Joint EDQM-EPAA Event

The future of pyrogenicity testing: phasing out the rabbit pyrogen test

14-15 February 2023

Joint EDQM-EPAA Event

The future of pyrogenicity testing: phasing out the rabbit pyrogen test
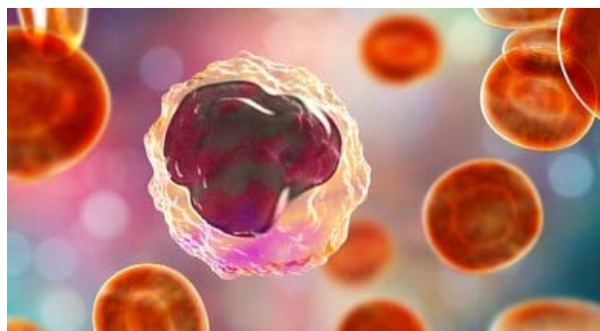
In-depth exploration of the monocyte-activation test (MAT)

THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

‣ **Ph. Eur. chapter 2.6.30**
Monocyte-activation test

Joint EDQM-EPAA Event on Pyrogenicity, Brussels 14-16 February 2023
The Future of Pyrogenicity testing: Phasing Out the Rabbit Pyrogen Test

Outline

▸ MAT in the new Ph. Eur. Pyrogenicity strategy

▸ Ph. Eur. chapter 2.6.30

▸ Ongoing revision of Ph. Eur. chapter 2.6.30

▸ MAT for vaccines containing inherently-pyrogenic components

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▸ MAT in the new Ph. Eur. Pyrogenicity strategy

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▸ MAT for vaccines containing inherently-pyrogenic components

Assays for pyrogens / endotoxins in the Ph. Eur.

1971

Pyrogens (2.6.8)
("Rabbit Pyrogen Test")

Pyrogen detection

1987

LAL is a lyophilised amoebocyte lysate obtained from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*)
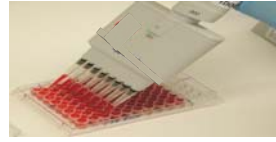BET (2.6.14) & Guidelines for using the BET (5.1.10)
▸ *BET using recombinant Factor C (2.6.32) [NEW]*

2020

Endotoxin detection
(e.g. LPS from Gram- bacteria)
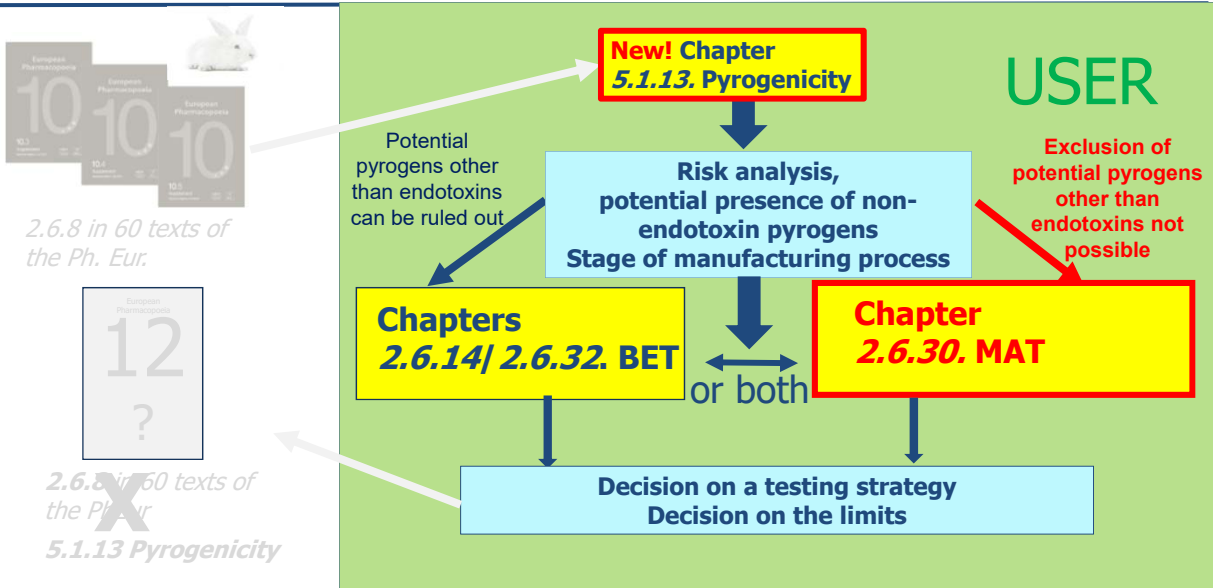
2010

Monocyte-activation test
(2.6.30)
Monocyte activation test for vaccines containing inherently pyrogenic components (2.6.40) [Draft Phpa 33.3 NEW]

2023?

Pyrogen detection

Replacement of chapter *2.6.8: proposed strategy*
Consolidated strategy approved by the European Pharmacopoeia Commission in June 2022

2.6.8 in 60 texts of the Ph. Eur.

2.6.8 in 60 texts of the Ph. Eur.
5.1.13 Pyrogenicity

New! Chapter
5.1.13. Pyrogenicity

USER

Potential pyrogens other than endotoxins can be ruled out

Risk analysis, potential presence of non-endotoxin pyrogens Stage of manufacturing process

Exclusion of potential pyrogens other than endotoxins not possible

Chapters
2.6.14/ 2.6.32. BET

or both

Chapter
2.6.30. MAT

**Decision on a testing strategy
Decision on the limits**
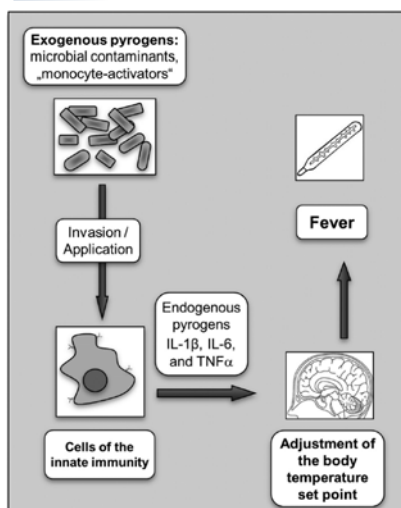
 https://go.edqm.eu/NewPyrogenicityStrategy

▸ MAT in the new Ph. Eur. Pyrogenicity strategy

▸ Ph. Eur. chapter 2.6.30

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Monocyte-Activation Test

Figure: Human fever reaction.
Source: Hasiwa et al. ALTEX 30, 2/13 2013

- <u>Principle</u>: Upon activation by pyrogens, human monocytes release mediators such as pro-inflammatory cytokines (e.g. IL-6, IL-1β, TNF-α), which are detected in an immunoassay (ELISA)

- Can detect **endotoxin and non-endotoxin pyrogens**

- Based on the human fever response (better prediction of pyrogenic activity in humans)

- Non-animal test

Monocyte-Activation Test

Figure: Principle of MAT.
Source: Hasiwa et al. ALTEX 30, 2/13 2013

MAT: EP 2.6.30

Unknown sample →
- Whole blood or cryo blood
- Isolated primary mononuclear cells (PBMC)
- Monocytic cell lines

→ IL-1β, IL-6, TNFα

Invasion / Application

Incubation → Detection

- Different variants of MAT depending on:
  - Source of human monocyte: whole blood (fresh or cryopreserved), PBMCs (fresh or cryo-preserved), human monocytic cell line
  - ELISA read-out: IL-6, IL-1β, TNF-α…

- 3 methods currently described in chapter 2.6.30:
  - Method A (Quantitative test): comparison of the preparation being examined with a standard endotoxin dose-response curve
  - Method B (Semi-quantitative test): comparison of the preparation being examined with standard endotoxin
  - Method C (Reference lot comparison test): comparison of the preparation being examined with a validated reference lot of that preparation

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Chapter 2.6.30 - Overview

07/2017:20630
corrected 11.0

2.6.30. MONOCYTE-ACTIVATION TEST

1. INTRODUCTION

The monocyte-activation test (MAT) is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines have a role in fever pathogenesis. Consequently, the MAT will detect the presence of pyrogens in the test sample. The MAT is suitable, after a product-specific validation, as a replacement for the rabbit pyrogen test.

Pharmaceutical [...] contain non-endotoxin pyrogenic [...] ants often show very steep or non [...] s in comparison with endotoxin d [...] ations that contain or may contain [...] ts have to be tested at a range of d [...] um dilution.

The following [...] n the present chapter.

Method A. [...]
Method B. S [...]
Method C. R [...]

In addition, [...] n practical aspects of the tests can [...] notes' section at the end of this g [...]

The test is c [...] a manner that avoids pyrogen contamination.

2.6.30 MONOCYTE-ACTIVATION TEST
1. INTRODUCTION
2. DEFINITIONS
3. GENERAL PROCEDURE
4. APPARATUS
5. CELL SOURCES AND QUALIFICATION
5-1. WHOLE BLOOD
5-2. PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)
5-3. QUALIFICATION OF BLOOD DONORS
5-4. QUALIFICATION OF CELLS POOLED FROM A NUMBER OF DONORS
5-5. QUALIFICATION OF CRYO-PRESERVED CELLS
5-6. MONOCYTIC CONTINUOUS CELL LINES
6. PREPARATORY TESTING
6-1. ASSURANCE OF CRITERIA FOR THE ENDOTOXIN STANDARD CURVE
6-2. TEST FOR INTERFERING FACTORS
6-3. METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS
6-4. INTERFERENCE IN THE DETECTION SYSTEM

7. METHODS
7-1. METHOD A: QUANTITATIVE TEST
7-2. METHOD B. SEMI-QUANTITATIVE TEST
7-3. METHOD C: REFERENCE LOT COMPARISON TEST
Test procedure, Calculation and interpretation, Pass/fail criteria of the preparation
Guidance notes

Guidance note
1. INTRODUCTION
2. METHODS
2-1. INFORMATION REGARDING THE CHOICE OF METHODS
2-2. CALCULATION OF CONTAMINANT LIMIT CONCENTRATION
2-3. INFORMATION REGARDING CRYO-PROTECTANTS
2-4. INTERFERENCE TESTING
2-5. CROSS-VALIDATION
3. REPLACEMENT OF THE RABBIT PYROGEN TEST BY THE MAT
4. VALIDATION OF ALTERNATIVE METHODS

European Pharmacopoeia 11

Determination of the MVD

- *Maximum Valid Dilution (MVD):* the maximum allowable dilution of a sample at which the contaminant limit concentration (CLC) can be determined

$$\frac{CLC \times C}{LOD}$$

  → *MVD is calculated for each product*

- *CLC* (endotoxin equivalents) → acceptance criterion for a pass/fail decision

  CLC = K / M

  - K= threshold pyrogenic dose per kilogram of body mass → *Values for K are given in Guidance notes*
  - M = maximum recommended bolus dose of product per kilogram of body mass

- *LOD*: concentration of endotoxin corresponding to the cut-off value ($\bar{x}$ + 3s)
  - $\bar{x}$ = mean of the 4 replicates for the responses to the blank
  - s = standard deviation of the 4 replicates of the responses to the blank

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Cell sources and qualification

- Qualification of blood donors
  - *Qualification criteria for blood donors (health, medication...)*

- Qualification of cell sources
  - Whole blood, PBMCs: obtained from single donors or from pooled whole blood, qualified according to the requirements described under sections [...] 5-4 (*Qualification of pools*), 5-5 (*Qualification of cryopreserved cells*) and where applicable, section 6-3 (*Method validation for non endotoxin monocyte-activating contaminants*)
  - Qualification of pools: *minimum number of donors, timeframe for use of blood after collection, criteria for dose-response curve, qualification for use for the detection of non-endotoxin contaminants, averaging effect*
  - Qualification of cryopreserved cells: *criteria for dose-response curve, qualification for use for the detection of non-endotoxin contaminants*

Cell sources and qualification (cont'd)

- Qualification of cell sources
  - Monocytic cell lines:
    - Test for mycoplasma contamination & check for identity and stability
    - Functional stability: *criteria for functional stability, testing the receptor expression*
    - *Criteria for dose-response curve, qualification for use for the detection of non-endotoxin contaminants*

Preparatory testing

- Assurance of criteria for the endotoxin standard curve
  - Standard curve: at least 4 endotoxin concentrations, and at least 4 replicates of each concentration
  - The basal release of the chosen read-out (blank) is optimised to be as low as possible
  - Acceptance criteria for the standard curve: 1) regression of responses on $\log_{10}$ dose shall be statistically significant ($p < 0.01$); 2) regression of responses on $\log_{10}$ dose must not deviate significantly from linearity ($p > 0.05$)

- Test for interfering factors *(for methods A and B)*
  - Aim: ensure that the preparation being examined does not interfere with the test
  - Concentration of endotoxin spike: usually equal to or near the estimated middle of the endotoxin standard curve (Method A) or twice the estimated LOD (Method B)
  - Test solution is considered free of interfering factors if the mean recovery of the spike is within 50-200%

- Determination of the optimal dilutions of the test and reference lots *(for method C)*
  - Dilutions of the test and reference lots depends on the type of analysis to make the comparison between the two (to be justified and validated for each product). *An example is given*

Preparatory testing (cont'd)

- ## Interference in the detection system
  - **Aim**: ensure that the preparation being examined does not interfere in the detection system
  - Preparation is tested for interference in the detection system (e.g. ELISA) for the chosen read-out (e.g. IL-6)
  - Agreement between a dilution series of the standard for the chosen read-out, in the presence and absence of the preparation being examined, is to be within, for example ± 20 per cent of the optical density

- ## Method validation for non-endotoxin monocyte-activating contaminants
  - **Aim**: show that the test system detects non-endotoxin pyrogens
  - Can be achieved using historic product batches found to be contaminated with non-endotoxin contaminants that caused positive responses in the rabbit pyrogen test or adverse events in man. Or, if not available, using at least 2 non-endotoxin ligands for TLRs reflecting the most likely contaminant(s), at least 1 of which is to be spiked into the preparation being examined, to validate the test system

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Method A: Quantitative test

- Comparison of the preparation being examined with a standard endotoxin dose-response curve
- To pass the test, the contaminant concentration of the prep. is to be < CLC

- Sol. A: dilution at which the test for interfering factors was carried out (highest concentration for which endotoxin recovery is within 50-200%)
- Sol. B: 2-fold dilution of sol. A, not exceeding the MVD
- Sol. C: 2-fold dilution of sol. B, not exceeding the MVD
- Sol. AS, BS, CS: sol. A, B & C spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve
- Sol. R_0: negative control
- Sol. R_1-R_4: sol. of standard endotoxin at the concentrations used in the test for interfering factors

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/f	None	4
B	Test solution/$2 \times f$	None	4
C	Test solution/$4 \times f$	None	4
AS	Test solution/f	Middle dose from endotoxin standard curve (R_3)	4
BS	Test solution/$2 \times f$	Middle dose from endotoxin standard curve (R_3)	4
CS	Test solution/$4 \times f$	Middle dose from endotoxin standard curve (R_3)	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1-R_4	Pyrogen-free saline or test diluent	4 concentrations of standard endotoxin	4 of each concentration

Method A: Quantitative test (cont'd)

- Data included in the analysis relate to cells for which the 2 criteria for the endotoxin standard curve are satisfied

- Calculate the concentration of endotoxin equivalents in each of the replicates of solutions A, B and C and solutions AS, BS and CS using the standard curve R_1-R_4

- Validity criteria: endotoxin recovery for spiked samples (AS, BS & CS) is within 50-200%. Dilutions not fulfilling the criterion are not valid and excluded from further evaluation.

- The preparation complies with the test if the mean concentrations of endotoxin equivalents in the replicates of sol. A, B and C, after correction for dilution and concentration, are all < CLC

- One valid dilution is the minimum required for a valid test

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Method B: Semi-quantitative test

- Comparison of the preparation being examined with standard endotoxin
- To pass the test, the contaminant concentration of the prep. is to be < CLC

- Solution A is chosen for the pass decision (unless otherwise authorised)

- Sol. A: dilution at which the test for interfering factors was carried out
- Sol. B & C: dilutions chosen after review of data from product-specific validation, not exceeding the MVD (e.g. 1:2 x MVD & MVD)
- Sol. AS, BS, CS: sol. A, B & C spiked with standard endotoxin at 2 × estimated LOD
- Sol. R_0: negative control
- Sol. R_1: standard endotoxin at 0.5 × estimated LOD
- Sol. R_2: standard endotoxin at 1 × estimated LOD
- Sol. R_3: standard endotoxin at 2 × estimated LOD
- Sol. R_4: standard endotoxin at 4 × estimated LOD

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/f	None	4
B	Test solution/f_1	None	4
C	Test solution/f_2	None	4
AS	Test solution/f	Standard endotoxin at 2 × estimated LOD for the test system	4
BS	Test solution/f_1	Standard endotoxin at 2 × estimated LOD for the test system	4
CS	Test solution/f_2	Standard endotoxin at 2 × estimated LOD for the test system	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1	Pyrogen-free saline or test diluent	Standard endotoxin at 0.5 × estimated LOD for the test system	4
R_2	Pyrogen-free saline or test diluent	Standard endotoxin at 1 × estimated LOD for the test system	4
R_3	Pyrogen-free saline or test diluent	Standard endotoxin at 2 × estimated LOD for the test system	4
R_4	Pyrogen-free saline or test diluent	Standard endotoxin at 4 × estimated LOD for the test system	4

Method B: Semi-quantitative test (cont'd)

- Data included in the analysis relate to cells for which mean responses to solutions R_0-R_4 increase progressively. For each cell source, the mean response to sol. R_2 is > a positive cut off value

- Data < cut off value are considered negative

- For each negative solution of the prep. (A, B and C), the mean response to the corresponding spiked solution (AS, BS or CS) is compared with the mean response to R_3 to determine the spike recovery

- The contaminant concentration of the preparation is < CLC if the solution of the preparation designated for the pass/fail-decision and the dilutions below give negative results, and the spike recovery is within 50-200%

Method C: Reference lot comparison test

- Comparison of the preparation being examined with a validated reference lot

- The type of analysis to compare the two is to be justified and validated for each product
- The reference lot is selected according to criteria that have been justified and authorised
- Method intended to be performed where a prep. shows marked interference but cannot be diluted within the MVD to overcome the interference or because it contains or is believed to contain non-endotoxin contaminants

Solution	Solution/dilution factor	Number of replicates
A	Solution of reference lot/f_1	4
B	Solution of reference lot/f_2	4
C	Solution of reference lot/f_3	4
D	Solution of preparation being examined/f_1	4
E	Solution of preparation being examined/f_2	4
F	Solution of preparation being examined/f_3	4
G	Positive control (standard endotoxin)	4
R_0	Diluent (negative control)	4

- Sol. A, B and C: reference lot diluted by dilution factors determined in the test for interfering factors
- Sol. D, E and F: prep. being examined diluted by the same dilution factors
- Sol. G: positive test control for the viability of the cells (standard endotoxin concentration that gives a clear positive response)
- Sol. R_0: negative control (diluent used to dilute the prep.)

Method C: Reference lot comparison test
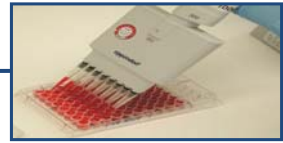
- Data included in the analysis relate to cells for which sol. G and at least one of sol. A, B and C give a response that is greater than the basal release of the read-out (sol. R_0)

- Calculate the mean responses of the replicates of sol. A-F using the standard curve for the read-out. Divide the sum of the mean responses to solutions D, E and F by the sum of the mean responses to solutions A, B and C. The preparation complies if the resulting value complies with a defined acceptance criterion not exceeding a justified value

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Pass/fail criteria of the preparation

- When cells from individual donors are used:
  - The prep. is required to comply with the test with the cells from each of 4 different donors
  - If the prep. passes the test with cells from 3 of the 4 donors, the test is continued with cells from a further 4 donors, and the prep. is required to pass the test with cells from 7 of the 8 different donors

- When cells pooled from a number of individual donors are used:
  - The prep. is required to pass the test with 1 pool of cells

- Where a human monocytic cell line is used:
 - The prep. is required to pass the test with 1 qualified passage of cells

▸ MAT in the new Ph. Eur. Pyrogenicity strategy

▸ Ph. Eur. chapter 2.6.30

▸ Ongoing revision of Ph. Eur. chapter 2.6.30

▸ MAT for vaccines containing inherently-pyrogenic components

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Evolution of Ph. Eur. chapter 2.6.30

Recommendations of ECVAM Workshop 43 (2001)

First version published in 2010,
(*Supplement 6.7*)

EDQM survey (2013)
on implementation of MAT & applicability of 2.6.30

➢ **Outcome**:
- MAT uses: for product release, to rule out the presence of NEPs, for in-process testing, for trouble-shooting
- Chapter 2.6.30 is useful however some technical guidance for successful performance of the test are required

Revised chapter published in 2017
(*Supplement 9.2*)

Improvements

WORK IN PROGRESS

- Situation in the field has evolved since 2010 (e.g. accrued user experience, kits based on different approaches are available...)
- **New revision of chapter 2.6.30 to reflect accrued experience, take into account user feedback**

Draft revised chapter released for consultation **in Pharmeuropa 34.2**
Review of stakeholder comments

Improvements

Ongoing revision of chapter 2.6.30

WORK IN PROGRESS Proposed changes to chapter 2.6.30 include:

- MVD calculation: Replacement of LOD by assay sensitivity in the calculation of the MVD. The assay sensitivity is an actual point on the standard curve rather than a calculated value. Allows a consistent calculation of the MVD and a better comparability between different MAT setups

- Validity criteria for the endotoxin standard curve:
  - Allow the use of non-linear regression models
  - Less strict validity criteria for the endotoxin standard curve (e.g. requirement for parallelism removed)

Ongoing revision of chapter 2.6.30

- Methods: Proposal to merge Methods A and B into a single test ("Method 1")

- Test for interfering factors: Spiking with 2x LOD in Method B considered too low to enable spike recovery in the range of 50-200%. The spike conc. for Method 1 is equal to or near the middle of the endotoxin standard curve

- Dilutions in the routine assay: in Method 1, a specific dilution factor is not imposed for sol. B and C – the dilution is chosen after reviewing the data from the product-specific validation. An example of dilution is provided.

- Cell lines: Proposal to clarify that cell lines meeting the requirements of chapter 2.6.30 are appropriate for the detection of endotoxins and NEPs, after successful qualification

Ongoing revision of chapter 2.6.30

- This revision is also part of the exercise aimed at suppressing the rabbit pyrogen test (RPT) from the Ph. Eur. (removal of references to the RPT)

→ Revision primarily aimed at addressing certain difficulties reported by users of methods A and B, in order to facilitate the wider implementation of the MAT

→ In view of the planned suppression of the RPT from the Ph. Eur., important revision to keep chapter 2.6.30 up-to-date and support all users!

▸ MAT in the new Ph. Eur. Pyrogenicity strategy

▸ Ph. Eur. chapter 2.6.30

▸ Ongoing revision of Ph. Eur. chapter 2.6.30

▸ MAT for vaccines containing inherently-pyrogenic components

MAT for inherently-pyrogenic vaccines
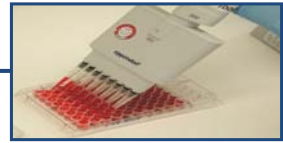
- New Chapter 2.6.40, under finalisation

- To cover the use of MAT to monitor the consistent pyrogenicity of a vaccine where pyrogens are an integral part of the product (→ *use as consistency test rather than safety test*)

- Aim: Facilitate the implementation of MAT method C (*Reference lot comparison test*) for inherently pyrogenic vaccines

- Intended to complement the information given in the MAT chapter 2.6.30

- At present, using the mother chapter, users have to read between the lines to understand how to apply the test in this context

- Standalone chapter

Thank you for your attention

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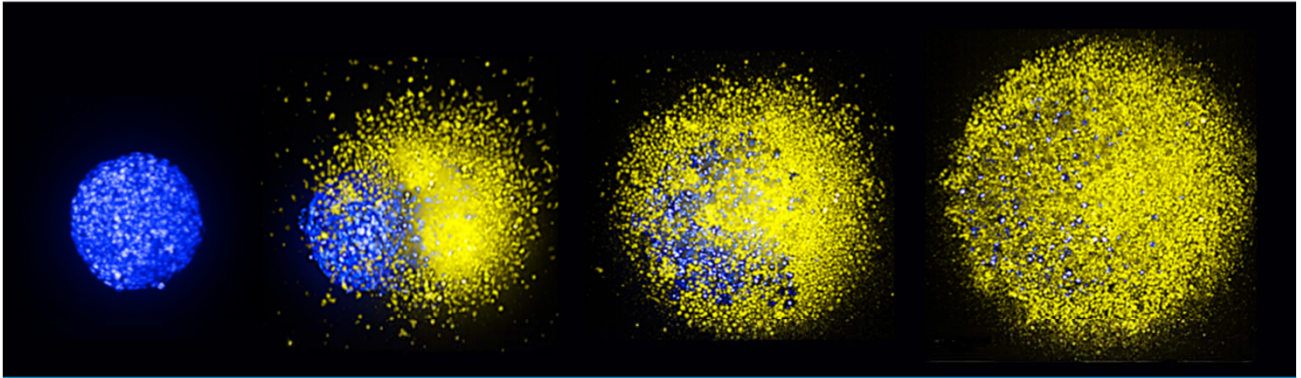
An industry-perspective on the validation and implementation of an in-house developed MAT

Ruth Daniels, PhD
Senior Scientist - Analytical Development, Microbiology CoE

Joint EDQM-EPAA Hybrid Event on Pyrogenicity
The future of pyrogenicity testing: phasing out the rabbit pyrogen test
Brussels, 14-16 February 2023

Vγ9Vδ2 T cells (shown in yellow) infiltrate and
kill HER2+ tumor spheroids (shown in blue)
following treatment with a Vγ9xHER2 redirector

Credit: Renata Gordon, Cell Therapy, Therapeutics Discovery
Laura Struzyna, Exploratory Biology, Therapeutics Discovery

Janssen Research & Development, LLC ©2022 JRD, LLC

Agenda

1   Selection and characterization of suitable cell sources

2   General GMP method qualification of in-house MAT method

3   Product-specific method validation acc. to Ph. Eur. 2.6.30 on therapeutic monoclonal antibodies

4   Test setup of batch analysis

5   Technical challenges

6   Industry perspective

7   Conclusions

Selection and characterization of suitable cell sources

Viral exacerbation at 40x magnification

Selection and characterization of suitable cell sources

✓ Evaluation of most suitable cell sources

  ✓ Whole blood (cryopreserved, pooled)
  ✓ Monomac 6
  ✓ Peripheral Blood Mononuclear Cells (PBMC) (cryopreserved, pooled)

✓ Availability human PBMCs

  ✓ Partnership with well-experienced blood bank

  ✓ Blood collection & isolation, characterization and cryopreservation PBMC's
  ✓ Pooled PBMCs to account for donor variability to mimic human fever response

  ✓ Dedicated batches of PBMC for J&J, compliant with EP and internal J&J requirements
  ✓ New PBMC batches require full characterization and comparability study to previous batch

NEP	Concentration
FLA	3.13 ng/mL
	6.25 ng/mL
	12.5 ng/mL
	25 ng/mL
PAM	0.625 ng/mL
	1.25 ng/mL
	2.5 ng/mL
	5 ng/mL
PGN	1.25 µg/mL
	2.5 µg/mL
	5 µg/mL
	7.5 µg/mL
HKSA	0.06×10^6 cells/mL
	0.125×10^6 cells/mL
	0.25×10^6 cells/mL
	0.5×10^6 cells/mL

General GMP method qualification of in-house MAT method

Viral exacerbation at 40x magnification

General GMP method qualification

✓ Non-product specific
✓ European Pharmacopoeia 2.6.30 - Method A (= Method 1) (semi-quantitative)
  ✓ LOD (limit of detection)
  ✓ Basal release of IL-6
  ✓ Averaging effect of pooled PBMC
✓ Complementary in-house qualification parameters
  ✓ LOQ (limit of quantification)
  ✓ Range
  ✓ e.g. accuracy, precision, robustness

✓ Test setup
  ✓ 6 different endotoxin concentrations
  ✓ 4 different non-endotoxin pyrogens (NEPs); each at 4 different concentrations
  ✓ 3 PBMC batches
  ✓ 7 dose endotoxin standard curve
  ✓ IL-6 readout
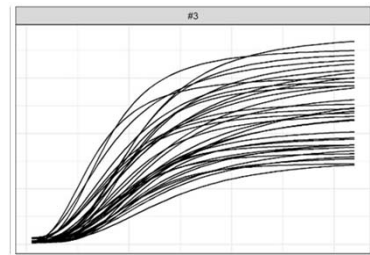
6

General GMP method qualification

✓ Successful qualification using standard endotoxin and non-endotoxin pyrogens (NEPs) compliant with Ph. Eur. 2.6.30 and in-house requirements

  ✓ LOD = 0.03 EE/mL (with human AB serum)
  ✓ Basal release OD < 0.1
  ✓ No averaging effect of pooling cells
  ✓ LOQ = 0.08 EE/mL
  ✓ Range = 0.08 EE/mL - 0.32 EE/mL
  ✓ e.g. accuracy, precision, robustness
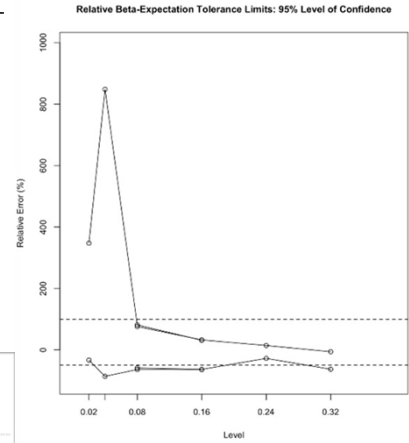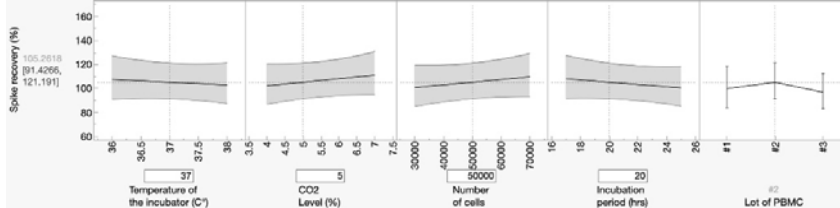
Product-specific method validation acc. to Ph. Eur. 2.6.30
on therapeutic monoclonal antibodies

Viral exacerbation at 40x magnification

Product-specific method validation

✓ Product-specific validation includes:
  • Screening to determine appropriate dilution = f
  • Spiking of diluted product at 3 dilutions with NEP
  • Test for interference with IL-6

✓ Test setup:
  • 2 different non-endotoxin pyrogens at EC_{50} (PGN and FLA)
  • At least 1 operator
  • 1 PBMC batch
  • 3 PPQ drug product batches (mAb)
  • 7 dose endotoxin standard curve
  • Positive control spiked in diluted product and medium
  • IL-6 readout
  • MVD calculation: MVD = CLC/LOQ (LOD not used) (more stringent than in Pharmacopoeia)

✓ All Ph. Eur. 2.6.30 requirements were met

✓ To date: 7 therapeutic mAb-based products validated

Test setup of batch analysis

Viral exacerbation at 40x magnification

Test setup of batch analysis

✓ Test setup
- 1 PBMC batch
- 3 PPQ drug product batches (mAb)
- Positive control spiked in diluted product and medium
- n = 3
- IL-6 readout
- Reportable results:
  - Highest result within a plate and between plates is reported
  - Values < 0.08 EE/mL reported as "< LOQ"

Technical challenges

Viral exacerbation at 40x magnification

Technical challenges

– Lack of international standards for Non-Endotoxin Pyrogens (NEPs)

– Source of serum (foetal calf *vs.* human AB serum)

– When the Critical Limit Concentration (CLC) is very narrow, the Maximal Valid Dilution (MVD) is extremely small ➔ it may be challenging for certain matrices showing interferences to have validated dilutions within the MVD

– For certain matrices showing high interference, it may only be possible to validate 1 or 2 dilutions (i.e., higher risk for an invalid assay during routine testing)

– If a 4-PL model is fitted to the endotoxin standard curve, the observed curve must not deviate significantly from the theoretical (S-shaped) curve *(requirement added in upcoming Ph. Eur. 2.6.30 update).*

– It should not be assumed that responses to non-endotoxin ligands for toll-like receptors (TLRs) will dilute parallel to the standard endotoxin curve *(requirement removed in upcoming Ph. Eur. 2.6.30 update).*

Industry perspective

Viral exacerbation at 40x magnification

Industry perspective

✓ MAT is a valid *in vitro* alternative to RPT

✓ Regulatory uncertainty: some countries/regions may not accept MAT data and require RPT data as part of commercial licensing applications (e.g., US FDA CFR610.13b requires RPT data)

✓ RPT would be needed (incl. licensed testing facilities) within the EU to support RPT requirements in other regions

✓ Platform Method A ('Method 1') (e.g., PBMC with IL-6) may not always be suitable to overcome product interference
  ➜ No clarity on number of alternatives to the platform method which must be developed & optimized (e.g., different cytokines, different cell sources,…)
  ➜ Platform Method C (= 'Method 2') needed

Conclusions

Viral exacerbation at 40x magnification

Conclusions

✓ Successful internal development of MAT 'Method A' ('Method 1') using pooled, cryopreserved PBMCs in combination with ELISA IL-6 read-out

✓ MAT 'Method A' ('Method 1') proven to be suitable to detect (spiked) pyrogens in 7 therapeutic mAb-based drug products (to date)

✓ 3 approved MAA submissions with MAT instead of RPT data (to date)

✓ Need for clarity on MAT back-up scenario's

✓ Need for harmonized Health Authority requirements to eliminate concurrent RPT and MAT

Questions?

Contact details:
Ruth Daniels
rdanie22@its.jnj.com

Further reading:
Daniels et al. (2022). Validation of the monocyte activation test with three therapeutic monoclonal antibodies *Alternatives to animal experimentation (ALTEX)* doi: 10.14573/altex.2111301

Acknowledgments:

Marijke W.A. Molenaar-de Backer (Sanquin, NL)
Eelo Gitz (Sanquin, NL)

Stephen Poole (independent consultant, former NIBSC, UK)

Mehul Patel (Janssen R&D, US)
Wim Van der Elst (Janssen R&D, BE)
Nele Dieltjens (Janssen R&D, BE)
Tinne Appels (Janssen R&D, BE)
Chi Kwong So (Janssen R&D, US)
Thomas Nys (Janssen R&D, BE)
Liesbeth Voeten (Janssen R&D, BE)
Philip Breugelmans (Janssen R&D, BE)

Viral exacerbation at 40x magnification

Implementing the MAT @ Microcoat

Dr. Johannes Reich

EDQM-EPAA Pyrogenicity Event: The future of pyrogenicity testing: phasing out the rabbit pyrogen test
Brussels, Belgium, 14-Feb-2023

Disclaimer

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Slide 3

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Contract laboratory for Endotoxin and Pyrogen testing

Microcoat Biotechnologie GmbH
Am Neuland 3
82347 Bernried/Munich
Germany

FOCUS

- Product specific evaluation of new test methods/procedures

- Development/Improvement of test protocols (eg.; Interference, LER)

- Endotoxin/Pyrogen Removal studies

- GMP-Release testing of medicinal products

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Slide 4

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Methods @ Microcoat

- **Limulus Amebocyte Lysate (LAL) Tests**
  - Kinetic chromogenic LAL (Lonza, ACC, Charles River)
  - Kinetic turbidimetric LAL (Lonza, ACC, Charles River)
  - Gel-clot test (ACC)
  - Cartridge System (Charles River)

- **Limulus-based ß-Glucan test (ACC)**

- **Recombinant Factor C Tests**
  - Endpoint fluorescence rFC (Lonza, Hyglos/bioMérieux)
  - Kinetic chromogenic rBET (ACC/Seikagaku)

- **Monocyte Activation Tests (MAT)**
  - **Cryo-blood + IL 1ß (Merck)**
  - **PBMC + IL 6 (MAT Research, Sanquin/Lonza, Haemochrom)**
  - **Cell-line + IL 6 (Merck)**
  - **Combination of different cell systems with multiple read-out (multiplex)**
- Platforms:

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Germany

Agilent   MSD   Protein Simple   Olink   Quanterix   etc.

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Slide 5

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Pyrogen testing

https://www.aerzte-gegen-tierversuche.de/de/helfen/35-projekte/stellungnahmen/3202-pyrogentest-unendlich-viel-tierleid-trotz-vorhandener-tierversuchsfreier-methoden
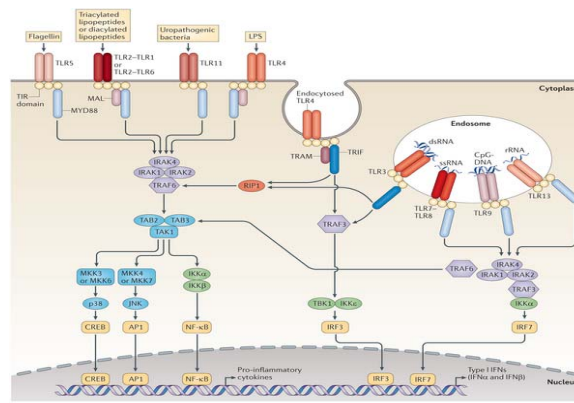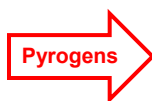
https://www.researchgate.net/publication/285586315_Limitations_of_the_Rabbit_Pyrogen_Test_for_Assessing_Meningococcal_OMV_based_Vaccines/figures
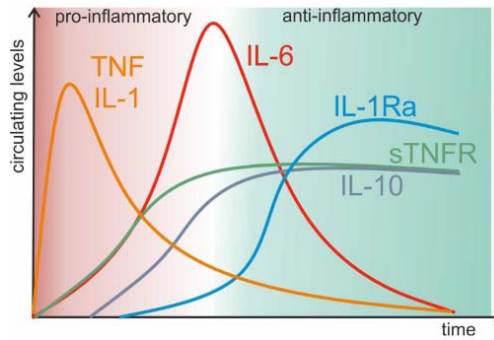
The MAT, an alternative to the Rabbit Pyrogen Test?

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Slide 6

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The principle: Activation of the Human Immune System

Pyrogens

Cytokines

| 6

Background information

Cytokine kinetics (in sepsis)

Tumor necrosis factor (TNF) and interleukin (IL-)1 are the first cytokines to be released in sepsis and promote the secretion of IL-6.
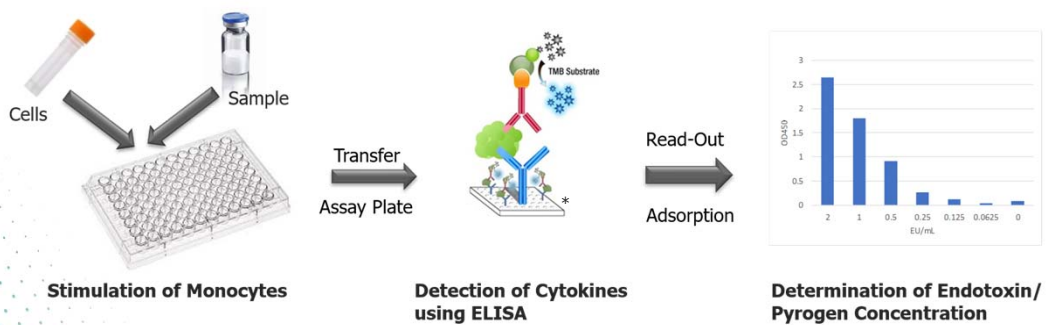

- Model based on human immune system
- Model allows detection of endotoxin <u>and</u> other pyrogens
➔ Big advantage compared to BET
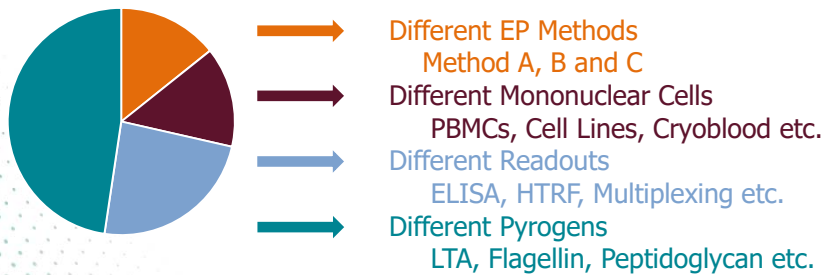
| 7

The Monocyte Activation Test (MAT)

MAT work-flow

Stimulation of Monocytes   **Detection of Cytokines using ELISA**   **Determination of Endotoxin/ Pyrogen Concentration**
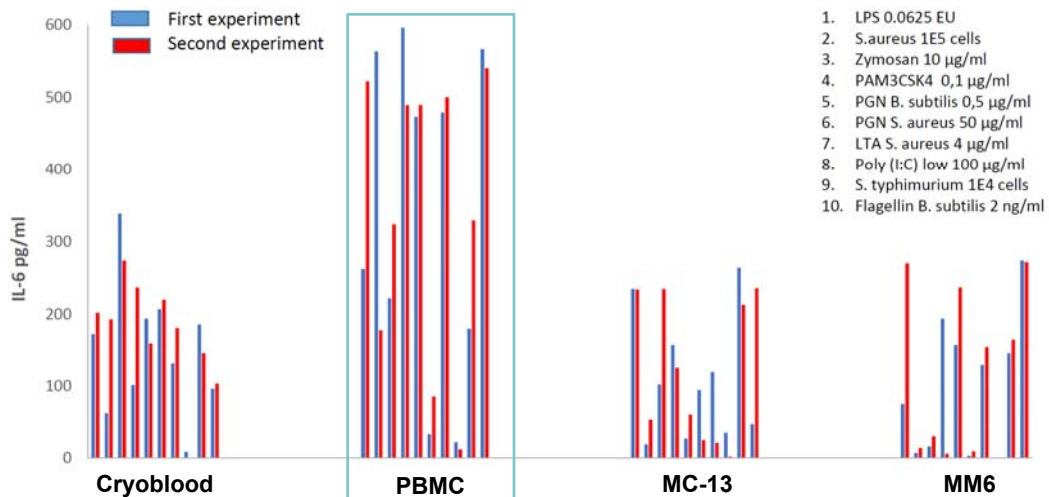
| 8

Starting the MAT

Since the introduction of the MAT in the European Pharmacopeia (EP) in 2010, numerous of different variants have been developed due to the large variety of variable parameters, making it substantially difficult to select an appropriate test method.
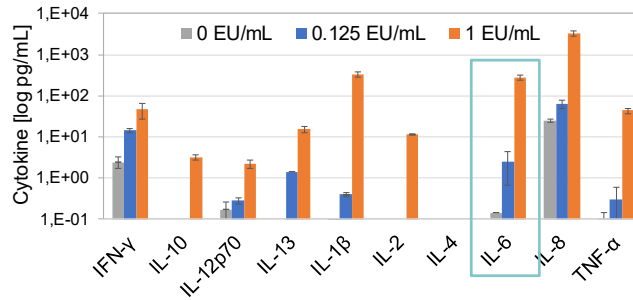
Different EP Methods
Method A, B and C
Different Mononuclear Cells
PBMCs, Cell Lines, Cryoblood etc.
Different Readouts
ELISA, HTRF, Multiplexing etc.
Different Pyrogens
LTA, Flagellin, Peptidoglycan etc.

Different Mononuclear Cells

Legend:
- First experiment
- Second experiment

1. LPS 0.0625 EU
2. S.aureus 1E5 cells
3. Zymosan 10 µg/ml
4. PAM3CSK4 0,1 µg/ml
5. PGN B. subtilis 0,5 µg/ml
6. PGN S. aureus 50 µg/ml
7. LTA S. aureus 4 µg/ml
8. Poly (I:C) low 100 µg/ml
9. S. typhimurium 1E4 cells
10. Flagellin B. subtilis 2 ng/ml

Categories: Cryoblood, PBMC, MC-13, MM6

Y-axis: IL-6 pg/ml

- Variations between the different cell sources
- Variations between experiments

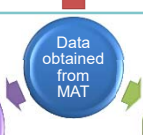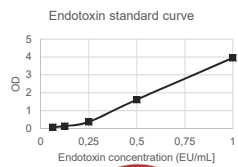# Detection of released cytokines

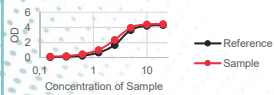## Comparison of different cytokines (based on endotoxin stimulation)

- Big difference in amount of expressed Cytokine (4 log)
- Measurable concentration dependent stimulation of Cytokine Expression (except IL-4)
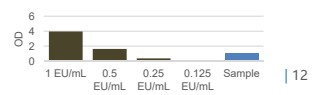- Comparable sensitivity of different Cytokines
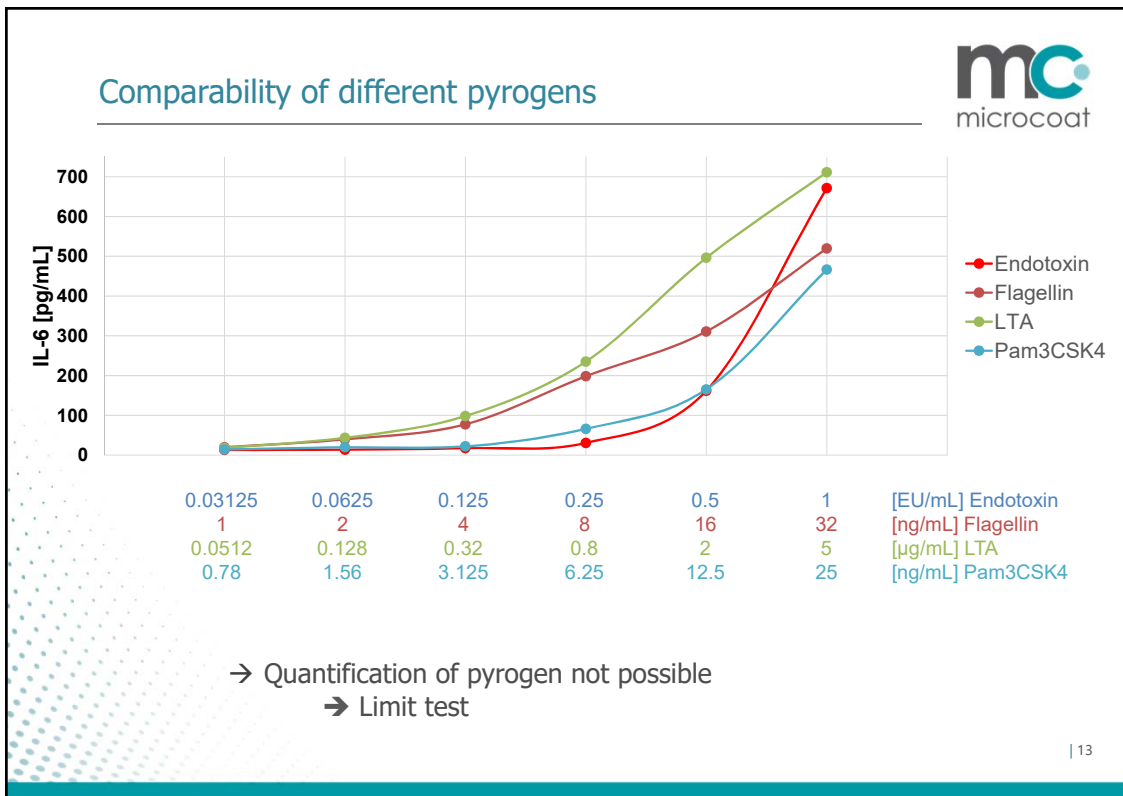
Different EP methods

Quantitative Analysis

Batch Comparison

Semi-Quantitative Analysis

Comparability of different pyrogens

0.03125	0.0625	0.125	0.25	0.5	1	[EU/mL] Endotoxin
1	2	4	8	16	32	[ng/mL] Flagellin
0.0512	0.128	0.32	0.8	2	5	[µg/mL] LTA
0.78	1.56	3.125	6.25	12.5	25	[ng/mL] Pam3CSK4

➔ Quantification of pyrogen not possible
    ➔ Limit test

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Summary

Monocyte Activation Test is an adequate method to detect pyrogens

Standard conditions: -PBMCs + IL6 read-out
                          -Qualitative interpretation

Application of the MAT under **GMP?**

➔ Need for **standardization**: Home brew or commercial ready to use kit?

    - Home brew tests require additional skills, controls, time, costs, etc.

    - Various commercial kits for MAT are available

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Assay sensitivities

Standard curve (Sample)

MAT/Provider	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vendor 1	0.16	0.08	0.04	0.02	0.01	-
Vendor 2	1.0	0.5	0.25	0.125	0.06125	-
Vendor 3	0.8	0.4	0.2	0.1	0.05	0.025
Vendor 4	0.4	0.2	0.1	0.05	0.025	-
Vendor 5	0.4	0.2	0.1	0.05	0.025	-

Standard curve (concentration/well)

MAT/Provider	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vendor 1	0.08	0.04	0.02	0.01	0.005	-
Vendor 2	0.1	0.05	0.025	0.0125	0.006125	-
Vendor 3	0.16	0.08	0.04	0.02	0.01	0.005
Vendor 4	0.1	0.05	0.025	0.0125	0.00625	-
Vendor 5	0.1	0.05	0.025	0.0125	0.00625	-

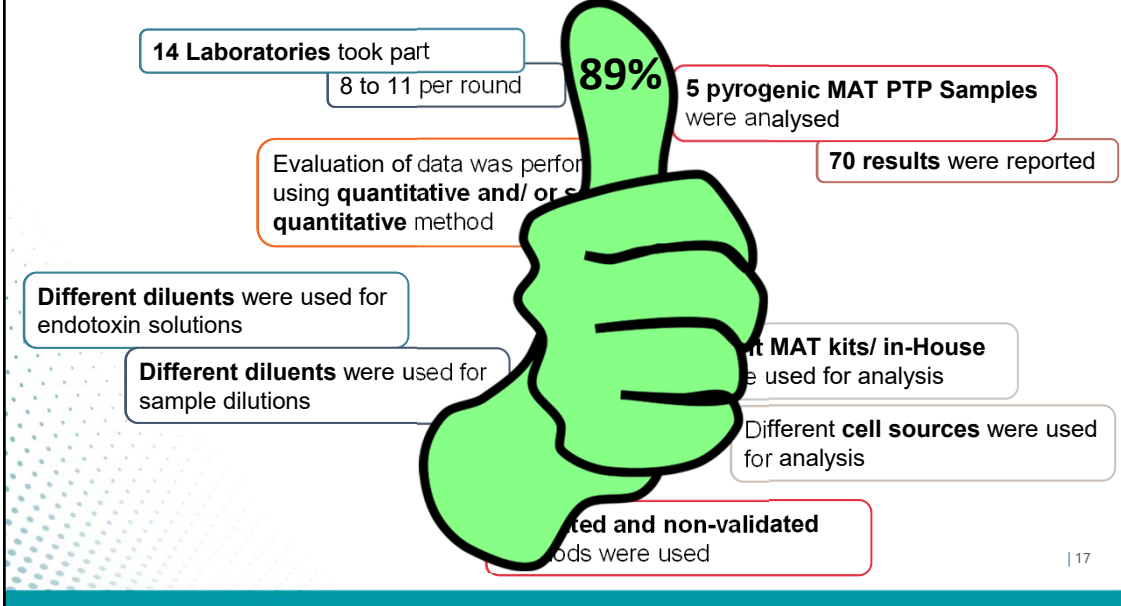## Proficiency Testing Program (PTP)

Goals of the MAT Proficiency Testing Program

- Assure qualification of MAT systems

- Assure qualification of laboratory personnel

- Test comparability of results between laboratories and MAT systems

Get a MAT PTP Sample with an unknown potential contamination → Analysis with routine MAT → Evaluation of Data → Reporting of Data → Certificate for MAT PTP

Proficiency Testing Program (PTP)

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Benchmarks of three years PTP

14 Laboratories took part
8 to 11 per round

89%

5 pyrogenic MAT PTP Samples were analysed

Evaluation of data was perfor... using **quantitative and/ or s...**
quantitative method

70 results were reported

Different diluents were used for endotoxin solutions

Different diluents were used for sample dilutions

...t **MAT kits/ in-House** ...e used for analysis

Different **cell sources** were used for analysis

...ted and non-validated ...ods were used

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The Monocyte Activation Test

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Conclusions

- MAT is able to correctly identify contaminations

- MAT is more and more implemented and validated in pharmaceutical QC labs

- (Current) State of the art @ Microcoat: Stimulation of PBMCs overnight → IL6-Readout

- **Benefits of Monocyte Activation Test:**
  - 3R: Replace of animal testing
  - Application of controls (positive product controls)
  - Validation of method
  - Trouble shooting
  - Broad application

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Some Lessons learned

10 Years MAT in a contract lab

Past	Today
Logistics of cells was often inadequate --> inoperable	No problem, logistics are under control → reliable cells available
Home brew kits require high maintenance (preps, qualification, costs,…)	Various vendors with excellent quality available
Lack of routine testing	Today, MAT is a routine test procedure
Limited knowledge	Experience (specific handling, data evaluation, statistics, etc.)
Limited comparability of tests	Increasing amounts of products and studies available (PTP)
Limited training opportunities	Training courses

Next training session @ Microcoat:

Acknowledgment- Endotoxin Service