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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Pyrogenicity testing in Europe

MAT: workflow, technical tips&tricks

Methods in Ph. Eur: what’s new?

MAT: a practical application
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
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)

Eliana M. Coccia (Head of MAT Unit)
Marilena P. Etna (Expert of MAT Unit)
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May 25th, 2023
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Pyrogenicity testing in Europe

MAT: workflow, technical tips&tricks

Methods in Ph. Eur: what’s new?

MAT: a practical application
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].

**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].

PYROGENS (2.6.8 Chapter) [RABBIT PYROGEN TEST] 60 TEXTS
DIRECTIVE 2010/63
Substitution with non-animal technologies is mandatory in EU
BIG CHANGES in the Ph. Eur.
Newsroom
European Pharmacopoeia to put an end to the rabbit pyrogen test

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 Pharmedeuropa.
REPLACEMENT OF CHAPTER 2.6.8: proposed strategy:

https://go.edqm.eu/NewPyrogenicityStrategy
Pyrogen detection method with a risk-based approach

Exclusion of potential NEP is not clear

MAT: EP 2.6.30
Is the risk under control that non-endotoxin pyrogens are present

NO

YES

Endotoxins detection

Exclusion of potential pyrogens other than endotoxins (NEP)

BET Guideline: EP 5.1.10
rFC: EP 2.6.32
LAL: EP 2.6.14

1 Substitute by the BET test as a release
2 Replace by the MAT test as a release

From EDQM-EPAA RPT event 14-16 February 2023
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

PRODUCT i.e. VACCINE

22 h ± 1 h

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

<table>
<thead>
<tr>
<th></th>
<th>Whole Blood</th>
<th>PBMCs</th>
<th>Monocytic Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor variability</td>
<td>Very low variability</td>
<td>For unspecified pyrogens</td>
<td>Donor variability</td>
</tr>
<tr>
<td>For unspecified pyrogens</td>
<td>For unspecified pyrogens</td>
<td>For known pyrogens</td>
<td></td>
</tr>
<tr>
<td>Presence of cytokines and Abs in plasma</td>
<td>Basal activation due to PBMC isolation procedures</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[Peripheral Blood Mononuclear Cells]
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

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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**)

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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

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

Whole blood Buffy Coats

Liquid N₂

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**Tips & Tricks**

**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

**Commercially ready-to-use**
- With Fetal Bovine Serum (FBS)
- Synthetic [Animal-free]

---

**Workflow Diagram**

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

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**Tips & Tricks**

**Controlled freezing rate**

Slow freezing is critical to ensure cell viability and integrity.

- Controlled-rate freezer
- Isopropanol freezing container

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

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

Transfer in liquid nitrogen tank
Reagents & Equipment
[critical]

- Serum (FBS or HS)
- Cryoprotectant
- Cryovials resistant to very low temperature
- Liquid nitrogen tank
- Cryopreservation Medium
- Ready-to-use freezing media
- Freezing containers

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# 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

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

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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

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.

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

Cells are now ready for use in downstream applications.
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.
METHOD TO DETECT THE READ-OUT

- Enzyme-linked immunosorbent assay [ELISA]-

Microwells coated with capture Ab

Cell secreted endogenous pyrogen (Antigen)

Incubation with Detection Ab

Incubation with Streptavidin

Incubation with Substrate

Adding of STOP Solution

Microplate washer employed along ELISA procedure

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Critical factors for a successful ELISA procedure

- Plate coating
- Sample Incubation
- Secondary Ab Incubation

Reagents (Abs, substrate etc.)

Incubation time

Temperature of incubation along the procedure

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# Type of Antigens used in Licensed Vaccines

<table>
<thead>
<tr>
<th>Type of Antigen</th>
<th>Features</th>
<th>Examples of Vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live-attenuated Vaccines</td>
<td>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 disease.</td>
<td>Measles, Rubella, Parotitis, Varicella, Yellow Fever and Mycobacterium tuberculosis vaccines</td>
</tr>
<tr>
<td>Inactivated (non-replicating) Vaccines</td>
<td>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.</td>
<td>Hepatitis A virus, Poliomyelitis virus, and Influenza &quot;split&quot; or &quot;fragmented&quot; virus</td>
</tr>
<tr>
<td>Subunit Vaccines</td>
<td>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,</td>
<td>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</td>
</tr>
<tr>
<td>Anatoxin/Toxoid Vaccines</td>
<td>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.</td>
<td>Tetanus vaccine and Diphtheria vaccine</td>
</tr>
<tr>
<td>Viral Vector Vaccines</td>
<td>Harmless virus genetically engineered to deliver to host cells the genetic code of an antigen against which immune system respond to fight the pathogen</td>
<td>Ebola vaccine, COVID-19 vaccine</td>
</tr>
<tr>
<td>Messenger RNA Vaccine</td>
<td>Composed of proprietary lipid nanoparticle delivery systems and mRNA optimized for stability and translation. They are based on a very adaptable technology.</td>
<td>COVID-19 Vaccine</td>
</tr>
</tbody>
</table>
### Adjuvants used in Licensed Vaccines

Classification of adjuvants according to their main mechanism of action

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

Facciò A. et al, Vaccines 2022 9. doi.org/10.3390/vaccines10050819
Other ingredients can be present in vaccine final formulation

<table>
<thead>
<tr>
<th>Stabilizers</th>
<th>Adjuvants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purpose:</strong> To keep the vaccine effective after manufacturing</td>
<td><strong>Purpose:</strong> To help boost the body's response to the vaccine</td>
</tr>
<tr>
<td><strong>Most commonly found in:</strong></td>
<td><strong>Most commonly found in:</strong></td>
</tr>
<tr>
<td>jell-O®, naturally in the body</td>
<td>Drinking water, infant formula, and some health products such as antacids, buffered aspirin, and antiperspirants</td>
</tr>
<tr>
<td><strong>Examples:</strong> Sugars, gelatin</td>
<td><strong>Examples:</strong> Aluminum salts</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residual inactivating ingredients</th>
<th>Residual cell culture materials</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purpose:</strong> To kill viruses or inactivate toxins during the manufacturing process</td>
<td><strong>Purpose:</strong> To grow enough of the virus or bacteria to make the vaccine</td>
</tr>
<tr>
<td><strong>Most commonly found in:</strong></td>
<td><strong>Most commonly found in:</strong></td>
</tr>
<tr>
<td>Naturally in the human body, fruit, household furnishings (carpets, upholstering)</td>
<td>Eggs, and foods that contain eggs</td>
</tr>
<tr>
<td><strong>Example:</strong> Formaldehyde†</td>
<td><strong>Examples:</strong> Egg protein†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residual antibiotics</th>
<th>Preservatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purpose:</strong> To prevent contamination by bacteria during the vaccine manufacturing process</td>
<td><strong>Purpose:</strong> To prevent contamination</td>
</tr>
<tr>
<td><strong>Most commonly found in:</strong></td>
<td><strong>Most commonly found in:</strong></td>
</tr>
<tr>
<td>Common antibiotics</td>
<td>Some kinds of fish</td>
</tr>
<tr>
<td>Antibiotics that people are most likely to be allergic to—like penicillin—aren't used in vaccines.</td>
<td>Example: Thimerosal (only in multi-dose vials of flu vaccine)*</td>
</tr>
<tr>
<td><strong>Examples:</strong> Neomycin, Kanamycin, Streptomycin</td>
<td></td>
</tr>
</tbody>
</table>

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.
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METHOD A
QUANTITATIVE TEST

METHOD B
SEMI-QUANTITATIVE TEST

METHOD C
REFERENCE LOT COMPARISON

From European Pharmacopoeia, Chapter 2.6.30 v.11

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May 25th, 2023
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).

\[
\text{CLC} = \frac{K}{M}
\]

- \(K\) = 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

\[ \text{MVD} = \frac{\text{CLC} \times C}{\text{LOD}} \]

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**

*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**

*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

---

**PASS/FAIL CRITERIA**

- The endotoxin equivalent content of the preparation being examined should be less than 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

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May 25th, 2023

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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>
<thead>
<tr>
<th>Solution</th>
<th>Solution/dilution factor</th>
<th>Number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Solution of reference lot/f</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>Solution of reference lot/f</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>Solution of reference lot/f</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>Solution of preparation being examined/f</td>
<td>4</td>
</tr>
<tr>
<td>E</td>
<td>Solution of preparation being examined/f</td>
<td>4</td>
</tr>
<tr>
<td>F</td>
<td>Solution of preparation being examined/f</td>
<td>4</td>
</tr>
<tr>
<td>G</td>
<td>Positive control (standard endotoxin)</td>
<td>4</td>
</tr>
<tr>
<td>Rn</td>
<td>Diluent (negative control)</td>
<td>4</td>
</tr>
</tbody>
</table>

**PASS/FAIL CRITERIA**

Sum the mean response to solution A, B and C and the mean response to solution 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.

\[
\frac{\text{mean}_D + \text{mean}_E + \text{mean}_F}{(\text{PREPARATION BEING EXAMINED})} > \frac{\text{mean}_A + \text{mean}_B + \text{mean}_C}{(\text{REFERENCE LOT})}
\]
METHOD A
QUANTITATIVE TEST

METHOD B
SEMI-QUANTITATIVE TEST

METHOD C
REFERENCE LOT COMPARISON

MAT METHODS

Not-intrinsically pyrogenic products

Inherently pyrogenic products

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May 25th, 2023
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) [1]

**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

![Method 1 (A+B) Semi-quantitative Method](image)

For inherently pyrogenic products

![Method 2 Lot/Lot comparison](image)

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

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May 25th, 2023
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.I3): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.I8): maximum of 0.1 g/l.

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

Sterility (2.6.I). 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
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.

Reference:
Barrett et al., 2008 (2)
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).

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

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
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

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

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**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.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Standard+V1</th>
<th>Standard+V2</th>
<th>Standard+V3</th>
<th>Standard+V4</th>
<th>Standard+V5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/mL</td>
<td>OD</td>
<td>OD</td>
<td>Interference (%)</td>
<td>OD</td>
<td>Interference (%)</td>
</tr>
<tr>
<td>600</td>
<td>2.629</td>
<td>2.549</td>
<td>-3.4%</td>
<td>2.507</td>
<td>-4.6%</td>
</tr>
<tr>
<td>300</td>
<td>1.788</td>
<td>1.770</td>
<td>-1.0%</td>
<td>1.818</td>
<td>+1.6%</td>
</tr>
<tr>
<td>150</td>
<td>1.095</td>
<td>1.061</td>
<td>-3.1%</td>
<td>1.024</td>
<td>-6.4%</td>
</tr>
<tr>
<td>75</td>
<td>0.593</td>
<td>0.555</td>
<td>-6.4%</td>
<td>0.565</td>
<td>-4.7%</td>
</tr>
<tr>
<td>37.5</td>
<td>0.294</td>
<td>0.240</td>
<td>-18.3%</td>
<td>0.291</td>
<td>-1.0%</td>
</tr>
<tr>
<td>18.8</td>
<td>0.140</td>
<td>0.131</td>
<td>-6.4%</td>
<td>0.130</td>
<td>-7.1%</td>
</tr>
<tr>
<td>9.38</td>
<td>0.076</td>
<td>0.073</td>
<td>-3.9%</td>
<td>0.062</td>
<td>-18.4%</td>
</tr>
</tbody>
</table>


Acceptance criterium: Interference fall within +/- 20%
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

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

May 25th, 2023
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

<table>
<thead>
<tr>
<th>TLR agonist</th>
<th>LOD</th>
<th>AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSE (EU/ml)</td>
<td>0.040</td>
<td>0.100</td>
</tr>
<tr>
<td>R-848 (µg/ml)</td>
<td>0.050</td>
<td>0.150</td>
</tr>
<tr>
<td>FSL-1 (ng/ml)</td>
<td>0.003</td>
<td>0.009</td>
</tr>
</tbody>
</table>

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)

\[
\text{CLC} = \frac{K}{M} = 100 \text{ EU/ml}
\]
\[
\text{MVD}^* = \frac{\text{CLC}}{\text{LOD}} = 2700
\]
\[
\text{MVD}^\circ = \frac{\text{CLC}}{\text{AS}} = 1000
\]

* As described in European Pharmacopoeia

° Proposed new calculation


www.iss.it/malattie-infettive

May 25th, 2023
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.

Research Article

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

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