Joint EDQM-EPAA Event

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

14-15 February 2023

epaa The European Partnership for Alternative Approaches to Animal Testing

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

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

Pulling the rabbit out of the hat: Industry perspectives

epaa The European Partnership for Alternative Approaches to Animal Testing

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Microcoat's experience with the MAT over the last decade

Dr. Johannes Reich

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

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Expertise and Focus

Example for support by Microcoat for measuring your sample in the MAT:

- Check test inhibition and enhancement (Endotoxin)
- Check test inhibition and enhancement (Non-Endotoxin Pyrogens)
- **Improve methods/protocols**

Potential Solutions:

- Different methods/kits
- Different read-out parameters
  → different cytokine
- Different cell system
  → whole blood vs. cell line vs. PBMCs
- Different MAT setup
  → ratio of cells to medium

→ Development of dedicated MAT setup

Guidelines
EP 2.6.30, FDA (Q&A): alternative test
For method validation: ICH Q2 (R1), USP <1225>, USP <1223>

Routine Testing ← Validation ← Protocol ← = Exploratory

Case study 1 – Basic workflow

Getting ready for testing a biological drug product with MAT

Product feasibility → Generic validation → Product-specific validation → Release testing

Standard plate layout (product-specific validation):

	1	2	3	4	5	6	7	8	9	10	11	12
A	Endotoxin STD 1				sample dilution 1				samplpe dilution 2 + LTA			
B	Endotoxin STD 2				sample dilution 1 + RSE				samplpe dilution 2 + Flagellin			
C	Endotoxin STD 3				sample dilution 1 + LTA				samplpe dilution 3			
D	Endotoxin STD 4				sample dilution 1 + Flagellin				sample dilution 3 + RSE			
E	Endotoxin STD 5				sample dilution 2				sample dilution 3 + LTA			
F	Blank (medium)				sample dilution 2 + RSE				sample dilution 3 + Flagellin			
G	LTA – 0.5x spike				LTA – 1x spike				LTA – 2x spike			
H	Flagellin – 0.5x spike				Flagellin -1x spike				Flagellin – 2x spike			

Standard read-out: IL-6

Standard interpretation: 2.6.30, Method B (semi-qualitative)

Reference: van den Berg I. et al., Generic Method and Specific Product Validation of the Monocyte Activation Test, Pharmaleb 2022

Case study 1 – Basic workflow

Getting ready for testing a biological drug product with MAT

Product feasibility → Generic validation → Product-specific validation → Release testing

Example of Product A:

- MAT release testing performed with the pilot product
- Pyrogens used: RSE
- Product batches: 3

Product dilution	Release criteria		Pass/Fail
1 x MVD	< 1 x LOQ	< OD(0.05EU/mL)	Pass
0.5 x MVD	< 2 x LOQ	< OD(0.1EU/mL)	Pass
0.3 x MVD	< 3.4 x LOQ	< OD(0.17EU/mL)	Pass

Result:

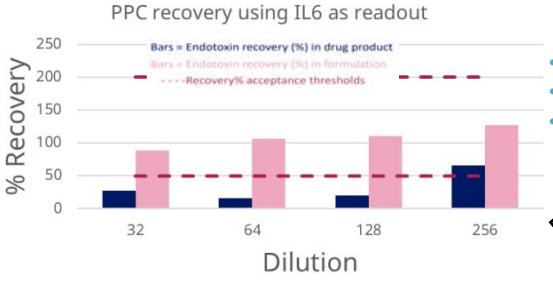
- **MAT passed, no pyrogens detected**
- **In parallel, the RPT also did not detect any pyrogens**

Conclusion: To fulfil global regulatory requirements, full method und product validation needed.

Reference: Van den Berg I. et al., Generic Method and Specific Product Validation of the Monocyte Activation Test, Pharmalab 2022

Case study 2 – Deviation from routine test

Product X interferes with standard MAT



- Valid recovery at MVD only
- Strong interference detectable
- Product X needs another readout than IL-6

IL-1ß Read-out

- Valid PPC recoveries for all dilutions and spikes
- Values for samples w/o spike below LOQ (0.05 EU/mL RSE)
- **BUT:** ELISA lot-to-lot variances with respect to BLK value

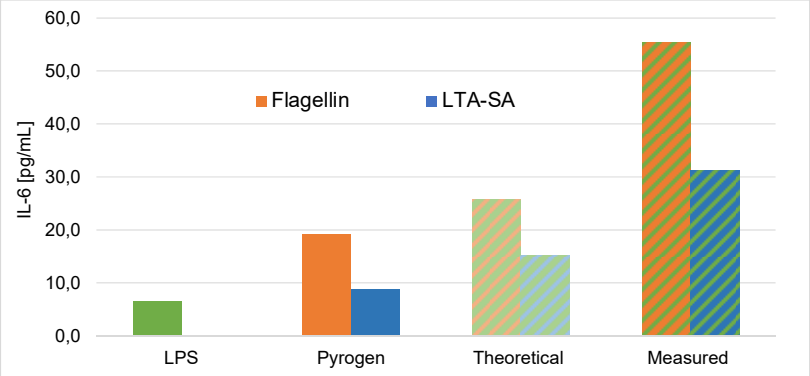
Dilution	sample [OD^P]	RSE Spike Recovery [OD^S-OD^P]	LTA Spike Recovery [OD^S-OD^P]	Flagellin Spike Recovery [OD^S-OD^P]
0.25x MVD	0.078	0.05	0.41	0.31
0.5x MVD	0.090	0.04	0.46	0.36
MVD	0.076	0.06	0.43	0.30

OD^P = measured OD value in sample without spike OD^S = measured OD value in sample with spike

Reference: Orving R., Monocyte activation test with an antiinflammatory product, Pharmalab 2022

Case study 3 - Synergistic Effect

Mixing of pyrogens



- Synergistic effect of Flagellin and Endotoxin
- Synergistic effect of LTA-SA and Endotoxin

Case study 3 – Synergistic effects

Spike recovery > 200 %

Dilution	Measured [OD^P]	Endotoxin Spike [OD^S-OD^P]	LTA Spike [OD^S-OD^P]	Fla Spike [OD^S-OD^P]	Status
Dilution 1	0.059	0.208	0.246	2.050	Invalid
Dilution 2	0.056	0.243	0.149	1.325	Invalid
Dilution 3	0.119	0.164	0.077	1.571	Invalid

Spike of **Product B** with Endotoxin, Lipoteichoic acid (LTA) and Flagellin (FLA)

OD-BLK	0.5 x Spike	1 x Spike	2 x Spike
Endotoxin	0.094	0.266	1.414
LTA	0.016	0.033	0.150
Fla	0.147	0.581	1.056

Spike of **Media** with Endotoxin, Lipoteichoic acid (LTA) and Flagellin (FLA)

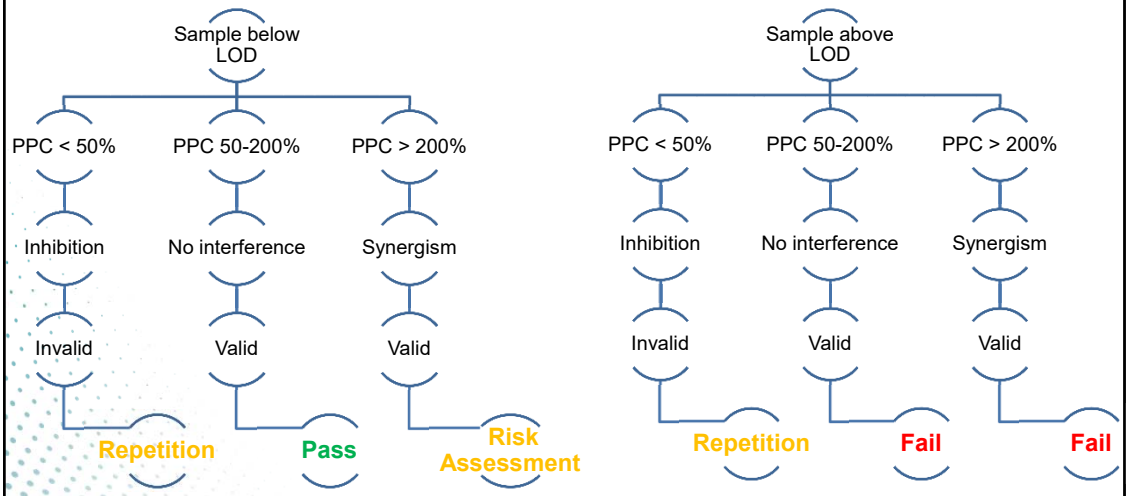
- Unspiked Product B: OD < OD(LOD)
- RSE: Valid recoveries with all tested dilutions
- LTA: Invalid recovery with dilution 1, valid recoveries with dilution 2 and 3
- Fla: Invalid recoveries for all tested dilutions (OD > OD(2x spike))

- Product B may contain low level pyrogens (ie., below detection limit)
  - → Synergistic effects in a sample are not predictable
  - ➔ Synergistic effects may cause PPC recovery > 200 %

OD^P = measured OD value in sample without spike OD^S = measured OD value in sample with spike

Case study 3 – Synergistic effects

Assessment of PPC results



Case study 4 – An alternative to BET

The challenge: Analysis of VLPs

Endotoxin test	Units	VLP batch			
		VLP1	VLP2	VLP3	VLP4
		Test results			
Gel clot (Method A)	EU/mg	30-60	Not determined	≤350; >273	≥350; >983
Turbidimetric kinetic (Method C)		3	154.8	3-17; 3,817	8.5-30
Chromogenic kinetic (Method D)		504	1,037	Not determined	Not determined

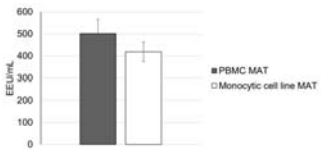
→ BET not reliable/robust for given test articles

Analysis of growth medium with MAT and LAL

Test	Average values	SD
Method D: Chromogenic kinetic method (Ph. Eur. 2.6.14.)	3,630 EU/mL	1,103
PBMC MAT (Ph. Eur. 2.6.30.)	32 EEU/mL	5

→ MAT reliable/robust for given test articles

Comparison of two different MAT methods



➔ MAT is the preferred test method

Reference: Scott C., Challenges of Endotoxin Detection During Development of a Novel VLP Vaccine, ECA Microbiology, 2022

Case study 5 – Low Endotoxin Recovery

LER Study – MAT (quantitative Interpretation) vs. LAL

Results from LER study in LAL at **day 0**

Dilution	Measured value in DP [EU/mL]	EU/mL x Dilution in DP	Recovery to theor. value in DP [%]	PPC in DP [%]
1:12.5	0.103	1.29	86	136
1:25	0.0563	1.41	93	155
1:50	0.0241	1.21	81	100

Results from LER study in LAL at **day 2**

Dilution	Measured value in DP [EU/mL]	EU/mL x Dilution in DP	Recovery to theor. value in DP [%]	PPC in DP [%]
1:12.5	0.0115	0.144	9	125
1:25	0.00648	0.162	11	99
1:50	< 0.00330	< 0.165	< 11	97

Results from LER study in MAT at **day 0**

Dilution	Measured value in DP [EU/mL]	EU/mL x Dilution in DP	Recovery to theor. value in DP [%]	PPC in DP [%]
1:1	0.654	0.654	44	86 *
1:2	0.619	1.24	83	142
1:4	0.412	1.65	110	119
1:8	0.202	1.62	108	100

Results from LER study in MAT at **day 2**

Dilution	Measured value in DP [EU/mL]	EU/mL x Dilution in DP	Recovery to theor. value in DP [%]	PPC in DP [%]
1:1	< 0.125	< 0.125	< 8	104
1:2	0.193 *	0.386	26	36
1:4	< 0.125	< 0.500	< 33	103
1:8	< 0.125	< 1.00	< 67	111

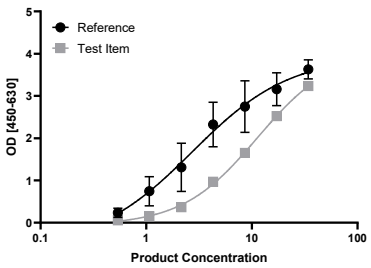
*CV > 30 %; Note: Spiked water controls were stable ie., 50-200 % over time (data not shown).

- In Product DP, a LER-effect was detected with MAT and LAL

Case study 6 – Comparability testing

Method C – An example

- Product with inherent pyrogenicity
- Multiple product batches needed
- Analysis of dose-response curves
- Definition of EC-50 criteria



- Method shows low intra-assay variability
- **Method allows for assessment of batch-to-batch comparability**

Take home message

- Broad application of MAT possible
- Robust MAT kits available, however adequate sample preparation needed
- MAT allows analysis of complex samples/matrices
- Synergistic effects may require additional assessments
- To fulfil global regulatory requirements, full method und product validation needed

Acknowledgment- Endotoxin Service

Microcoat Endotoxin/Pyrogen test team

Collaboration partners

Rene Orving, Novo Nordisk, Jonas van den Berg, Roche Diagnostics, Sven Deutschmann, Roche Diagnostics, Callum Scott, Allergy Therapeutics and all of our valued clients.

Thank You!

Pyrogenicity Testing from the Biopharma and Plasma Industry Perspective

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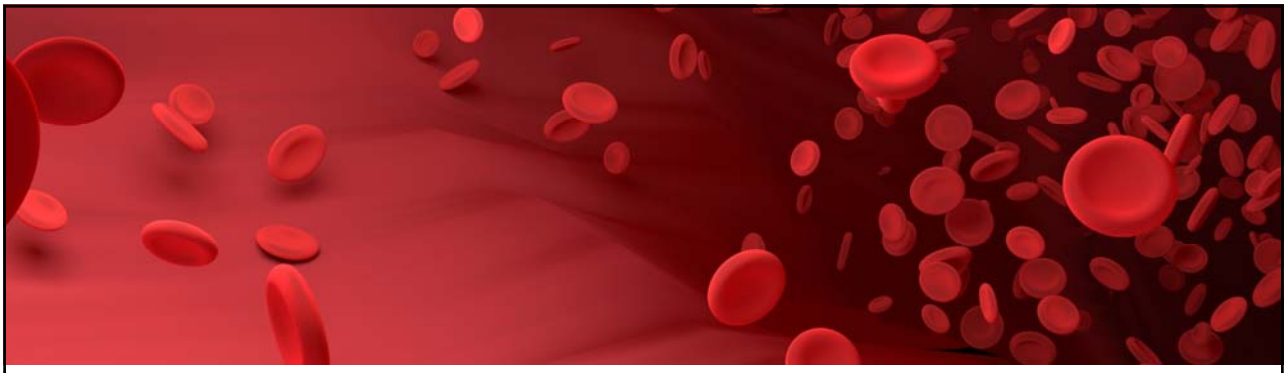
Better Health, Brighter Future

Disclosure

- Peter L. Turecek is a full-time employee and a stock owner of Takeda

Agenda

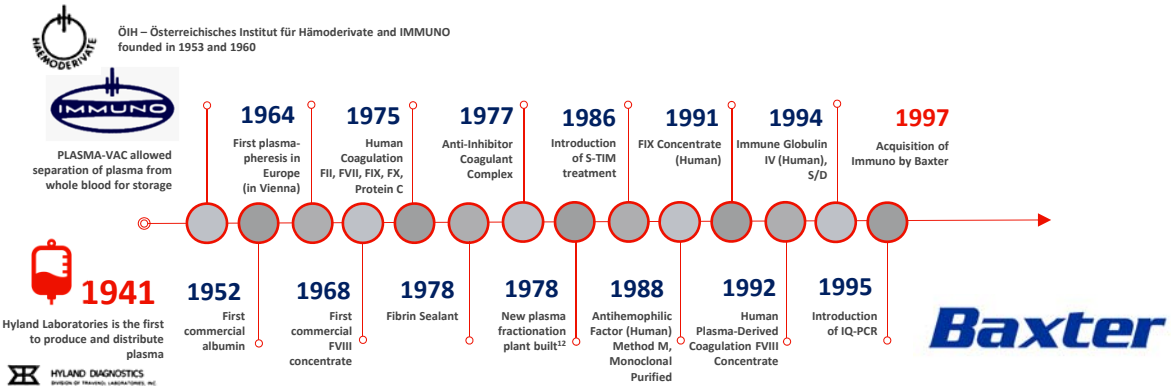
- History of Pyrogen Testing from an Industry Perspective
- The Eau Claire Incident
- Plasma Industry Status
- Future Pyrogen Testing Strategy
- Summary and Conclusions
- Q&A



History of Pyrogen Testing from an Industry Perspective

Better Health, Brighter Future

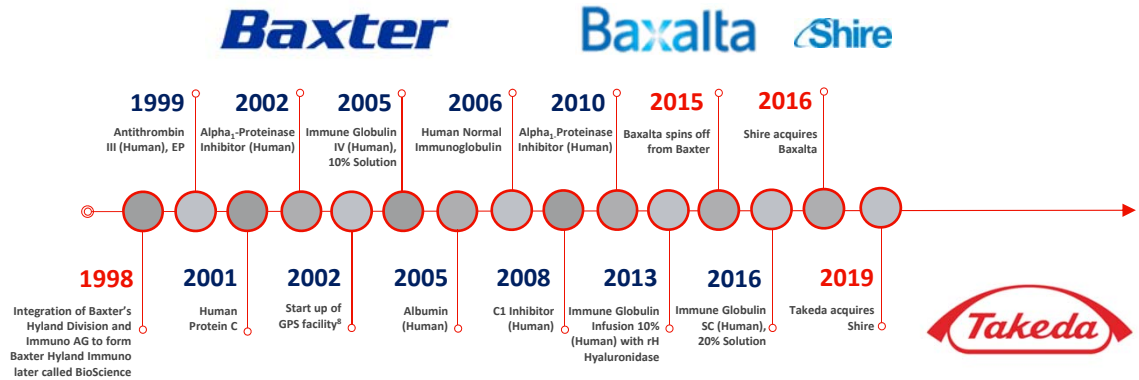
A long history in plasma-derived therapies (1/2)[1–12]



IV, intravenous; PCR, polymerase chain reaction; S/D, solvent/detergent-treated.

1. Takeda. Available at: www.Takeda.com/who-we-are/company-information/ Accessed November 2022; 2. Kim J. 2019. Available at: https://www.takeda.com/4ab4df/siteassets/system/investors/report/quarterlyannouncements/fy2019/pdt_20191115.pdf Accessed November 2022; 3. Negrier C, Gomperts ED. *Haemophilia*. 2006;2-3; 4. Clinical Drug Experience Knowledgebase. Immuno AG. Available at: https://www.cdek.liu.edu/org/190/ Accessed November 2022; 5. Pharma Boardroom. Directory: Baxter Austria. Available at: https://pharmaboardroom.com/directory/baxter-austria/ Accessed November 2022. 6. Baxter International. Available at: https://investor.baxter.com/investors/sec-filings/default.aspx Accessed November 2022. 7. Baxter Healthcare. Available at: https://www.baxterhealthcare.co.uk/our-story/our-history Accessed November 2022 ; 8. BioLife Austria: Available at: https://www.plasmazentrum.at/en/ueber-uns/ Accessed November 2022; 9. Takeda. Available at: https://www.takeda.com/who-we-are/company-information/history/foundation-modernization/ Accessed November 2022; 10. Esposito S, et al. *PLOSone* 2016;11(4):e0151533; 11. Storch H, et al. *Beitr Infusionsther Transfusionsmed* 1997;34:31–6; 12. Speaker's experience.

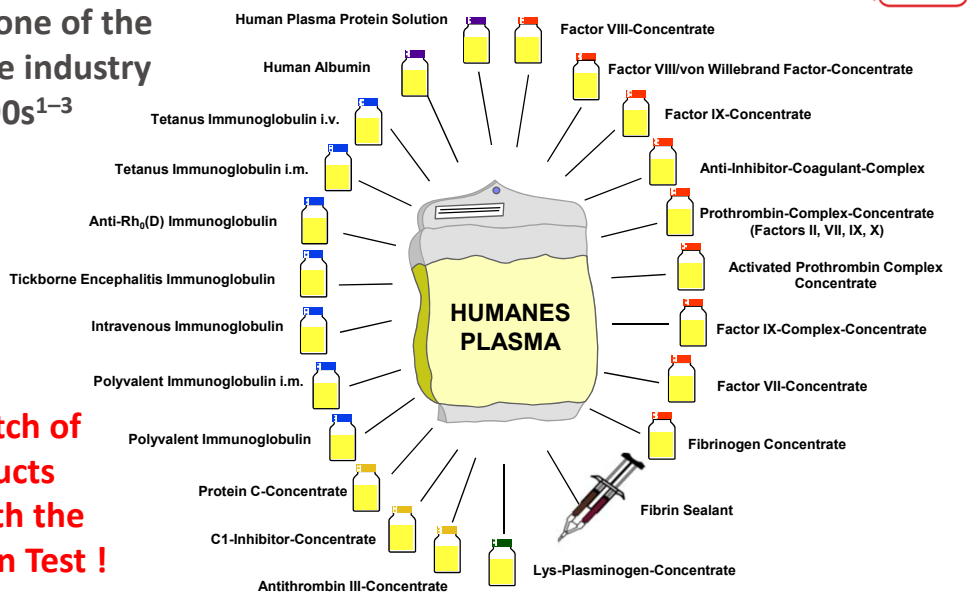
A long history in plasma-derived therapies (2/2)[1–8]



GPS, Global Pathogen Safety; IV, intravenous; rH, recombinant human; SC, subcutaneous.

1. Takeda. Available at: www.Takeda.com/who-we-are/company-information/ Accessed August 2021; 2. Kim J. 2019. Available at: https://www.takeda.com/4ab4df/siteassets/system/investors/report/quarterlyannouncements/fy2019/pdt_20191115.pdf Accessed November 2022; 3. Negrier C, Gomperts ED. Haemophilia. 2006;2-3; 4. Baxter 10-K Annual Report 1998. Available at: https://investor.baxter.com/investors/sec-filings/sec-filings-details/default.aspx?FilingId=168431 Accessed November 2022. 5. Federal Trade Commission. 20011176. Available at: https://www.ftc.gov/legal-library/browse/early-termination-notices/20011176 Accessed November 2022; 6. BioLife Austria: Available at: https://www.plasmazentrum.at/en/ueber-uns/ Accessed November 2022; 7. Curling J, et al. Chapter 1. 2012. In: Bertolini J, et al. (eds) Production of plasma proteins for therapeutic use. Wiley; 8. Speaker's experience.

Takeda´s plasma product portfolio was one of the broadest in the industry in the late 1990s[1–3] and still is!



…and each batch of all these products was tested with the Rabbit Pyrogen Test !

Product portfolio in the 1990s. Not all products currently available.
1. Speaker's knowledge; 2. Curling J, et al. Chapter 1. 2012. In: Bertolini J, et al. (eds) Production of plasma proteins for therapeutic use. Wiley; 3. Kim J. 2019. Available at: https://www.takeda.com/4ab4df/siteassets/system/investors/report/quarterlyannouncements/fy2019/pdt_20191115.pdf Accessed November 2022

Control and Limitation of Animal Experimentation is an Ongoing Task since the 1950s /1

Essentially the task of limitation of animal experimentation is described with the definition of 3 Rs:

3R

Reduction - fewer animals

Refinement - less painful

Replacement - alternative techniques

… and resulted in the **Declaration of Bologna on Reduction, Refinement and Replacement, and Alternatives in Laboratory Animal Procedures,**

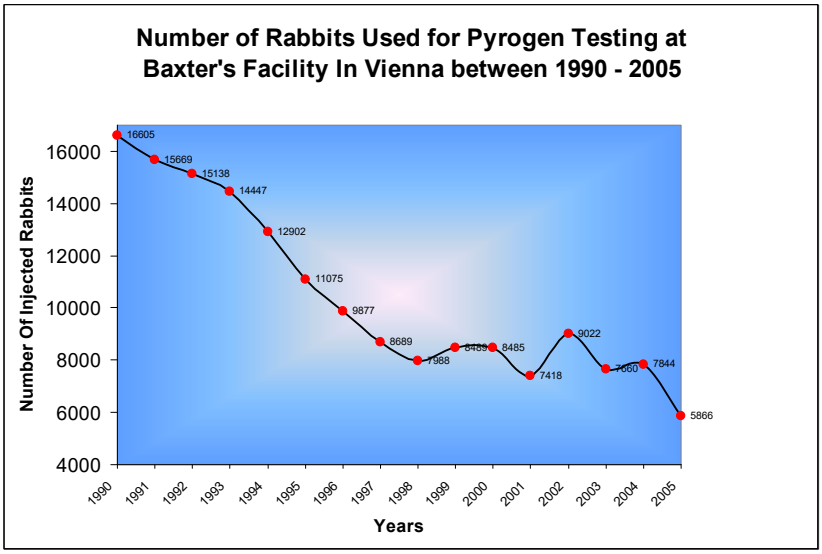
which was adopted and issued by the **3rd World Congress on Alternatives and Animal Use in the Life Sciences in Bologna, Italy on August 31st, 1999.**

Control and Limitation of Animal Experimentation is an Ongoing Task since the 1950s /2

- **Companies like Immuno AG (successor Takeda) had been consistently working on reduction of animal experiments.**
  - **For example:**
    From the early 1980s over 15 years Immuno AG replaced, reduced or refined animal tests for vaccine products release from 4,278 to 2,011 per average vaccine lot with a total savings of animals of around 50,000 per year!

Schober-Bendixen S., Application of the 3 R.
ALTEX. 1997;14(3):99-106.

Rabbit Pyrogen Testing at Immuno / Baxter, now Takeda



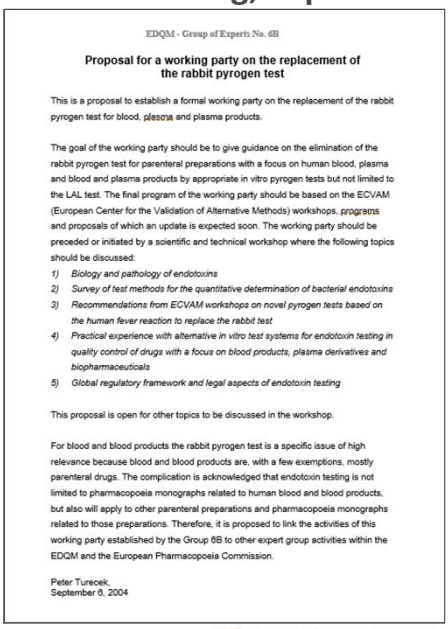
Takeda Data on File

Ph. Eur. Monographs on Plasma and Blood Related Products Consequences in 2004

- → ***The monographs related to plasma and plasma products did not explicitly allow alternatives for the rabbit pyrogens test***
- → ***Estimates said that by 2004 approx. 200,000 rabbits per year were used worldwide for the pyrogen test only***

Proposal to Ph. Eur. Group of Experts 6B in the 74th Meeting, Sep 2004

Proposal for a working party on the replacement of the rabbit pyrogen test



EDQM - Group of Experts No. 6B

Proposal for a working party on the replacement of the rabbit pyrogen test

This is a proposal to establish a formal working party on the replacement of the rabbit pyrogen test for blood, plasma and plasma products.

The goal of the working party should be to give guidance on the elimination of the rabbit pyrogen test for parenteral preparations with a focus on human blood, plasma and blood and plasma products by appropriate in vitro pyrogen tests but not limited to the LAL test. The final program of the working party should be based on the ECVAM (European Center for the Validation of Alternative Methods) workshops, programs and proposals of which an update is expected soon. The working party should be preceded or initiated by a scientific and technical workshop where the following topics should be discussed:

1) *Biology and pathology of endotoxins*
2) *Survey of test methods for the quantitative determination of bacterial endotoxins*
3) *Recommendations from ECVAM workshops on novel pyrogen tests based on the human fever reaction to replace the rabbit test*
4) *Practical experience with alternative in vitro test systems for endotoxin testing in quality control of drugs with a focus on blood products, plasma derivatives and biopharmaceuticals*
5) *Global regulatory framework and legal aspects of endotoxin testing*

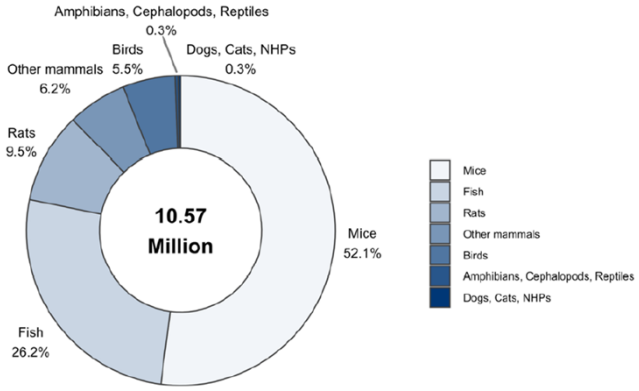
This proposal is open for other topics to be discussed in the workshop.

For blood and blood products the rabbit pyrogen test is a specific issue of high relevance because blood and blood products are, with a few exemptions, mostly parenteral drugs. The complication is acknowledged that endotoxin testing is not limited to pharmacopoeia monographs related to human blood and blood products, but also will apply to other parenteral preparations and pharmacopoeia monographs related to those preparations. Therefore, it is proposed to link the activities of this working party established by the Group 6B to other expert group activities within the EDQM and the European Pharmacopoeia Commission.

Peter Turecek,
September 6, 2004

Animals for scientific purposes in the Member States of the European Union and Norway (status 2018, reported by EC July 2021)

Numbers of animals used for the first time



Mammal category

	2018
Mice	5,505,169
Rats	999,246
Guinea-Pigs	129,931
Other rodents	35,967
Rabbits	342,788
Cats	1,554
Dogs	17,711
Other carnivores	6,082
Farm animals	137,234
Non-human primates	8,583
Other mammals	5,944
Total	7,190,209

- **Rabbits account for < 5% of all animals used for scientific purposes in the EU**

https://ec.europa.eu/environment/chemicals/lab_animals/reports_en.htm

Use of rabbits for pyrogenicity testing in the Member States of the European Union and Norway (status 2018, reported by EC July 2021)

- **Quality control related uses represented 1.08 million uses**
- **In 2018, QC related uses decreased (-5%) with a decrease for pyrogenicity testing (-13%)**

Quality control related uses by type of use

	2018
Batch potency testing	859,797
Batch safety testing	145,769
Other quality controls	43,043
Pyrogenicity testing	30,453
Total	1,079,062

Between 2015 and 2018, pyrogenicity testing decreased steadily (-35%)

- **"Collaborative efforts need to continue in areas where alternative methods are available for regulatory testing, such as for the use of animals for pyrogenicity testing …"**
- **"Such use can only be authorised if the project applicant provides robust scientific evidence why the use of alternatives is not possible."**

https://ec.europa.eu/environment/chemicals/lab_animals/reports_en.htm

Rabbit Pyrogen Test in the Ph. Eur. is most relevant for Plasma Products and Vaccines

General monographs (3)

- Substances for pharmaceutical use (2034)
- Radiopharmaceutical preparations (0125)
- Immunosera for human use, animal (0084)

Dosage form monographs (3)

- Parenteral preparations (0520)
- Preparations for irrigation (1116)
- Intravesical preparations (2811)

Pyrogens (2.6.8)
(Rabbit Pyrogen Test)
59 texts

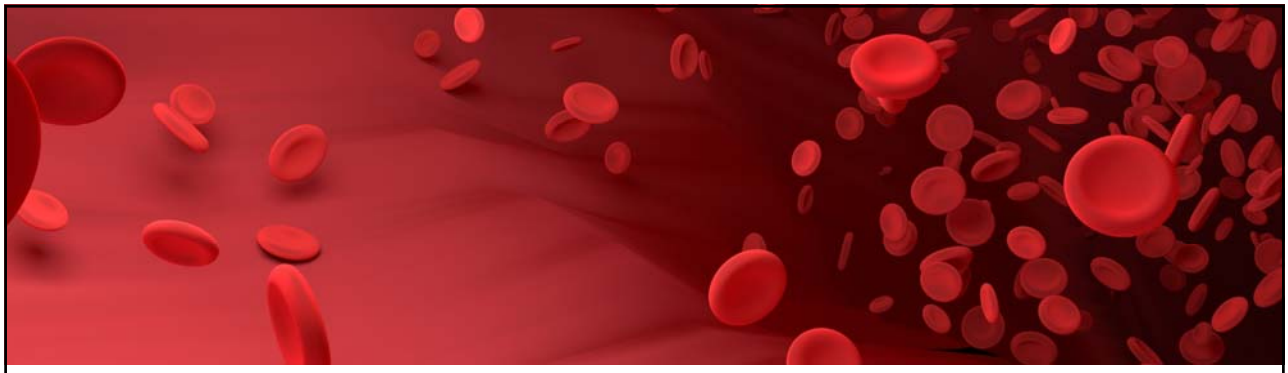
General chapters (3)

Plastics
- Sterile plastic containers for human blood and blood components plastic materials (3.3.4)
- Sets for the transfusion of blood and blood components (3.3.7)

Vaccines for human use
- Carrier proteins for the production of conjugated polysaccharide vaccines for human use (5.2.11)

Individual monographs (50)

- Solutions (4)
- Vaccines for human use (**17**)
- **Plasma products** (**17**)
- Antibiotics (8)
- other chemical substances (4)



The Eau Claire Incident

Takeda

Better Health, Brighter Future

What had happened?

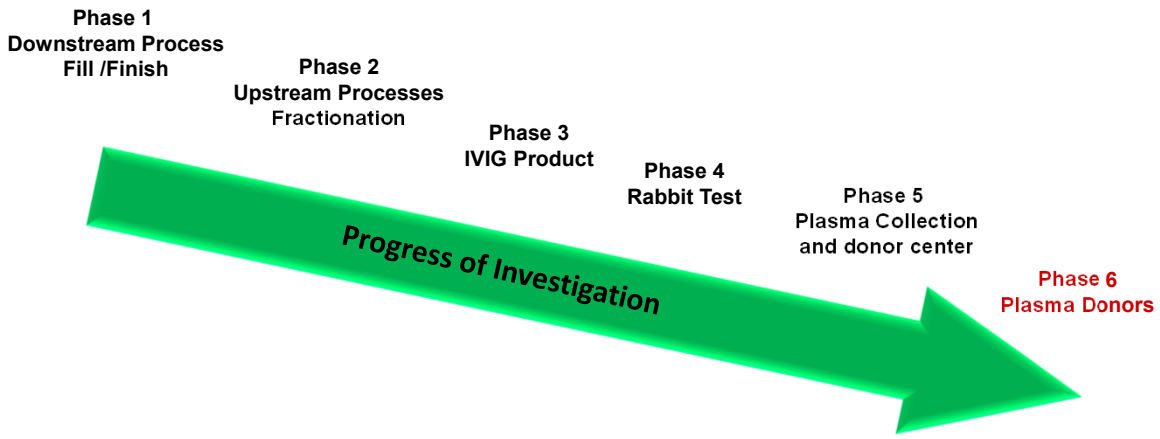
Problem description:

- In March 2007, an IVIG 10% lot was tested for pyrogen (rabbit test) by the QC-laboratory at the LE plant and found to fail test requirements.
- Subsequently eleven (11) other IVIG 10% batches failed the rabbit pyrogen test.

Investigation:

- CAPA investigation was initiated to identify the root cause in the manufacturing plant LE at the formulation/filling and downstream processes.
  - Following thorough investigation any influence through the processes in LE could be ruled out. All analytical investigations on the product proved that the cause was not coming from the LE processes.
- Another CAPA was conducted in the VI plant for detailed investigations in the upstream process.
  - Following thorough investigation, no root cause in the upstream processes could be identified. This proved that the cause is not coming from the upstream processes.
- As next step CAPA was conducted on Division level (in charge of all plants) to focus on investigations in plasma sourcing and handling side.

Flow of Investigations



Investigations–Downstream Process & Fill Finish

All involved downstream and pharmaceutical production processes were assessed against potential sources of root cause:

- **Environmental monitoring**
  - routine LAL (WFI, before sterile filtration, downstream processes, raw materials) all results (Jan to Jun 2007) met acceptance criteria
  - extraordinarily LAL final product testing: all results < 1EU/ml
  - bioburden (equipments, in process samples during bulk production, WFI, demineralized water) did not show any correlation to pyrogenicity
  - depyrogenization/sterilisation processes incl sterile filter integrity were checked O.K.
  - peptidoglycan testing of final product: no correlation found to pyrogenicity and all values found far below pyrogenicity causing concentration
- **Bulk Manufacturing**
  - pyrogenicity is not production line specific, not time specific, not successively produced
  - no correlation with cleaning processes
  - no correlation with column storages, cycle times, or other in process parameter
- **Fill/Finishing operations**
  - no correlation with particles, garments and operation trendings,
  - no correlation with process parameters or cycle times
  - no correlation to any in process test or release test parameter
- **Raw Materials**
  - no correlation to raw material lots
- **Equiment calibration/maintenance & training**
  - no unusual observations or events regarding maintenance and calibration of equipment or personal training

- **No observation which could explain the pyrogenicity of the product**
- **No root cause could be identified**

Takeda Data on File

Investigations – Upstream Processes

All involved processes (Plasma Fractionation, Central Warehouse, Receiving & Inspections Dpt.) were assessed against all potential sources of root cause which include people, materials, methods, environment, and equipment:

- **Data analysis**
  bioburden, LAL, in process testing, process parameters, quality control testing
- **CIP and Cleaning Processes**
  manual and automated cleaning of equipment and room cleaning
- **Maintenance**
  fractionation operations, support systems, employees
- **Raw Materials**
  chemicals, auxiliaries
- **Process**
  production equipment, parameters, personal processes (shifts,..)
- **Additional samples drawn and analyzed**
  in process bioburden and LAL, cleaning processes (last rinse, swabs, cleaning agents)

- **The investigations and the data analysis did not reveal any correlation or observation, which could explain the pyrogenicity of the affected IVIG lots**
- **No root cause could be identified in the upstream manufacturing processes**

Takeda Data on File

Investigations – IVIG Product Analysis

- TLR 2 and TLR4 assays conducted by FDA
- Ouchterlony double immunodiffusion assay
- IgG structural analysis: FT IF, differential scanning calorimetry, 2-dimensional electrophoresis, size exclusion chromatography
- Anti-rabbit ELISA, anti-LPS ELISA
- Elements, chemicals: ICP MS, GC
- Protein analytics: LC-MS, GC-MS, SDS-PAGE
- Forensic screening of final container product for neuroleptic drugs (120 drugs)
- Fluorescence spectroscopy pattern
- Cytokine/chemokine in product: human cytokines and rabbit cytokines
- Bronchospastic guinea pig and hypotensive rat
- Monkey (cynomolgus) study: IV application of most pyrogenic final products: no difference in body temperature/blood cytokine level
- PBMC cytokine release assays (high/low density cells)
- Toll Like receptor assay in THP-1 blue CD14 cells
- Human granulocyte cell line (HL60) activation assay
- Co-culture human saphenous vein (endothelial) Cell + PBMC

➢ **The investigations did not reveal any useful correlation to pyrogenicity**

➢ **No discrimination test (pyrogenic versus non pyrogenic lots) could be found**

Investigations – Pyrogen Test

All involved processes were assessed against potential sources of root cause:

❖ **Animal facilities**
- no unusual observations, events at facility
- no correlation to rabbit source
- no correlation to materials used for testing
- no correlation to environmental conditions at facility

➢ **The investigations did not reveal any correlation or observation, which could explain the pyrogenicity of the product lots**

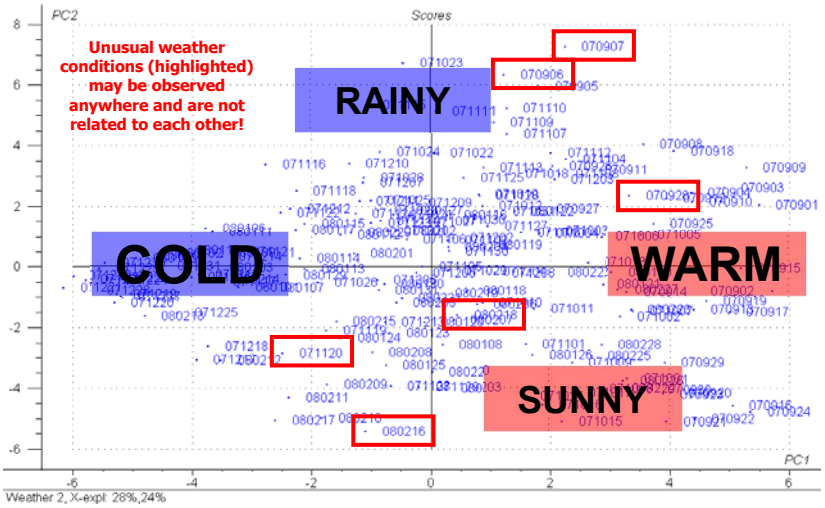
❖ **Supplemental Rabbit Studies**
- Post pyrogen test: cytokines/blood picture and –chemistry
- Pre rabbit test: rabbit whole blood cell adsorption experiment
- Rabbit tissue study: cross-reactivity study in a rabbit model system using immunohistochemistry on cryo tissue sections

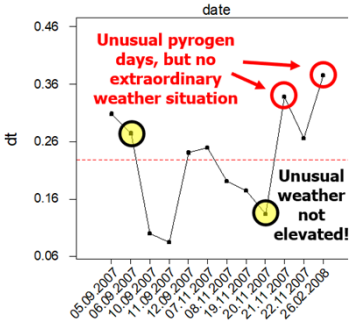
➢ **The investigations did not reveal any correlation to pyrogenicity**

➢ **No discriminatory test could be found**

Multivariate data analysis was performed to correlate weather conditions at the animal testing facility with pyrogen reaction!

Weather conditions 09/2007-11/2007





➢ **No correlation found**

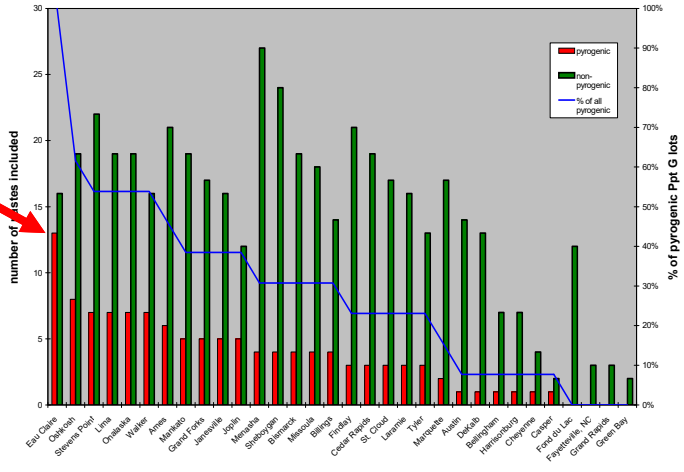
Takeda Data on File

Investigation was extended to the raw material, plasma for fractionation

- Look Back Procedure enabled effective investigation
- Problem: lots are manufactured from plasma pools

➢ **Plasma from a plasma donation center in Wisconsin/USA showed somewhat higher frequency in resulting in pyrogenic IVIG lots**

Eau Claire



Takeda Data on File

The Eau Claire Investigation

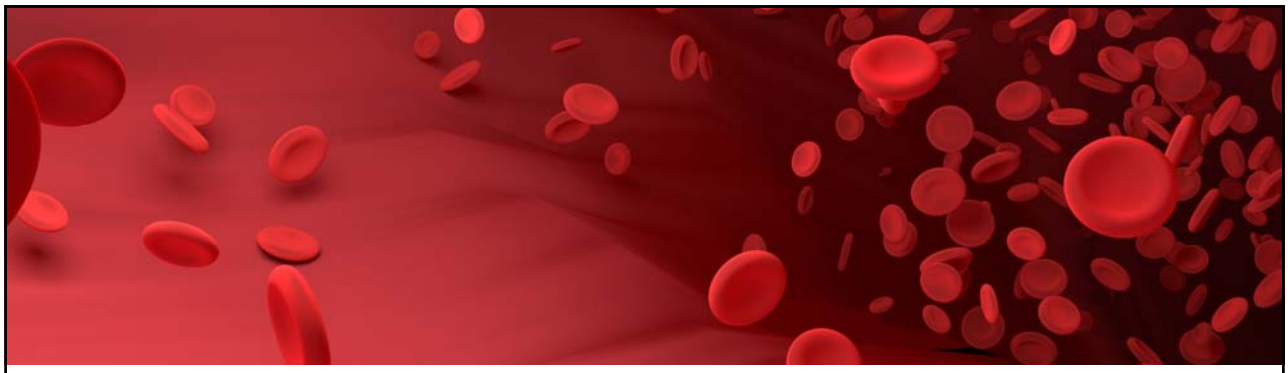
- All potential root causes for contamination of plasma with pyrogenic substances were investigated in greatest detail
  - Contamination of collection bags
  - Environmental contamination
  - Unfavorable storage and/or transport
  - Plasmapheresis equipment and machines
  - Plasma donation process
  - Plasma donors
- **Statistical analysis identified 28 donors from the plasma center as potential contributors to the RPT failures**
  - 11 of the 28 donors plasma were available for testing
- **One individual's plasma was positive in the RPT test at a dilution between 100 to 1000 times higher than control donors!**
  - This was a young woman
  - Review of her medical history, a subsequent enhanced medical history and clinical laboratory testing focused on immune function did not identify any unusual factors
  - The donor was and remains in robust good health

Takeda Data on File

The Eau Claire Incident is not Unique in Plasma Industry

- Grifols recently published a similar case
  - Immunoglobulin G from a single plasma donor in IVIG 10% caused false positive pyrogen test
  - All microbe-related testing, including LAL test for endotoxin, proved negative, and no deficiencies were discovered in manufacturing
  - A single plasma donor ("Donor X″) was common to all pyrogenic IVIG lots
  - One unit of "Donor X″ plasma in a pool of ~4500 units was sufficient to cause lot failure in the rabbit pyrogen test
  - Whole plasma and Protein A-purified IgG from "Donor X″ caused a temperature increase in rabbits; however, all IgG samples tested pyrogen-negative in two in vitro cell-based pyrogen tests (incl. MAT)
  - Flow cytometry showed that "Donor X″ IgG bound strongly to rabbit white blood cells but minimally to human WBC
  - Unusual specificity present in "Donor X″ IgG towards an antigen on rabbit WBC triggers release of a pyrogenic cytokine from these cells that, in turn, triggers a febrile response in rabbits
  - Exclusion of "Donor X″ plasma from manufacturing marked the end of IVIG lots registering positive in the rabbit pyrogen test
- **Confirmed our results indicating that the pyrogenic agent is an IgG or a substance very tightly associated with antibodies only reactive in rabbits!**

Zervos C et al. Biologicals. 2019 May;59:12-19.



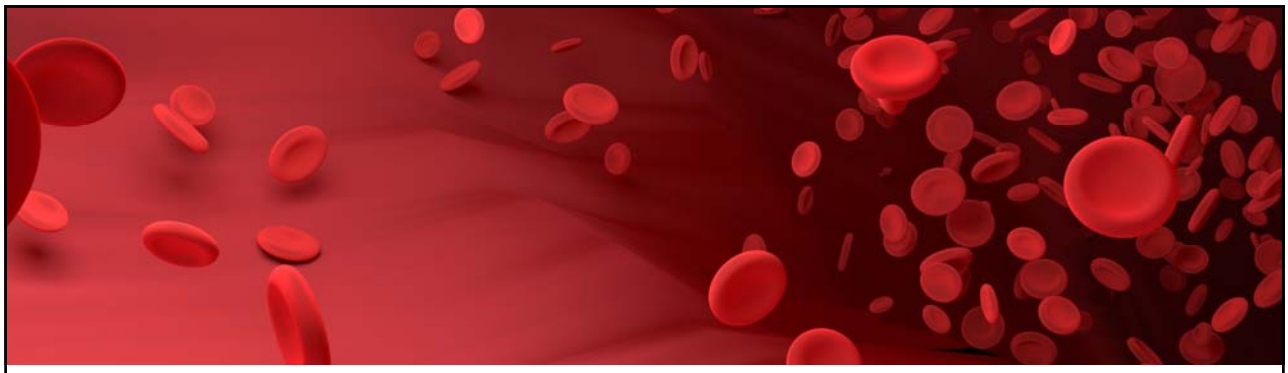
Plasma Industry Status

Better Health, Brighter Future

Pyrogen Testing in the Plasma Industry – Results from a Recent Survey

- All plasma companies (6/6) work on replacement
  - 5/6 successful with alternative LAL test
  - 4/6 companies moved either all (1/6), 70% (1/6) or majority of products (2/6) over from pyrogen test to LAL
- European regulators and FDA accepted switch from pyrogen to LAL test
  - 3/ 6 companies received notices to cease pyrogen testing in Ireland and Germany
  - Remaining authorities are in-progress
- Main challenge is with ROW markets including Latin America where the pyrogen tests is still required by regulators (Peru, Brazil), and for ALL companies (6/6) in SE Asia
  - Authorities in Japan, Korea, Malaysia and China did not accept this variation, thus, tests are now performed in country's local contract lab
- MAT: 2/6 companies received challenges from European regulators about the use of the MAT rather than LAL
  - 1/6 manufacturers addressed this by conducting a risk assessment which has been accepted for those products that have gone through the change process
  - Other manufacturers did not receive any questions from US FDA or other regulators on MAT; switching to LAL has been accepted.

Survey conducted by PPTA in 2018, updated in 2023.



Future Pyrogen Testing Strategy

Better Health, Brighter Future

Rabbit Pyrogen Test (RPT) – Ph. Eur. Chapter 2.6.8 - Pyrogens

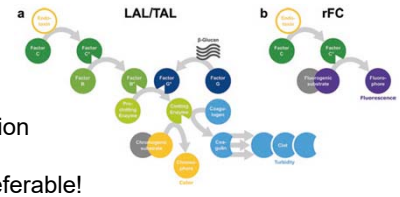
- In Takeda programs to reduce or eliminate animal tests were initiated in the 1990s, which already included the RPT
- Between 2014 and 2017 Takeda systematically replaced the RPT by the Bacterial Endotoxin Test (BET) for all plasma derived products wherever possible
  - Vast majority of countries have accepted and approved the change from RPT to BET
  - Few countries in Asia remained on RPT: South Korea, Japan, China
  - In these countries RPT is performed locally, no more RPT conducted in Europe
  - Takeda currently performs ~ 50.000 BET per year only for plasma derived therapies
- Takeda continues working on complete elimination of RPT globally
  - Asian countries are observing developments of Ph. Eur.
  - It is hoped that revision of monographs with elimination of RPT would lead the way to switching to non-animal alternatives also there

Takeda Data on File

Replacement of Bacterial Endotoxin Test (monograph 2.6.14) by BET using recombinant Factor C (according to monograph 2.6.32)

- LAL (or TAL) is a lyophilised product obtained from amoebocyte lysate from the horseshoe crab (*Limulus polyphemus or Tachypleus tridentatus*)
  - Classical LAL/TAL test is an indirect animal test
  - In 2020 >500,000 horseshoe crabs were harvested for biomedical production
  - Horseshoe crabs harvested for LAL are returned-to-sea with a mortality of 15%
  - Although horseshoe crabs are released after medical production or studies, they would become vulnerable, and their mortality rate might increase; female horseshoe crabs released back to the sea encounter difficulties in spawning

- Besides animal welfare considerations the high variability of "classic" reagents used for BET testing on plasmatic samples is the most common cause of invalid results
  - The complexity of reactions can easily result in false positive results due to enhancement effect
  - Variability intra lots for cartridges or endotoxin/lysate reagents
  - Influence of dilution buffer
- In contrast to LAL/TAL, rFC assays solely rely on the enzymatic function of Factor C
- Though more expensive, BET using recombinant Factor C seems preferable!

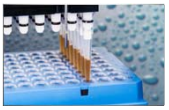


- Gauvry GA et al. LAL/TAL and Animal-Free rFC-Based Endotoxin Tests: Their Characteristics and Impact on the Horseshoe Crab Populations in the United States and Asia. in J. T. Tanacredi et al. (eds.), International Horseshoe Crab Conservation and Research Efforts: 2007-2020, Springer Interna Publishing, 2022
- Gorman R. Atlantic Horseshoe Crabs and Endotoxin Testing: Perspectives on Alternatives, sustainable Methods, and the 3Rs (Replacement, Reduction, and Refinement). Front Mar Sci. 2020 Sep 30;7:fmars.2020.582132.
- Anderson RL, Watson WH 3rd, Chabot CC. Sublethal behavioral and physiological effects of the biomedical bleeding process on the American horseshoe crab, Limulus polyphemus. Biol Bull. 2013 Dec;225(3):137-51.

Takeda is Evaluating the Most Feasible Options to Replace BET by BET using recombinant Factor C

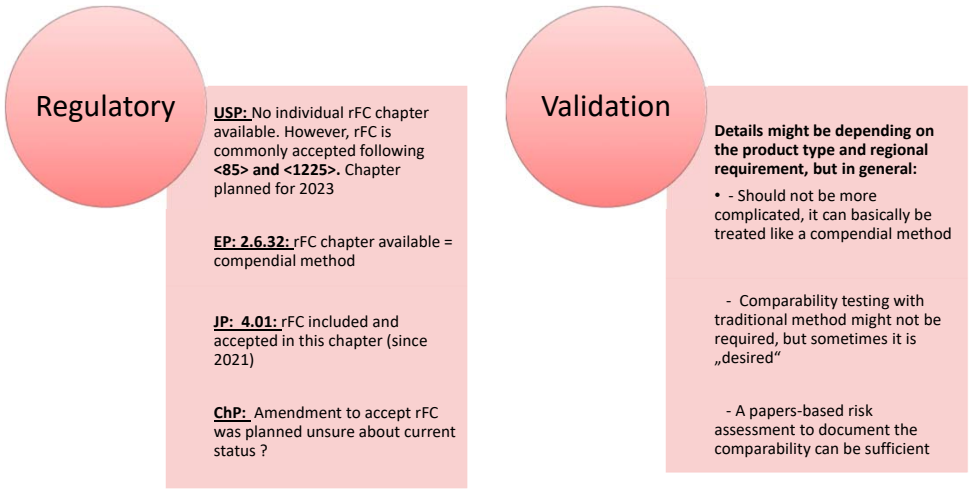
	Chrom. / Turb. Kinetic	PTS™ / MCS™ Cartridges	Endozyme® II GO Strips (rFC)	EndoLisa® (rFC)
Source of the Reagents	Animal based (variable)	Animal based (variable)	Recombinant (standardized)	Recombinant (standardized)
Sensitivity (EU/ml)	0.005 EU/ml	0.05 EU/ml	0.05 EU/ml	0.05 EU/ml (lower dilutions possible)
Standard Curve	Manual	Archived	Pre-coated (minimal preparation)	Pre-coated (minimal preparation)
Interfering Factors	Multiple	Multiple	Low	Low
False positive due to ß-Glucan	Yes	Yes	No	No
Reading	Kinetic	Kinetic	Endpoint	Endpoint
Time to Result 21 Samples	50 min – 90 min	90 min (MCS only, PTS too low capacity to compare)	50 min	180 min
Hands-on Time 21 Samples	20 -30 min	60 – 80 min	20 -30 min	20 -30 min
Throughput	High to very high if automated	Low to high if automated with Nexus	High to very high if automated	High

- Preference for ELISA type assays



Takeda Data on File

BET using recombinant Factor C: Validation and Regulatory Aspects



- Needs to be carefully evaluated as impact is huge
  - Takeda performs approx. 50.000 BET per year only for plasma derived therapies

Monocyte Activation Test, MAT – Ph. Eur. Chapters 2.6.30/2.6.40 /1

- Takeda has vast and long-term experience with MAT test
  - Active participation in *IN VITRO* PYROGEN TEST WORKSHOP, organized by EDQM in Sep 2005
  - Based on in-house experience Baxter (now Takeda) endorsed Human Monocyte Cytokine Release Assays to be included in Ph. Eur. as an alternative method to RPT as long a reference method is specified
  - Prerequisite is that assay can be validated and shows suitability of cytokine release assay for a particular product type
  - Company position in 2005 was, that both BET (LAL) and MAT could replace RPT and both tests should be used alternatively or in combination depending on product/substance specific assessments

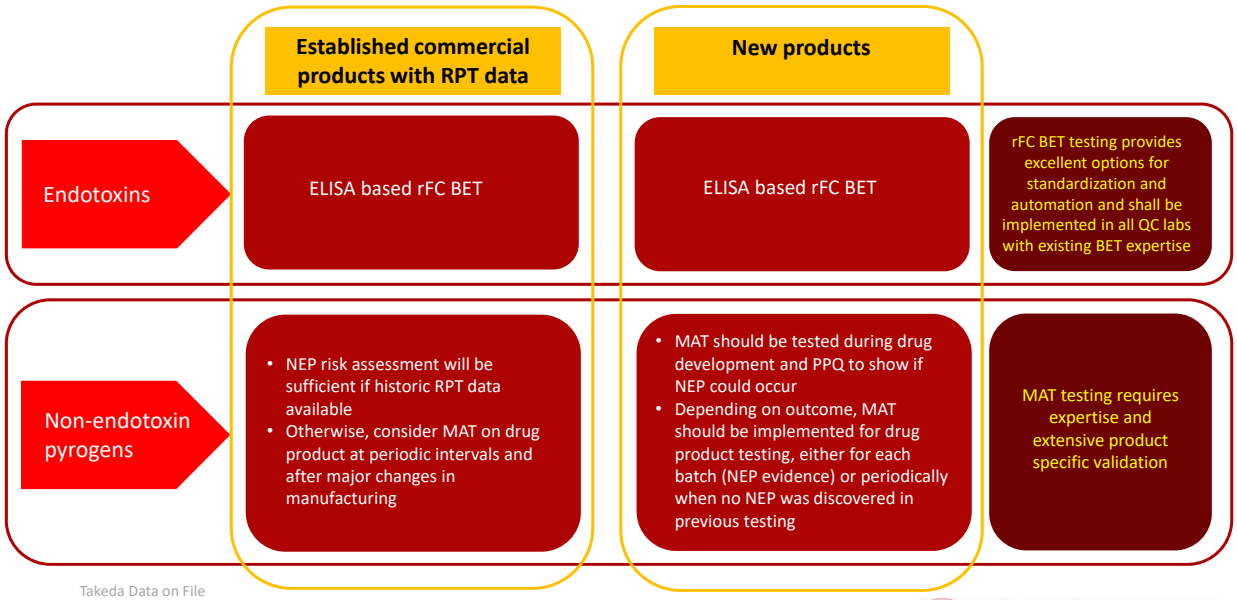
Monocyte Activation Test, MAT – Ph. Eur. Chapters 2.6.30/2.6.40 /2

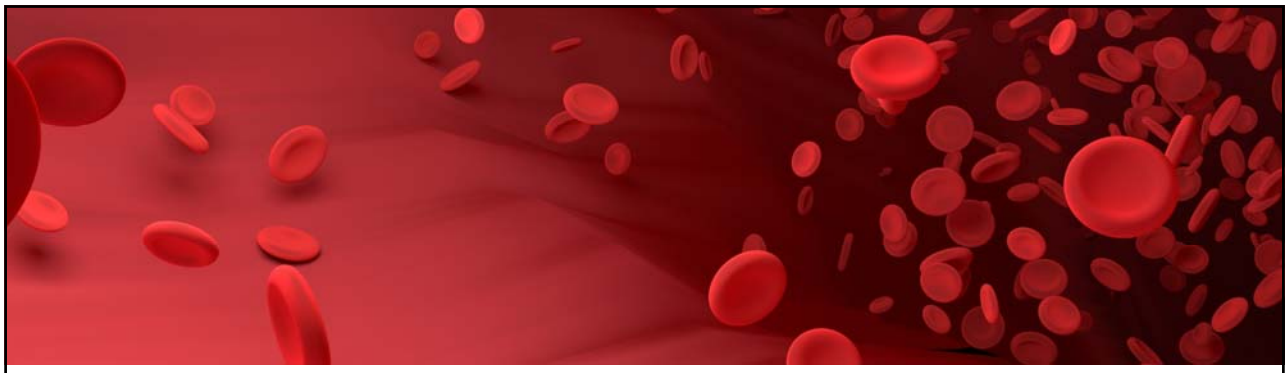
- Takeda´s position is also reflected in new Chapter 5.1.13 Pyrogenicity
  - Requiring risk analysis of potential presence of non-endotoxin pyrogens and the respective manufacturing process

Caution:

- European regulators, FDA, and in most other legislations switch from pyrogen to LAL test accepted
- Implementation of a new test system always requires extensive validation
- Difficult to perform for a pharmaceutical company manufacturing biologicals for rare disease
  - Some products are rarely produced – sometimes only 1 or 2 batches per year
  - Validation requires statistically significant number of batches

Future Pyrogen Testing Strategy





Summary and Conclusions

Better Health, Brighter Future

Removal of the Rabbit Pyrogen Test from Ph. Eur. is Highly Appreciated

- Biopharmaceutical drug products are very valuable materials
- Examples of false positive RPT results
  - Caused withdrawal of drug products derived from human plasma
  - Waste of highly valuable materials, not only from a monetary but also ethical point-of-view
- Takeda worked on replacement of animal testing incl. RPT for > 30 years
- If RPT would not still be requested by some regulatory authorities around the world, the industry would have switched entirely to alternative methods
- BET seems easier than MAT to implement and to gain regulatory approval
- New Ph. Eur. Chapter 5.1.13 Pyrogenicity is aligned with approaches taken by the bio-pharmaceutical and plasma industry
  - Product- and manufacturing process specific risk analysis of potential presence of non-endotoxin pyrogens will ensure patient safety while optimizing resources

Acknowledgment

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Alfred Weber
Wolfgang Teschner

Takeda Corporate Affairs Plasma Derived Therapies
Deborah Hibbett

Plasma Protein Therapeutics Association PPTA
Dominika Misztela

Thank you for your attention!

Questions ?

Takeda

Takeda Pharmaceutical Company Limited

Comparison of Pyrogenicity Assays for Products Exhibiting Low Endotoxin Recovery

14 February 2023

Ned Mozier

Pfizer

Topics

1. Origin Story – genesis of the “idea” for a study
2. Study goals – main objective / ancillary objectives
3. Study design – the “devil in the details”
4. Study execution – a single “shot on goal”
5. Results – *what does the data say*?
6. Interpretation – *what did we learn?*
7. Broader Takeaways / *Future Study Ideas*

Pfizer

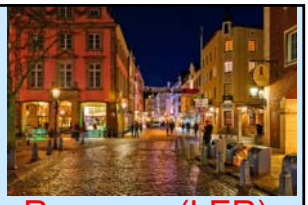
Origin / Ideation Story – Nov ‘16

or “a night in Dusseldorf” after the Pharmalab main event

Just prior to the US election

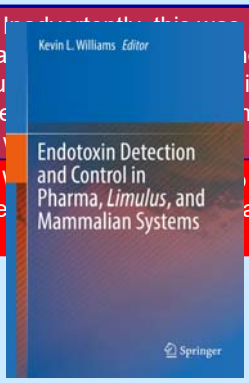
3 years before PDA TR82 was published

No good solutions existed for samples with Low Endotoxin Recovery (LER)



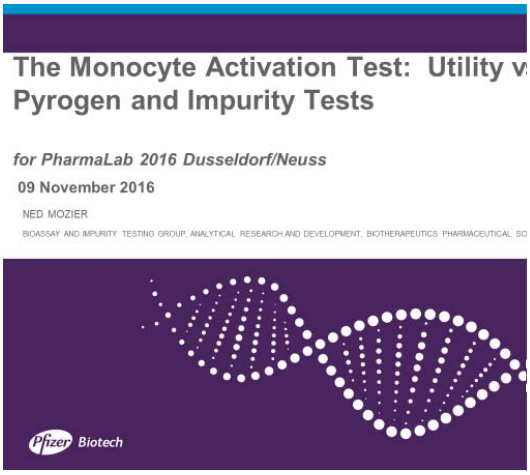
FDA-CDER-OBP ~2014/2015

USP LAL	Rabbit Pyrogen Test		Endotoxin Control Strategy
	Non- spiked finished product	Spiked finished product	
No LER	Non pyrogenic	-	Microbial control during manufacturing; USP LAL is suitable as a release test method.
LER	Non-pyrogenic	Pyrogenic	Microbial control during manufacturing; Rabbit Pyrogen test as release test (interim measure); PMC to develop a suitable *in vitro* test method.
LER	Non-pyrogenic	Non-pyrogenic	Microbial control during manufacturing; Risk assessment; Endotoxin specification at the BDS stage prior to PS addition; Microbial control of input materials; PMC to develop a suitable *in vitro* test method.
LER	Pyrogenic	-	Reject product

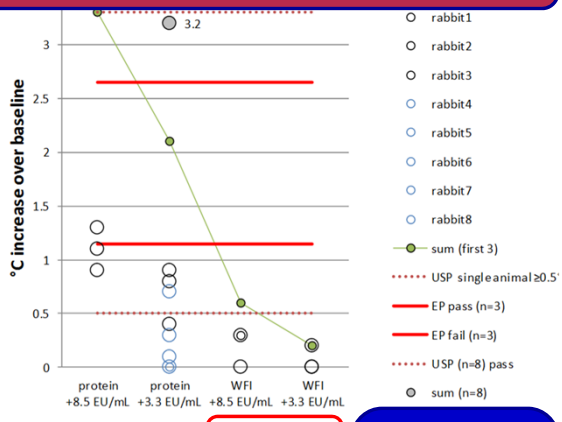


Pfizer

Shown in 2016



1. Was our sample and/or endotoxin unique?
2. Obvious Practical Challenges with Study Design
3. NOE ≠ RSE in MAT



EU/kg, nomial (spiked)	30.7	9.6	22.2	9.5
measured BET (EU/kg), F/T	9.8	2.0	15.7	4.6
measured BET (EU/kg), retains	6.1	0.5	4.2	1.1

[RSE]: nominal vs “actual” not pyrogenic at up to 4X the 5 EU/kg limit in WFI.

Consistent with other informal reports of high LPS passing RPT

"Ring Trial" Study Goals – Questions we Hoped to Answer

1. Do products with LER behave similarly? (vs product-specific phenomena)

2. Is the MAT inferior, superior or equivalent to the RPT in samples of known [endotoxin] when "masked" in the BET (i.e. LER)?

3. Are rabbits sensitive to very high levels purified endotoxin?
   - The CRO was different than the one used for the prior Pfizer study

4. How do results compare when samples are tested in Whole Blood (WB) vs PBMCs in MAT?

   - Would have preferred to test >1 cytokine / cell type (as in the prior Pfizer study), but impractical at the CRO for our study (vendor kit format used)

Whole Blood	IL-1β
PBMCs	IL-6

Pfizer

PDA Low Endotoxin Recovery Technica

Dayue Chen, PhD, Eli Lilly and Company, C
Friedrich von Wintzingerode, PhD, Genente
Julie Barlasov-Brown, Merck & Co.
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Jessica Hankins, PhD, U.S. Food and Drug A
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Cheryl Platco, Merck & Co. (retired)
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Stijn Seels, Sanofi
Anders Thorn, Novo Nordisk A/S
Masakazu Tsuchiya, PhD, Charles River Labo
René Ørving, Biogen

Pfizer

5.2.2 Product Dosage

Another major consideration in conducting animal or in vitro studies is the dose of the product tested. For the traditional pyrogen test in rabbits with unspiked product, animals are administered a quantity of drug equivalent to the maximum dose per kg of body weight of a human subject. For example, a fixed dose of 1 mL of a 150 mg/mL drug product given subcutaneously to human subjects weighing 50-100 kg would be calculated as follows:

150 mg per 50 kg (Smallest Patient) = Max Exposure of 3 mg/kg

In compendial RPT methods, administration of very small-volume doses is neither recommended nor practical, so dilution is required prior to treatment. This is complicated by the fact that LER samples are expected to be spiked "neat," that is, undiluted. A guide for conducting the LER hold-time and preparation of dosing solutions for pyrogen test in rabbits, based on an assumed human dose of 3 mg/kg active pharmaceutical ingredient containing a targeted 35 EU/kg of endotoxin, and matching per kg human dosing, is as follows:

1. Use protein sample of 150 mg/mL
2. Reconstitute RSE (10,000 EU) with 1 mL LRW to achieve 10,000 EU/mL
3. Combine and mix 0.876 mL (876 µL) of stock RSE with 5 mL of protein sample
   a. (10,000 EU/mL) * (0.876 mL/[0.876+5.0 mL]) = 1491 EU/mL
   b. (150 mg/mL) * (5.0 mL/[5.0 mL+0.876mL]) = 128 mg/mL
4. If sample is to be held three (3) days, test these samples immediately (T_0) and after hold time
   a. Dilute 0.78 mL of spiked LRW or sample in 99 mL of PBS
   b. Protein concentration = (128 mg/mL) / (0.78 mL/[0.78 mL + 99 mL]) = 1.0 mg/mL
   c. RSE concentration = (1491 EU/mL) / (0.78 mL/[0.78 mL + 99 mL]) = 11.6 EU/mL
   d. For a 3.0 kg rabbit, administer 9 mL of the above (4a-4d)
   e. Check
   i. 1.0 mg/mL protein * 9 mL = 9 mg protein
      - 9 mg/3 kg = 3 mg/kg protein dose
   ii. 11.6 EU/mL RSE * 9 mL = 105 EU
      - 105 EU/3 kg = 35 EU/kg RSE dose
   f. Based on individual rabbit weights, administer 3 mL/kg for the RPT

Before conducting LER studies in animals, ensure that the unspiked sample in the RPT is not inherently pyrogenic. The same should be done before conducting the MAT design described in **Section 5.2.3.** Data analysis for both the RPT and MAT LER studies is discussed in **Section 5.3.**

l Approach was
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nical Report No. 82

Endotoxin Recovery

Study design – Key Features

1. Avoid the sample handling challenges by using one facility for all tests
2. Test appropriate samples and compare MAT to RPT
   - Select products previously shown to be non-pyrogenic in RPT
   - Select products shown to exhibit LER in 3 days or less
   - Compare to an identically prepared water control
   - Use RSE as source of LPS as spiking solution
3. Test from same vial at the same time in the same laboratory
   - Control for sample handling to directly compare assays
4. Pre-dilute each spiked product and water to achieve:
   - Target mg protein per kg in RPT based on human dose
5. Dilute pre-dilutions identically for BET & MAT

Pfizer used same drug substance tested for TR82 and the preliminary correlation study

Product 1	T0	T3
Product 2	T0	T3
Product 3	T0	T3
Water	T0	T3
Total	8 samples	

Pfizer

Study execution

1. No **single** facility was **proficient in all** 3 assays: RPT, MAT & BET
2. The company chosen struggled to perform MAT, so we split the study
   - One company did the RPT
   - Another did both BET & MAT
3. Pfizer person observed to assure samples prepared identically at both sites
   - This gave us high confidence that results could be compared
4. The 3 Product doses varied widely, so initial spiking was varied to achieve 35 EU/kg and product-specific (mg/kg) goals in the final dosing solution
5. First samples prepared 3 days earlier (T3) and fresh (T0), then all 8 samples tested at the same time (the “Reverse Mode” as per TR82)
6. Execution of Study was Flawless – “right the first time”
   - ✓ **All assays performed as close in time as humanly possible**

Product 1	T0	T3
Product 2	T0	T3
Product 3	T0	T3
Water	T0	T3
Total	8 samples	

Pfizer

Study Completed in 2018, published last year

ALTEX, accepted manuscript
published June 24, 2022
doi:10.14573/altex.2202021

Research Article

Comparison of Pyrogen Assays by Testing Products Exhibiting Low Endotoxin Recovery

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[1]Pfizer, Chesterfield, MO, USA; [2]CJLahti Consulting Services, Albany, CA, USA; [3]Amgen, Thousand Oaks, CA, USA; [4]EirGenix, Inc., Taiwan, Republic of China; [5]Genentech, a Member of the Roche Group, South San Francisco, CA, USA; [6]Roche Diagnostics GmbH, Penzberg, Germany

Abstract
The use of pyrogen tests to assess the risk of endotoxin in biological products has increased recently due to concerns of some regulatory authorities about products exhibiting low endotoxin recovery (LER). Manufacturers increasingly seek to reduce the use of animals unless essential to assure patient safety. The current study compares the ability of the monocyte activation test (MAT) and the bacterial endotoxin test (BET) to the rabbit pyrogen test (RPT) to detect endotoxin spikes in samples of products shown to exhibit LER. Product samples or water were spiked

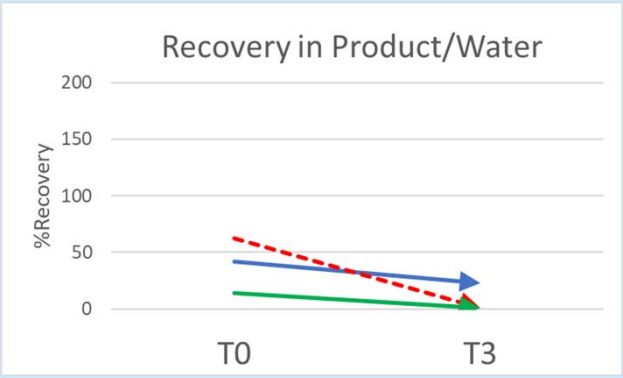
BET Results

Tab. 1: Bacterial endotoxin testing results (values are average of n=2)
Percentage spike recovery <50% (marked in red) indicates LER.

	Dilution	EU/mL	
		T0	T3
LRW	10	8.6449	6.294
	40	13.9082	10.3684
	400	23.3872	19.8752
	Mean	**15.3**	**12.2**

Good recovery in LRW vs. nominal (19.7 EU/mL)

Recovery in products calculated vs measured EU/mL in LRW at each timepoint



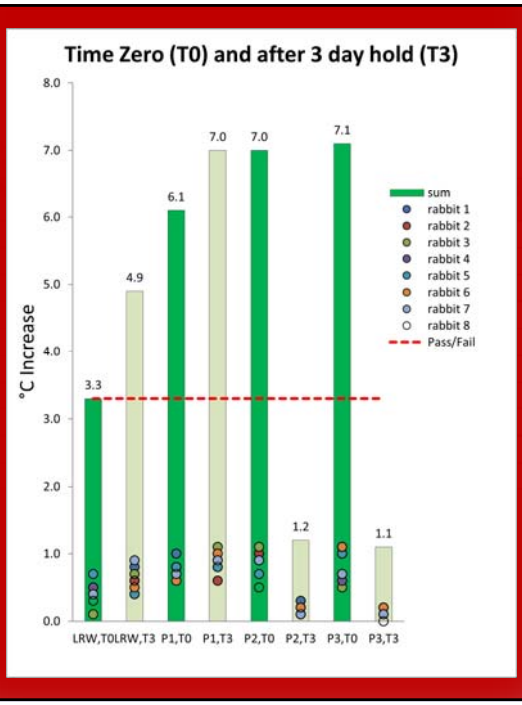
The expected LER confirmed, and it begins quickly (at T0) for 2 of 3 products

Pfizer

RPT Results

Increased body temperature ≥0.5°C and sum of in

	LRW T0	LRW T3	Product 3 T3
rabbit 1	**0.5**	**0.8**	0.2
rabbit 2	0.4	**0.6**	0.1
rabbit 3	0.1	**0.7**	0.2
rabbit 4	**0.5**	0.4	0.2
rabbit 5	**0.7**	0.4	0.1
rabbit 6	0.4	**0.5**	0.2
rabbit 7	0.4	**0.9**	0.1
rabbit 8	0.3	**0.6**	0
sum (n=8 rabbits)	**3.3**	**4.9**	1.1
RPT result	FAIL	FAIL	PASS



Pfizer

MAT Design Summary

Whole Blood IL-1β
PBMCs IL-6

All samples (and water) have the identical amount of endotoxin and are diluted to this common concentration, then all diluted in exactly the same volumes to be in range of the MAT

Tab. S5: Preparation of Samples for MAT using 1.97 EU/mL predilutions from Table S3

Assay type	Total Fold Dilution	49-fold	66-fold	99-fold	131-fold	197-fold	263-fold
MAT	Expected Result (EU/mL)	0.40	0.30	0.20	0.15	0.10	0.075
	sample (mL)	0.100	0.100	0.050	0.050	0.050	0.050
	Kit-specific media (mL)	0.390	0.560	0.445	0.605	0.935	1.265
	Total Volume (mL)	0.490	0.660	0.495	0.655	0.985	1.315

	1	2	3	4	5	6	11	12
A	standard 1			Product 1: 49-fold dilutio			uct 3: 49-fold dilution	
B				Product 1: 66-fold dilutio			uct 3: 66-fold dilution	
C				Product 1: 99-fold dilutio			uct 3: 99-fold dilution	
D				Product 1: 131-fold dilutio			uct 3: 131-fold dilution	
E				Product 1: 197-fold dilutio			uct 3: 197-fold dilution	
F				Product 1: 263-fold dilutio			uct 3: 263-fold dilution	
G				LRW: 49-fold dilution			W: 197-fold dilution	
H				LRW:66-fold dilution			RW: 263-fold dilution	

Same lot of RSE that was used for hold time spike study

For discussion
Within-in plate replicate strategy

n=2,3 or 4?

Final sample diluent is "Kit-specific media"

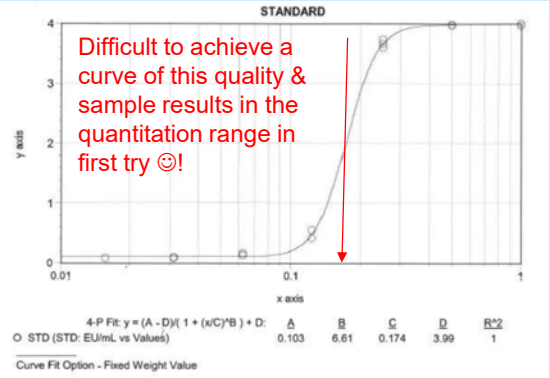
The endotoxin concentration calculated **before** adding to cells+media is how we report [RSE]

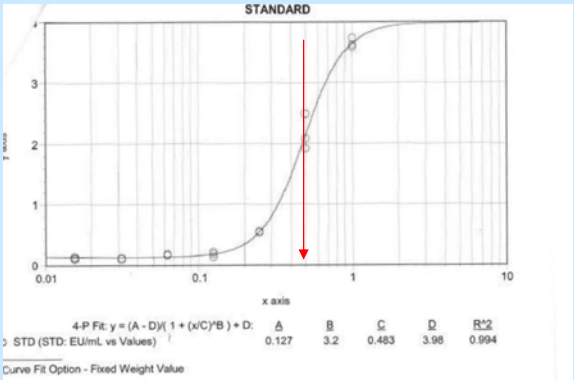
Secreted cytokines measured, cells not intentionally disrupted secreted

One plate for all four T0 Samples, another for the four T3 samples

Pfizer

MAT RSE Standard Curve; IL-6 by PBMCs



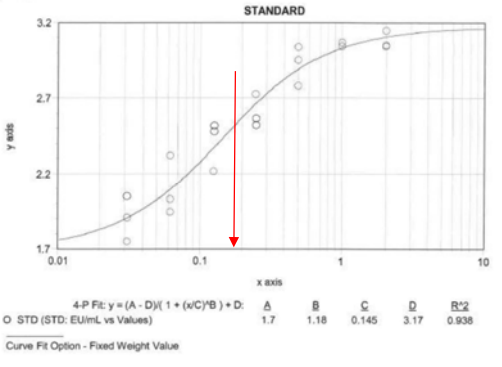


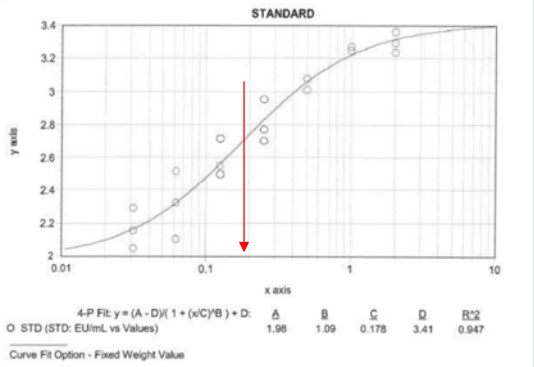
1. **Close agreement between replicates (n=3)**
2. **Steep slope ("on / off") but achieved good dose response**
3. **Fairly good upper & lower asymptotes**
4. **EC_{50} varies by >>2X between plates**

This is why within-plate comparisons of samples are most powerful (e.g. recovery in sample vs water)

Pfizer

MAT RSE Standard Curve; IL-1β in Whole Blood





1. **Poor agreement between replicates (n=3)**
2. **Poorly defined asymptotes (wider range of concentrations needed)**
3. **Similar but shallow Slopes**
4. **EC_{50} similar between plates**

Pfizer

MAT Results – RSE (LPS) in water

1. **Nominal LPS is 19.7 EU/mL**

Tab. 3: MAT PBMCs, IL-6 and MAT WB, IL-1β; values are average of n=3

	Dilution	EEU/mL PBMCs, IL-6		EEU/mL WB, IL-1β	
		T0	T3	T0	T3
LRW	49	49	31.4	11.4	14.2
	66	20.5	35.8	12.1	18.9
	99	21.6	31.3	13.1	11.4
	131	23.1	39.7	15.9	18.5
	197	15.2	33.6	8.9	12.4
	263	32.1	42.1	8.5	19.2
	Mean	22	36	12	16

This kind of variability (182% recovery) is not unusual, could be the standard curve prep for this particular plate (raw data suggests). No one believes that endotoxin in water is increasing over the 3 days! This is why **comparing results within a plate** (e.g. recovery in sample vs in water) is best and why **plate to plate comparison of raw numbers is can be misleading.**

Pfizer

Comparative Results for 4 Assays (2X MAT)

Results (T0 vs T3) not statistically different for any assay but RPT & MAT trend together in LRW

Tab. 4: Summary of data from BET, RPT and MAT assays
Values indicating <50% recovery (LER) or passing RPT (n=8, USP<151>) are marked in red.

	BET [EU/mL]	RPT (sum n=8) [°C]	MAT PBMC-IL-6 [EEU/mL]	MAT WB-IL1β [EEU/mL]
LRW (T0)	15.3	3.3	22	12
LRW (T3)	12.2	4.9	36	16
Product 1 (T0)	6.4	6.1	18	34
Product 1 (T3)	2.8	7.0	21	49
Product 2 (T0)	9.4	7.0	<1	<2
Product 2 (T3)	0.2	1.2	<1	<2
Product 3 (T0)	2.1	7.1	9	4
Product 3 (T3)	0.1	1.1	<1	<2

MAT & RPT trend together but not statistically different (prior MAT suggestive)

Pfizer

Major Findings / Conclusions

1. At both CROs, Rabbits require >>>>>>> 5 EU/kg RSE to cause pyrogenicity
2. RSE in water is recovered well in BET & both MATs, less so with RPT
   - BET & MAT are ≥ 100X more sensitive to RSE than the RPT
3. LER-Prone Products **at T3** show positive correlations of MAT to RPT

	EU/mL **BET**	Σ°C, n=8 **RPT**	EEU/mL **MAT**/PBMC/IL6	EEU/mL **MAT**/WB/IL1β
Product 1	**2.8**	**7.0** (Fail)		
Product 2	**0.2**	**1.2** (pass)		
Product 3	**0.1**	**1.1** (pass)		

In hindsight, for MAT should have put 2 samples / plate (T0 & T3 for each)!

4. RPT adds no additional information beyond MAT as a pyrogenicity test for LER resolution for relevant samples of these 3 products

MAT is not easy – careful design, superb execution and clear standards of assay performance are necessary to make sense of the data!

Pfizer

Research Article

Comparison of Pyrogen Assays by Testing Products Exhibiting Low Endotoxin Recovery

Tammy L. Thurman[1], Carol J. Lahti[2], Jeanne M. Mateffy[3], Ren-Yo Forng[4], Friedrich von Wintzingerode[5], Lindsey M. Silva[5], Sven M. Deutschmann[6] and Ned M. Mozier[1]

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Abstract

The use of pyrogen tests to assess the risk of endotoxin in biological products has increased recently due to concerns of some regulatory authorities about products exhibiting low endotoxin recovery (LER). Manufacturers increasingly seek to reduce the use of animals unless essential to assure patient safety. The current study compares the ability of the monocyte activation test (MAT) and the bacterial endotoxin test (BET) to the rabbit pyrogen test (RPT) to detect endotoxin spikes in samples of products shown to exhibit LER. Product samples or water were spiked with endotoxin and held for three days or tested immediately in the BET, the RPT and two variations of the MAT at the same time. Results show high sensitivity to endotoxin of both the BET and MAT and much lower sensitivity in the RPT, indicating that much higher levels of reference standard endotoxin are required to induce pyrogenicity in the RPT than the 5 endotoxin units (EU) per kg common threshold. The results of the BET and MAT correlated well for the detection of endotoxin spike in water. We also show that LER (masking of endotoxin) found in the BET is also seen in the MAT and RPT, suggesting that the products themselves elicit a biological inactivation of spiked endotoxin over time, thereby rendering it less or non-pyrogenic. We conclude the non-animal MAT option is a suitable replacement for the RPT to measure spiked endotoxin in biopharmaceuticals.

Broader Take-aways

Back to the Original Purpose of our Study

FDA-CDER-OBP ~2014/2015

USP LAL	Rabbit Pyrogen Test		Endotoxin Control Strategy
	Non- spiked finished product	Spiked finished product	
No LER	Non pyrogenic	-	Microbial control during manufacturing; USP LAL is suitable as a release test method.
LER	Non-pyrogenic	Pyrogenic	Microbial control during manufacturing; Rabbit Pyrogen test as release test (interim measure); PMC to develop a suitable *in vitro* test method.
LER	Non-pyrogenic	Non-pyrogenic	Microbial control during manufacturing; Risk assessment; Endotoxin specification at the BDS stage prior to PS addition; Microbial control of input materials; PMC to develop a suitable *in vitro* test method.
LER	Pyrogenic	-	Reject product

"We conclude the non-animal MAT option is a suitable replacement for the RPT to measure spiked endotoxin in biopharmaceuticals"

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Pfizer

Acknowledgements

- Fellow brainstormers that night in Dusseldorf
- Tammy Thurman
- Carol Lahti
- John Dubczak, Jack Levin
- Thomas Hartung
- And our other Altex coauthors:

Tammy L. Thurman[1], Carol J. Lahti[2], Jeanne M. Mateffy[3], Ren-Yo Forng[4], Friedrich von Wintzingerode[5], Lindsey M. Silva[5], Sven M. Deutschmann[6] and Ned M. Mozier[1]

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Exploiting the Monocyte Activation test for assessing the pyrogenicity of vaccines: instances from industry

Liliana Alleri
GSK, Rosia, Italy

The future of pyrogenicity testing
February 15th , 2023

This work is sponsored by GlaxoSmithKline Biologicals SA. Liliana Alleri is employed by the GSK group of companies

gsk.com

Outlook

- GSK 3R strategy
- MAT assay overview
- MAT assay for intrinsically pyrogenic product: the example of Bexsero*
- MAT assay for all other products: instances of semi-quantitative and quantitive methods
- Risk-based approach for phasing out RPT

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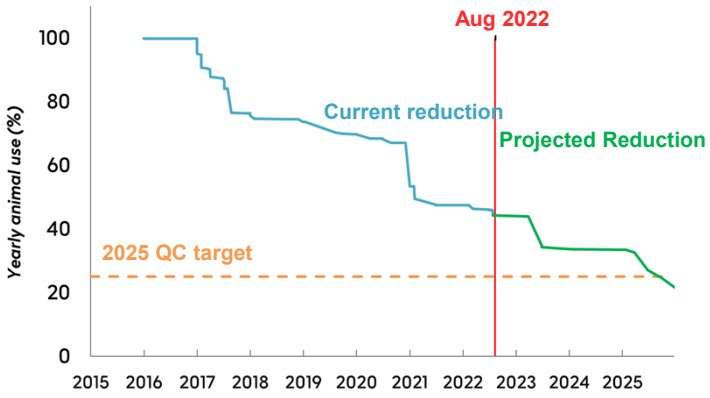
** Bexsero is a trademark owned by the GSK group of companies.*

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

Animal use in lifecycle has been already reduced by 75% from 2007 to 2015

More information @ GSK web site **The 3R at GSK**



Conducting animal studies with high standards of **humane care and treatment is** GSK's **moral responsibility** and priority.

Historically **refinement** had the highest **priority**. Having those high animal welfare standards and the availability of **new non-animal-technologies** allows a **focus on replacement**

3R Portfolio in QC

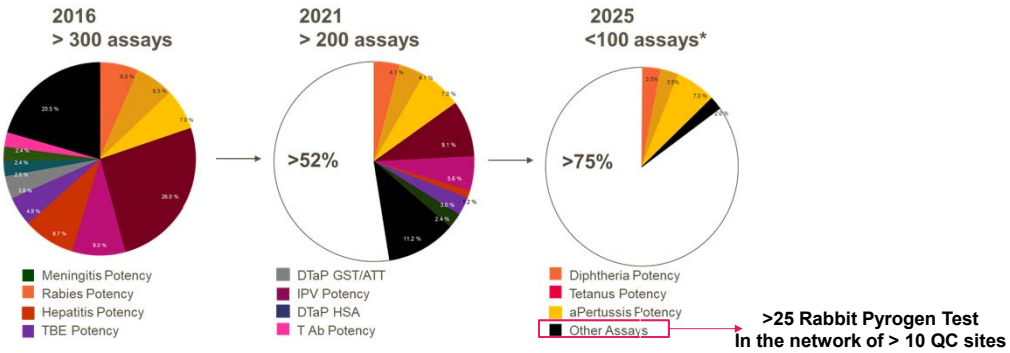
Replacement 80%

Reduction 15%

Refinement 5%

3R & GSK: Animal assays are conducted in Release stability and for process changes in Quality

A substitution at all three stages simultaneously is time consuming.



The RPT is a small but crucial part of GSK 3R strategy to ensure patient safety without animal testing.

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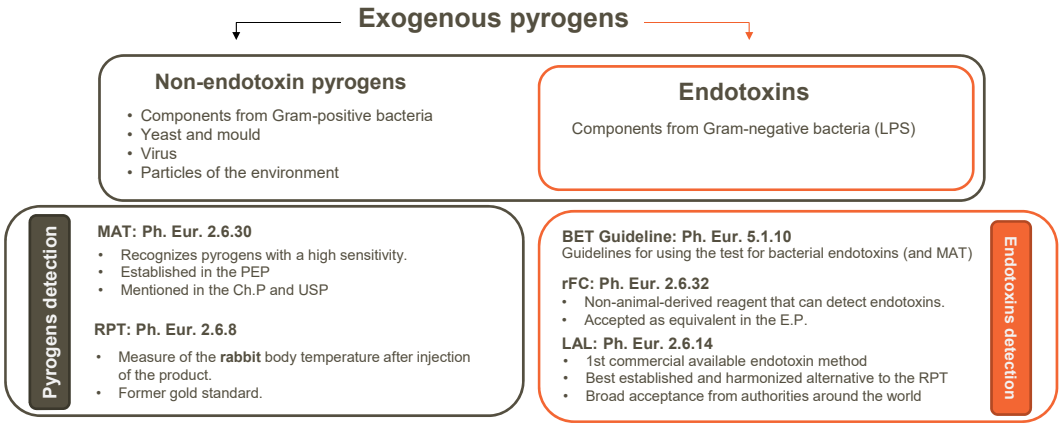
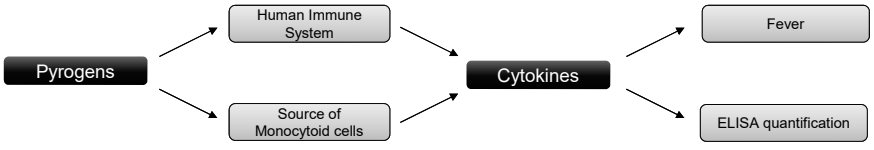
Pyrogen detection

Several techniques are available allowing animal-free detection of pyrogens

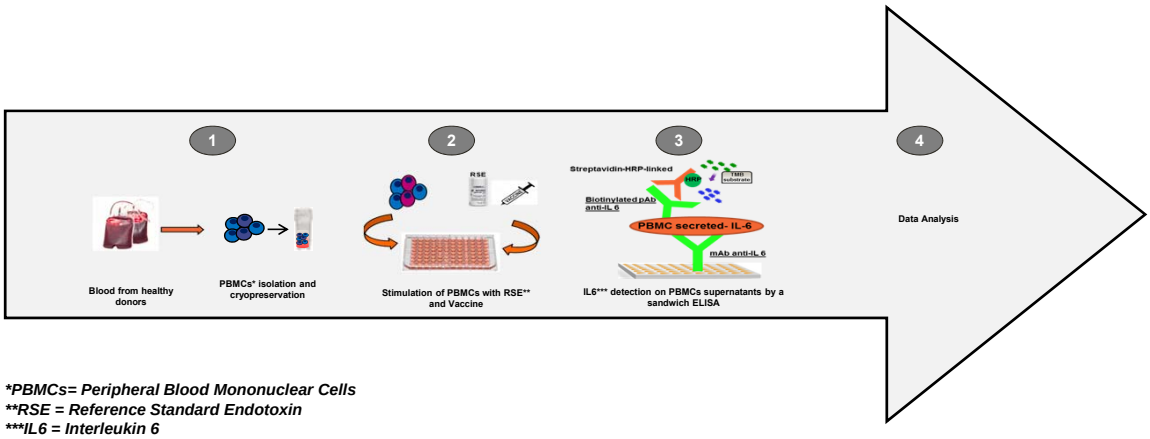


The Monocyte Activation Test (MAT): the human cell-based tool to predict pyrogenic content of products

- The MAT is an *in-vitro* cell-based assay.
- Principle of test: human monocytic cells secrete considerable amounts of fever-inducing mediators (proinflammatory cytokines) in response to any contact with exogenous pyrogens (fever-causing agents).
- Advantages of using MAT in a QC environment:
  - A much lower variability as compared to the *in-vivo* methods with higher sensitivity
  - Reduced time for lot release testing for a sustainable supply
  - Fully alignment with 3Rs principles ((Refine, Reduce, Replacement)



GSK QC release assay design of MAT



PBMCs= Peripheral Blood Mononuclear Cells
RSE = Reference Standard Endotoxin
****IL6 = Interleukin 6***

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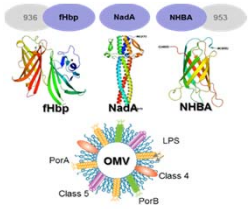
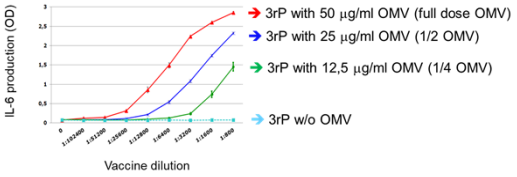
The Bexsero vaccine elicits cytokines production in the cells

- ✓ 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
- ✓ The test replaces both LAL and RPT tests on Bexsero DP for EU/RoW while it replaces RPT only for US



Bexsero components

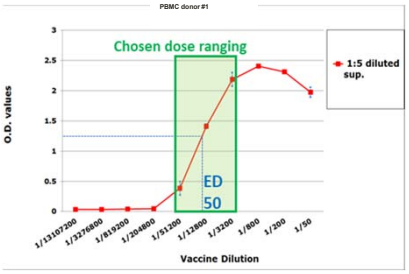


IL-6 production in hPBMC stimulated with ad hoc Bexsero formulations containing different quantities of OMV components.

A 'Reference lot comparison test' using a Bexsero lot as Reference (Ph. Eur. 2.6.30) is applied for QC release of lots

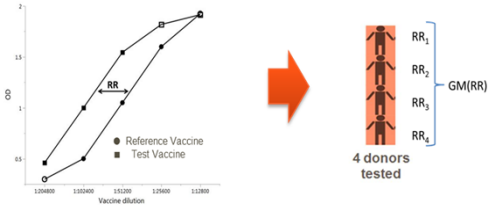
1 ***Donor qualification step***

- ➢ 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



2 ***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. N° of donors can be selected based on the desired assay variability



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GSK

15 February 2023

Quantitative and semi-quantitative MAT assays

- Quantitative and semi-quantitative MAT methods foresee the comparison of the tested preparation with the Standard Endotoxin which is used as Reference Standard in the test.
- The Contaminant Limit Concentration (CLC) is used as Specification Limit

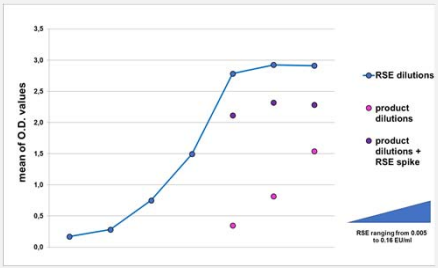
MAT assay	Characteristics
Quantitative Test (Method A of Eu.Ph. 2.6.30)	• Comparison of the tested preparation with a **dose-response curve** of Standard Endotoxin • Appropriate when dose-response curve (expressed in endotoxin equivalents per milliliter) of a preparation is broadly parallel to that of standard endotoxin • Results are provided in Endotoxin Equivalents per ml
Semi-quantitative Test (Method B of Eu.Ph. 2.6.30)	• Comparison of the tested preparation with Standard Endotoxin (**4 endotoxin concentrations** close to the Limit of Detection of the assay) • Parallelism between Endotoxin and the preparation not required • PASS/FAIL test (Result has to be <CLC) • Applicable to intrinsically non-pyrogenic product

Product-specific Validation: Monocyte Activation Tests

Quantitative and Semi-Quantitative Tests: final layout

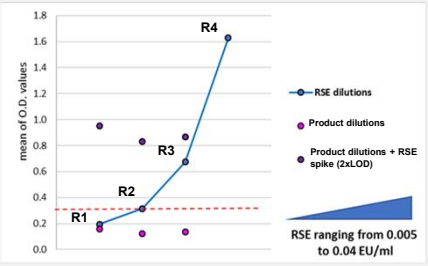
Quantitative Test

- ✓ 8 RSE concentrations
- ✓ 3 Product dilutions: optimum dilution* (Sol.A), ½ Sol.A (Sol. B), ½ Sol B
- ✓ RSE spikes at a concentration corresponding to ½ dose of RSE curve



Semi-Quantitative Test

- ✓ 4 RSE concentrations
- ✓ 3 Product dilutions: optimum dilution*, ½ MVD, MVD
- ✓ RSE spikes at a concentration corresponding to 2xLOD



Optimum dilution = first dilution of the product for which the endotoxin recovery (in the test for interfering factor) is centered within the validity range 50-200%

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Substitute RPT with a risk-based approach

Avoid test duplication and rely on the most effectual test

1 **Remove and rely on controls at other steps**

A B +C

Lifecycle based on the **consistency approach**:

- Based upon the principle that the **quality of a biologic** is the result of the **strict application of a quality system and consistent production.**
- Subsequent **batches are determined to be similar to clinically evaluated batches** and therefore acceptable for release

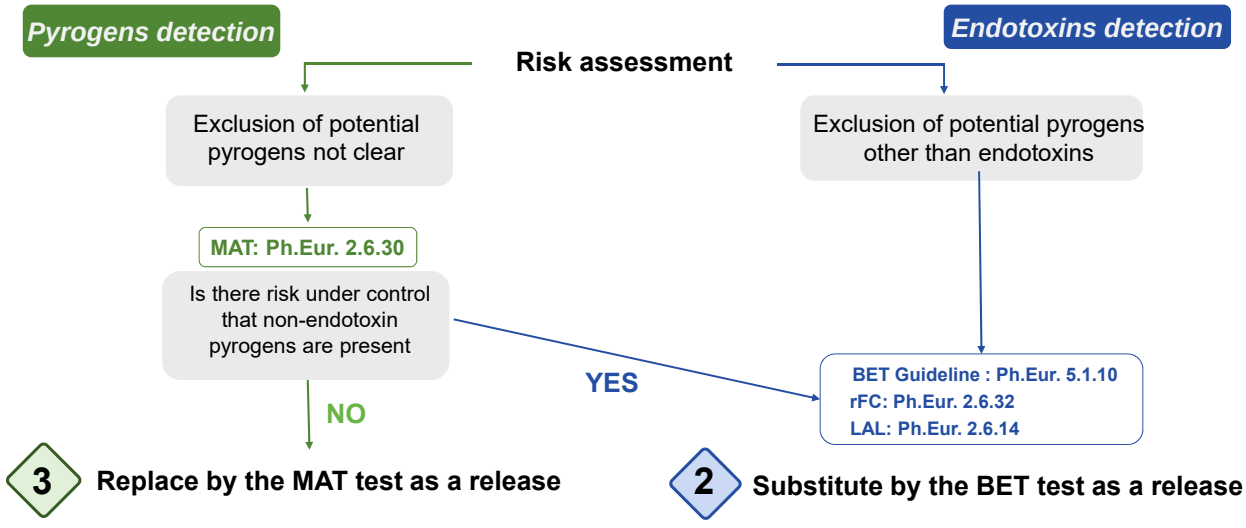
Pyrogen testing in Incoming Material (e.g. Antibiotics)

Pyrogenicity is

- ✓ Reduced in the manufacturing process
- ✓ Tested at intermediate drug product level
- ✓ Analyzed by the Supplier and part of the CoA
- ✓ Evaluated again at a later manufacturing steps

Pyrogen detection method should depend on a risk-based approach

Without the use of RPT



Will GSK abolish the RPT?

RPT use has been drastically reduced. An abolition in lifecycle is a realistic target

R&D = NO

- GSK always proposes *in vitro* test if applicable
  This is not always accepted by all authorities
- If RPT is requested, GKS aims to have RPT as interim release test while proving equivalence or superiority of alternatives

RPT use in vaccines

~5%	2016
~50%	2022
100%	2025

~95%	2016
~50%	2022
0%	2025

QC = YES

- The most suitable alternatives have been identified, validated, or even submitted for approval
- Target: all approvals before 2025
- If specific authorities request RPT, it will be reintroduced for the region

2016	> 25 RPT
2022	< 5 RPT
2025	0 RPT

Exemplary benefits for a replacement

<0.1% animal, Ethics, Compliance
>10d reduced test time, 25-75k€ ingross gains

Conclusions and future perspective

Conclusions

- The MAT assay is a robust and effective method which allows to effectively guarantee the safety of vaccine products. Indeed, the method:
  1. Enables a better evaluation of critical quality attributes of products
  2. Reduces complexity/limitations of existing methods
     - much lower variability as compared to the in vivo method with higher sensitivity
     - reduced time for lot release testing
     - full alignment with 3Rs principles (replacement, refinement, reduction)
  3. Ensures the reliably detection of pyrogen contaminants (if present) in the product
- The use of MAT assay is particularly strategic in the field of vaccines which could be complex in matrix structure (the case of the Bexsero vaccine is an example).
- MAT is approved worldwide for Bexsero (Method C), approved for Encepur in Europe (Method B), under review for other products (Method B and A).

Future perspective

- Abolition of RPT for QC testing before 2025
- RPT not proposed for pyrogenicity for new GSK products. Proposal of Equivalent or superior *in-vitro* alternatives for pyrogenicity assessment on new products.

Thank you for your attention

QUESTIONS?

Development roadmap for quantitative and semi-quantitative MAT assays

Generic Assay set up

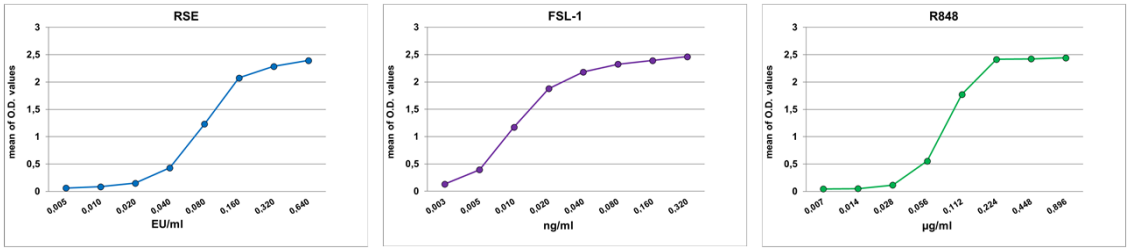
1. Confirmation of endotoxin and non-endotoxin pyrogens detection on donors
2. Limit of Detection (LOD) estimation
3. Setting up of a donor qualification procedure

Product-specific Validation

1. Calculation of CLC (Contaminant Limit Concentration) and MVD (Maximum Valid Dilution) for the product;
2. Verification of the potential interference of the product on:
   - Endotoxin and non-endotoxin spikes
   - MAT detection system (IL-6);
3. Assessment of parallelism between product and Endotoxin
4. Final test layout
5. Assessment of pyrogens detectability with the final method format

Generic Assay Set Up: PBMCs respond to both endotoxin and non-endotoxin pyrogens (NEPs)

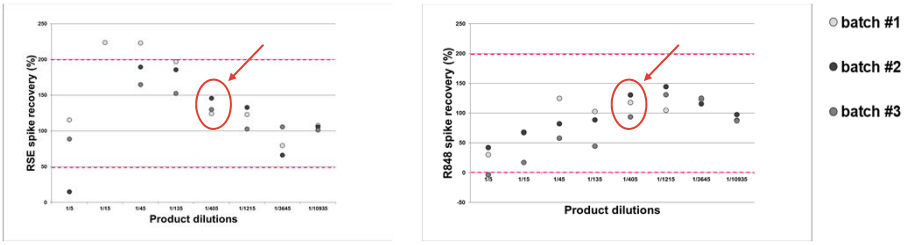
- Comparable response observed in single donors and pool of 8 donors
- Limit of Detection (LOD) calculated as concentration corresponding to *cut-off value**
  - **RSE (Reference Standard Endotoxin) LOD: 0.01 EU/ml**
  - **FSL-1 (mycoplasmal lipopeptide) LOD: 0.005 ng/ml (NEP)**
  - **R848 (imidazoquinoline) LOD: 0.014 µg/ml (NEP)**



**cut-off value* was calculated by applying the following formula: x + 3*s where x is the mean of the 4 replicates for the responses to the cell blank, S is the correlated standard deviation

Product Specific Validation: Tests for interfering factors reveal which product dilutions do not interfere with the test

- Tests for interfering factors are conducted with both RSE and at least 1 non-endotoxin pyrogens (R848 or FSL1) based on product features
- The knowledge of the product and its reaction on the cells are fundamental to the application of the proper method.
- The product is spiked with RSE and the non-endotoxin pyrogen at twice the stimulus estimated LOD (method B) or to its ½ dose of the dose response curve (method A)
- Spike at 2XLOD is applicable to product with neglect or low matrix interference while spikes at ½ dose of the dose response curve enable to overcome matrix interference effects



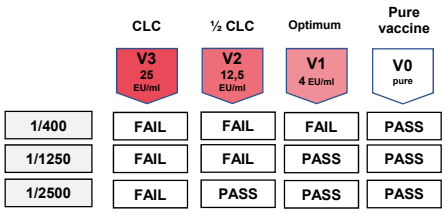
Product dilution showing no potential matrix interference on endotoxin and non-endotoxin pyrogens (e.g. 1/405 for this product) is tested for interference on the MAT detection System (IL-6 specific ELISA) as well.

Semi-Quantitative MAT assay: assessment of pyrogens detectability with the final method format

- The correlation between CLC of a product and dilutions of that product is described in Ph.Eur., i.e. MVD = CLC/eLOD.
- Contaminant concentration (in EU/ml) can be thus calculated also for other dilutions tested for a product. Contaminant concentration = Product dilution* eLOD

Examples of product Dilution	LOD of the assay	Contaminant concentration correspondance
400 (optimum dilution)	0.01 EU/ml	4 EU/ml
1250 (1/2 MVD)		12.5 EU/ml
2500 (MVD)		**25 EU/ml**

- Pure vaccine samples can be spiked with RSE as a surrogate for a contaminated final product
- Samples are then tested in MAT final format to assess pyrogenic content in the final product



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PYROGENICITY TESTING OF VACCINES : No future for the Rabbit Pyrogen Test (RPT)

Emmanuelle Coppens

Stéphanie Richard

EDQM-EPAA 15 February 2023

AGENDA

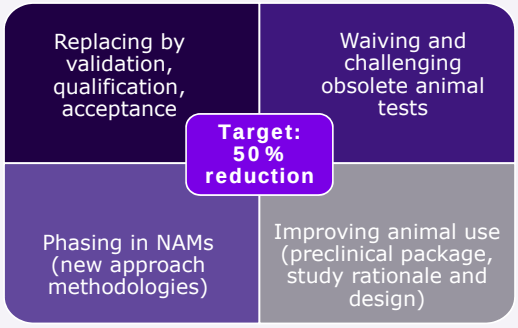
1 Sanofi 3Rs strategy

Integrated Research and Testing Strategy (IRTS)

IRTS is Sanofi strategy that lays out our guidelines to affirm rigorous, state-of-the-art science as key criteria to select the best available, feasible, and translatable models to address scientific questions and adhering to regulatory requirements, most importantly with the primary aim to relieve Sanofi of reliance on live animals.

Sanofi Animal Use (2021)

Objective : 50 % global reduction of animal use in 10 years
- ***Between 2020 and 2030***
- ***Internal and external use***



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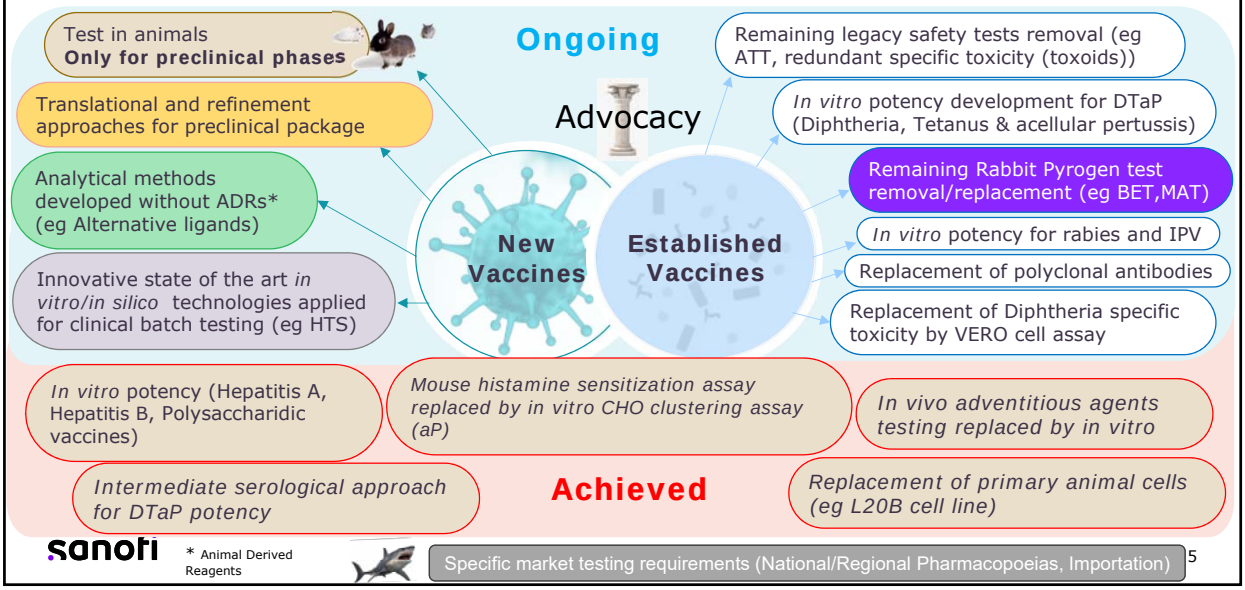
Sanofi's ambition for vaccines : no animal-based analytical testing in Quality Control



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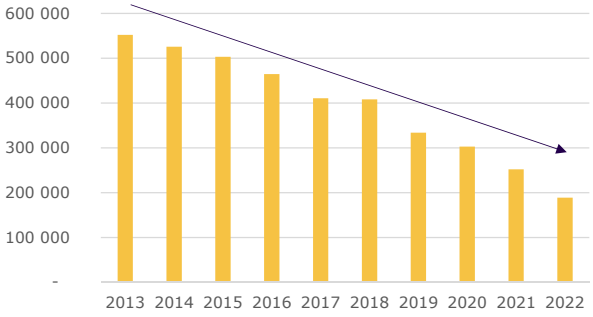
* Animal Derived Reagents

Specific market testing requirements (National/Regional Pharmacopoeias, Importation)

Evolution of animal internal use (2013-2022)

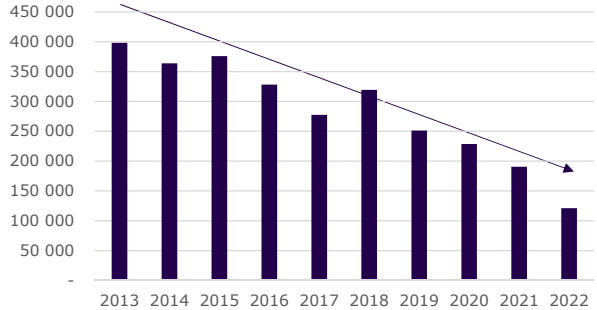
Over 90 % are rodents

Sanofi Global Use



Constant decrease of animal use :
- 45 % between 2013 and 2020
- 38 % between 2020 and 2022

Global QC animal use for vaccines



Overall decrease of animal use :
- 70 % between 2013 and 2022

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2 Which pyrogenicity testing for what pyrogens?

Pyrogens – which test methods for their detection

Comparison of the methods described in pharmacopeias

	Rabbit Pyrogen Test (RPT) Ph. Eur. 2.6.8 / USP <151> / JP 4.04 / Russian Ph GPM 1.2.4.0005.15 / ChP <1142> / Indian Ph 2.2.8 : Not harmonized	**Bacterial Endotoxin Tests (BET)** *LAL*: Ph. Eur. 2.6.14 / USP <85> /JP 4.01: harmonized (ICH 2001)/ ChP 1143 (TAL) *rFC*: Ph. Eur. 2.6.32 / ChP guideline 9251 / JP guideline JP G4-4-180	**Monocyte Activation Test (MAT)** Ph. Eur. 2.6.30 (and 2.6.40) IP 22 2.2.25* ChP draft "Gene Reporter Assay"
Principle	Body temperature elevation post IV injection	Hemolymph clotting in contact with endotoxins / or recombinant reagent	Mimic the first step of fever mechanism – uses human cells
Method	Limit Assay (0.5 IU/mL/kg)	Detection or Quantitative Assay shown to be sensitive to 0.005 IU/mL	A) Quantitative Assay (but plan to be deleted) B) Limit Assay C) Lot-to-lot comparison
Goal	Safety Test Product/Process Consistency	Safety Test Product/Process Consistency	Safety Test Product/Process Consistency
Advantages	Compendial method (US, Eur and JP but not harmonized) Sensitive to all pyrogens	Compendial method harmonized for LAL based BET (US, EU, JP) Sensitive and fast rFC compendial only in Ph. Eur.	*In vitro* – Compendial method (EU) Sensitive to pyrogens Based on human cells
Drawbacks	*In vivo* *Not harmonized through Pharmacopeias* Variable Not representative of human biology Injection route Dilution of the product (vaccine) **Intended to be deleted in Ph. Eur. (2026)**	*Ex vivo (*horseshoe crab is an endangered species) « Only sensitive to endotoxins from Gram negative bacteria » rFC approach not compendial in USP and JP and China	**Compendial method only for Ph. Eur. since 2010**

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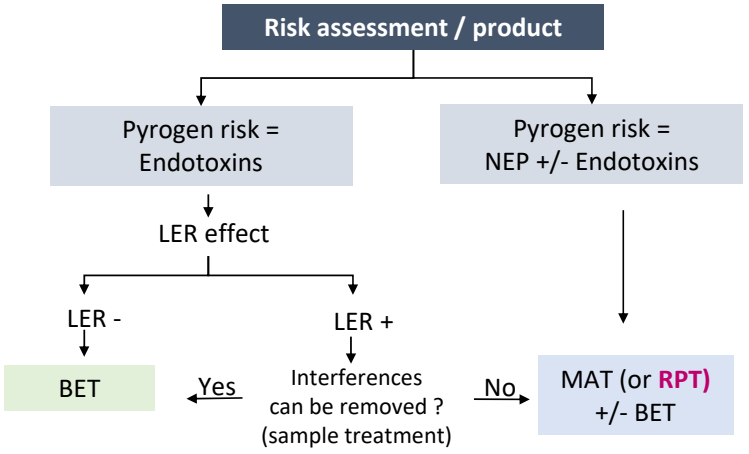
In vitro alternatives are not compendial methods outside Europe*

*exception

Pyrogens – which test methods for their detection

A risk assessment is performed to identify which is the candidate method for pyrogens detection depending on the nature of pyrogens



NEP= Non Endotoxin Pyrogen

LER = Low Endotoxin Recovery

RPT Intended to be replaced

3 Sanofi Vaccines : ongoing strategy to phase out RPT

Sanofi overall strategy for new and established vaccines

DP release testing

BET (rFC) solely performed for release on licensed product

Under implementation : *For each manufacturing platform MAT (method 1/B) performed on 3 industrial batches of a representative product as a* ***one-off characterization***

Substitution by BET (LAL)
BET already performed-> RPT deletion
BET not in place -> LAL implementation

Product identified with ***endotoxin risk only***

Risk assessment based on process knowledge and contamination control strategy to justify product safety in terms of potential pyrogens and **MAT testing**

New Vaccines

Established Vaccines

Risk assessment based on process knowledge and contamination control strategy and **historical data** to justify product safety in terms of potential pyrogens

Product identified as ***inherently pyrogenic*** *:*
MAT (method 2/C or 2.6.40)

MAT and BET (rFC) performed for release on licensed product

Substitution by MAT

RPT- current situation

Remaining RPT release testing on established product

- Products with endotoxin risk only :
  - Only 1 remaining (LAL implementation ongoing)
  - Challenge for the use of BET as sole method to assess pyrogenicity from some Health Authorities outside Europe
- Products inherently pyrogenic : MAT development ongoing

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4 MAT format selected by Sanofi for vaccines

Introduction to the Monocyte Activation Test (MAT)

In vitro alternative to the Rabbit Pyrogen Test

Based on the first step of fever mechanism

Based on **human** monocytic cells & pro-inflammatory cytokine quantification

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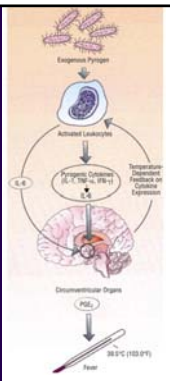
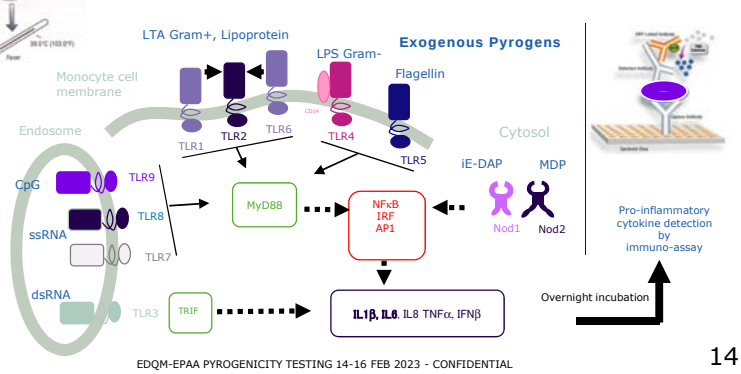


Pharmacopoeia chapters & Guidance

Ph Eur. 2.6.30 (method 1 and 2)-under revision
Ph Eur. 2.6.40 (vaccine dedicated)-under creation
Indian Ph. 22 2.2.25
No other Pharmacopoeia text
US text-Guidance 2012

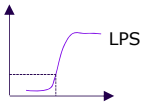


Introduction to the Monocyte Activation Test (MAT)

General Chapter 2.6.30 evolution (Pharmeuropa 34.2)

Methods to be justified regarding the product and the goal of the testing

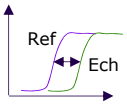
Method 1 (A+B)= semi quantitative method



ECL (Endotoxin Concentration Limit) as specification
Purified *E. coli* standard
EE/mL (Equivalent Endotoxin/ml) as reportable value
Conclusion : Pass /Fail

To be used when no NEP is expected (Product Characterization)

Method 2 (C) = lot to lot comparison



Use when matrix interference in method 1
Or

If the pyrogen content of the product is inherently high, it may be more appropriate to carry out, for example, a parallel-line analysis on the dose-response curves for the test and reference lots. In this situation, solutions of the preparations are tested at 3 or more geometric dilutions which cover the range of the dose-response curve used for the validated analysis (see chapter 5.3. *Statistical analysis*).

To be used when the pyrogen content (NEPs and/or endotoxin) of the product to be tested is high (inherently pyrogenic)

MAT format: Source of Monocytes & pro-inflammatory cytokines

PBMCs (pool) / IL-6 as a universal format across Sanofi

Human monocyte cell source

- **Primary cell** (PBMCs or Whole Blood), individual or pooled, fresh or frozen
- **Cell lines,**

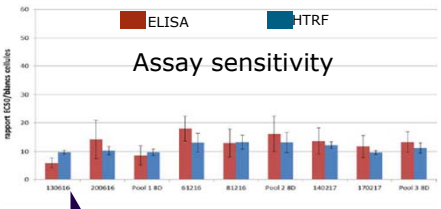
Pro-inflammatory cytokines

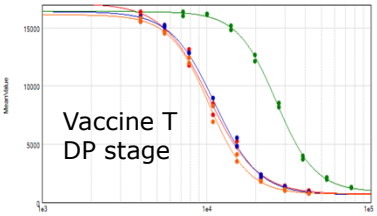
- IL-1β, IL-6, TNFα, IL-8

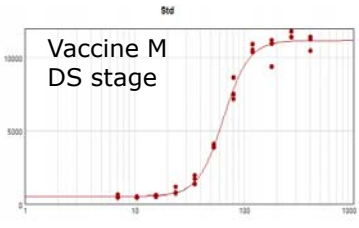
	Pros	Cons
Cell line MonoMac6 / IL-6	Ready to use Test Reproducibility improved	Restricted access (Merck- Worldwide licence) Not always well accepted by MAT European experts Abnormal cell TLR expression characterization required
Whole Blood/IL-1β	/	Intra/Inter batch variability Not used anymore
PBMCs/IL-6 (Pheripheral Blood Mononuclear Cells)	A better representative of what may occur in vivo than the cell line Several suppliers worldwide ECVAM validation Pool of donors (reduces variability) Currently used by ANSM	High cost Complex reagent management

Which technology to detect IL-6 : ELISA or HTRF ?

ELISA	HTRF (Homogenous Time Resolve Fluorescence)
- The most frequently used in MAT - Sensitivity suitable for method 1	**- High dynamic range** (full curve-4PL) -Sensitive enough for consistency approach -Faster than ELISA
- **not suitable for MAT for vaccines containing pyrogens** (high signal magnitude deep slope-4PL analysis)	-New technology for Sanofi's QC







HTRF technology has been chosen for IL-6 detection (method 1 or 2)

Summary of MAT systems in place in Sanofi

MAT format	cryoPBMCs (Pool) / IL-6	
Goal	Absence of pyrogens	Product containing pyrogens – consistency approach
Method	2.6.30 Method 1	2.6.30 Method 2 or 2.6.40
IL6 detection technology	HTRF*	HTRF

* If sensitivity is suitable for method 1

5 MAT case studies for inherently pyrogenic vaccines

Pediatric combo vaccine P

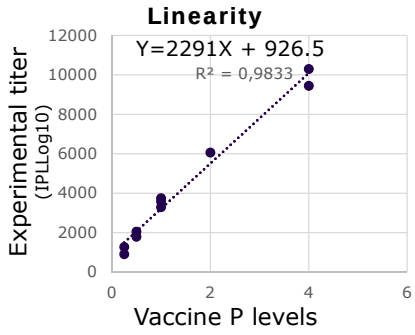
Lot to lot comparison method C

Licenced product currently released with RPT

Development of the MAT to switch in the coming years

MAT – Product-specific Performance

- 2 operators
- 3 series with 3 independant determinations
- 5 levels of concentration
  - 25-50-100-200-400%



Accuracy
An acceptable global accuracy of the test has been documented during Linearity and Intermediate Precision study

Intermediate precision (level 100%)

Reportable values were found to have a **%CV of 1,32** (N=8)

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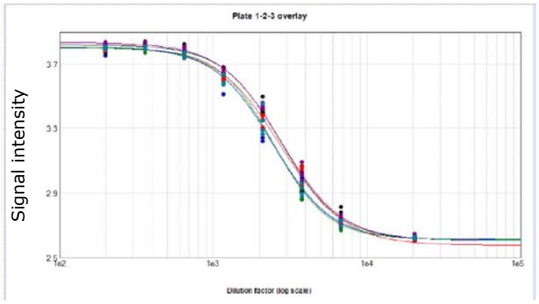
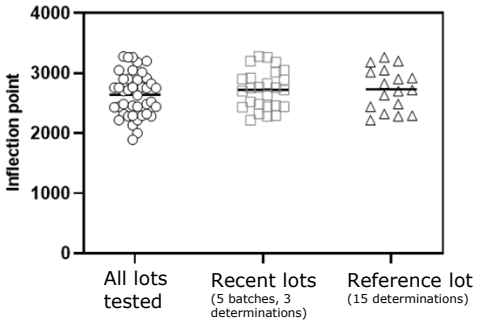
Pyrogenicity consistency on vaccine P demonstrated by MAT





MAT method C is applicable to Vaccine P at DP stage

Preliminary results demonstrate an appropriate process consistency

Pediatric vaccine carrying intrinsic pyrogens vaccine M DS stage

Lot to lot comparison
Method C

Clinical phase I/IIa

Characterization Test during – clinical process devlpt
- Major process change in the future

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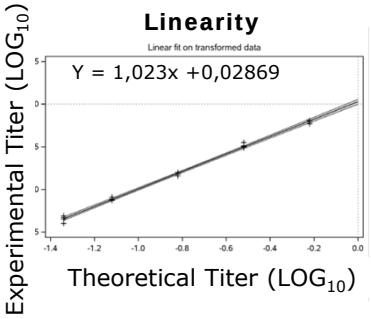
MAT – Product-specific Performance

- 2 operators
- 4 series
- 5 levels of concentration
  - 50-75-100-150-200%

Linearity



Accuracy (recovery)

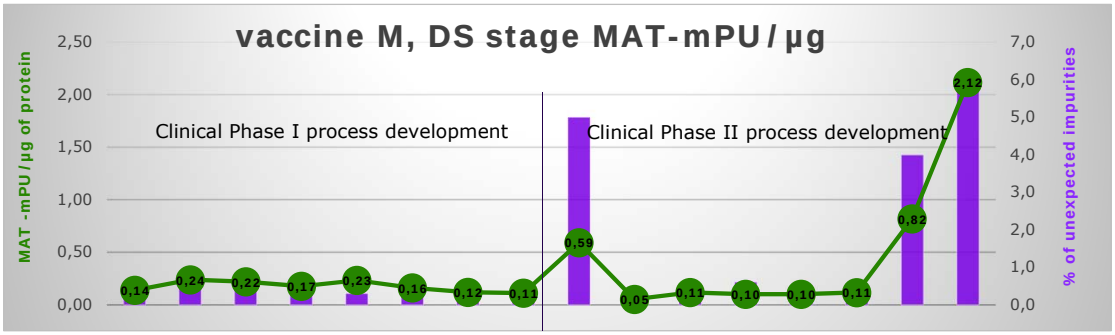
Exactitude MAT
Trueness/Difference of centering study - Recovery mean (%)

Concentration level	Recovery mean (%) (90% confidence interval)
Overall	101.077 [99.211 ; 102.943]
30	98.500 [93.830 ; 103.170]
50	101.250 [96.580 ; 105.920]
100	100.000 [97.184 ; 102.816]
200	106.500 [101.830 ; 111.170]
400	101.000 [95.607 ; 106.393]

Intermediate precision (routine level)

N	GMean	Repeatability GCV(%) (95% one-sided upper confidence limit)	Intermediate precision GCV(%) (95% one-sided upper confidence limit)
11	0.152324	3.572 (6.511)	5.110 (14.577)

Example of process support by MAT



Within the toolbox of analytical methods, MAT is a suitable and important method driving process development of vaccine M at DS stage.

6 Conclusion

Conclusion (1)

- **Removing RPT is a clear ambition in alignment with Ph Eur evolution strategy**
  - RPT is almost completely removed for non-NEP-containing marketed vaccines with substitution by BET as the sole method used to assess pyrogenicity

- **MAT implementation is ongoing :**
  - **For inherently pyrogenic products (Method 2/C):**
    - QC transfer of the assay for release :ongoing for 1 product, planned within 2 years for another one
    - Used in R&D as characterization tool
  - **For products under development with no expected NEPs (Method 1/A+B):**
    - Under implementation as characterization tool

Conclusion (2)

- **Challenges foreseen with MAT as alternative to RPT**

  - **Acceptance of MAT outside Europe**
    - As a compendial test with only product specific validation as described in 2.6.30 and without comparative *in vivo/in vitro* studies
    - As a release test for clinical batches during clinical development

  - **Reference selection & Product acceptance criteria definition with method 2/C (lot to lot comparison)**
    - Choice of the first reference lot
    - How to define acceptance criteria for clinical batch release using MAT ?

  - **Use of MAT as a routine Quality Control test**
    - Complex Cell-based assay over two days
    - Expensive assay

Acknowledgements :
K. Thibault-Duprey, H. Chausse, T. Bonnevay, K. Mathews, M. Fowlers, S. Vydelingum, S. Mlatese, C. Mallet, N. Nougarede

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