PYROGEN TESTING OF HUMAN VACCINE BY MONOCYTE ACTIVATION TEST

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COCCIA'S TEAM INVESTIGATES:

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- ✓ 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.

"VACCINE BATCH TO VACCINE BATCH COMPARISON BY CONSISTENCY TESTING" PROJECT (VAC2VAC)

OBJECTIVES AND AMBITION

Proof of concept of **consistency approach** for **batch release testing** of established vaccines using sets of ***in vitro*** and analytical methods

Report on pyrogenicity assessment of human Tick-borne encephalitis virus (TBEV) vaccine (ENCEPUR®) using monocyte activation test (MAT) in human PBMC.

- 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 (h-PBMC).

✓ Develop, optimise & evaluate non-animal methods that cover key-parameters for demonstrating batch consistency, safety and efficacy

✓ (Pre-)validate methods and define with regulators guidance for regulatory approval and routine use

TICK-BORNE ENCEPHALITIS VIRUS (TBEV)

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

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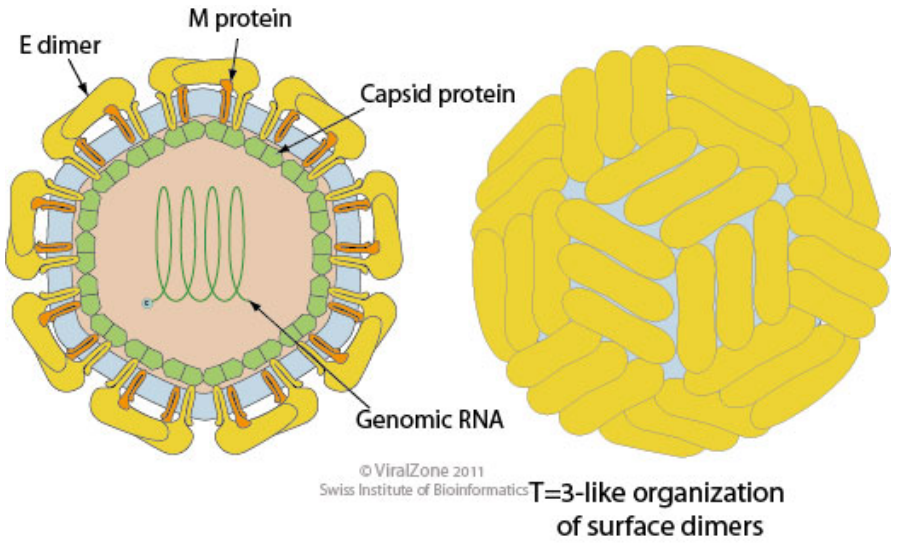
NO INTRINSIC PYROGENICITY



TICK-BORNE ENCEPHALITIS VACCINE (INACTIVATED)

Vaccinum encephalitidis ixodibus advectae inactivatum

Vaccines

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.

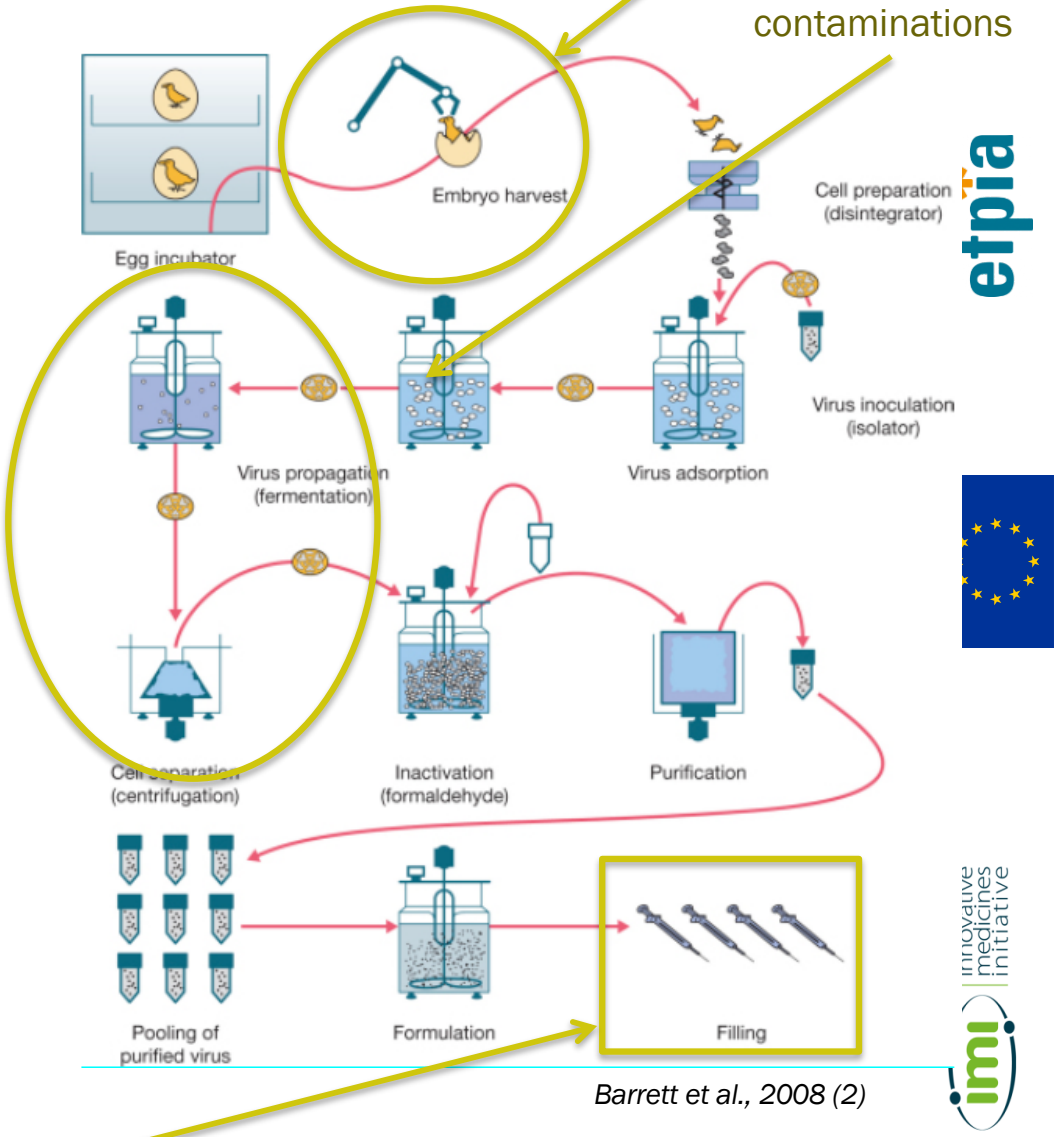
Bovine serum albumin. If bovine serum albumin has been used during production, the vaccine contains not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

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.

PRODUCTION PROCESS

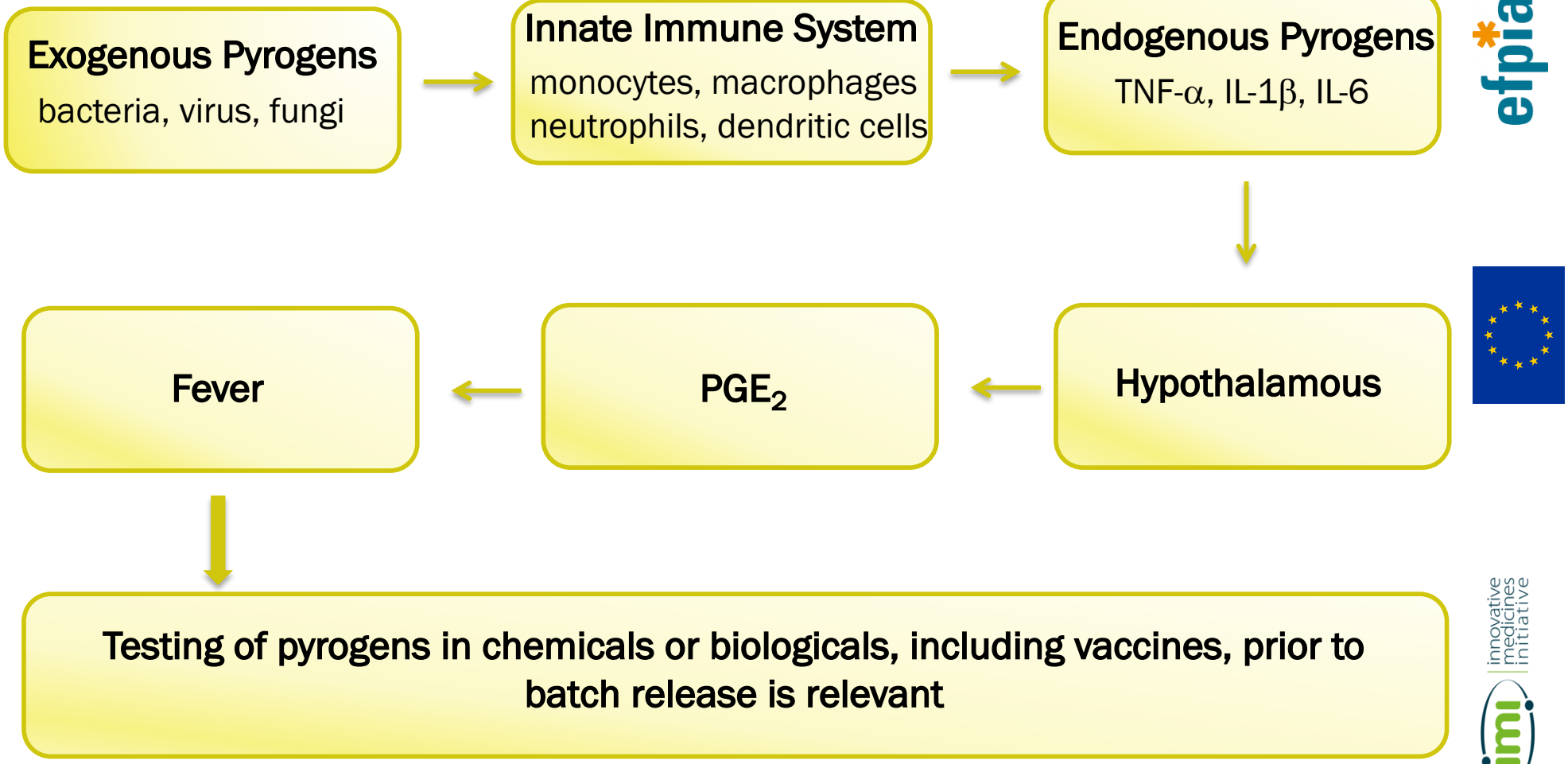
Risk of cellular, viral, bacterial and fungal contaminations



Barrett et al., 2008 (2)

(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]

PYROGENS: WHAT THEY TRIGGER?

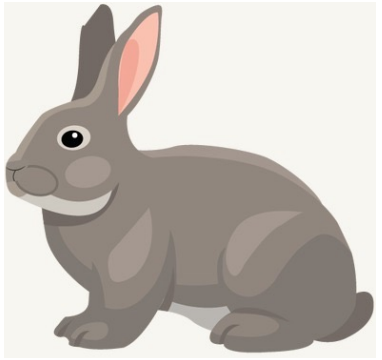


PYROGEN/ENDOTOXIN TESTS (I)

RPT- Rabbit pyrogen test

(Qualitative measurement of endotoxin and non-endotoxin pyrogens)

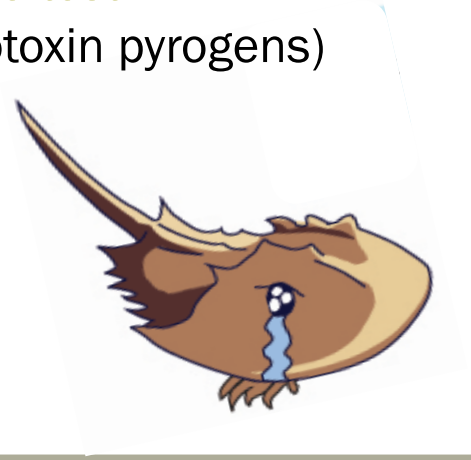
"The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined" (Chapter 2.6.8 Ph. Eur.).



BET-Bacterial endotoxin test / LAL – Limulus amebocyte lysate test

(Limit /quantitative test of endotoxin; does not detect not-endotoxin pyrogens)

"The test is used to detect or quantify endotoxin from gram-negative bacteria using amebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*)" (Chapter 2.6.14 Ph. Eur.).

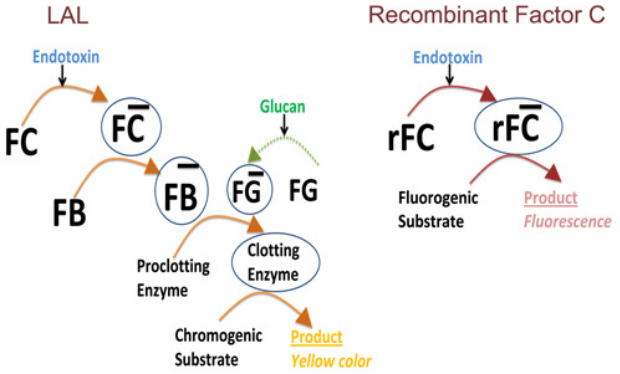


PYROGEN/ENDOTOXIN TESTS (II)

➔rFC- Recombinant factor C test

(Quantitative measurement of endotoxin)

The test is used to quantify endotoxin from gram-negative bacteria by mean of a non-animal-derived reagent namely Recombinant Factor C. (Coming soon as chapter 2.6.32 Ph. Eur.).



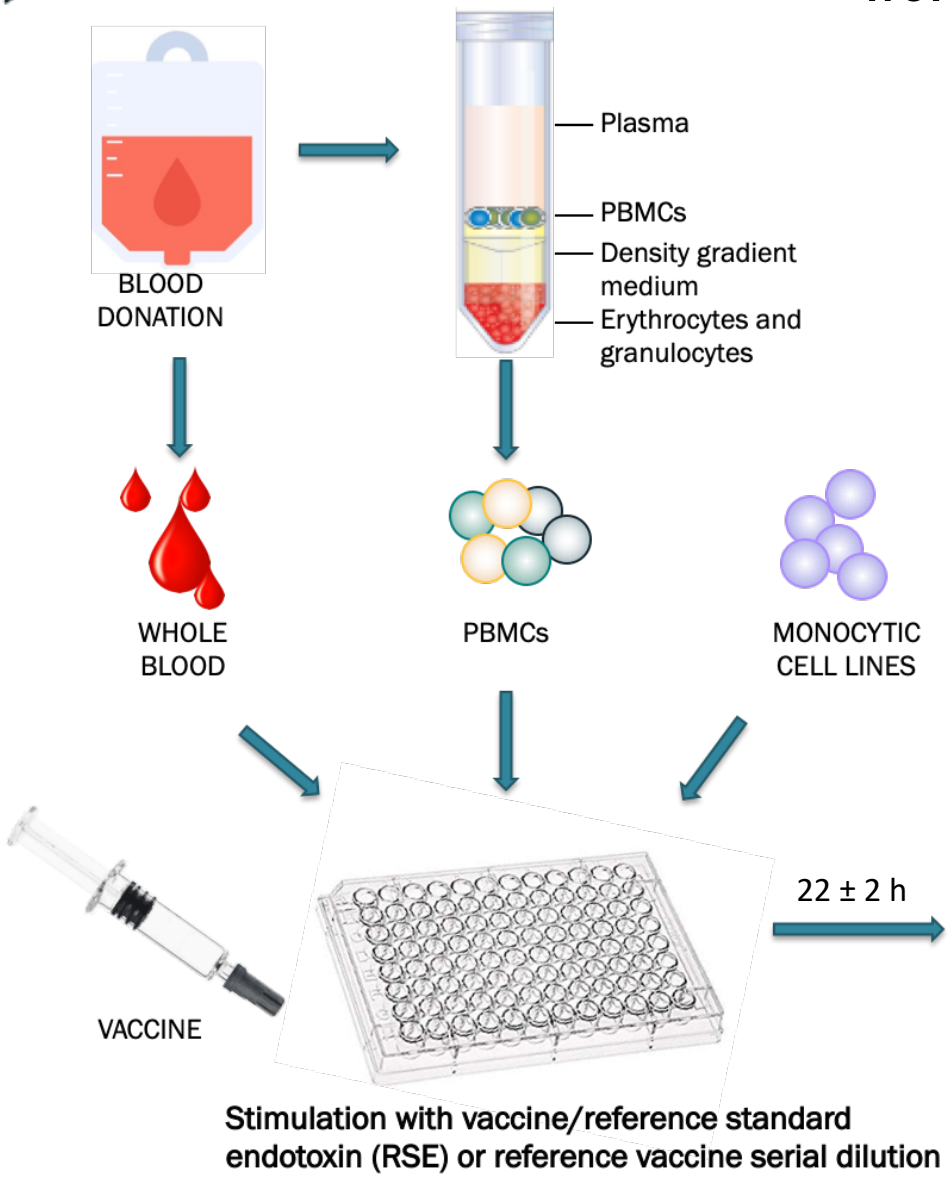
➔MAT- Monocyte activation test

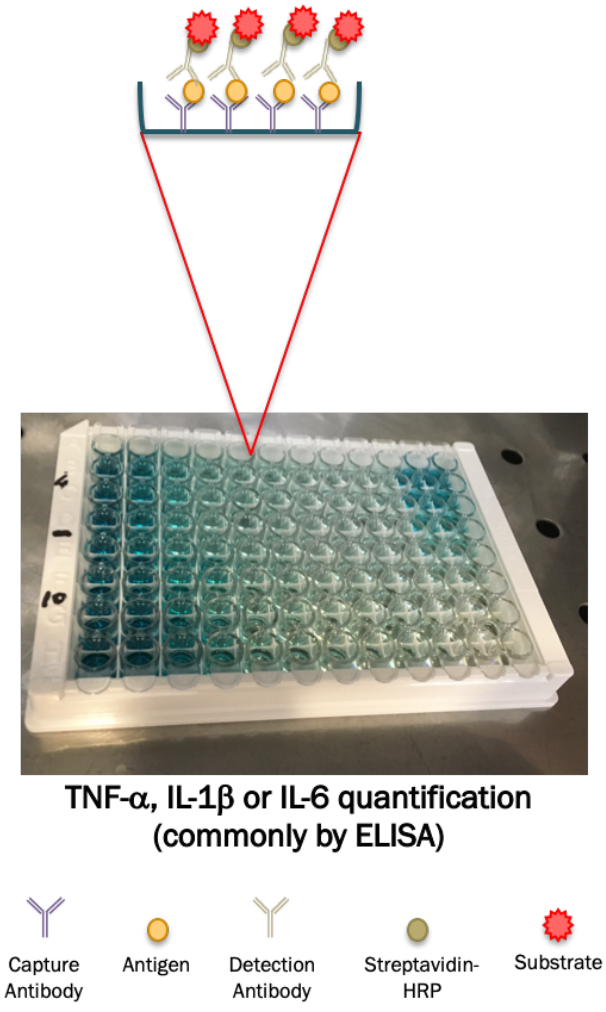
(Semi-quantitative/quantitative measurement of endotoxin and non-endotoxin pyrogens)

"The 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 TNF-α, IL-1β and IL-6" (Chapter 2.6.30 Ph. Eur.).

MONOCYTE ACTIVATION TEST

- workflow -

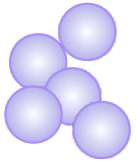




CELL SOURCE FEATURES

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 antibodies in plasma	Basal activation due to PBMC isolation procedures	

RABBIT PYROGEN TEST (RPT) VS MONOCYTE ACTIVATION TEST (MAT) (I)

STATE OF ART FOR VACCINE TESTING

- ✓ **RPT**: multivalent DTwP-HepB vaccine, vaccines against HepB, rabies, TBEV, pneumococcal and meningococcal polysaccharide vaccine;

- ✓ **MAT**: *Neisseria meningitidis* group B vaccine (BEXSERO®); Salmonella vaccine (Typhim Vi® - ANSM communications to OMCL annual meeting – Sarajevo 2018);

↓

MAT is not applied so far for the batch release of other vaccines

RPT VS MAT (II)
PROS AND CONS

✓ MAT is a non-animal alternative to the RPT (in agreement with the 3Rs principle);

✓Since RPT was originally developed to test pyrogens in parenterals (administered intravenously in large volume), the method is not appropriated for testing pyrogens in intramuscularly or subcutaneously administered vaccines (dilution is needed);

✓MAT execution (from purchase of material/animals to data report) is quicker with respect to the RPT;

✓ MAT allows the testing of human vaccine in human setting;

✓ MAT incubation time (22 ± 2 hours) is longer than RPT one (3 hours), thus allowing the detection of delayed inflammatory response.

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.

CHARACTERISTICS OF ANTIGENS AND ADJUVANTS USED IN LICENSED VACCINES

Table 1. Characteristics of adjuvants used in licensed vaccines.

Adjuvant	Composition			Major Immune Effects
(vaccines where used)	Component	Origin	Other Uses	
Aluminum (D, T, pertussis, IPV, hepatitis A & B, HPV, meningococcal and pneumococcal)	Aluminum as salts mixed with antigen (adsorption)	Naturally occurring present in soil, water, air	Medicines, cosmetics, food industry	Increases local inflammation, improves antigen update by APCs. Acts to increase antibody production
Virosomes (Hepatitis and influenza)	Vesicles where influenza antigens in aqueous volume are enclosed within a standard phospholipid cell membrane bilayer	Natural phospholipids, Seasonal influenza glycoproteins	None	Increases uptake by APCs. May interact with B cells leading to T-cell activation.
AS04 (Hepatitis B, HPV)	(3-deacyl-monophosphoryl lipid A) derived from LPS from *Salmonella Minnesota*, *Aluminum salts*	Natural exposure to LPS from Gram-negative bacteria occurs frequently	None	Directly stimulates TLR-4 increasing APC maturation and Th1 responses.
MF59® (Influenza-seasonal and pandemic)	Squalene	Animal source (shark liver oil). Found naturally in human tissues: adipose tissues, skin, arterial walls, skeleton, muscles, lymph nodes	Cosmetics, moisturizers	Increases APC recruitment and activation. Promotes antigen uptake and migration of cells to lymph nodes.
AS03 (Influenza-pandemic)	• Vitamin E (α-Tocopherol) • Surfactant polysorbate 80 • Squalene	• Naturally occurring in humans. • Surfactant and emulsifier • Animal source (shark liver oil). See above	• Vitamin • Used in foods, eye drops & intravenous injections • Naturally occurring. See above	Promotes local production of cytokines and recruitment of innate cells.
Thermo-reversible oil-in-water (Influenza-pandemic)	Squalene	Animal source (shark liver oil). See above	Naturally occurring. See above	Not reported
ISA51 (therapeutic vaccine NSCLC)	Mineral oil DRAKEOL 6 VR Surfactant mannide-mono-oleate	Refined mineral oil of vegetable origin	Food industry	Strongly immunogenic

D = diphtheria, T = tetanus, IPV = inactivated poliomyelitis vaccine, HPV = human papilloma virus, LPS = lipopolysaccharide, APC = antigen presenting cells, TLR = toll-like receptor, NSCLC = non-small cell lung cancer, MPL = monophosphoryl lipid A.

Alberta Di Pasquale et al. Vaccines doi:10.3390/vaccines3020320

MAT METHODS

METHOD A QUANTITATIVE TEST

Method A involves a **comparison of the product examined with standard endotoxin dose-response curve**. The contaminant limit concentration (CLC) of the preparation being examined is to be less than the contaminant limit concentration to pass the test.

METHOD B SEMI-QUANTITATIVE TEST

Method B involves a **comparison of the product examined with standard endotoxin**. The contaminant concentration of the **product** is to be **less than the CLC** to pass the test. The highest product concentration must be chosen for the pass decision, unless otherwise justified and authorized.

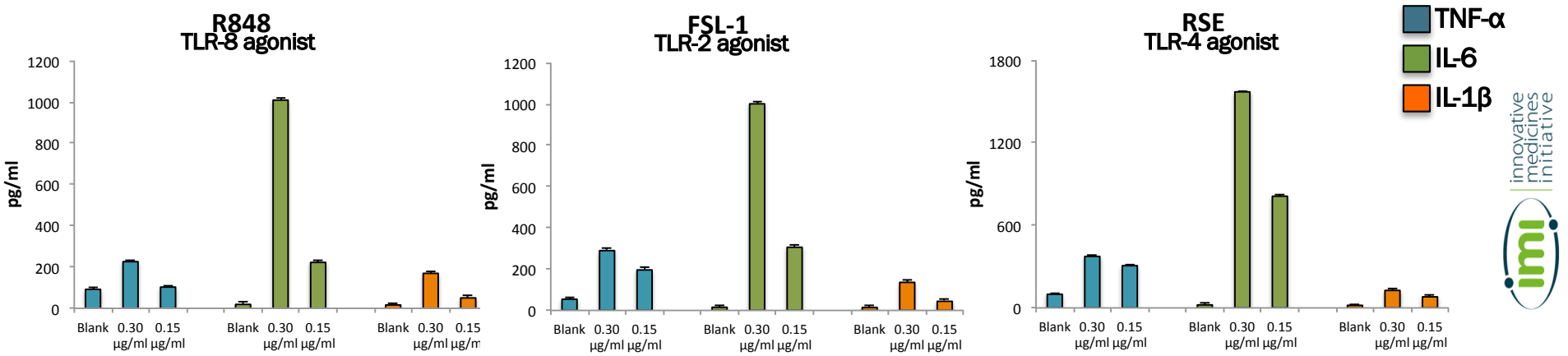
METHOD C REFERENCE LOT COMPARISON

Developed to address extreme donor variability in response to certain product containing high level of pyrogen contaminants. Method C involves a **comparison of the preparation being examined with a validated reference lot of that preparation**.

SETTING OF MAT CONDITIONS FOR THE TICK-BORNE ENCEPHALITIS VIRUS (TBEV) VACCINE (I)

- ✓ 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:
  - PBMCs remain viable (≥ 95%) when stored at -196°C up to 18 months;
  - Reproducibility of the response to scalar doses of reference standard endotoxin (RSE) at 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 (II)

- ASSURANCE OF CRITERIA FOR ENDOTOXIN STANDARD CURVE
- INTERFERENCE IN THE DETECTION SYSTEM
- TEST FOR INTERFERING FACTORS
- METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS

SETTING OF MAT CONDITIONS FOR THE TBEV VACCINE (III): PLATE LAYOUT

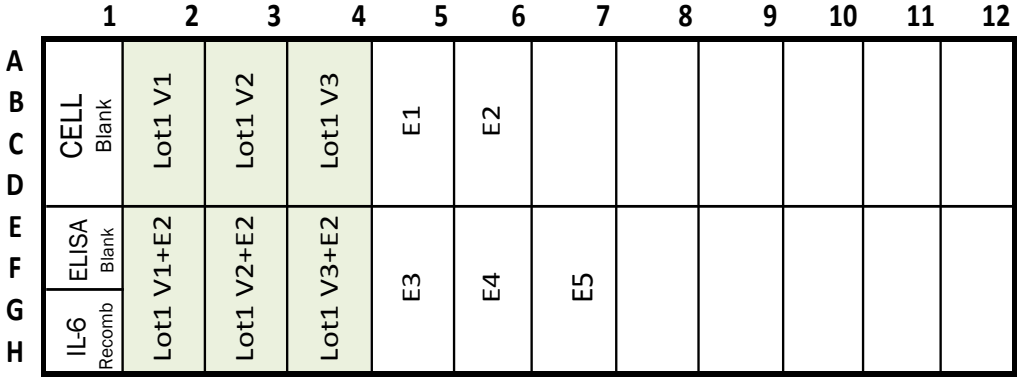
ACTIVE SUBSTANCE: TBEV inactivated by formaldehyde - ENCEPUR®-;
EXCIPIENTS: Aluminum hydroxide, TRIS buffer, sucrose. Traces of tetracycline, gentamicine, neomycine and formaldehyde.

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.



APPLICATION OF MAT TO MENIGOCOCCAL B (MenB) VACCINE

- ✓ First application of MAT to a vaccine;
- ✓ Recombinant fusion proteins NHBA and fHbp and recombinant protein NadA of MenB; MenB outer membrane vesicles (OMV);
- ✓ OMV contain: endotoxin, porins, peptigloglycan, muramylpeptides, lipoproteins (highly pyrogenic);
- ✓ To test MenB vaccine by RPT, higher dilutions are needed then in MAT;
- ✓ Successful application of Method C: data expressed as “Relative Pyrogen Units” with respect to a reference vaccine lot.

MenB VACCINE BATCH RELEASE OUTSIDE EUROPE

EUROPEAN NCLs PERFORMING MAT

Norwegian Medicines Agency

NIBSC Confidence in Biological Medicines

ISTITVTO SVPERIORE DI SANITÀ

- ✓ MenB vaccine is distributed in several countries, which do not perform local testing for batch release. In these countries (Australia, Argentine, Canada, Chile, Brazil, Turkey, Israel, New Zealand, Hong Kong), the OCABR released by European NCLs is sufficient to get market authorization for a MenB vaccine lot. In addition, the company provides the summary protocol, including all the results obtained on the distributed lot, through internal testing;

- ✓ FDA and USP position remains vague, although recently, MAT and RPT comparison test on the same vaccine lots has been requested.

WHO REQUIREMENTS FOR RPT

- State of art -

PRE-QUALIFIED VACCINES	TRS N°	Stage of RPT execution
D, T, aP, wP, HepB, IPV, Hib single or combined	980/Annex 6/2014 978/Annex 4/2013 980/Annex 4/2012	RPT or LAL on intermediate production stage and final lot
HPV (bi-, nine- and quadri-valent)	999/Annex 4/2016	If there is interference with LAL, RPT on final lot
JE (inactivated)	963/Annex 1/2007	RPT on final lot
MenA	962/Annex 2/2011	RPT on final lot
MenAC	924/Annex 2/2004	If there is interference with LAL, RPT on final lot
MenACYW-135	594/Annex 2/ 1975	RPT on final lot
PCV	977/Annex 32013	RPT on intermediate production stage; RPT or LAL on final lot
Rabies	941/Annex 2/2007	RPT on final lot
ViCPS	840/Annex 1/1992	RPT on final lot

OTHER VACCINES	TRS N°	Stage of RPT execution
HepE	WHO/BS/2018.2348	RPT on final lot
Ebola	1011/Annex 2/2018	RPT or LAL on final lot
HFRS (inactivated)	848/Annex 2/1993	RPT on final lot
RTS (Malaria)	980/Annex 3/2014	RPT or LAL on final lot
TBEV	889/Annex 2/1997	RPT on final lot

SUMMARY AND CONCLUSIONS

- ✓ MAT is intended as a **replacement** of the rabbit pyrogen test;
- ✓ The method has been already described in the general chapter of the Ph. Eur. and therefore does not require re-validation *per se* while tests for **product (vaccine)-specific optimization** are needed;
- ✓ MAT represents a human setting for testing human vaccines;
- ✓ **MAT sensitivity could be adjusted to face the heterogenicity of vaccine formulation**: ranging from the possibility to chose between primary cell or monocytic cell to three different methods of analysis;
- ✓ To rule out the presence of endotoxin and non-endotoxin pyrogens in vaccines, the MAT could be a useful tool during development of the production process (R&D), manufacturing process or for batch release;
- ✓ **Pharmacopoeia harmonization is not too far** since China has announced MAT implementation in the Pharmacopeia for 2020 while Health Canada and the National Institute of Health Science in Japan are on the way.

ACKNOWLEDGEMENT

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

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