EXPLOITING MONOCYTE ACTIVATION TEST: The experience of Istituto Superiore di Sanità

Eliana M. Coccia and Marilena P. Etna
Department of Infectious Diseases

May 25th, 2023

- Overview Istituto Superiore di Sanità
- Pyrogenicity testing in Europe
- MAT: workflow, technical tips&tricks
- Methods in Ph. Eur: what's new?
- MAT: a practical application

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ISTITUTO SUPERIORE DI SANITÀ [ISS]

MISSION: Promotion and protection of national and international public health through research, surveillance, regulation, control, prevention, communication, counseling and training activities.

Dept of Infectious Diseases (DMI)

7 Units (250 researchers & technicians)

DMI research ranges from the fundamental biology to the development of new approaches for the prevention, diagnosis and treatment of viral, bacterial, fungal and parasitic infections

Technical-scientific services
Biological Service
Core facilities
Grant office and technology transfer
Research coordination and support
Statistics

Departments
Cardiovascular, dysmetabolic and ageing-associated diseases
Environment and health
Food safety, nutrition and veterinary public health
Infectious diseases
Neurosciences
Oncology and molecular medicine

IMMUNOLOGY UNIT

Ongoing studies:

- ✓ **Immune-pathogenic mechanisms of infectious diseases and escaping strategies evolved by pathogens;**
- ✓ **gene expression in response to infectious agents;**
- ✓ **immunotherapy of infectious diseases;**
- ✓ **alternative experimental model to test in vitro vaccine pyrogenicity and potency.**

MAT at the
Italian National Institute of Health
(Istituto Superiore di Sanità)

DEPARTMENT
INFECTIOUS DISEASES

MAT Unit

Eliana M. Coccia (Head of MAT Unit)
Marilena P. Etna (Expert of MAT Unit)
Fabiana Rizzo (Analyst)
Ramona Ilari (Analyst)

CENTRO NAZIONALE
CONTROLLO
E VALUTAZIONE DEI FARMACI

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PYROGENS IN THE Ph. Eur.

GENERAL MONOGRAPHS

- Substances for pharmaceutical use [2034].
- Radiopharmaceutical preparations [0125].
- Immunosera for human use, animal [0084].

DOSAGE FORM MONOGRAPHS

- Parenteral preparations [0520].
- Preparations for irrigation [1116].
- Intravesical preparations [2811].

PYROGENS (2.6.8 Chapter)
[RABBIT PYROGEN TEST]
60 TEXTS

GENERAL CHAPTERS

PLASTICS

- Sterile plastic. Containers for human blood and blood components [3.3.4].
- Sets for the transfusion of blood and blood components [3.3.7].

VACCINES FOR HUMAN USE

- Carrier proteins for conjugated polysaccharide vaccines for human use [5.2.11].

INDIVIDUAL MONOGRAPHS

- Solutions [4].
- Blood products [17].
- Vaccines for human use [17].
- Antibiotics [8].
- Other chemical substances [4].

DIRECTIVE 2010/63

Substitution with non-animal technologies is mandatory in EU

20.10.2010 EN Official Journal of the European Union L 276/33

DIRECTIVES

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

of 22 September 2010

on the protection of animals used for scientific purposes

(Text with EEA relevance)

BIG CHANGES in the Ph. Eur.

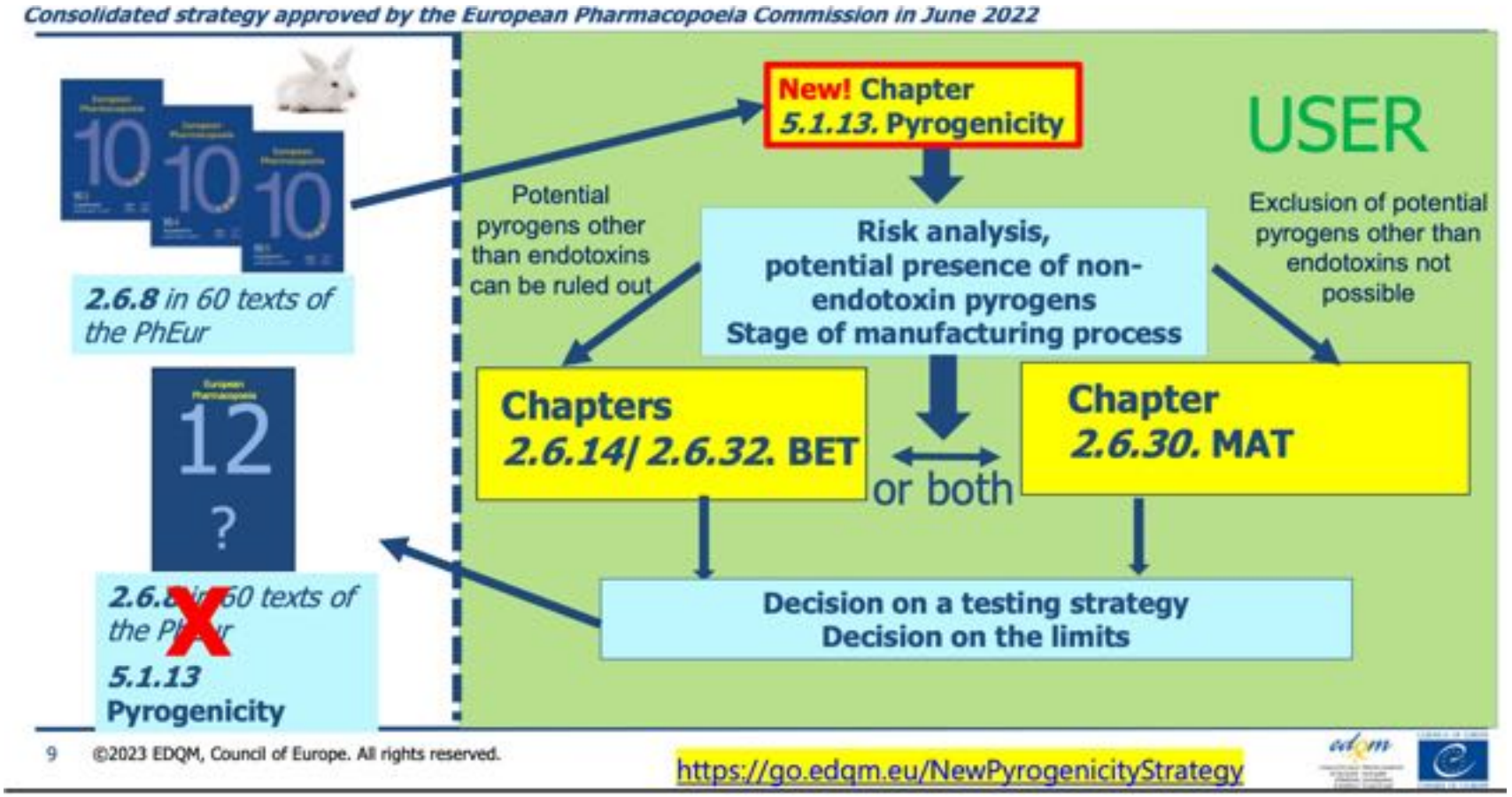
- ***5.1.13*** Pyrogenicity
- ***2034*** *Substances for pharmaceutical use*
- ***5.1.10*** *Guidelines for using the test for bacterial endotoxins*

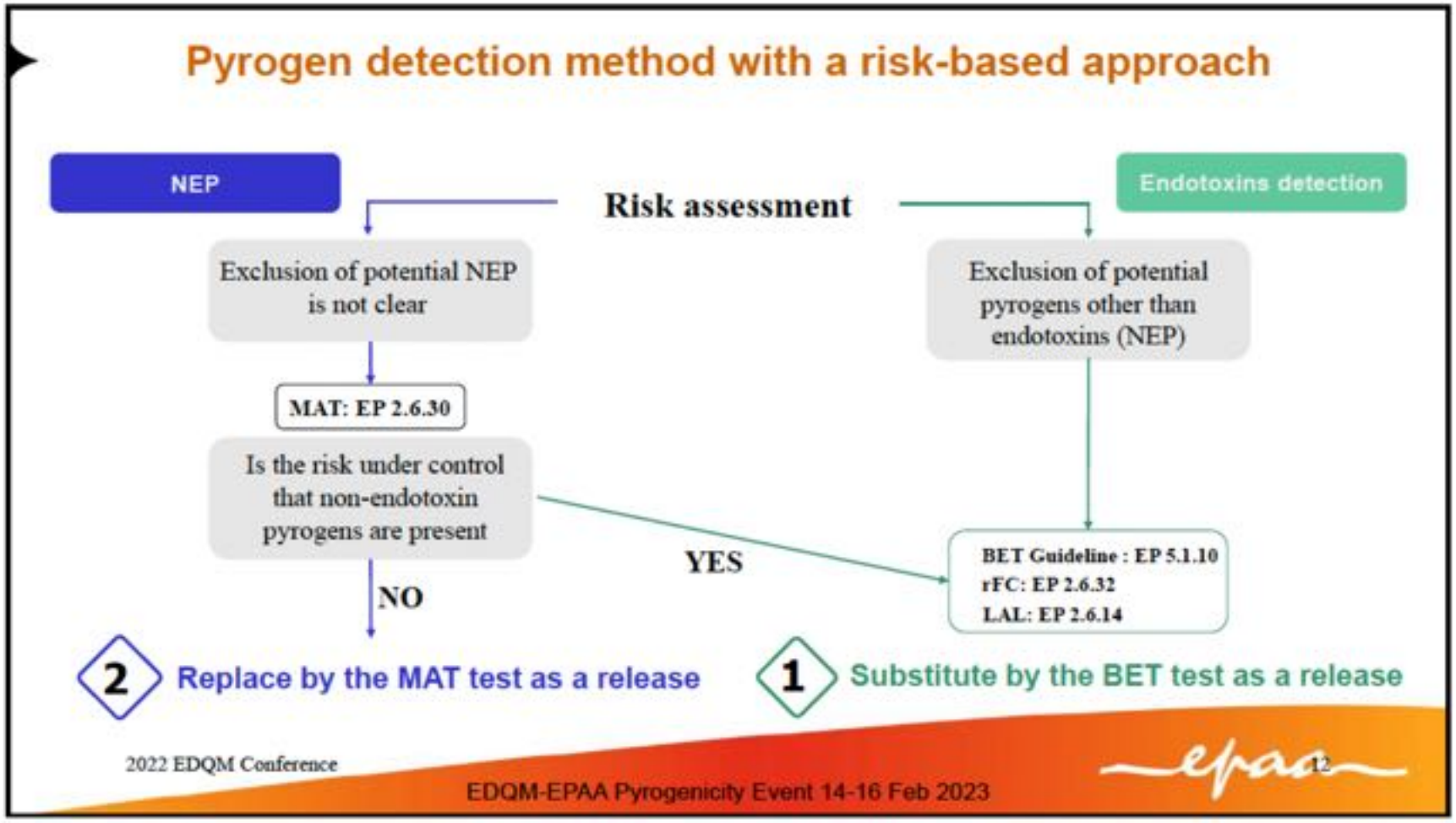
Texts have now been published in Pharmeuropa!

There are currently 59 Ph. Eur. texts – covering a variety of topics including vaccines for human use, blood products, antibiotics, radiopharmaceuticals and containers – that refer to the RPT and will be affected. The Ph. Eur. is committed, for all these texts, to replacing the test for pyrogens with a suitable in-vitro alternative, ultimately leading to the complete elimination of the RPT. In the meantime, users are actively encouraged to seek alternatives to chapter 2.6.8, the best option being the MAT.

Throughout the process, users will have the opportunity to comment on a case-by-case basis, since each of the texts concerned will go through the standard public enquiry in Pharmeuropa.

REPLACEMENT OF CHAPTER 2.6.8: proposed strategy:





From EDQM-EPAA RPT event 14-16 February 2023

- Overview Istituto Superiore di Sanità
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- Methods in Ph. Eur: what's new?
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2.6.30. MONOCYTE-ACTIVATION TEST

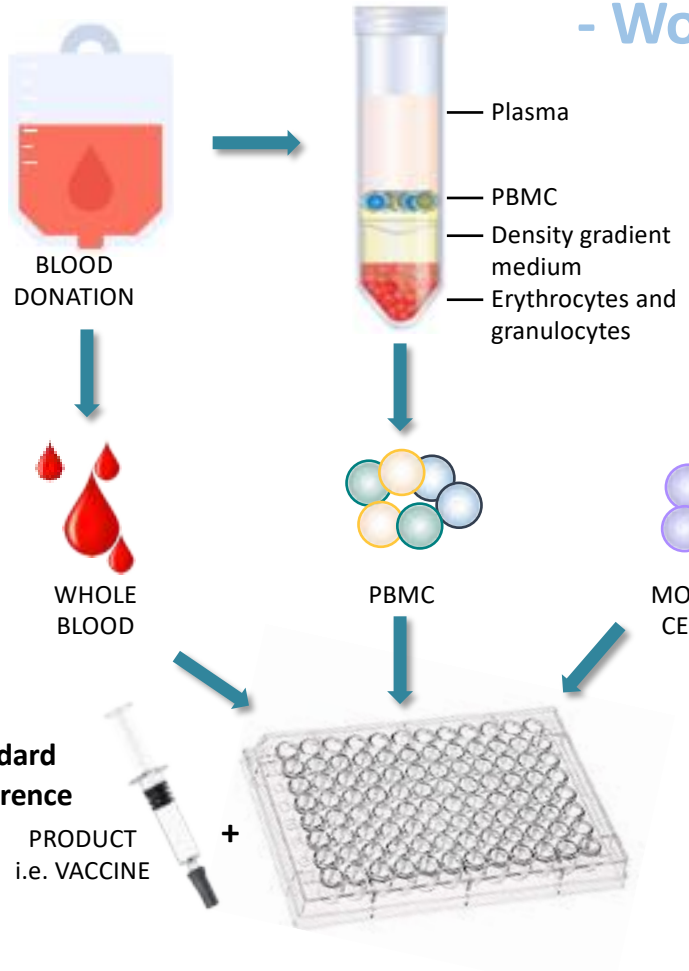
1. INTRODUCTION

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.

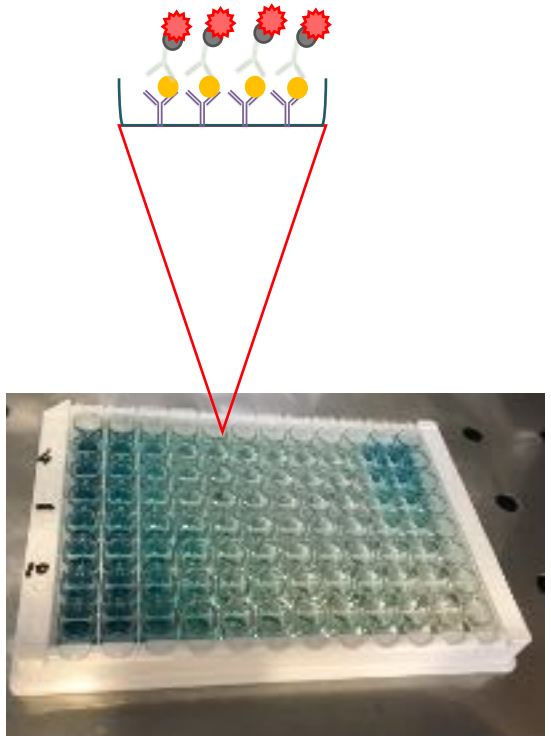
Pharmaceutical products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants often show very steep or non-linear dose-response curves in comparison with endotoxin dose-response curves. Preparations that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

MONOCYTE ACTIVATION TEST

- Workflow -

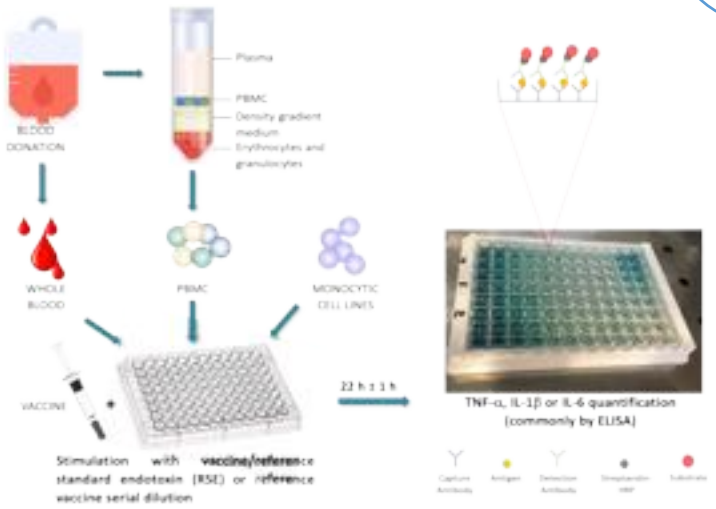


Stimulation with vaccine/reference standard endotoxin (RSE) or reference vaccine serial dilution



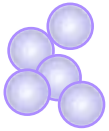
TNF-α, IL-1β or IL-6 detection (by immuno-assay, commonly ELISA)

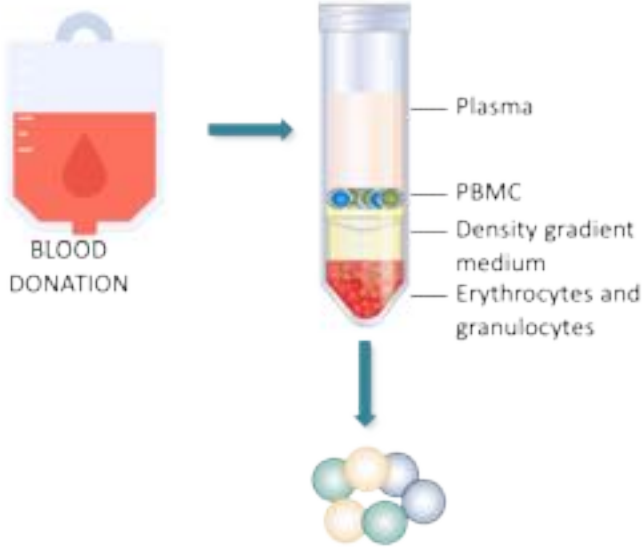
VARIABLE PARAMETERS



- Cell source
- Read-out
- Method to detect the read-out
- Product (i.e. vaccine)

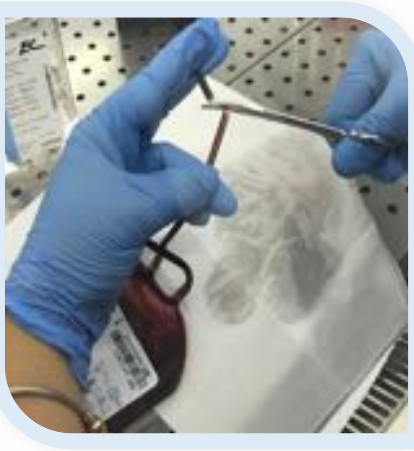
CELL SOURCE

 WHOLE BLOOD	 PBMCs	 MONOCYTIC CELL LINES
[POLIMORFONUCLEAR AND MONONUCLEAR CELLS]	[MONONUCLEAR CELLS]	[MONO-MAC-6 AND THP1]
Donor variability	Donor variability	Very low variability
For unspecified pyrogens	For unspecified pyrogens	For known pyrogens
Presence of cytokines and Abs in plasma	Basal activation due to PBMC isolation procedures	



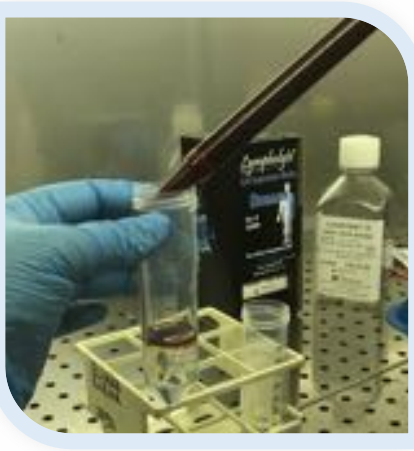
PBMC ISOLATION (I)

Blood bag



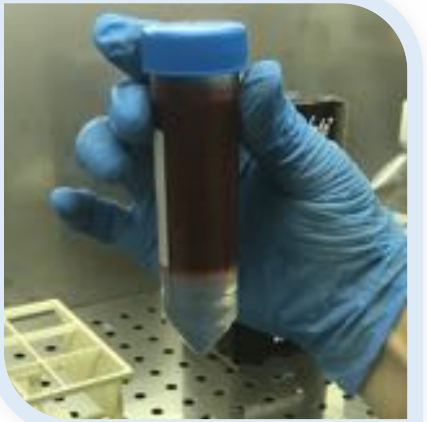
Blood bag cleaning and opening

Blood transfer in a 50 ml tube



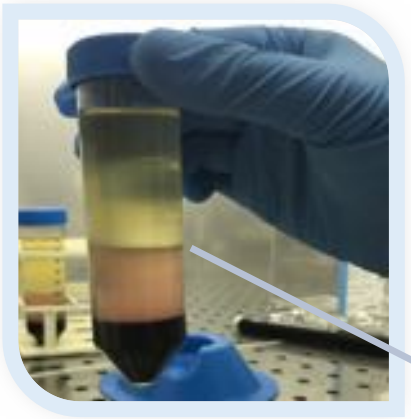
Blood stratification on a density gradient separation medium

PBMC ISOLATION (II)

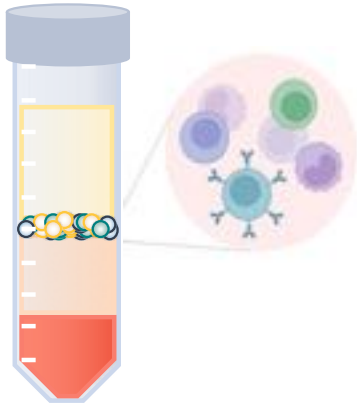


Tube after blood stratification ready for centrifugation

Centrifugation with swing-bucket rotors adapters



Blood stratified soon after centrifugation



PBMC

How to face donor variability when using primary cells

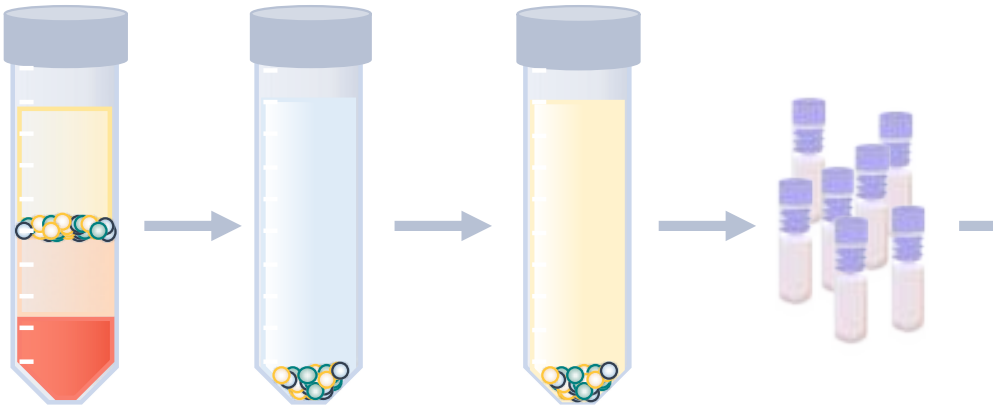
- Preserve qualified cells (-> preliminary assays to **assess the quality of cells**)
- Ensure the long-term use of a cell source with robust results (**Creation of cell banks**)

Before starting…

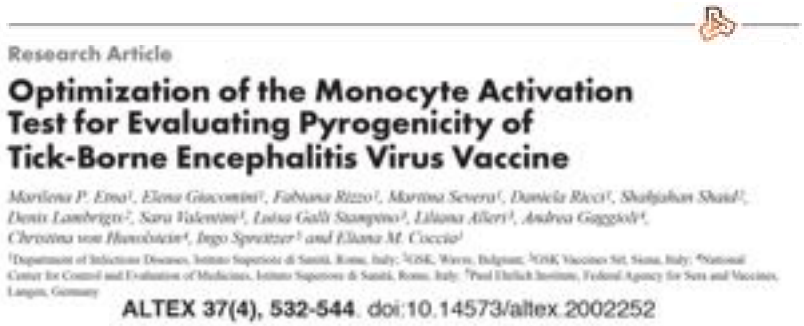
1. Ensure aseptic conditions while freezing cells
2. Define the optimal number of cells per vial to avoid low cell viability as well as unwanted cell clump (for PBMC 5 - 20 x 10^6 cells/mL)

PBMC freezing workflow

Whole blood
Buffy Coats



1	2	3	4	5	6
Separate PBMC	Harvest Cells	Resuspend PBMC in freezing media	Aliquot in cryovials	Place cryo-freezing container at -80°C overnight	Transfer in liquid nitrogen tank



Research Article

Optimization of the Monocyte Activation Test for Evaluating Pyrogenicity of Tick-Borne Encephalitis Virus Vaccine

Marilena P. Ema[1], Elena Giacomini[1], Fabiana Rizzo[1], Martina Severa[1], Daniela Ricci[1], Shahjahan Shaid[1], Denis Lambrigts[2], Sara Valentini[1], Luisa Galli Stampino[3], Liliana Alleri[3], Andrea Gaggioli[4], Christina von Hanckstein[4], Ingo Spreitzer[5] and Eliana M. Coccia[1]

[1]Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy; [2]GSK, Wavre, Belgium; [3]GSK Vaccines Srl, Siena, Italy; [4]National Center for Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome, Italy; [5]Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany

ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

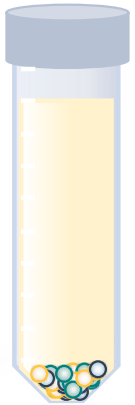
Tips & Tricks [I]

Home-made

⇨ Fetal Bovine Serum (FBS) + Cryoprotectant agent* (DMSO)

⇨ Human Serum (HS) + Cryoprotectant agent* (DMSO) [Animal-free]

*avoids: ice crystal formation/ osmotic stress/ membrane damage



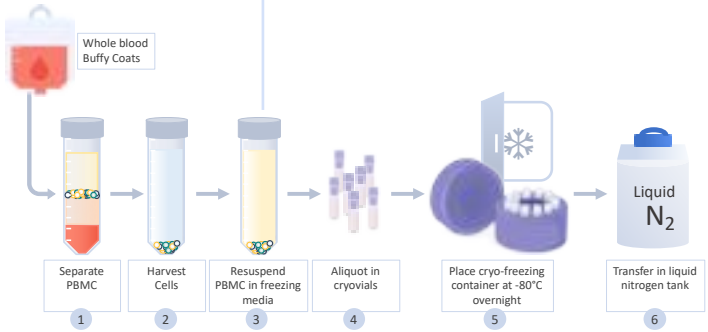
3

Resuspend PBMC in freezing media

Commercially ready-to-use

⇨ With Fetal Bovine Serum (FBS)

⇨ Synthetic [Animal-free]



Tips & Tricks [II]

5

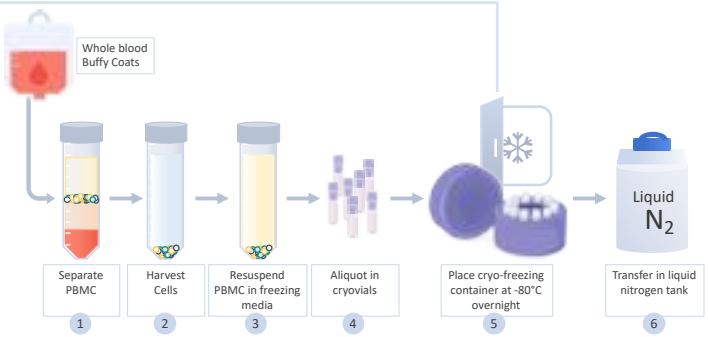
Place cryo-freezing container at -80°C overnight

Controlled freezing rate

Slow freezing is critical to ensure cell viability and integrity

⇨ Controlled-rate freezer

⇨ Isopropanol freezing container



Tips & Tricks [III]

Transfer in liquid nitrogen tank

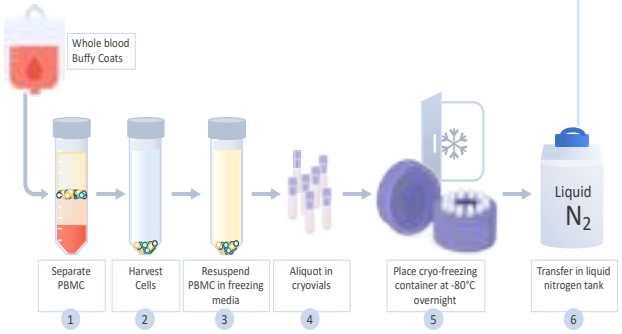
6

Storage & Recording

⇨ Very low temperature (-135°C to -196°C) for long-term storage

⇨ Alcohol/liquid nitrogen-resistant markers or printed cryo-labels

⇨ Inventory of cell bank



Reagents & Equipment

[critical]

Cryopreservation Medium

Serum (FBS or HS)
Cryoprotectant

Ready-to-use
freezing media

Cryovials resistant to very low temperature

Freezing containers

Liquid nitrogen tank

PBMC Thawing: before starting…

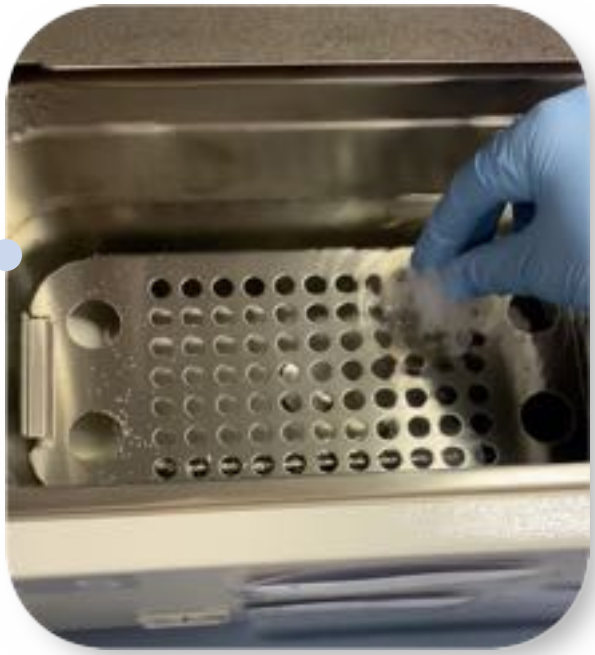
1. Ensure aseptic conditions while thawing cells
2. Warm thawing medium of choice in a 37°C water bath
3. Put warmed thawing medium in a 15 ml conic tube (sufficient for one vial of PBMC)

Rapid thawing helps to minimize any impact on cell recovery

PBMC thawing workflow [I]

1. Remove the cryovial from liquid nitrogen storage tank and place it in dry ice (no exposure to room temperature until thawing)

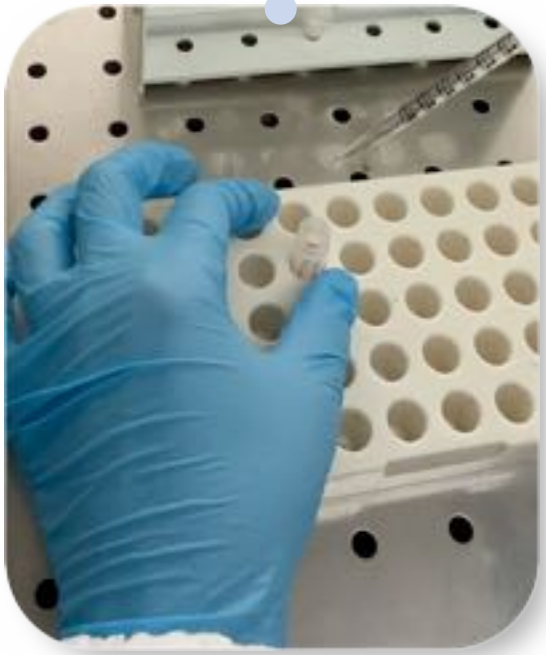
2. Place cryovial in 37°C water bath and thaw cells by swirling it until a little piece of ice remains

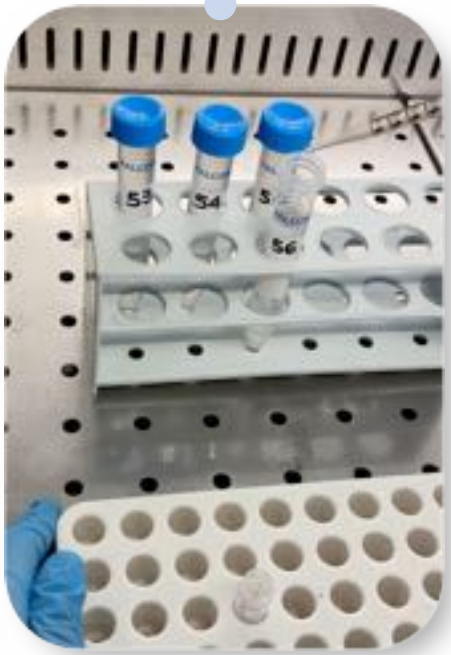


3. Wipe the outside of the cryovial with 70% ethanol or isopropanol before transferring into the biosafety hood

PBMC thawing workflow [II]

4 Add 1 ml of thawing medium (warm) **dropwise** directly into the cryovial then slowly transfer cell suspension in the conic tube pre-filled with warm thawing medium

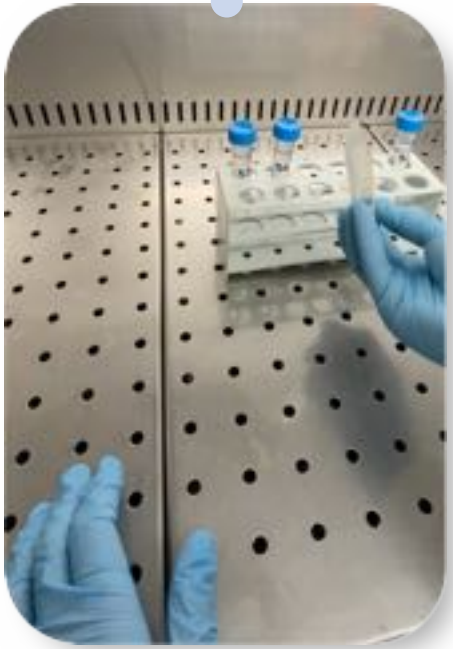




5 Wash the cryovial with an additional 1 ml of thawing medium and add it to the conic tube

PBMC thawing workflow [III]

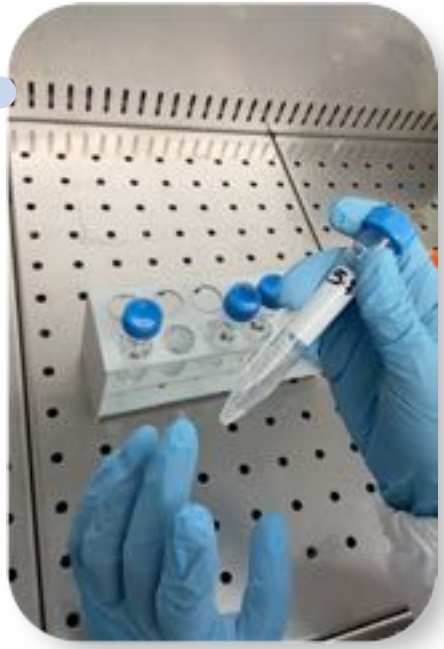
6 Mix by inverting the tube several times and then centrifuge at room temperature at 300 g for 10 minutes



7 Discard the supernatants and leave a small amount of medium to ensure the cell pellet is not disturbed

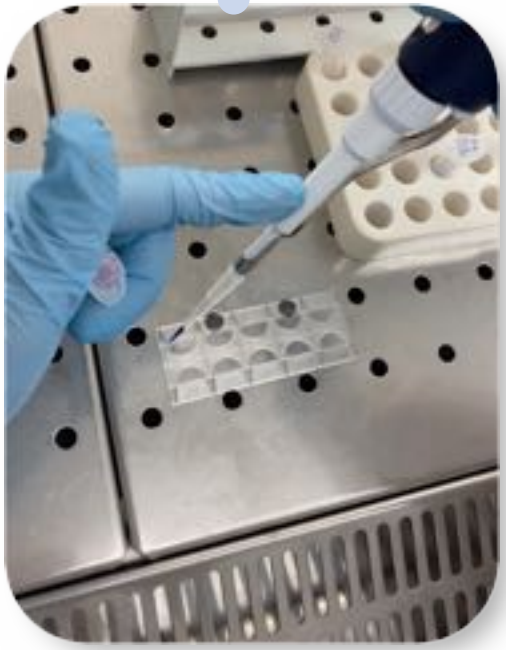
8 Resuspend the pellet by gently flicking the tube and then add thawing medium and repeat the washing step

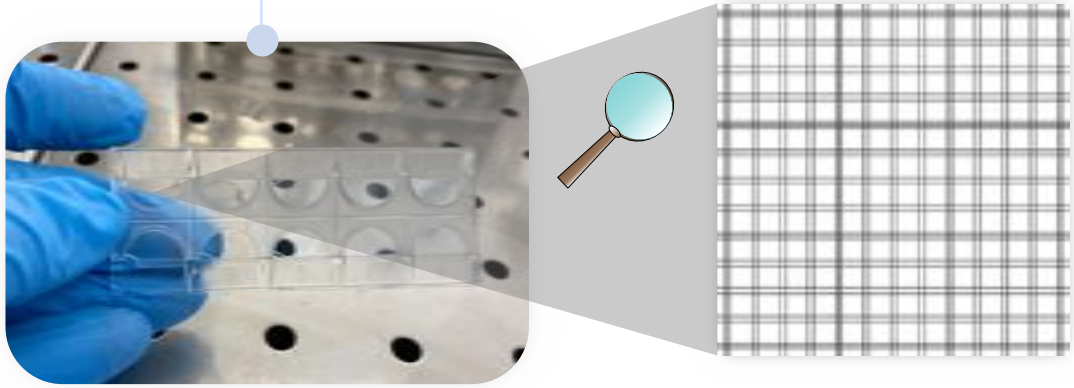
9 Repeat action n°7 and then add an established volume of cell culture medium. Take a small aliquot for cell viability count



PBMC thawing workflow [IV]

10 Dilute cell suspension with trypan blue exclusion dye and, by using a hemocytometer, count 3 small squares of the chamber to calculate the cell number





Counting Chamber

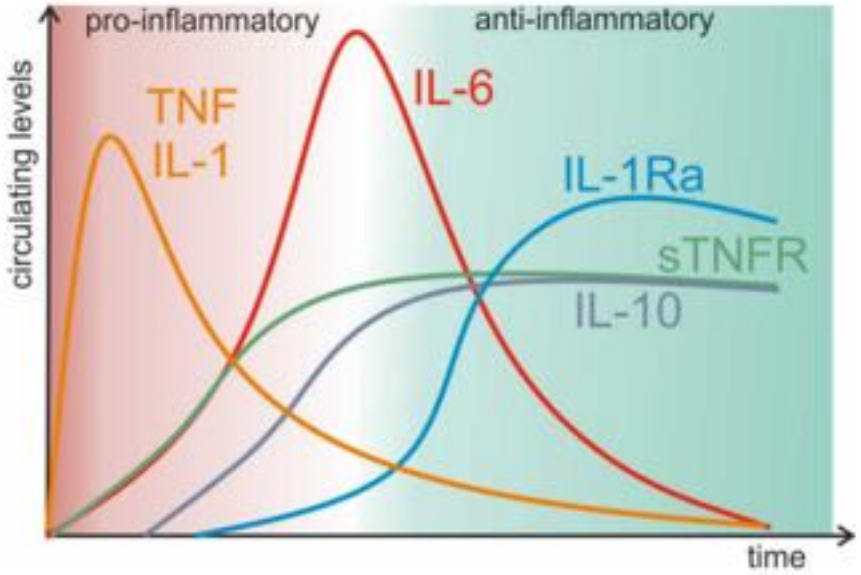
Counting Grid

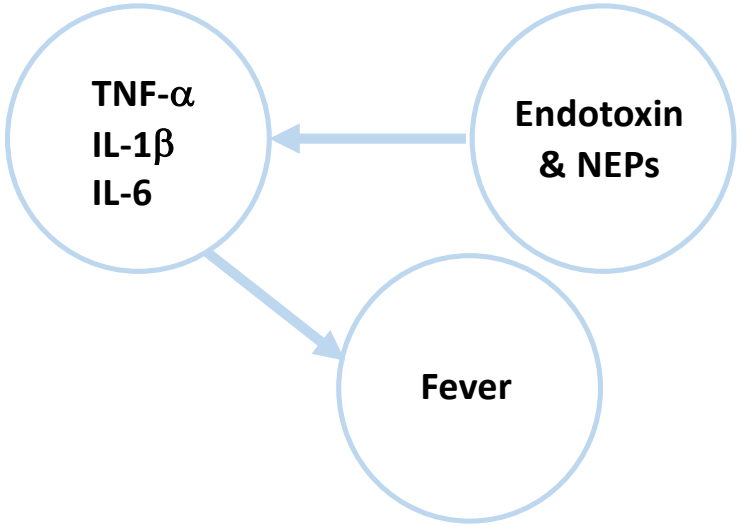
12 Adjust cell volume to the desired cell concentration with cell culture medium

Cells are now ready for use in downstream applications

READ-OUTS

Tumor Necrosis Factor [TNF] and interleukin [IL]-1 are the first cytokines to be released in sepsis and promote the secretion of IL-6. Together, these cytokines are the orchestrators during the pro-inflammatory phase in sepsis.

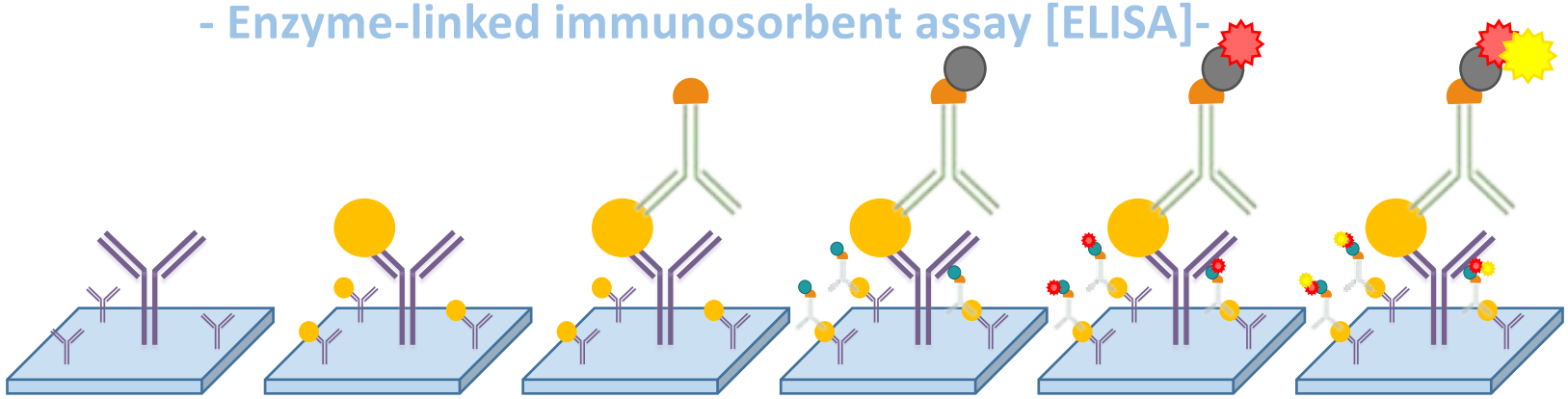




Int. J. Mol. Sci. 2018, 19, 1442, ; doi:10.3390/ijms19051442

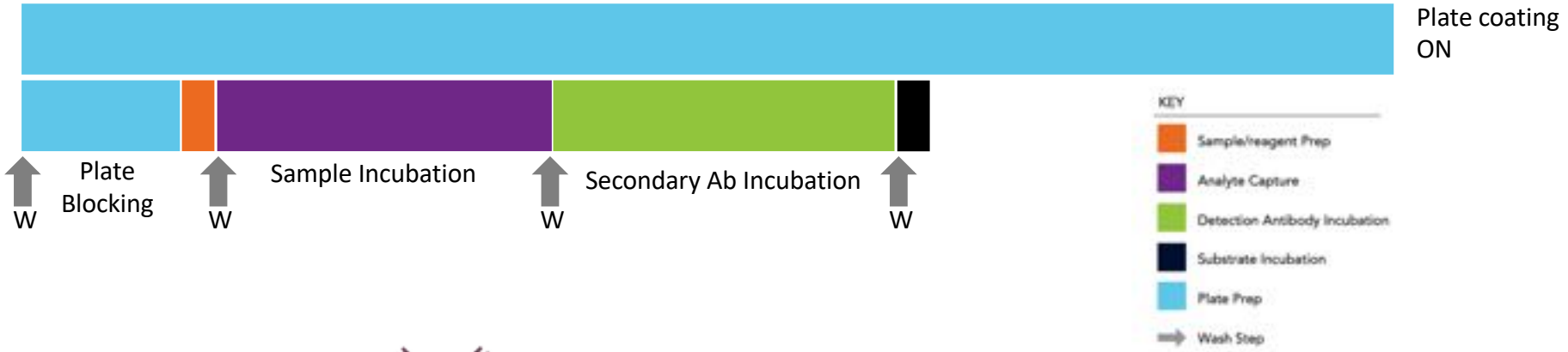
METHOD TO DETECT THE READ-OUT

- Enzyme-linked immunosorbent assay [ELISA]-



Microplate washer employed along ELISA procedure

Critical factors for a successful ELISA procedure



Reagents (Abs, substrate etc..)

Incubation time

Temperature of incubation along the procedure

Type of Antigens used in Licensed Vaccines

Type of Antigen	Features	Examples of Vaccines
Live-attenuated Vaccines	Live-attenuated vaccines contain live pathogens from either a bacteria or a virus that have been "attenuated," or weakened. They are produced by selecting strains of a bacteria or virus that still produce a robust immune response but that does not cause	Measles, Rubella, Parotitis, Varicella, Yellow Fever and Mycobacterium tuberculosis vaccines
Inactivated (non-replicating) Vaccines	Composed by inactivated or killed pathogens. They establish a strong immune response without causing disease. Multi-dose are often needed to build full protection. They can be easily produced without excessive cost	Hepatitis A virus, Poliomyelitis virus, and Influenza "split" or "fragmented" virus)
Subunit Vaccines	Produced through purification techniques of the bacterium/virus components interacting with the organism (they do not contain any live pathogen). They are suitable for people who should not receive "live" vaccines,	Haemophilus influenzae type B (Hib) vaccine (conjugate), Pneumococcal vaccine (polysaccharide or conjugate), Hepatitis B (recombinant protein), Acellular Pertussis, MenACWY (conjugate) and those containing the antigens of the influenza virus defined as
Anatoxin/Toxoid Vaccines	Inactivated toxins produced in most cases from proteins released by the micro-organism (toxins) to target the toxic activity created by the bacteria, rather than targeting the bacteria itself.	Tetanus vaccine and Diphtheria vaccine
Viral Vector Vaccines	Harmless virus genetically engineered to deliver to host cells the genetic code of an antigen against which immune system respond to fight the pathogen	Ebola vaccine, COVID-19 vaccine
Messenger RNA Vaccine	Composed of proprietary lipid nanoparticle delivery systems and mRNA optimized for stability and translation. They are based on a very adaptable technology.	COVID-19 Vaccine

Adjuvants used in Licensed Vaccines

Classification of adjuvants according to their main mechanism of action

Adjuvant Groups	Types of Adjuvants
Delivery systems	
Mineral Salts	Aluminium salts
Emulsions	Freund's adjuvants
	MF59
	AS03
Microparticles	Virus-like particles
	Virosomes
	PLA/PLGA
Immune Potentiators	
TLR1/2 agonists	L-pampo, MALP-2, Pam2CSK4 and Pam3CSK4
TLR3 agonists	Poly(I:C) (polyinosinic:polycytidylic acid)
	Poly-ICLC
TLR4 agonists	Monophosphoryl lipid A (MPL)
TLR5 agonists	Flagellin
TLR7/8 agonists	Imiquimod (R837; 1-(2-methylpropyl)-1H-imidazo [4,5-c]quinolin-4-amine) and resiquimod (R848, 4-amino-2-(etoximetil)-a,a-dimethyl-1H-imidazo [4,5-c]quinoline-1-ethanol)
TLR9 agonists	CpG ODNs
Combined adjuvants	AS01 and AS02
	AS04
Mucosal adjuvants	Cholera toxin (CT)
	Heat-labile enterotoxin (LTK3 and LTR72)
	Chitosan

Facciolà A. et al, Vaccines 2022 9. doi.org/10.3390/vaccines10050819

Other ingredients can be present in vaccine final formulation

Stabilizers

Purpose: To keep the vaccine effective after manufacturing

Most commonly found in: Jell-O®, naturally in the body

Examples: Sugars, gelatin

Adjuvants

Purpose: To help boost the body's response to the vaccine

Most commonly found in: Drinking water, infant formula, and some health products such as antacids, buffered aspirin, and antiperspirants

Examples: Aluminum salts

Residual inactivating ingredients

Purpose: To kill viruses or inactivate toxins during the manufacturing process

Most commonly found in: Naturally in the human body, fruit, household furnishings (carpets, upholstering)

Example: Formaldehyde†

Residual cell culture materials

Purpose: To grow enough of the virus or bacteria to make the vaccine

Most commonly found in: Eggs, and foods that contain eggs

Examples: Egg protein^

Residual antibiotics

Purpose: To prevent contamination by bacteria during the vaccine manufacturing process

Most commonly found in: Common antibiotics. Antibiotics that people are most likely to be allergic to—like penicillin—aren't used in vaccines.

Examples: Neomycin, Kanamycin, Streptomycin

Preservatives

Purpose: To prevent contamination

Most commonly found in: Some kinds of fish

Example: Thimerosal (only in multi-dose vials of flu vaccine)*

https://www.cdc.gov/vaccines/vac-gen/additives.htm

- The type of antigen determines **whether the product possesses or not intrinsic pyrogenicity**

- Based on product properties, different analysis methods may be employed to optimize **a product-specific MAT assay**

- Overview Istituto Superiore di Sanità
- Pyrogenicity testing in Europe
- MAT: workflow, technical tips&tricks
- Methods in Ph. Eur: what's new?
- MAT: a practical application

MAT METHODS

- **METHOD A QUANTITATIVE TEST**
- **METHOD B SEMI-QUANTITATIVE TEST**
- **METHOD C REFERENCE LOT COMPARISON**

From European Pharmacopoeia, Chapter 2.6.30 v.11

Definition of Limit of Detection [LOD]

The lowest analyte concentration to be reliably distinguished from the assay blank and at which detection is feasible

LOD in MAT

- Identified as the endotoxin concentration corresponding to the cut-off value: mean[OD(blank cells)] + 3SD[OD(blank cells)]
- LOD must be calculated also for non endotoxin pyrogens (NEPs)

Definition of Assay Sensitivity [AS]

- The lowest endotoxin or NEP concentration detected in samples from several donors or pools
- AS corresponds to the beginning of the linear part of the endotoxin or NEP standard curve

Definition of Contaminant Limit Concentration [CLC]

- CLC is the criterion for pass/fail decision
- CLC is expressed as endotoxin equivalent with respect to the product to be examined (ml, mg or Units)

$$CLC = \frac{K}{M}$$

K= threshold of pyrogenic dose per kilogram of body mass
M= maximum recommended bolus dose of product per kilogram of body mass

Definition of Maximum Valid Dilution [MVD]

The maximum valid dilution of a product at which the CLC can be determined

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

C = concentration of test solution

METHOD A: QUANTITATIVE TEST

- It is intended for products/vaccines showing a parallel response respect to the dilutions of standard endotoxin.

- Method A foresees a comparison of the preparation being examined with a standard endotoxin dose-response curve.

From European Pharmacopoeia, Chapter 2.6.30

METHOD A: QUANTITATIVE TEST

TEST CONDITIONS

Table 2.6.30.-1

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/*f*	None	4
B	Test solution/2 × *f*	None	4
C	Test solution/4 × *f*	None	4
AS	Test solution/*f*	Middle dose from endotoxin standard curve (R_1)	4
BS	Test solution/2 × *f*	Middle dose from endotoxin standard curve (R_1)	4
CS	Test solution/4 × *f*	Middle dose from endotoxin standard curve (R_1)	4
R_3	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

PASS/FAIL CRITERIA

PASS FAIL

- Criteria for endotoxin standard curve;
- The **endotoxin equivalent content** of the preparation being examined should be **less then the contaminant limit concentration** (CLC)*;
- The recovery of endotoxin in spiked test samples should fall within 50-200 %.

* The CLC is defined by considering the product dose, the route of administration and the sensitivity of the set-up MAT assay

From European Pharmacopoeia, Chapter 2.6.30

METHOD B: SEMI-QUANTITATIVE TEST

- For products/vaccines showing a not parallel response respect to the dilutions of standard endotoxin.

- Method B is based on the comparison between the preparation being examined and the standard endotoxin.

From European Pharmacopoeia, Chapter 2.6.30

METHOD B: SEMI-QUANTITATIVE TEST

TEST CONDITIONS

Table 2.6.30.-2

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/	None	4
B	Test solution/(	None	4
C	Test solution/(	None	4
AS	Test solution/	Standard endotoxin at 2 × estimated LOD for the test system	4
BS	Test solution/(	Standard endotoxin at 2 × estimated LOD for the test system	4
CS	Test solution/(	Standard endotoxin at 2 × estimated LOD for the test system	4
R_1	Pyrogen-free saline or test diluent	None (negative control)	4
R_2	Pyrogen-free saline or test diluent	Standard endotoxin at 0.5 × estimated LOD for the test system	4
R_3	Pyrogen-free saline or test diluent	Standard endotoxin at 1 × estimated LOD for the test system	4
R_4	Pyrogen-free saline or test diluent	Standard endotoxin at 2 × estimated LOD for the test system	4
R_5	Pyrogen-free saline or test diluent	Standard endotoxin at 4 × estimated LOD for the test system	4

PASS/FAIL CRITERIA

PASS

FAIL

- The endotoxin equivalent content of the preparation being examined should be less then the CLC*;
- The response to solution R2 should be higher than an established cut-off value;
- To determine spike-in recovery, the mean response of the spiked solution is compared with the mean response to R3 (should fall within 50-200 %).

* The CLC is defined by considering the product dose, the route of administration and the sensitivity of the set-up MAT assay

From European Pharmacopoeia, Chapter 2.6.30

METHOD C: REFERENCE LOT COMPARISON

- Developed to address extreme donor variability in response to product containing endotoxin and/or non-endotoxin pyrogens (NEPs).
- Method C compares the preparation being examined with a validated reference lot of that preparation. The type of analysis selected to compare the two is to be justified and validated for each product and is to include assay validity criteria.

From European Pharmacopoeia, Chapter 2.6.30

METHOD C: REFERENCE LOT COMPARISON

TEST CONDITIONS

Table 2.6.30.-3

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_w	Diluent (negative control)	4

PASS/FAIL CRITERIA

PASS

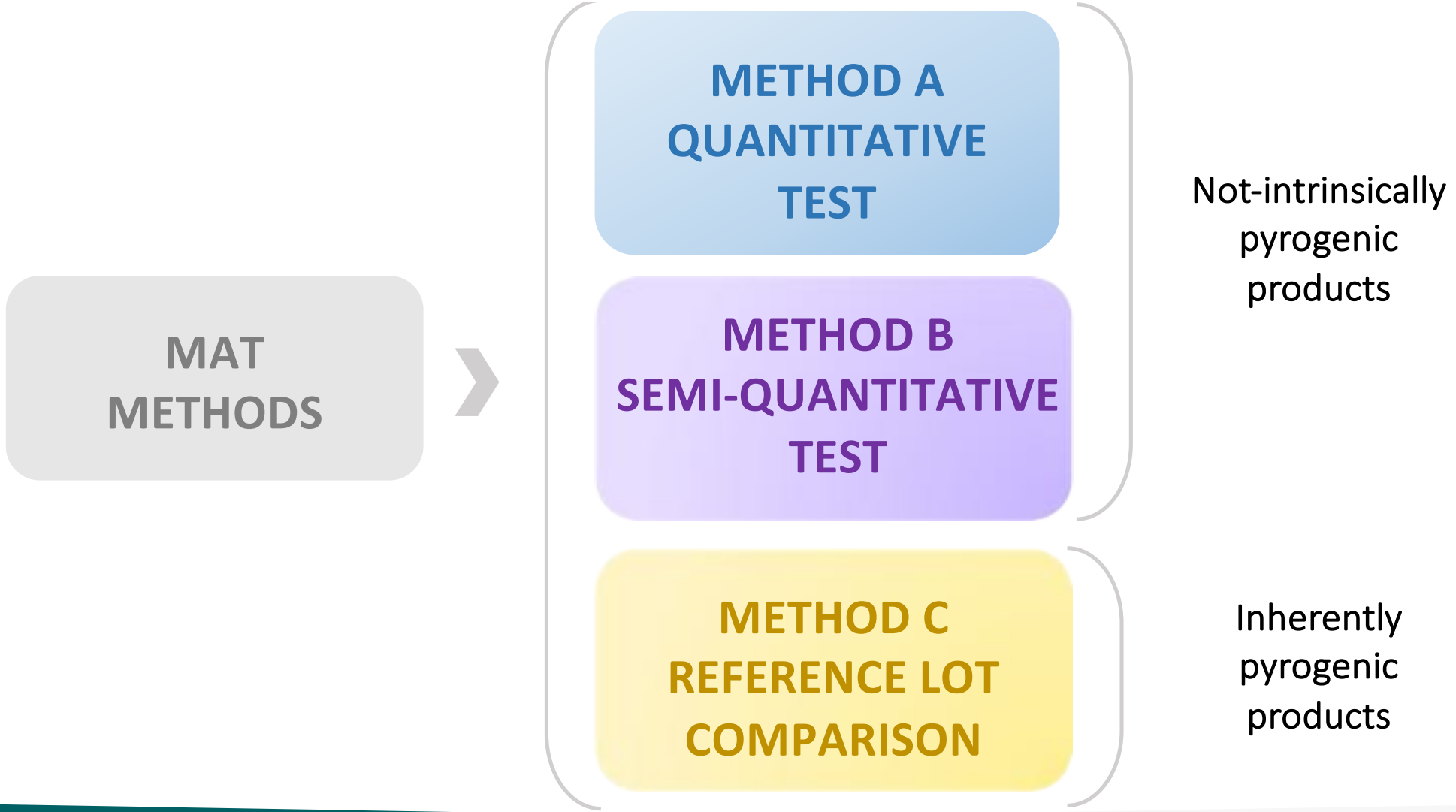
FAIL

Sum the mean response to solution A, B and C and the mean response to solution D, E and F. Divide the sum of D, E and F with the sum of A, B and C. The preparation being examined complies with the test if the resulting value complies with a defined acceptance criterion.

$$\frac{\text{mean}D + \text{mean}E + \text{mean}F}{\text{mean}A + \text{mean}B + \text{mean}C}$$

(PREPARATION BEING EXAMINED) / (REFERENCE LOT)

From European Pharmacopoeia, Chapter 2.6.30


MAT METHODS
METHOD A QUANTITATIVE TEST
METHOD B SEMI-QUANTITATIVE TEST
METHOD C REFERENCE LOT COMPARISON
Not-intrinsically pyrogenic products
Inherently pyrogenic products

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 products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants often show very steep or non-linear dose-response curves in comparison with endotoxin dose-response curves. Preparations that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

WHAT'S NEW?

Revision of MAT monograph is ongoing to address certain difficulties reported by users and to facilitate the widest implementation of the MAT

Ongoing revision of MAT in Ph. Eur (chapter 2.6.30) [I]

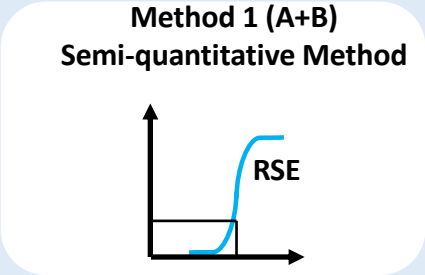
- **MVD calculation:** To allow consistent calculation and better comparability among different MAT setups, it has been proposed to replace LOD with AS [AS is a point of standard curve and not a calculated value]

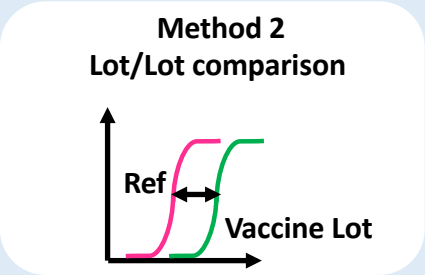
- **Validity criteria Endotoxin standard curve:** Use of non-linear regression model and less strict criteria for the standard curve (i.e., parallelism requirement deleted)

Ongoing revision of MAT in Ph. Eur (chapter 2.6.30) [II]

To **merge Methods A** and **B** into a single Method ("Method 1")

For products not inherently pyrogenic





For inherently pyrogenic products

Test for interfering factors: Replacement of current spiking dose 2xLOD with an amount of endotoxin equal or near to the middle of endotoxin standard curve

- Overview Istituto Superiore di Sanità
- Pyrogenicity testing in Europe
- MAT: workflow, technical tips&tricks
- Methods in Ph. Eur: what's new?
- MAT: a practical application

TICK-BORNE ENCEPHALITIS VACCINE (INACTIVATED)

Vaccinum encephalitidis ixodibus advectae inactivatum

DEFINITION

Tick-borne encephalitis vaccine (inactivated) is a liquid preparation of a suitable strain of tick-borne encephalitis virus grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by a suitable, validated method.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, bovine serum albumin (where applicable) and pyrogens and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain tick-borne encephalitis virus antigen by a suitable immunochemical method (*2.7.1*) using specific antibodies or by the mouse immunogenicity test described under Assay.

TESTS

Aluminium (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (*2.4.18*): maximum of 0.1 g/l.

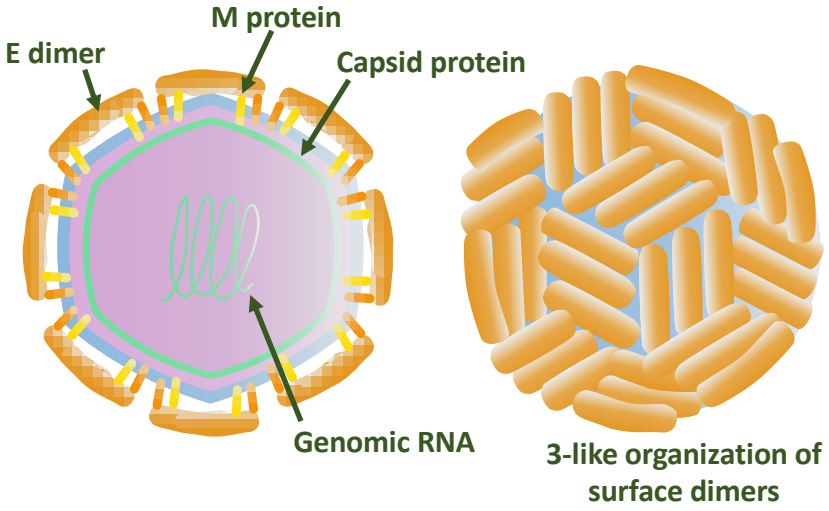
Bovine serum albumin. If bovine serum albumin has been used during production, the vaccine contains not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

Sterility (*2.6.1*). The vaccine complies with the test for sterility.

Pyrogens (*2.6.8*). The vaccine complies with the test for pyrogens. Inject into each rabbit, per kilogram of body mass, one dose of vaccine.

Tick-borne encephalitis virus [TBEV]

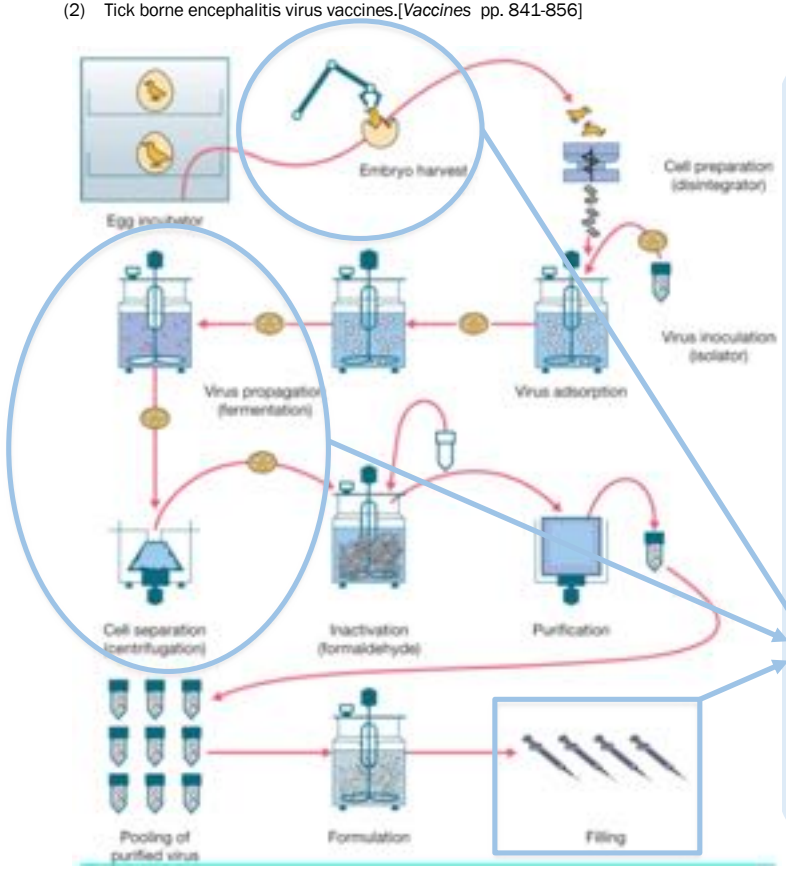
- Flavivirus
- Small enveloped virus
- Positive-sense, single-stranded RNA
- 3 structural proteins



NO INTRINSIC PYROGENICITY

Production process of TBEV vaccine

(1) *Background Document on Vaccines and Vaccination against Tick-borne Encephalitis* [Vaccine. 2011 ;29(48):8769-70]
(2) Tick borne encephalitis virus vaccines.[*Vaccines* pp. 841-856]



Barrett et al., 2008 (2)

TBEV (active substance) is cultivated chicken embryo cells, clarified by centrifugation, inactivated with formalin and then purified to produce the vaccine virus stock. Pools of different purified virus stock were formulated with aluminum hydroxide (adjuvant)

Embryo harvest from chicken eggs or the virus propagation could entail the risk of bacterial, viral or cellular contaminants entering the final product

vac2vac

- http://www.imi.europa.eu/
- http://www.vac2vac.eu/

VAC2VAC - Vaccine batch to vaccine batch comparison by consistency testing

One of the objective of VAC2VAC project was the development and the optimization of cellular assays based on analysis of human tick-borne encephalitis virus (TBEV) vaccine-induced activation of primary APC.

↓

To replace the existing pyrogenicity test in rabbit by performing the monocyte activation test MAT assay described in the European Pharmacopoeia by using human peripheral blood mononuclear cells (PBMC).

Setting of MAT conditions for the TBEV vaccine [I]

- Qualification of cell source -

The MAT optimized for the TBEV vaccine was set-up by using as cell source cryopreserved **peripheral blood mononuclear cells (PBMCs)**. According to Ph.Eur., human PBMCs have been qualified by assessing:

- PBMC viability
- Reproducibility of the response to scalar doses of reference standard endotoxin (RSE)

Setting of MAT conditions for the TBEV vaccine [I]

- Qualification of cell source -

Tab. 1: Follow-up study on cell viability and responsiveness to RSE in cryopreserved PBMC
Stability and responsiveness of cryopreserved peripheral blood mononuclear cells (PBMC) were assayed by testing cell viability and IL-6 release after stimulation with RSE in cells thawed after 6, 12 and 18 months storage in liquid nitrogen.

Etna MP et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

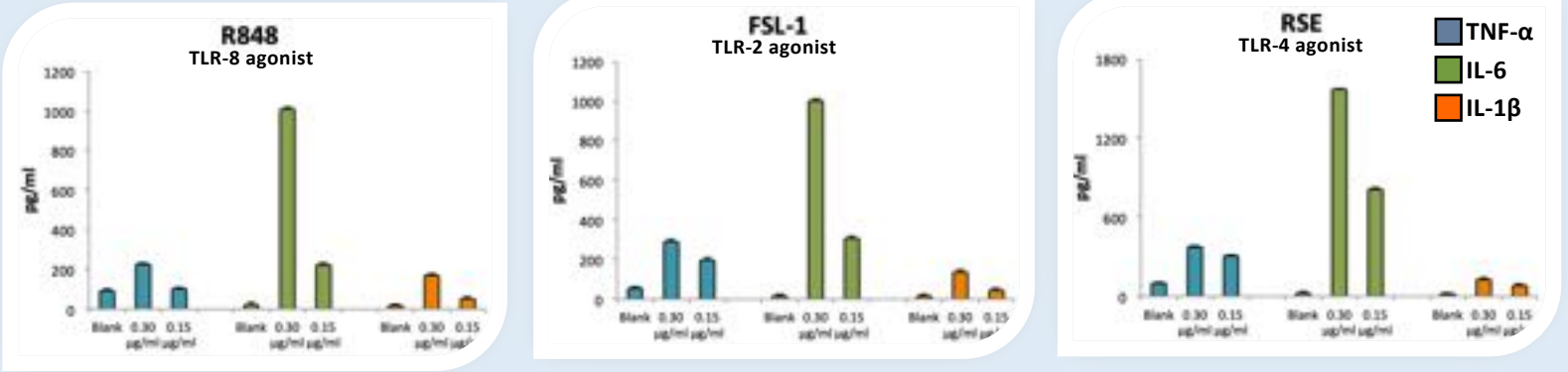
	Cell viability (%)			RSE 0.2 EU/mL (IL-6 pg/mL)			RSE 0.4 EU/mL (IL-6 pg/mL)		
	6 months	12 months	18 months	6 months	12 months	18 months	6 months	12 months	18 months
PBMC_Donor 1	89.0	93.0	93.0	566	480	400	1200	1324	896
PBMC_Donor 2	91.2	95.5	95.5	500	455	475	1000	1000	1000
PBMC_Donor 3	95.0	92.0	91.0	410	370	430	1200	813	1200
PBMC_Donor 4	91.0	93.7	90.0	502	500	394	1213	1000	924

- PBMCs remain viable (≥ 90%) when stored at -196°C up to 18 months
- Response to scalar doses of RSE is reproducible and stable up to 12 and 18 months after PBMC freezing

Setting of MAT conditions for the TBEV vaccine [II]

- Choice of the Read-out-

IL-6 was chosen as read-out providing the robust production as compared to TNF-α and IL-1β after PBMCs stimulation with RSE, and the two non-endotoxin TLR agonists R-848 and FSL-1.



Setting of MAT conditions for the TBEV vaccine [III]

- Preparatory Tests -

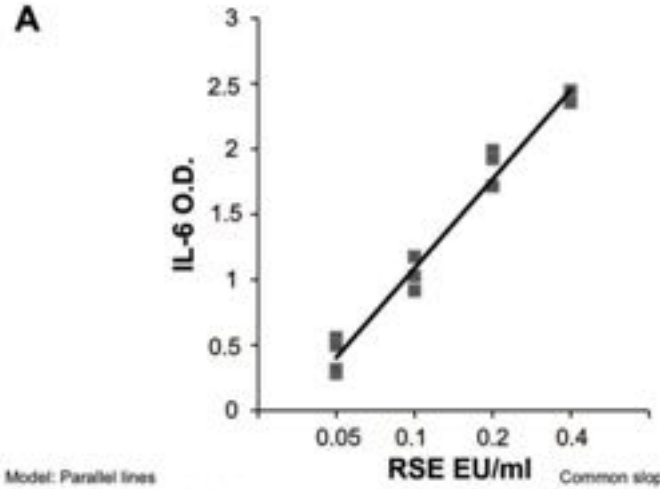
ASSURANCE OF CRITERIA FOR ENDOTOXIN STANDARD CURVE

- The test is performed with 4 replicates of **at least 4 standard endotoxin concentrations**
- The purpose is to verify whether the experimental conditions employed for the assay ensure a **linear response to different RSE doses**:
  - A statistically significant regression of response on Log_{10} dose of RSE ($p < 0.01$)
  - Not significant deviation of RSE Log_{10} dose from linearity ($p > 0.05$)

Setting of MAT conditions for the TBEV vaccine [III]

- Preparatory Tests -

ASSURANCE OF CRITERIA FOR ENDOTOXIN STANDARD CURVE



Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
* Regression	1	9.26024	9.26024	801.166	0.000 (***)
* Non-linearity	2	0.0881724	0.0440862	3.814	0.052
Treatments	3	9.34842	3.11614	269.598	0.000 (***)
Residual error	12	0.138702	0.0115585		
Total	15	9.48712	0.632475		

Modified from Etna MP et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Setting of MAT conditions for the TBEV vaccine [IV]

- Preparatory Tests -

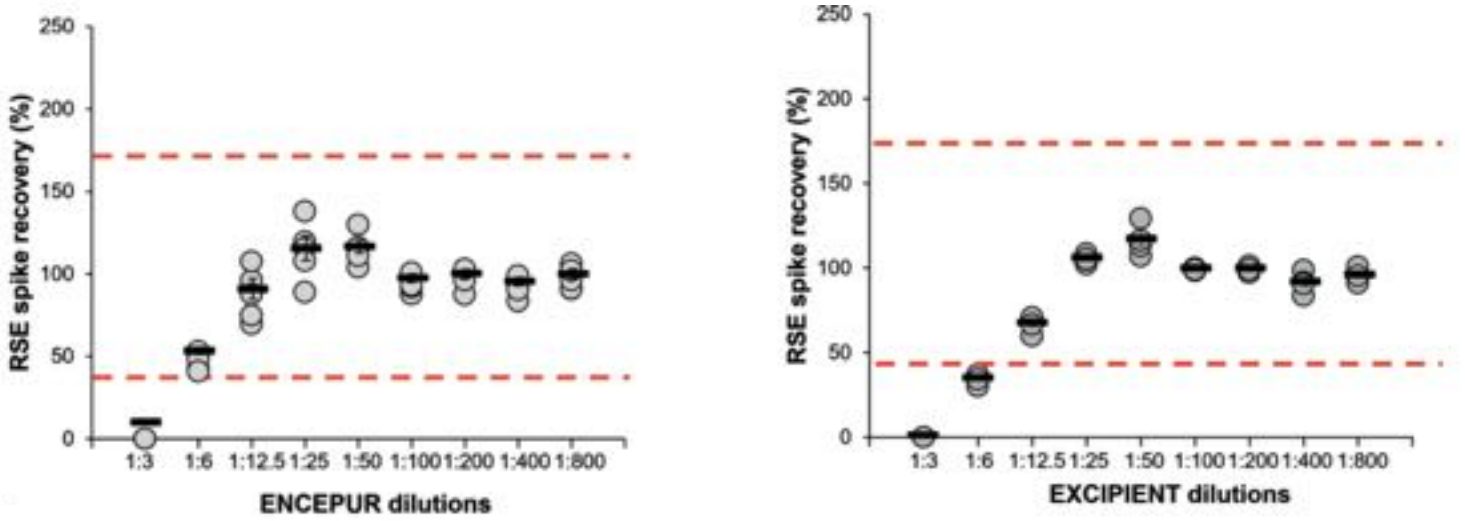
TEST FOR INTERFERING FACTORS

- The aim of the test is to verify **whether the product** to be tested (i.e., the vaccine) **interferes with the detection of endotoxin contaminants**
- The test is conducted by spiking a justified and fixed concentration of RSE into different dilution of the product (vaccine).

Setting of MAT conditions for the TBEV vaccine [IV]

- Preparatory Tests -

TEST FOR INTERFERING FACTORS



Modified from Etna MP et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Setting of MAT conditions for the TBEV vaccine [V]

- Preparatory Tests -

INTERFERENCE IN THE DETECTION SYSTEM

- The aim of the test is to verify **whether the product** to be tested (i.e., the vaccine) **interferes** (at technical level) **with ELISA procedure.**
- The test is conducted by spiking a fixed amount of the product into the standard recombinant protein curve inserted in the ELISA plate.

Setting of MAT conditions for the TBEV vaccine [V]

- Preparatory Tests -

INTERFERENCE IN THE DETECTION SYSTEM

Tab. 2: Evaluation of Encepur interference with the ELISA procedure

Five doses of Encepur (V1 = 1 µg/mL [1:3], V2 = 0.5 µg/mL [1:6], V3 = 0.25 µg/mL [1:12.5], V4 = 0.125 µg/mL [1:25] and V5 = 0.0625 µg/mL [1:50]) were added to the IL-6 standard curve. The interference of Encepur with the ELISA procedure was evaluated by considering optical density values of the IL-6 standard alone or in combination with the vaccine.

Standard		Standard+V1		Standard+V2		Standard+V3		Standard+V4		Standard+V5	
pg/mL	OD	OD	Interference (%)	OD	Interference (%)	OD	Interference (%)	OD	Interference (%)	OD	Interference (%)
600	2.629	2.549	-3.4%	2.507	-4.6%	2.580	-1.86%	2.415	-8.1%	2.535	-3.5%
300	1.788	1.770	-1.0%	1.818	+1.6%	1.579	-11.6%	1.719	-3.8%	1.662	-7.0%
150	1.095	1.061	-3.1%	1.024	-6.4%	0.924	-15.6%	1.001	-8.5%	0.980	-10.5%
75	0.593	0.555	-6.4%	0.565	-4.7%	0.500	-15.6%	0.562	-5.2%	0.526	-11.2%
37.5	0.294	0.240	-18.3%	0.291	-1.0%	0.244	-17.0%	0.276	-6.1%	0.265	-9.8%
18.8	0.140	0.131	-6.4%	0.130	-7.1%	0.111	-20.0%	0.116	-17.1%	0.120	-14.2%
9.38	0.076	0.073	-3.9%	0.062	-18.4%	0.068	-10.5%	0.068	-10.5%	0.066	-13.1%

Etna MP et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Acceptance criterium: Interference fall within +/- 20%

Setting of MAT conditions for the TBEV vaccine [VI]

- Preparatory Tests -

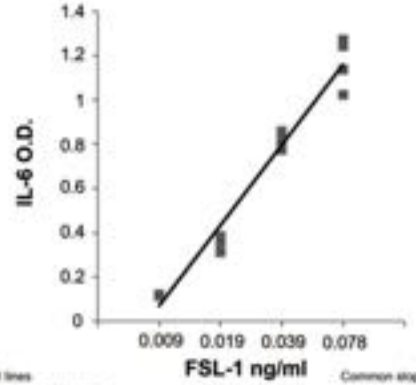
METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS

- The purpose is to verify whether the experimental conditions employed for the assay ensure a **linear response to different doses of 2 NEPs** (non-endotoxin pyrogens)
- Moreover, the test aims to verify **whether the product** to be tested (i.e., the vaccine) **interferes with the detection of NEPs**

Setting of MAT conditions for the TBEV vaccine [VI]

- Preparatory Tests -

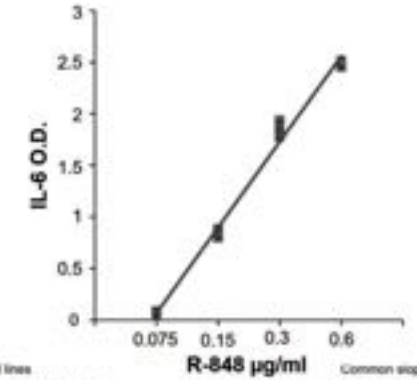
METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS



Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = 0.512265 (0.464511 to 0.560019)
Correlation | r |: 0.977391

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	1	2.52157	2.52157	365.536	0.000
Non-linearity	2	0.0352286	0.0176143	2.553	0.119
Treatments	3	2.55679	0.852265	123.548	0.000
Residual error	12	0.0827787	0.00689823		
Total	15	2.63957	0.175971		



Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = 1.09410 (1.02826 to 1.15995)
Correlation | r |: 0.989451

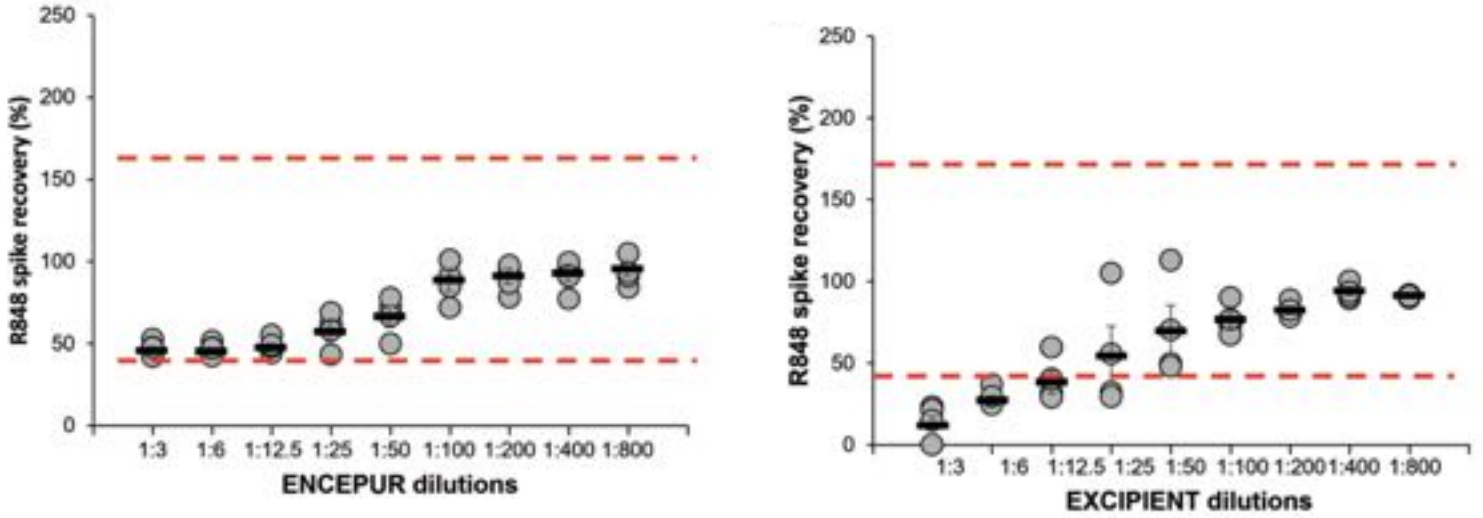
Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	1	11.5027	11.5027	877.090	0.000
Non-linearity	2	0.0891884	0.0445992	3.401	0.068
Treatments	3	11.5919	3.86395	294.631	0.000
Residual error	12	0.157375	0.0131146		
Total	15	11.7492	0.783282		

Modified from Etna MP et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Setting of MAT conditions for the TBEV vaccine [VI]

- Preparatory Tests -

METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS



Modified from Etna MP et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Determination of LOD, AS, MVD and CLC for MAT optimized for the TBEV vaccine

TLR agonist	LOD	AS
RSE (EU/ml)	0.040	0.100
R-848 (μg/ml)	0.050	0.150
FSL-1 (ng/ml)	0.003	0.009

MVD and CLC Calculation

- K= 5 EU/kg (as for any parenteral administration);
- M = dose (ml)/body mass (kg) where dose = 0,25 ml and body mass= 5 kg (since it is a pediatric vaccine);
- LOD (single PBMC donor) = 0,04 EU/ml
- Assay sensitivity [AS] = 0,1 EU/ml (the lowest or one of the lower concentration of RSE or NEPs close to the beginning of the linear part of the standard curve)

CLC = K/M = 100 EU/ml
MVD* = CLC/LOD = **2700**
MVD° = CLC/AS= **1000**

* As described in European Pharmacopoeia

° Proposed new calculation

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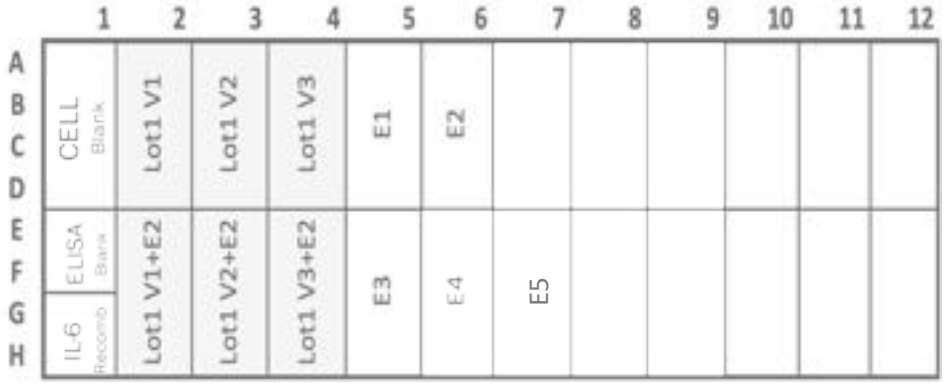
MAT setting for the pyrogenicity testing of TBEV vaccine

- Application of a modified version of Method B -

Active substance: TBEV inactivated by formaldehyde ENCEPUR®
Excipients: Aluminum hydroxide, TRIS buffer, sucrose. Traces of tetracycline, gentamicine, neomycine and formaldehyde.

Cell source: human peripheral blood mononuclear cells (PBMCs)
Read-out: IL-6 release



V1, V2, V3: Defined vaccine serial dilution; **E1, E2, E3, E4, E5:** RSE chosen serial dilutions showing a linear correlation.

Etna et al. 2020 ALTEX 37(4), 532-544. doi:10.14573/altex.2002252

Research Article

Optimization of the Monocyte Activation Test for Evaluating Pyrogenicity of Tick-Borne Encephalitis Virus Vaccine

Marilena P. Etna[1], Elena Giacomini[1], Fabiana Rizzo[1], Martina Severa[1], Daniela Ricci[1], Shahjahan Shaid[2], Denis Lambrigts[2], Sara Valentini[3], Luisa Galli Stampino[3], Liliana Alleri[3], Andrea Gaggioli[4], Christina von Hunolstein[4], Ingo Spreitzer[5] and Eliana M. Coccia[1]

[1]Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy; [2]GSK, Wavre, Belgium; [3]GSK Vaccines Srl, Siena, Italy; [4]National Center for Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome, Italy; [5]Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany

ALTEX 37(4), 532-544. doi:10.14573/altex.2002252