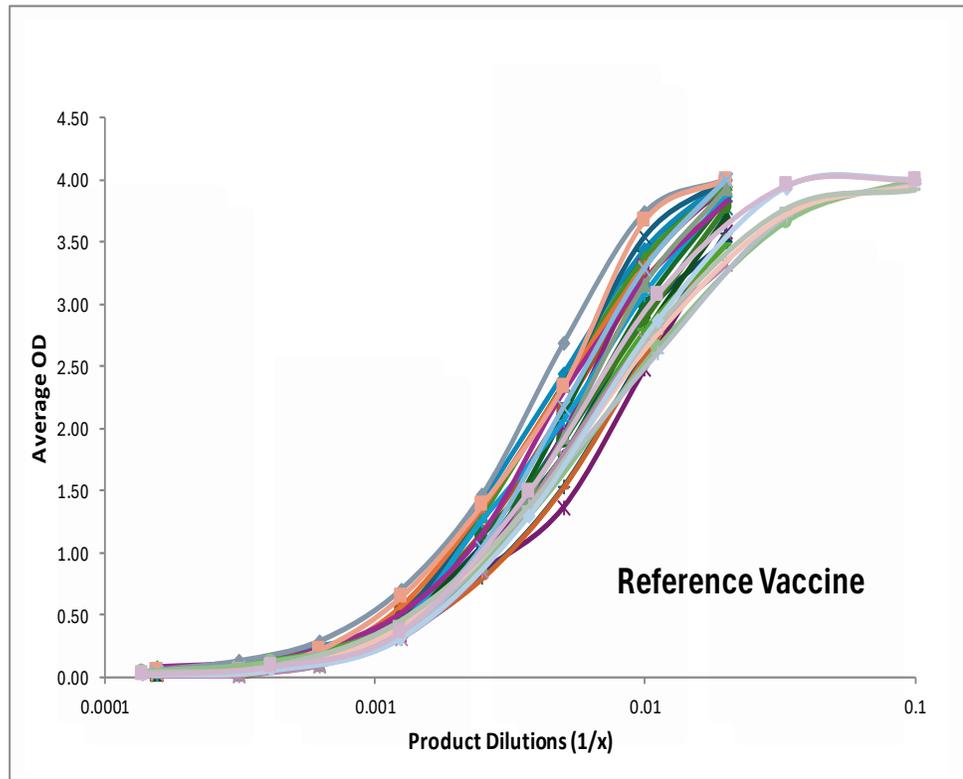


Monocyte Activation Test – Whole Blood – PBMCs – MM6

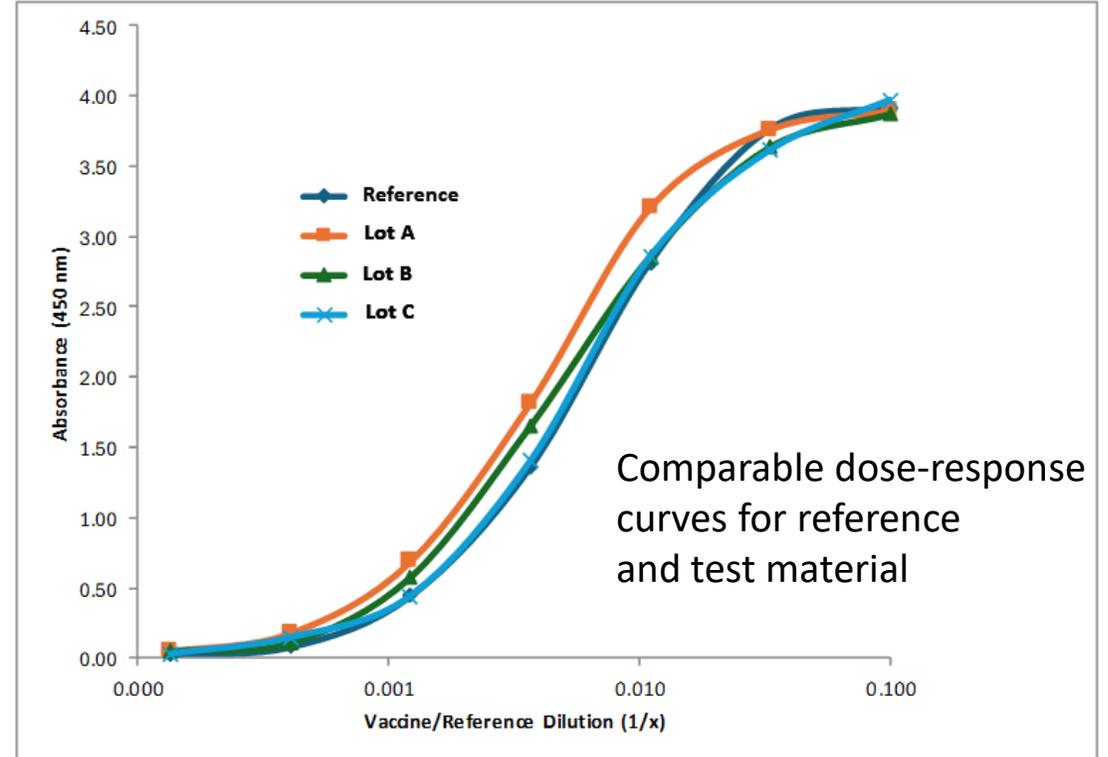
Feature	Human whole blood	Human PBMCs	Human monocytic cell line - MM6
What it detects	All pyrogens (endotoxins + NEPs)	All pyrogens (endotoxins + NEPs)	All pyrogens (endotoxins + NEPs)
Biological source	Fresh heparinized whole blood from human donors	Isolated peripheral blood mononuclear cells (PBMCs) from human donors	Human monocytic cell line cultured in vitro
Readout (typical markers)	Cytokines (e.g., IL-6, IL-1β, TNF-α)	Cytokines (e.g., IL-6, IL-1β, TNF-α)	Cytokines (commonly IL-6) via ELISA or multiplex
Human relevance	Highest (closest to in vivo milieu; includes plasma components and leukocyte crosstalk)	High (primary human immune cells without plasma components)	High (human lineage; standardized cell background)
Sensitivity to endotoxin	Moderate–high (assay-dependent)	Moderate–high	Moderate (improves with cell priming/optimized protocols)
Detects NEPs	Yes	Yes	Yes
Time to results	Hours (6–24 h)	Hours (6–24 h)	Hours (6–24 h)
Variability	Higher (strong donor-to-donor effects; matrix effects)	Moderate (donor-dependent, less matrix)	Lower (cell line standardization ; still biological)
Matrix tolerance / interference	Realistic matrix but higher risk of interference from plasma proteins/serum factors	Reduced plasma interference vs. whole blood	More Tolerant to diverse sample matrices
Controls needed	Endotoxin standard curves + spike recovery; donor qualification	Endotoxin standard curves + spike recovery; donor qualification & viability/phenotype checks as needed	Endotoxin standard curves + spike recovery, cell health/viability & responsiveness controls
			Routine, standardized, higher-throughput pyrogen screening across diverse products

Advantages of using a continuous cell line outweigh the advantages of using whole blood or PBMCs

Preliminary Assays



Relative pyrogenic activity of inherently pyrogenic sample lots in comparison to product specific reference material by parallel line analysis

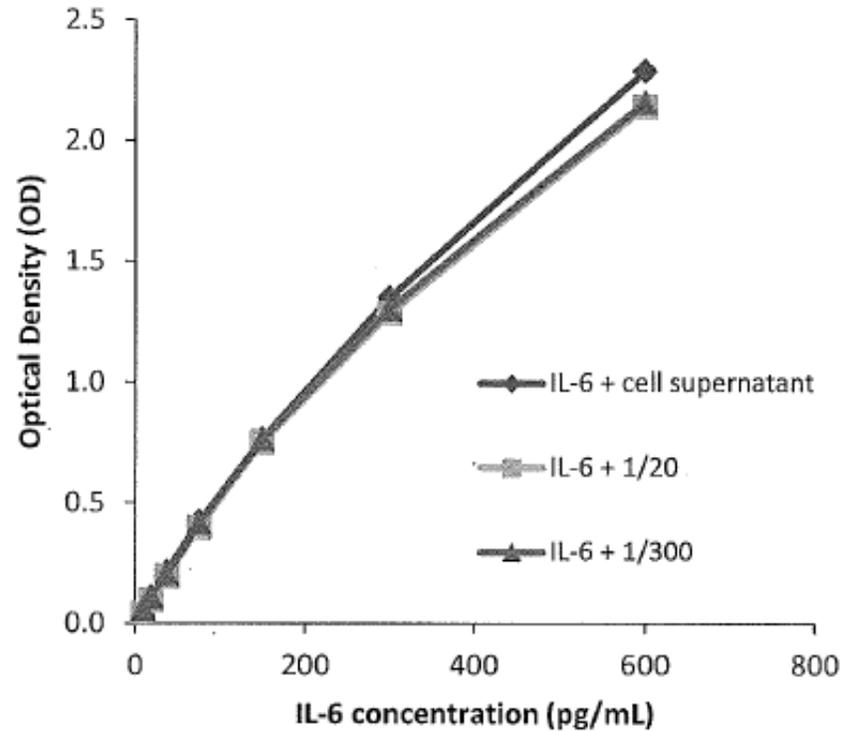


Low inter-assay variability of response by MM6 cells

Sample	Parallelism	Linearity	Slope	Slope Ratio	Relative Pyrogenic Activity
LOT A	Pass	Pass	0.825	0.962	1.34
LOT B	F (p<0.05)*	Pass	0.709	0.915	1.17
LOT C	Pass	Pass	0.824	0.948	1.01

*Passed on re-test

Verification of biologic product on IL-6 ELISA Detection System



Impact of the vaccine on the detection system (IL-6 ELISA)

MAT		
% difference to IL-6 Std + supernatant		
IL-6 (pg/mL)	+ 1/20	+ 1/300
600	-6.40	-5.67
300	-4.81	-3.41
150	-1.66	-0.09
75	-5.13	-0.55
37.5	-7.76	-2.80
18.75	-10.12	-0.30
9.375	-15.82	12.66

IL-6 standard in non-treated cell supernatant was tested in presence of vaccine sample; OD results are within $\pm 20\%$

Outcome of Product Specific Method Verification Study : Reference Lot Comparison Test (as per Eur. Ph. 2.6.30/2.6.40)

Performance Characteristic	Acceptance Criteria	Results	
Repeatability	RPE CV≤15%	2.3-12.3%	low intra-assay variability
Intermediate Precision	RPE CV≤15%	2-10%	low inter-assay variability
Linearity & Range	p>0.05 non-linearity/non-parallelism correlation coefficient >0.95	p>0.05 nonlinearity p>0.05 non-parallelism Correlation coefficient ≥ 0.95 % difference between expected and observed RPE ranged from 1-6% over dilution range	25-50%-100%-200% reference standard dilutions
Specificity	Difference of OD values in presence or absence of product ±20%	-2% to -16% (1/20 dilution) 0-12% (1/300 dilution)	Product does not interfere with IL-6 ELISA detection

RPE: Relative Pyrogen Estimate

Assay validity acceptance criteria were established for the product:

- No significant non-linearity or non-parallelism ($p > 0.05$) for reference and sample curves
- Significant regression between the reference standard and samples ($p < 0.05$)
- Correlation coefficient ≥ 0.95
- Average blank cells (control) OD ≤ 0.200

Vaccine acceptance criteria (provisional limits):

- Common Slope Limits : 0.271-0.534 (3σ of repeatability testing average)
- Relative Pyrogen Estimate upper limit: 1.668 (3σ of repeatability testing average)

The Monocyte Activation test is an acceptable and regulatory-recognized pyrogen test. Several advantages for detecting pyrogens as compared to the in vivo Rabbit Pyrogen Test.

- MAT provides a response that more accurately mirrors human physiology since it is based on human immune cells
- Stronger reliability and reproducibility
- High sensitivity to both endotoxins and non-endotoxin pyrogens
- Quantitative results when used with suitable reference standards and controls

The Monocyte Activation Test is an acceptable and regulatory-recognized pyrogen test but it has limitations:

- Requires product-specific validation
- Potential interference from Product Formulations (false positives/false negatives)
- Risk of cytokine suppression by product
- Complex test setup – more demanding compared to BET
- Higher costs compared to BET
- Does not replace BET – if non-endotoxin pyrogens are ruled out, BET is acceptable

Monocyte Activation Test is strongly recommended with demonstration of being Fit For Purpose

Acknowledgements

Dr. Gayle Pulle

Dr. Richard Isbrucker

Dr. Tong Wu

Dr. Richard Siggers

Thank you

MONOCYTE ACTIVATION TEST

1. PREPARATION OF TEST SAMPLES



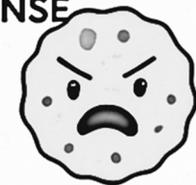
2. EXPOSURE OF MONOCYTES TO TEST SAMPLES



3. DETERMINATION OF CYTOKINE LEVELS



4. DETECTION OF PRO-INFLAMMATORY RESPONSE



A pro-inflammatory response indicates the presence of pyrogens.



Developments in the MAT since the EDQM-EPAA 2023 conference: what has happened, technical developments and new validation requirements

Pyrogen Testing 2.0: Ethical, Evolving and Eco-friendly
24-25 February 2026, European Commission, Albert Borschette Conference Center, Brussels

Dr. Ingo Spreitzer

Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines



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Disclaimer:

The views and opinions expressed in this presentation are those of the author and do not necessarily reflect the official policy or position of the Paul-Ehrlich-Institut

But there is an official statement of the PEI on RPT

<https://www.pei.de> > Newsroom > Positions > 14.12.2020 [Statement: Pyrogen Testing on Rabbits](#)



Wallpapers in this presentation by <https://pixabay.com> and StableDiffusion



Outlook

- The early MAT's and their european validation
- Newer MAT versions and how I see their validation status
- What all of us can do to support the global shift from RPT to MAT



To my best knowledge first MAT approval in 2002



In the early 1990, a life saving fermented drug (commercialized in US too) caused frequent reports of adverse drug responses (ADRs) **including fever**:

- **Both RPT and BET failed to identify the adverse batches**
- The affected drug company developed an **MAT** based on PBMC (4 donors in parallel) and IL-6, and **was able to identify contaminated batches** in QC; origin of MAT Method 2 (Reference lot comparison test)
- **The company validated their MAT, and received approval by FDA and other Health authorities for its use for QC of the affected product in 2002**
- Few details (pages 173-175) here:
Hasiwa, N.; Daneshian, M.; Bruegger, P.; Fennrich, S.; Hochadel, A.; Hoffmann, S. et al. (2013): Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test. In: *ALTEX*. 30 (2), S. 169–208.

Reference: 9

The European MAT-validations 2002-2006; separate RPT-data published



- Primary cells and cell-lines:
 - Fresh whole blood; cryo-whole blood; cryo PBMC, MM6, THP-1
- All detection systems (IL-1 β , IL-6 and Neopterin) were ELISA-based
- More than 1000 blinded samples with/without RSE
- Very limited NEP-data at that time

Primary
cells

Cell
lines

ELISA

References

[1] Hoffmann S, Peterbauer A, Schindler S, Fennrich S, Poole S, Mistry Y et al. International validation of novel pyrogen tests based on human monocytoïd cells. *J.Immunol.Methods* 2005;298(1-2):161–73.

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[4] Spreitzer I, Fischer M, Hartzsch K, Luderitz-Puchel U, Montag T. Comparative study of rabbit pyrogen test and human whole blood assay on human serum albumin. *ALTEX.* 2002;19 Suppl 1(0946-7785 (Print)):73–5.

Reference: 15, 20, 14, 23



Where and how to standardize/optimize the MAT

1

Controls:
WHO-LPS (tlr4)

NEP (recombinant or synthetic) for other tlr
2 NEP Reference candidates by MHRA in filling process

2

Incubation:
Cells
many new options,

Media

Supplements
Replacement of FCS by Platelet lysate

3

Detection / Analysis :
Detection systems mostly standardised
less pipetting
Faster assays
Reporter gene assay

Dedicated MAT-software missing

Automation may improve standardisation; facilitated by new MAT-versions

Reference: 13, 26

2: Cells, media, supplements



- **Users (even in the same organisation) have two conflicting wishes for the MAT:**
 - **R&D: cells should display the diversity of patients**
- vs.**
- **Batch release: the QC people want no cell batch variability**
- **Primary cell MAT „solve“ the issue by pooling of several donations for an „average“ and larger cell batch (up to 1000 vials).**
- **cell-line MAT-assays are available, displaying a single genetic background of a (often malign) cell**
- **iPSC-derived macrophages (maybe pooled) might have the potential to fulfil both wishes: representative for a population and reproducible (Biobanking)**
- **Media but especially Supplements (replace FCS by HSA or platelet lysate) are further options for improvement and standardisation**

Reference: 26, 1, 18, 11



3:Automation, Detection, calculation

- **Automation improves reproducibility and diminishes dependence on highly trained operators**
- **Methods with reduced pipetting: HTRF (heterogenous Time-resolved fluorescence), Lumit; reporter gene assays (RGA): smaller CV (quadruplicates still needed?); improved dynamic range; reduced time to result; RGA: no dependence on antibody quality**
- **Dedicated MAT-software still missing: very relevant for Pharma-users**

Newer MAT versions and how I see their validation status



Current MAT cell options published and / or available



Primary cells

Cryo-PBMC
Cryo whole blood

Pooled donors
or single genetical
Background
Donor variation

well known, many data, R&D +batch release

Common immortal Cell lines

MonoMac6
THP-1
HL-60

single genetical
background

engineered Cell Lines (GMO)

PBMC-derived
iPSC-derived
HEK239

single genetical
Background

new, fewer data, strong interest for QC

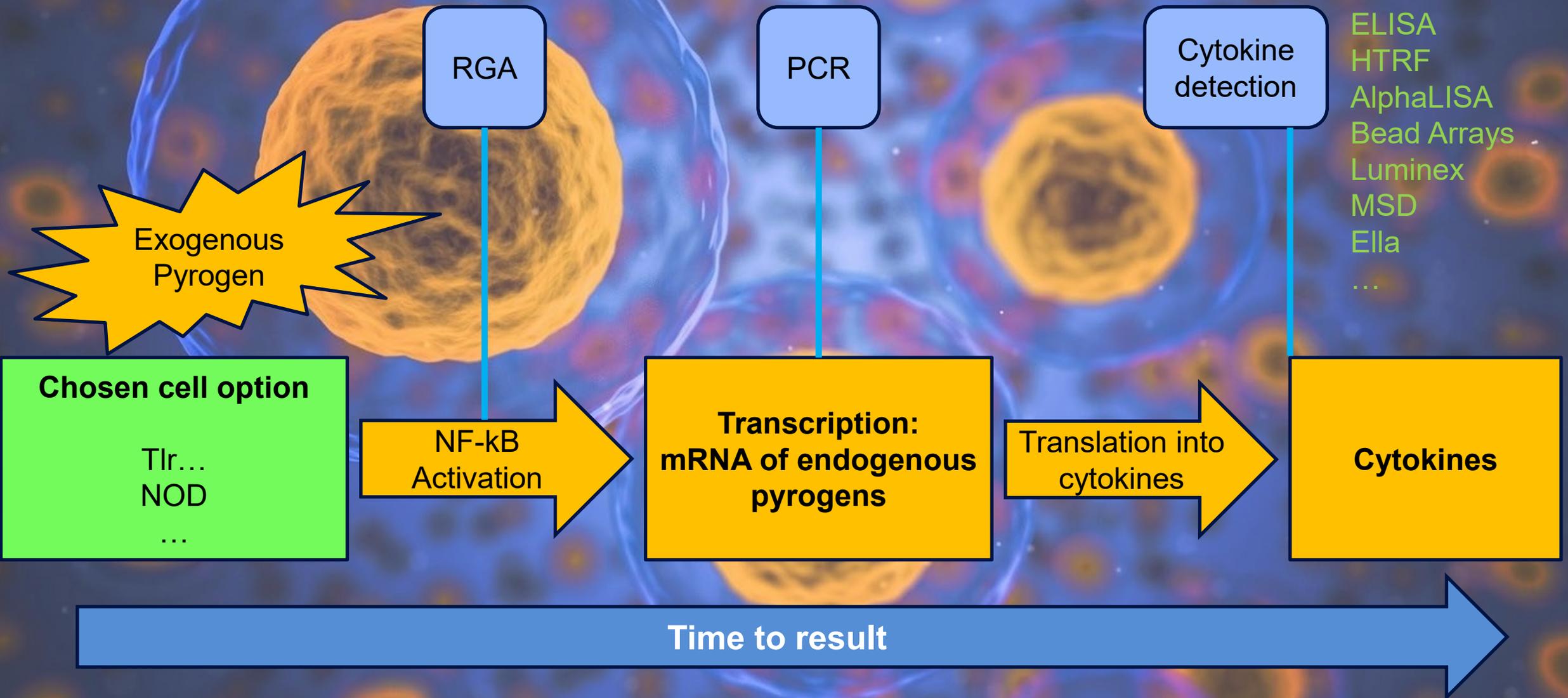
engineered Cell Lines with RGA (GMO)

Nomo
THP-1
RAW 264.7 (murine)

single genetical
background



Current MAT readout options



Is all of that compliant with 2.6.30.?

My guess: YES



3. GENERAL PROCEDURE

The test is carried out in a manner that avoids pyrogen contamination.

A solution of the preparation to be examined is incubated with a source of human monocytes or human monocytic cells, e.g. from anticoagulated human peripheral blood or a monocyte-containing fraction of that blood, such as human peripheral blood mononuclear cells (PBMC) isolated, e.g. by density-gradient centrifugation, or a human monocytic cell line. Human blood and PBMC should be processed within a validated timeframe that ensures sufficient reactivity of the cells towards pyrogens. Anticoagulated human peripheral blood is usually diluted, e.g. to 2-50 per cent V/V, with culture medium or saline. PBMC or monocytic cell lines are usually diluted in culture medium supplemented with either the donor's own plasma, human AB serum or foetal bovine serum to a final concentration of $0.1-1.0 \times 10^6$ cells/ mL.

The cell culture is incubated at 37 ± 1 °C, in an atmosphere appropriate for the culture medium, e.g. 5 per cent CO₂ in humidified air. The duration of the incubation must be sufficient to allow accumulation of the chosen read-out. The responses of the chosen read-out, e.g. a pro-inflammatory or pyrogenic cytokine, to a solution of the preparation to be examined are compared with responses to standard endotoxin (Method 1) or to a reference lot of the preparation to be examined (Method 2).

The basic methods have been validated 2002-2006, for the many new versions I expect (and encourage) users and kit suppliers to publish their validation efforts in peer-reviewed journals for the benefit of all.

We already see great examples for that, multiple authors here!

Yi, Sijia; Xu, Jenny; Song, Liping; Celeste, Frank; Wang, Christopher J.; Whiteman, Melissa C. (2025): Advancing Pyrogen Testing for Vaccines with Inherent Pyrogenicity: Development of a Novel Reporter Cell-Based Monocyte Activation Test (MAT). In: *Vaccines* 13 (10). DOI: 10.3390/ijms21124416.

Please refer to published RPT-data (some more will come)

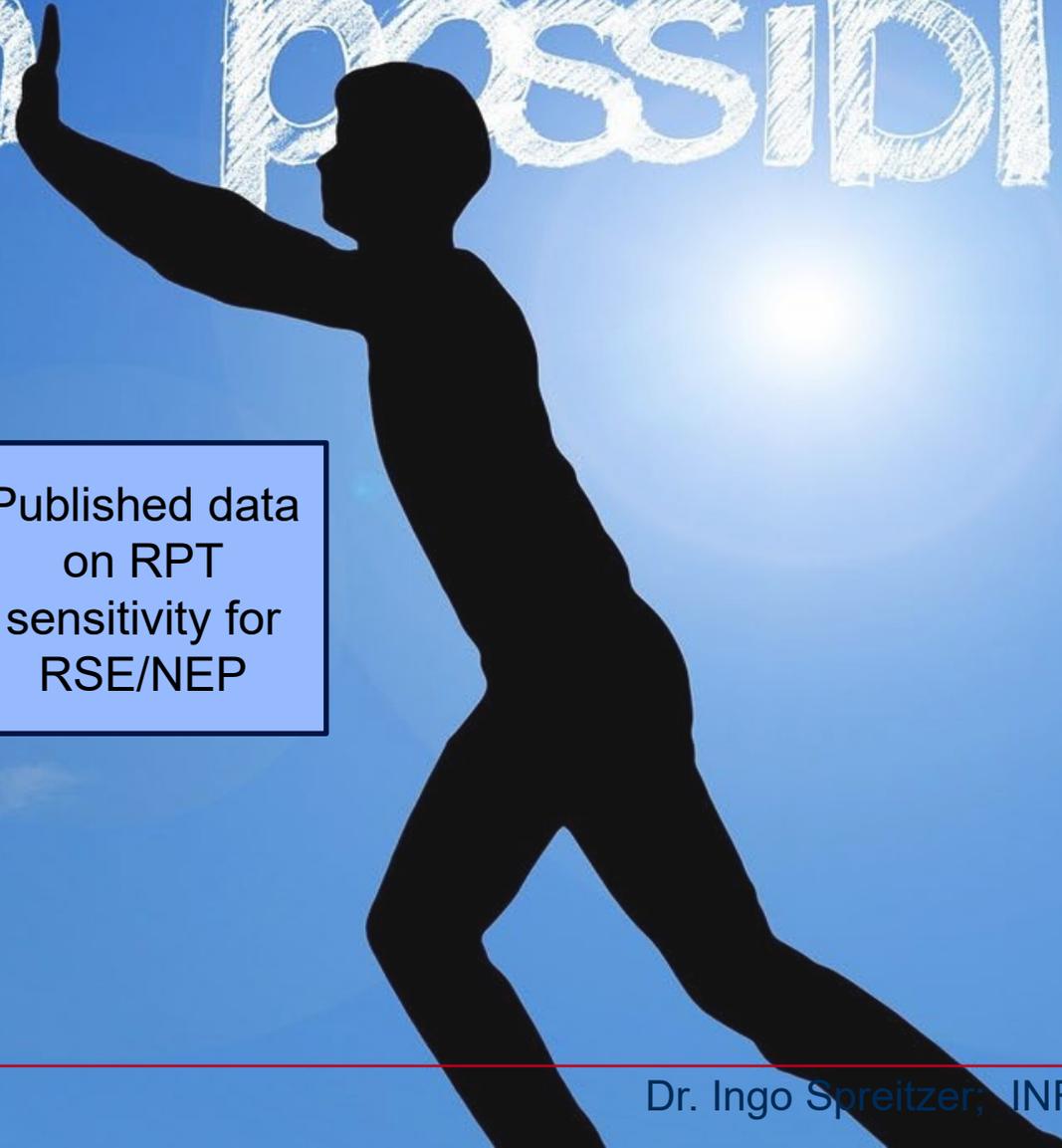
Burgmaier, Luisa; van den Berg, Jonas; Gajewi, Maria; Röder, Ruth; Reich, Johannes; Deutschmann, Sven M. (2025): Validation of the Monocyte Activation Test Demonstrating Equivalence to the Rabbit Pyrogen Test. In: *International journal of molecular sciences* 26 (22). DOI: 10.14573/altex.2111301.

Reference: 26,2,3,4

What all of us can do to support the global shift from RPT to MAT



impossible



Validate
your MAT-
setup



Published data
on RPT
sensitivity for
RSE/NEP



Peer-reviewed publication



New data to be generated by you

Kit-supplier:
Sensitivity for RSE
Sensitivity for at least 2 NEP
Quality assurance data for users

Customer +/- Kit-supplier

Validation of your chosen MAT
in conjunction with your product(s)

Refer to published RPT-data
on RSE/NEP-sensitivity

Publication in peer-reviewed journals

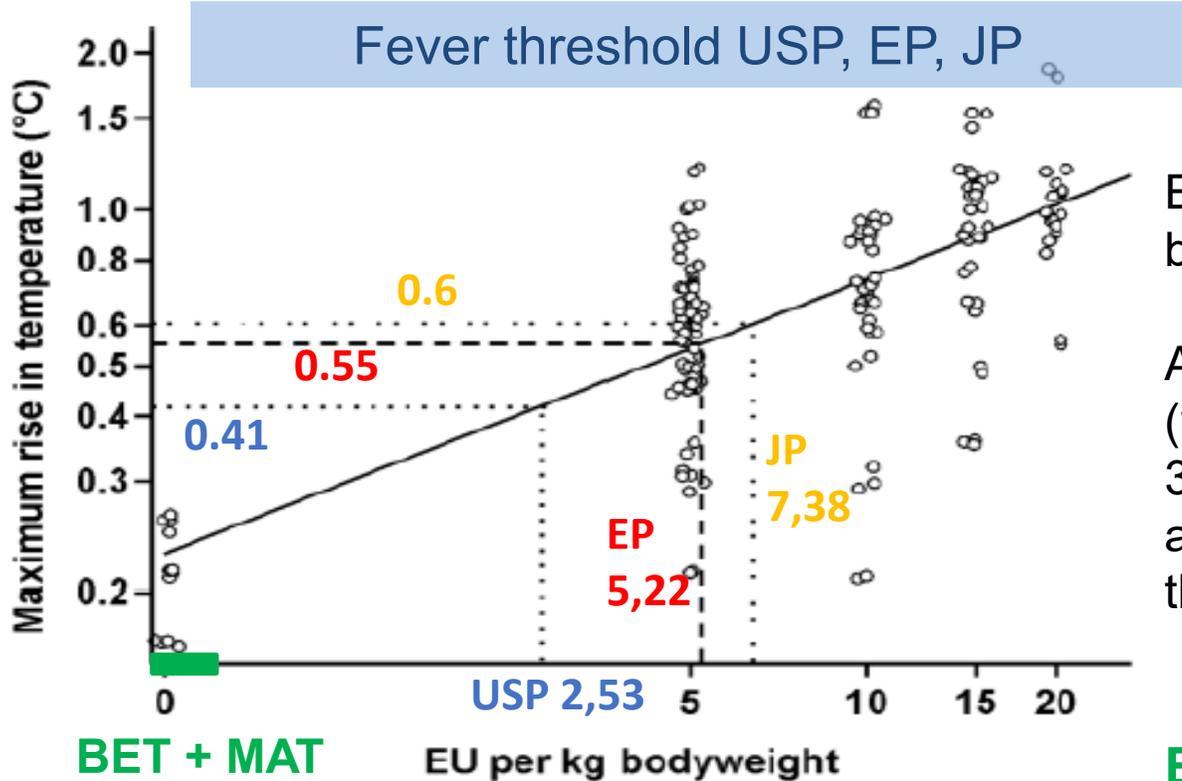
Implementation in QC

Published RPT-data on RSE/NEP-sensitivity

Europe:
No more RPT required or requested
for comparison

Rest of world?:
RSE- and NEP-data are published
and sufficient (more to come); request for
comparative product data in RPT seems
pointless as rabbit not target species or
state of art

Reference: 24, 9,14,17,19, 22, 5, 16



Even at 15 or 20 EU/kg individual rabbits are below 0.55°C (EP; pyrogenic threshold)

According to new data from masking experiments (you need a clearly positive control group) 35-40 EU/kg (in our experiments 50 EU/kg) are needed to get all three rabbits above the pyrogenic threshold

BET sensitivity: down to 0.001 EU/ml
MAT-sensitivity: at least down to 0.03 EU/ml
We defined a minimal sensitivity of 0.5 EU/ml for MAT (5EU/kg, divided by max. application volume of 10 ml/kg)

Reference: 14

in this **FDA & NIH Workshop on Reducing Animal Testing** from 07.07.2025 (<https://www.youtube.com/live/vz1uF42wnKQ>) in minute 13:55 **MAT/RFC** are mentioned as one of 6 examples of immediate actions FDA can take



Examples of immediate actions FDA can take (Identified in collaboration with NIH)



- **Rabies vaccine batch release testing.** Adopt ELISA assays to measure antigen content that have been validated in Europe & replace animal testing
- **Shellfish.** Replace the mouse bioassay to identify toxins in shellfish intended for human consumption with well-established non-animal methods that can better protect human health.
- **Leptospirosis vaccine potency testing.** Non-animal ELISA available from the USDA, but only four of six manufacturers have adopted its use. Encourage widespread and exclusive use.
- **Protein quality testing.** Replace rat digestibility test that has long been required for protein content claims with validated in vitro assays (e.g. pH-drop and pH-stat methods).
- **Pyrogen testing for parenteral products.** Replace rabbit pyrogen test and horseshoe crab-blood based endotoxin tests with in vitro Monocyte Activation Test and recombinant Bacterial Endotoxin Test methods.
- **Skin irritation testing.** Adopt ISO 10993-23 standard on skin irritation testing that gives preference to in vitro methods (e.g. for evaluating medical devices)

**INDs. AND THEN WIDELY
PUBLICIZE USE CASES WHERE**

Reference: 5, 6, 16



- EP replaced animal test (RPT) by superior *in-vitro* assay (MAT) predictive for humans

- EP endorsed rFC (soon in 2.6.14!)





F₄

U₁

T₁

U₁

R₁

E₁

Selection of relevant publications for this presentation



1. Abdin, S. M., Mansel, F. and Hashtchin, A. R. et al. (2024). Sensor macrophages derived from human induced pluripotent stem cells to assess pyrogenic contaminations in parenteral drugs. *Biofabrication* 16. doi:10.1088/1758-5090/ad4744.
2. Burgmaier, L., van den Berg, J. and Gajewi, M. et al. (2025). Validation of the Monocyte Activation Test Demonstrating Equivalence to the Rabbit Pyrogen Test. *International journal of molecular sciences* 26. doi:10.14573/altex.2111301.
3. Daniels, R., van der Elst, W. and Dieltjens, N. et al. (2022). Validation of the monocyte activation test with three therapeutic monoclonal antibodies. *ALTEX* 39, 621–635. doi:10.14573/altex.2111301.
4. Daniels, R., van der Elst, W. and K. So, C. et al. (2025). Fit for purpose testing and independent GMP validation of the monocyte activation test. *Current Research in Toxicology* 8, 100206. doi:10.1016/j.crtox.2024.100206.
5. Haile, L. A., Polumuri, S. K. and Rao, R. et al. (2017). Cell based assay identifies TLR2 and TLR4 stimulating impurities in Interferon beta. *Scientific reports* 7, 10490. doi:10.1038/s41598-017-09981-w.
6. Haile, L. A., Puig, M. and Kelley-Baker, L. et al. (2015). Detection of innate immune response modulating impurities in therapeutic proteins. *PLoS ONE* 10, e0125078. doi:10.1371/journal.pone.0125078.
7. Hartung, T. (2015). The human whole blood pyrogen test – lessons learned in twenty years. *ALTEX*, 79–100. doi:10.14573/altex.1503241.
8. Hartung, T. (2021). Pyrogen testing revisited on occasion of the 25th anniversary of the whole blood monocyte activation test. *ALTEX* 38, 3–19. doi:10.14573/altex.2101051.
9. Hasiwa, N., Daneshian, M. and Bruegger, P. et al. (2013). Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test. *ALTEX*. 30, 169–208.

Selection of relevant publications for this presentation



10. Hayashi, K., Ohya, K. and Kikuchi, Y. et al. (2024a). Uniform Suspension of Heat-Killed *Staphylococcus aureus* for a Positive Control Used in the Monocyte-Activation Test. *Biological & pharmaceutical bulletin* 47, 1321–1325. doi:10.1248/bpb.b24-00139.
11. Hayashi, K., Sano, M. and Kanayasu-Toyoda, T. et al. (2024b). Evaluation of the effect of cell freshness on pyrogen detection using a serum-free monocyte-activation test. *PLoS ONE* 19, e0316203. doi:10.14573/altex.2008261.
12. He, Q., Gao, H. and Xu, L.-M. et al. (2018). Analysis of IL-6 and IL-1 β release in cryopreserved pooled human whole blood stimulated with endotoxin. *Innate immunity* 24, 316–322. doi:10.1177/1753425918777596
13. He, Q., Yu, C.-F. and Wu, G. et al. (2024). A novel alternative for pyrogen detection based on a transgenic cell line. *Signal transduction and targeted therapy* 9, 1–9. doi:10.1038/s41392-024-01744-0.
14. Hoffmann, S., Luderitz-Puchel, U. and Montag, T. et al. (2005a). Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling. *J.Endotoxin.Res.* 11, 25–31.
15. Hoffmann, S., Peterbauer, A. and Schindler, S. et al. (2005b). International validation of novel pyrogen tests based on human monocytoïd cells. *J.Immunol.Methods* 298, 161–173.
16. Huang, L. Y., Dumontelle, J. L. and Zolodz, M. et al. (2009). Use of toll-like receptor assays to detect and identify microbial contaminants in biological products. *J.Clin.Microbiol.* 47, 3427–3434. doi:10.1128/JCM.00373-09.
17. Kim J-H, Jung K, Kim J, Lee J, Kim H, Song H et al. Development of a rabbit monocyte activation test as an alternative to the rabbit pyrogen test and its application in the analysis of plasma-derived products. *Biologicals* 2021;71:20–30.
18. Koryakina, A., Frey, E. and Bruegger, P. (2014). Cryopreservation of human monocytes for pharmacopoeial monocyte activation test. *Journal of immunological methods* 405, 181–191. doi:10.1016/j.jim.2014.01.005.

Selection of relevant publications for this presentation



19. Molenaar-de Backer, M. W. A., Doodeman, P. and Rezai, F. et al. (2023). In vitro alternative for reactogenicity assessment of outer membrane vesicle based vaccines. *Scientific reports* 13, 12675. doi:10.1016/j.vaccine.2015.01.063.
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21. Schindler, S., Spreitzer, I. and Loschner, B. et al. (2006). International validation of pyrogen tests based on cryopreserved human primary blood cells. *J.Immunol.Methods* 316, 42–51.
22. Solati, S., Aarden, L. and Zeerleder, S. et al. (2015). An improved monocyte activation test using cryopreserved pooled human mononuclear cells. *Innate immunity*. doi:10.1177/1753425915583365.
23. Spreitzer, I., Fischer, M. and Hartzsch, K. et al. (2002). Comparative study of rabbit pyrogen test and human whole blood assay on human serum albumin. *ALTEX*. 19 Suppl 1, 73–75.
24. Vipond, C., Findlay, L. and Feavers, I. et al. (2016). Limitations of the rabbit pyrogen test for assessing meningococcal OMV based vaccines. *ALTEX* 33, 47–53. doi:10.14573/altex.1509291.
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National Centre
for the Replacement
Refinement & Reduction
of Animals in Research

Working with the global biologics community to accelerate adoption of the 3Rs: MAT survey results and discussion

**Elliot Lilley – NC3Rs Programme Manager
– 3Rs in Vaccine Batch Release and
Quality Control Testing**

MAT survey

- Three sections
 - Demographics
 - Questions for those who perform pyrogenicity testing
 - Questions for MAT kit developers/suppliers
- Launched in October 2025
- Closed December 2025
- Preliminary data shared at PharmaLab in November
- Additional Mentimeter responses

PLEASE REVIEW THE ETHICAL APPROVAL AND PARTICIPANT INFORMATION SHEETS BEFORE COMPLETING THE SURVEY QUESTIONS

Please return the completed survey to **Dr Elliot Lilley**, by email: elliott.lilley@nc3rs.org.uk Closing date **12 December 2025**

Section 1. Demographic Information (* denotes optional information)

1a. If you agree to others contacting you regarding your survey responses please provide a name and email address*

1b. In which country are you located?

1c. Organisation type

Add other organisation type here:

In the following sections there are questions (section 2) that are specifically intended to be answered by those whose organisations perform pyrogenicity testing and (section 3) that are specifically intended to be answered by those whose organisations develop and market MAT testing kits or services. Please answer the questions that are most relevant to your organisation. If your organisation both performs pyrogenicity testing and develops and markets MAT testing kits or services, please answer all questions.

Section 2. For those organisations who perform pyrogenicity testing

2a. Does your organisation have a policy regarding replacement of in vivo assays with non-animal alternatives?

- Yes, (please indicate what the policy is and/or include a link to a public facing policy, if available)

- No

- Don't know

Where relevant, please provide information about the policy:

2b. Regarding pyrogenicity testing, please specify your testing strategy:

For clinical studies	
MAT	<input type="checkbox"/>
RPT	<input type="checkbox"/>
BET (please specify format e.g. LAL, TAL, rFC, rCR)	<input type="checkbox"/>
Other (please specify)	<input type="checkbox"/>
Not applicable	<input type="checkbox"/>

Add comments or additional information here:

For product development	
MAT	<input type="checkbox"/>
RPT	<input type="checkbox"/>

Guidance notes: Providing your name is optional and will only be used for follow-up purposes (see 'Ethical and Data Approval' tab for more information). Please choose one option that most closely describes your organisation, or add information into the 'other' comments space.

Here and throughout the survey the following abbreviations are used:
MAT - Monocyte Activation Test
RPT - Rabbit Pyrogen Test
BET - Bacterial Endotoxin Test
LAL - Limulus Amebocyte Lysate
TAL - Tachypleyus Amebocyte Lysate
rFC - Recombinant Factor C
rCR - Recombinant Cascade Reagent

MAT survey data highlights

- 49 responses from 21 countries (9 European)
- Responses from Pharmaceutical companies (20), CROs (9), NCLs (7), Kit manufacturers (6), NRAs (5) & Joint NRA/NCLs (2)
- BET dominates pyrogenicity testing based on risk-based approach
- MAT and RPT used for NEP testing
- Not using MAT - Expense, access to cells and lack of expertise
- What would increase MAT use? Consistent international regulatory requirements, inclusion of MAT as a compendial method in USP
- Many cell lines investigated. Many considered unsuitable due to insufficient sensitivity and issues with accessibility
- Majority support the need for new cell lines
- Unanimous support for NC3Rs collaborative project



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