How Industry is Implementing *in vitro* Alternatives for Pyrogenicity Testing

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Sanofi's strategy for vaccines : aiming at Quality Control with scientifically relevant non-animal based analytical testing

Ongoing

Test in **animals only for preclinical** package

Innovative **state of the art *in vitro/in silico* technologies** applied for **clinical batch testing** (eg High Throughput Sequencing) and **product characterization**

Licensure without *in vivo* QC testing

New Vaccines

Established Vaccines

Development of tetanus residual toxicity *in vitro* functional assay (BinACle) + removal of irreversibility test

Rabbit Pyrogen test replacement by *in vitro* functional assays (MAT/BET)

In vitro potency development for DTaP (Diphtheria, Tetanus & acellular Pertussis)

Achieved

Legacy or redundant safety tests removal eg Abnormal toxicity test (ATT) & specific toxicity tests

Cell based assays for toxins (Diphtheria, Pertussis)

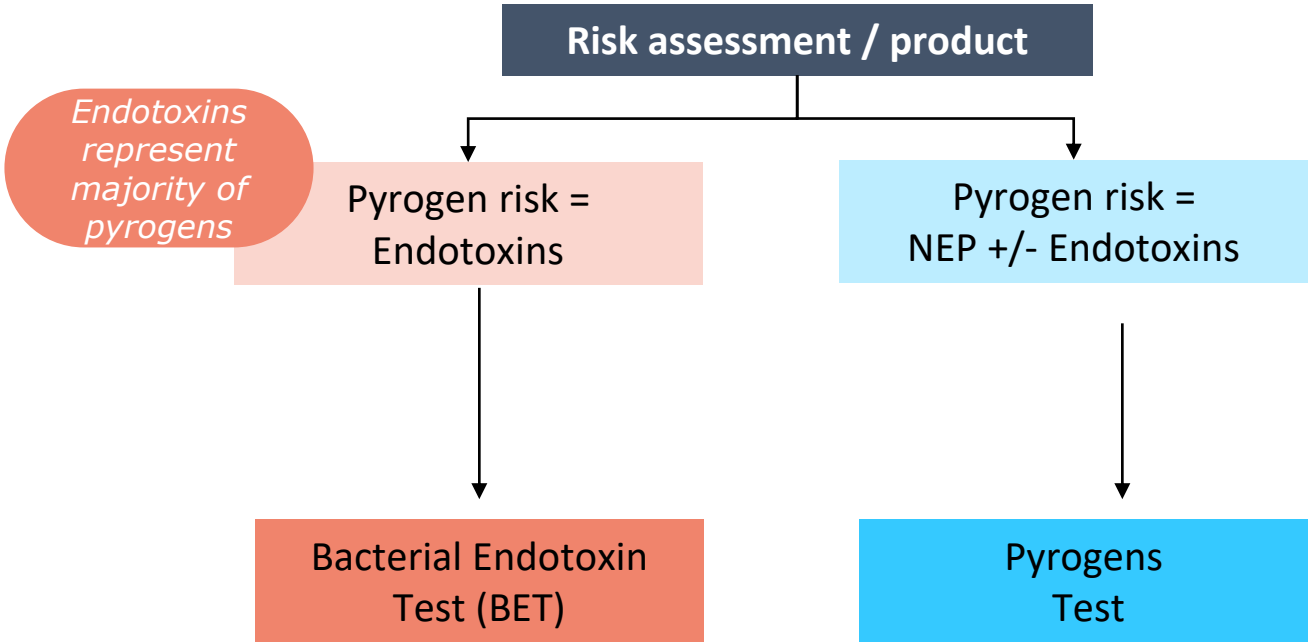
Full in vitro Adventitious agents testing

In vitro potency for viral and polysaccharide vaccines

Specific animal based testing requirements (National/Regional Pharmacopoeias, Importation)

Pyrogens – which test methods for their detection

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



NEP= Non Endotoxin Pyrogen

As per Ph. Eur. 5.1.13 Pyrogenicity (Guideline)

Pyrogenicity Testing Strategy : rFC for new vaccines & phasing out of Rabbit Pyrogen Test (RPT)

(Drug Product release testing)

rFC test solely performed for release on licensed product

*MAT Ph Eur 2.6.30 (method 1) performed on 3 industrial batches as a **one-off characterization***

BET test only

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

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

New Vaccines

Established Vaccines

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

*Product identified as **inherently pyrogenic** : MAT (Ph Eur 2.6.30 method 2/Ph Eur 2.6.40)*

MAT and rFC test performed for release on licensed product

MAT (Ongoing implementation)

rFC: Recombinant Factor C
MAT: Monocyte Activation Test
BET : Bacterial Endotoxin Test

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Overview of current of situation for pyrogenicity testing

Pyrogenicity Testing:

Choice of test according to a risk assessment

Replacement of horseshoe crab blood lysate :

- **Achieved for pharmaceutical water for some sites/ongoing for others**
- **Ongoing** for products

Rabbit pyrogen test phasing out :

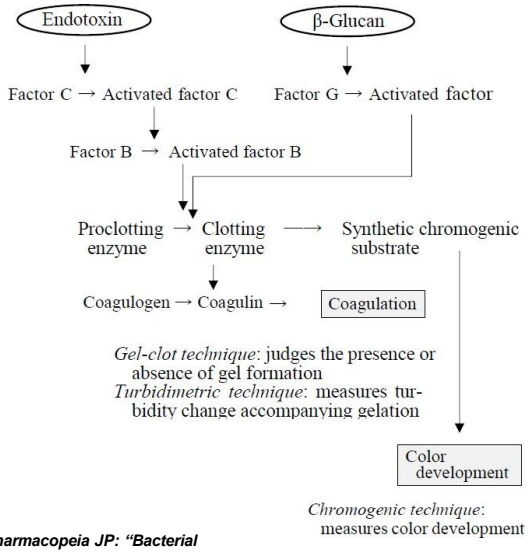
- **Few remaining RPT replacements with BET** are under worldwide approval for legacy products with an endotoxin risk only
- **Ongoing validation of MAT** for inherently pyrogenic vaccines in order to replace the RPT

RPT: Rabbit Pyrogen Test
MAT: Monocyte Activation Test
BET : Bacterial Endotoxin Test

Alternative to LAL/TAL tests: « rBETs »

- **rFC: detection and quantification of endotoxins**
  - recombinant protein-reagents for endotoxin assay as alternative methods, Main receptor is **rFC** instead of factor C from horseshoe crabs

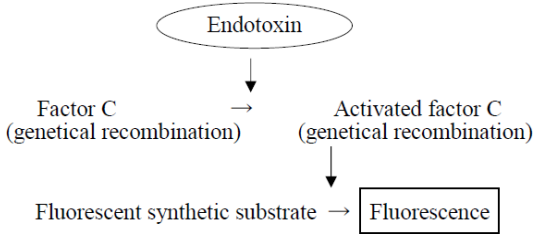
LAL/TAL based tests



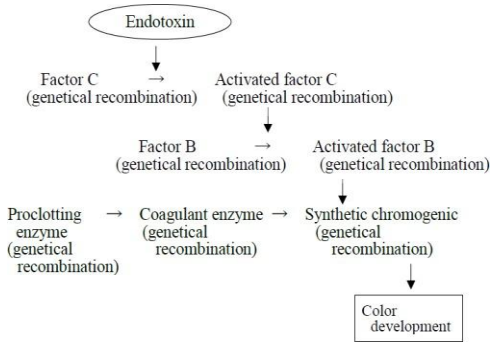
From Japanese Pharmacopeia JP: "Bacterial Endotoxins Test and alternative methods using recombinant protein reagents for endotoxin assay"

rBET

rFC

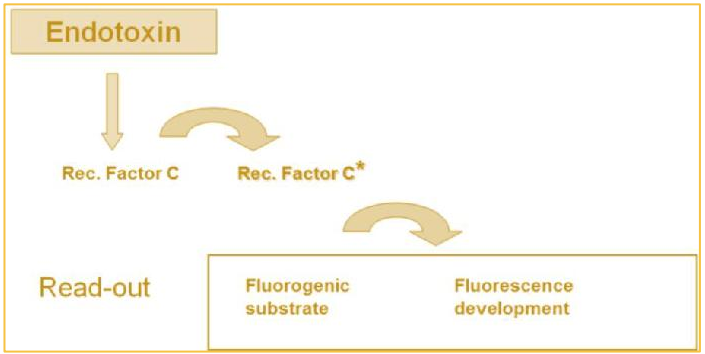


rCR (Full recombinant)



rFC Alternative chosen by sanofi has advantages

- 3Rs/ Compendial
- No ß-1,3-D-Glucan or LAL RM interference
- LPS-specific recombinant technology
- No biological variability
- Limitless source



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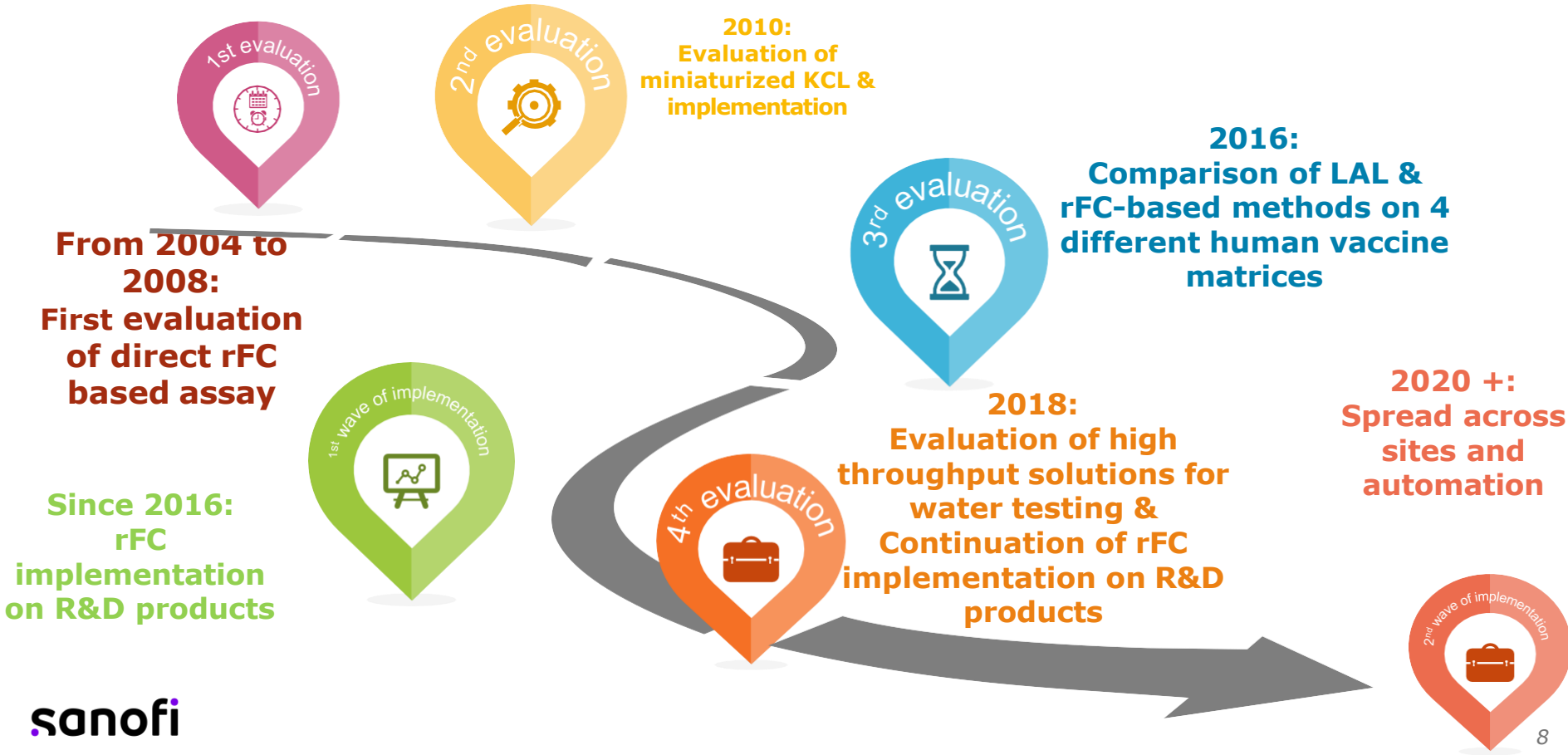
Sanofi experience on rFC evaluation



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Remaining challenges and next steps for rBET

Challenges

- All **pharmacopeias not aligned** on the topic
- **Outside EU need for full validation package**, as still considered as **an alternative method + comparability data with LAL**
- Use of **Fluorimeter for rFC** compared to Spectrophotometer for spectrophotometric method
- **Implementation roadmap** and regulatory pathway for licensed products
- **Competitive suppliers** in that field could generate **confusion between rFC / rCR**

Next steps

- Continue to **advocate for compendial harmonization** and follow all evolutions in terms of regulations
- Continue to semi automate and look into full **automation**
- **Licence** of first new vaccines with **rFC as release test**
- **Implement** on **commercialized products** : roadmap to be defined notably for regulatory submissions for PAC
- Start to **evaluate** available **rCR** kits

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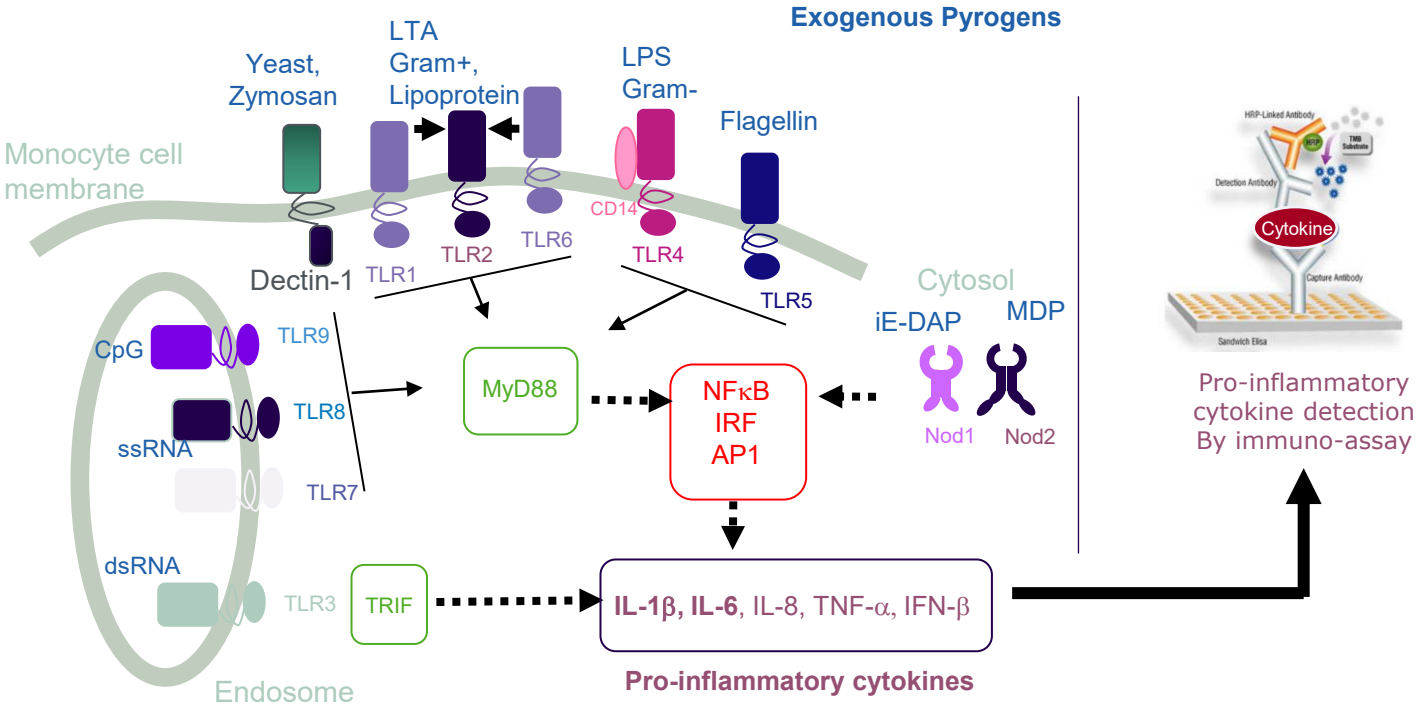
Illustrated MAT Principle

Human monocytic cells secrete pro-inflammatory cytokineS in response to the detection of exogenous pyrogens present in the product to be tested.



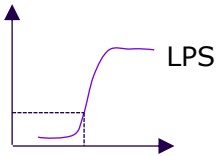
2 MAT Methods

Methods of MAT described in **Ph. Eur. 2.6.30** (method 1 and 2)

Method 1 : Limit Test / Semi-quantitative Test

- Comparison of the preparation being examined with endotoxin standard
- **Result: pass/fail (Endotoxin Equivalent/ml must be lower than the "Contaminant Limit Concentration"**)

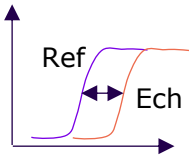
  - **→ used in Sanofi for product characterization when no NEPs are expected**



Method 2 : Reference Lot Comparison Test

- Comparison of the preparation being examined with a validated reference lot of that preparation
- **Result: Pyrogen Unit /mL or ratio (must be below a defined limit)**

  - **→ used in Sanofi when the pyrogen content (NEPs and/or endotoxin) of the vaccine to be tested is inherently high** (method according to **Ph. Eur. 2.6.40** « MAT for vaccines containing inherently pyrogenic components »)



Summary of MAT systems in place in Sanofi Vaccines

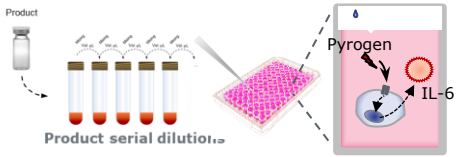
MAT format & Detection technology

cryoPBMCs (Pool) / IL-6

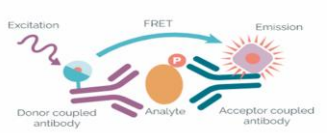
HTRF
(Homogeneous Time Resolved Fluorescence)

Per well : Human PBMCs,Media
+
Test & Ref samples & Controls

DAY 1:
Prepare *in vitro* assay
Incubate overnight (20-22h; 37°C / 5% CO_2 / humid atmosphere)



DAY 2:
3h incubation at room temperature (cell culture supernatant + anti-IL6 antibodies)
Measurement of cytokine production (IL-6)



Method & Applications		
	Ph. Eur. 2.6.30 Method 1	Ph. Eur. 2.6.30 Method 2 / 2.6.40
	New product characterization: demonstrate absence of NEPs	New product & Established vaccines: replacement of RPT for release

Which test methods are described in Pharmacopoeias

	Rabbit Pyrogen Test (RPT) **~~Ph. Eur. 2.6.8~~** / USP <151> / JP 4.04 / Russian Ph GPM 1.2.4.0005.15 / ChP <1142> / Indian Ph 2.2.8 : Not harmonized	**Bacterial Endotoxin Tests (BET)** ***LAL/TAL*: Ph. Eur. 2.6.14 / USP <85> / JP 4.01: harmonized (ICH 2001)/ ChP 1143** ***rFC*: Ph. Eur. 2.6.32 / ChP guideline 9251 / / EAEU 2.1.6.12 / South Korea Ph/ rFC/rCR: JP guideline JP G4-4-180 / USP <86>**	**Monocyte Activation Test (MAT)** **Ph. Eur. 2.6.30 revised and 2.6.40** **IP 22 2.2.25** **ChP supplement 2020 3309 "Reporter Gene Assay"** **ChP 2025 guideline <9301>** **Ph Korea guideline 2024** **Draft J Ph guideline 2024** **Brazil Ph 5.5.2.7.1**
Principle	Body temperature elevation post IV injection	Hemolymph clotting ~~in contact~~ with endotoxins / or recombinant reagent	Mimics the first step of fever mechanism – uses human cells
Method	Limit Assay (0.5 IU/mL/kg)	Detection or Quantitative Assay shown to be sensitive to 0.005 IU/mL	**Ph. Eur. method 1: Limit Assay (semi-quantitative)** **Ph. Eur. method 2: Reference lot comparison**
Goal	Safety Test Product/Process Consistency	Safety Test Product/Process Consistency	Safety Test Product/Process Consistency
Advantages	Compendial method (US, Eur and JP but not harmonized) Sensitive to all rabbit pyrogens	Compendial method harmonized for LAL based BET (US, EU, JP) Sensitive and fast	Compendial method (EU) Sensitive to all pyrogens
Drawbacks	*In vivo* Not harmonized through Pharmacopeias Variable Not representative of human biology Injection route Dilution of the product (vaccine) **Deleted in Ph. Eur. (effective Jan 2026)**	*Ex vivo (*horseshoe crab is an endangered (*Tachypleus*) or vulnerable (*Limulus*) species) « Only sensitive to endotoxins from Gram negative bacteria » **rBET compendial only in Ph. Eur. (rFC)**	*In vitro* - **Based on human cells** **Compendial method for Ph. Eur. since 2010**

MAT & rBET are generally not compendial methods outside Europe

Remaining challenges and next steps for MAT

Challenges

- All **pharmacopeias not aligned** on the topic
- Obtaining **acceptance outside EU vs obligation to phase out RPT in EU**
- Still seen as an **alternative method outside EU** → potential requirement for comparability data with RPT?
- Setting of **acceptance criteria**
- Technical challenge with **some matrices**

Next steps

- Fully implement **double sourcing for critical reagents** (PBMCs & Detection kits)
- Proceed with **validation, acceptance criteria setting and submission** for inherently pyrogenic commercialized products (Ph. Eur. method 2)
- Generate **characterization data** on new products (Ph. Eur. method 1)
- Continue to **advocate for compendial harmonization** and follow all evolutions in terms of regulations
- Assess **rapid MAT solutions** eg reporter gene approach
- Assess **serum free** cell culture media

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Emmanuelle Coppens is a Sanofi employee and may hold shares and/or stock options in the company.

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