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Microbiology chapters

Part 2

Endotoxin and Pyrogen Testing

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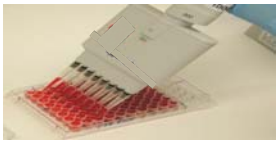
Assays for Pyrogens / Endotoxins in the Ph. Eur.

Risk-based assessment

When possible, replacement by in vitro test

or



Pyrogens (2.6.8)
(*"Rabbit Pyrogen Test"*)

LAL is a lyophilised product obtained from amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus)*

Monocyte-activation test (2.6.30)

BET (2.6.14) & Guidelines for using the BET (5.1.10)
- *BET using recombinant Factor C (2.6.32) [NEW]*

Pyrogen detection

Endotoxin detection
(e.g. LPS from Gram- bacteria)

Pyrogen detection

2.6.8 Pyrogens

("Rabbit Pyrogen Test")

Rabbit Pyrogen Test

- Principle: measure the rise in body temperature of rabbits following IV injection of the substance to be examined
- Historical test, can detect endotoxin and non-endotoxin pyrogens

  But: not quantitative, low sensitivity, animal-based (cost, method variability, animal welfare…)

2.6.8 Pyrogens

- General Monograph *Substances for pharmaceutical use*

  Pyrogens (2.6.8). If the test for pyrogens is justified rather than the test for bacterial endotoxins and if a pyrogen-free grade is offered, the substance for pharmaceutical use complies with the test for pyrogens. The limit and test method are stated in the individual monograph or approved by the competent authority. Based on appropriate test validation for bacterial endotoxins and pyrogens, the test for bacterial endotoxins may replace the test for pyrogens.

- General Monograph *Parenteral preparations*

  Bacterial endotoxins - pyrogens. A test for bacterial endotoxins (2.6.14) is carried out or, where justified and authorised, the test for pyrogens (2.6.8).

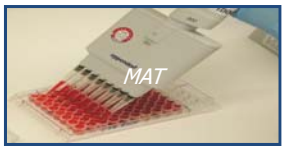
Replacement of the Rabbit Pyrogen Test

- Chapter 2.6.8 Pyrogens

  → Encourages the replacement of RPT by MAT

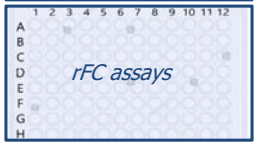
In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test (*2.6.30*).

Extract chapter 2.6.8



Replacement of the Rabbit Pyrogen Test

- Chapter 5.1.10 Guidelines for using the BET: Describes requirements for replacement of RPT by an alternative method.



Extract chapter 5.1.10

1. INTRODUCTION

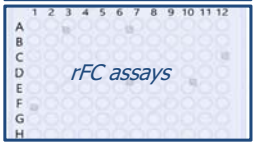
Replacement of the rabbit pyrogen test required in a pharmacopoeial monograph by an amoebocyte lysate test, or by other methods such as the monocyte-activation test or a test using recombinant factor C reagent as a replacement for the amoebocyte lysate, constitutes the use of an alternative method of analysis and hence requires demonstration that the method is appropriate for the given substance or product and gives a result consistent with that obtained with the prescribed method as described in the General Notices (see also section 12).

13. REPLACEMENT OF A METHOD PRESCRIBED IN A MONOGRAPH [Updated, Suppl. 10.3]

13-1. **REPLACEMENT BY ANOTHER METHOD DESCRIBED IN THE PH. EUR.**
Replacement of a method prescribed in a monograph by another method described in the Ph. Eur. is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices. The analyst has to demonstrate that a valid test can be carried out on the substance or product concerned. The alternative method does not have to be re-validated per se, other than in consideration of its use for a specific substance or product in a specific analytical environment and of its equivalence to the prescribed method.

Replacement of the Rabbit Pyrogen Test

- Chapter 5.1.10 Guidelines for using the BET: Decision to use the BET as the sole pyrogenicity test is made based on a risk assessment (assessment of the risk of the substance to contain NEPs).



Extract chapter 5.1.10

3. RISK ASSESSMENT

As stated in section 1 of this general chapter, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. To rule out the presence of non-endotoxin pyrogens in substances or products, the use of the monocyte-activation test (2.6.30) is recommended at release or during development of the production process; if any changes are made to the production process that could influence the quality of the product regarding pyrogenicity, the monocyte-activation test is repeated. Examples of such changes include the use of different raw materials, a different production site and different process parameters.

The decision to use the test for bacterial endotoxins as the sole pyrogenicity test is to be made after careful evaluation of the risk of the substance or product containing non-endotoxin pyrogens. The risk assessment is made with consideration given to any factor that could result in the inclusion of pyrogens not detected by the test for bacterial endotoxins. The items below constitute a non-exhaustive list of factors to be considered in the risk assessment.

2.6.14 Bacterial endotoxins

(General Chapter harmonised with JP and USP,
see Q4B Annex 14)

Test for bacterial endotoxins (BET)

- To detect or quantify endotoxins from gram-negative bacteria
- Uses amoebocyte lysate from the horseshoe crab ("LAL" reagent)
- Principle: cascade reaction of LAL in the presence of endotoxin.
- 3 techniques:
  - Gel-clot (gel formation)
  - Turbidimetric (development of turbidity after cleavage of a substrate)
  - Chromogenic (development of colour after cleavage of a substrate)

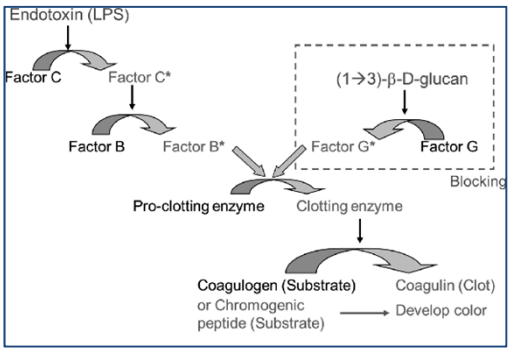


Figure: LAL cascade of endotoxin detection. *Source: JH Park, J Environ Health Sci, 2014; 40(4): 265-278*

Test for bacterial endotoxins (BET)

→ 6 methods are described in chapter 2.6.14:

- Method A. Gel-clot method: limit test
- Method B. Gel-clot method: semi-quantitative test

Gel-clot technique

- Method C. Turbidimetric kinetic method
- Method D. Chromogenic kinetic method
- Method E. Chromogenic end-point method
- Method F. Turbidimetric end-point method

Photometric quantitative techniques

"Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph."

2.6.14 BET… and 5.1.10 Guidelines for using the BET

Chapter 2.6.14 is to be read in conjunction with chapter 5.1.10 Guidelines for using the BET

2.6.14. BACTERIAL ENDOTOXINS	
1. APPARATUS	
2. REAGENTS	
3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION	
4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS	
5. PREPARATION OF THE TEST SOLUTIONS	
6. DETERMINATION OF THE MAXIMUM VALID DILUTION	
7. GEL-CLOT TECHNIQUE • *PREPARATORY TESTING* - Confirmation of the labelled lysate sensitivity - Test for interfering factors • *LIMIT TEST (METHOD A)* - Procedure - Interpretation • *QUANTITATIVE TEST (METHOD B)* - Procedure - Calculation and interpretation	8. PHOTOMETRIC QUANTITATIVE TECHNIQUES • *TURBIDIMETRIC TECHNIQUE (METHODS C AND F)* • *CHROMOGENIC TECHNIQUE (METHODS D AND E)* • *PREPARATORY TESTING* - Assurance of criteria for the standard curve - Test for interfering factors • *TEST* - Procedure - Calculation - Interpretation

5.1.10 GUIDELNES FOR USING THE BET
1. INTRODUCTION
2. METHOD AND ACCEPTANCE CRITERIA *2-1. METHODS AND PRECAUTIONS TO BE TAKEN* *2-2. ENDOTOXIN LIMIT CONCENTRATION* *2-3. CALCULATION OF THE ENDOTOXIN LIMIT* *2-4. CONSIDERATIONS WHEN ESTABLISHING AN ENDOTOXIN LIMIT FOR A SPECIFIC SUBSTANCE OR PRODUCT* *2-5. MAXIMUM VALID DILUTION*
3. RISK ASSESSMENT
4. REFERENCE MATERIAL
5. WATER FOR BET
6. pH OF THE MIXTURE
7. VALIDATION OF THE LYSATE
8. PRELIMINARY TEST FOR INTERFERING FACTORS
9. REMOVAL OF INTERFERING FACTORS
10. THE PURPOSE OF THE CONTROLS
11. READING AND INTERPRETATION OF RESULTS
12. IMPLEMENTATION OF METHODS DESCRIBED IN THE PH. EUR.
13. REPLACEMENT OF A METHOD PRESCRIBED IN A MONOGRAPH *13-1. BY ANOTHER METHOD DESCRIBED IN THE PH. EUR.* *13-2. BY AN ALTERNATIVE METHOD NOT DESCRIBED IN THE PH. EUR.*

Chapter 5.1.10:
- Explains the reason for requirements in 2.6.14
- Deals with reading and interpretation of results

2.6.14 BET

Apparatus

- Depyrogenated glassware and apparatus

Reagents

- LAL reagent with defined sensitivity λ (IU/mL), reconstituted in water for BET or buffer (as recommended by the lysate manufacturer)

Endotoxin reference standard

- Standard calibrated against the WHO IS, e.g. endotoxin standard BRP.
- Reconstitution/dilutions of standard using water for BET

Test solutions

- Dilutions of test samples using water for BET.
- pH adjustments may be necessary to fall within the pH range specified by the lysate manufacturer

2.6.14 BET

Determination of the endotoxin limit and the MVD

- *Endotoxin limit*: the endotoxin limit for active substances administered parenterally, defined on the basis of dose is equal to:

  Endotoxin limit = K / M → *Guidance on how to calculate the limit is given in Chapter 5.1.10*

  - K= threshold pyrogenic dose of endotoxin per kilogram of body mass. → *Values for K are given in Chapter 5.1.10*
  - M = maximum recommended bolus dose of product per kilogram of body mass

- *Maximum Valid Dilution (MVD):* the maximum allowable dilution of a sample at which the endotoxin limit can be determined.

$$\mathrm{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

→ *MVD is calculated for each product* → *Guidance on how to calculate the MVD is given in Chapter 5.1.10*

2.6.14 BET

Preparatory testing

Assurance of criteria for the standard curve [photometric techniques]

- Generate a standard curve from at least 3 endotoxin concentrations within the range indicated by the lysate manufacturer;
- The absolute value of the correlation coefficient | r | must be ≥ 0.980.

2.6.14 BET

Test for interfering factors [photometric techniques]

- Prepare solutions A, B, C, D (→ *cf. table*)
- Test valid if:
  - | r | ≥ 0.980 (standard curve generated with solution C)
  - The result with solution D does not exceed the limit of the blank value required in the description of the lysate reagent, or it is less than the endotoxin detection limit of the lysate employed
- Calculate mean recovery (B-A)
- Test solution is considered free of interfering factors if endotoxin recovery is within 50-200%

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

2.6.14 BET

Routine test

- Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by solution C
- Test valid if:
  - The results obtained with solution C comply with the requirements for standard curve;
  - Endotoxin recovery (B-A) is within 50-200%;
  - The result with solution D does not exceed the limit of the blank value required in the description of the lysate, or it is less than the endotoxin detection limit of the lysate.

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

- Preparation complies if the mean endotoxin concentration of the replicates of solution A, is less than the endotoxin limit for the product

BET

I have read chapter 2.6.14 BET but I am unclear on how to calculate the endotoxin limit for my product. Where can I find further guidance?

Further guidance, including values for K and a practical example, is given in chapter 5.1.10 Guidelines for using the BET.

2.6.32 Recombinant Factor C

NEW!

2.6.32 BET using recombinant Factor C

- New General Chapter
- Standalone chapter, not referenced in any monograph
- Describes a BET that uses a rFC based on the gene sequence of the horseshoe crab, and a fluorimetric end-point detection method
  - For now, only the fluorimetric method is described as the rFC kits currently available on the European market and most of the available scientific data are based on this method
- Topic of rFC assays is not new for the Ph. Eur.
  - rFC assays are mentioned in chapter 5.1.10 *Guidelines for using the BET* since 2016, allowing rFC assays to be used as alternative to classical LAL assays
- Chapter 2.6.32 is a significant development in a context where the world relies on horseshoe crabs as a single source of reagent

2.6.32 BET using recombinant Factor C

Background to Chapter 2.6.32

- Project resumed in 2017, in light of new developments, including:
  - rFC assay kits from several suppliers are available (kits from 2 manufacturers in Europe)
  - Increasing range of products on which validation has been performed
  - Independent data were published by JP (collaborative study results in Kikuchi, et al. 2017, comparison of 3 rFC and 3 LAL-based kits on 18 commercially available LPS types and 11 NOEs in water)
  - Other publications with rFC/LAL comparability data e.g. articles from Eli Lilly (Bolden, et al. 2017, data on medicinal products)
  - First medicinal product released using an rFC assay, approved by FDA (2018)

(*non-exhaustive list*)

2.6.32 BET using recombinant Factor C

Timelines

European Pharmacopoeia Commission
Decision to add project for new chapter on work program

↓

BET Working Party
Elaboration of draft chapter

↓

Public consultation in Pharmeuropa 31.1 (Jan-May 2019)

↓

BET Working Party
Review of comments

↓

European Pharmacopoeia Commission
Adoption (Nov 2019)

Next: **Publication in Ph. Eur. Suppl. 10.3: July 2020**

2.6.32 BET using recombinant Factor C

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2.6.32. TEST FOR BACTERIAL ENDOTOXINS USING RECOMBINANT FACTOR C
1. EQUIPMENT
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4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS
5. PREPARATION OF THE TEST SOLUTIONS
6. DETERMINATION OF THE MAXIMUM VALID DILUTION
7. FLUOROMETRIC QUANTITATIVE TECHNIQUE
8. PREPARATORY TESTING - Standard curve criteria - Interfering factors
9. TEST - Procedure - Calculation - Interpretation

2.6.14. BACTERIAL ENDOTOXINS	
1. APPARATUS	
2. REAGENTS	
3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION	
4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS	
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Chapter 2.6.32 is to be read in conjunction with chapter 5.1.10 Guidelines for using the BET

5.1.10 Guidelines for using the BET

- Revised to reflect the adoption of chapter 2.6.32 and clarify requirements for the introduction of rFC assays by users of the Ph. Eur. (Publication in Suppl. 10.3)
- Implication for users: facilitated implementation
  - With the new chapter 2.6.32, rFC assays will be described in the Ph. Eur. As a Ph. Eur. method, they will not have to re-validated, other than in consideration of their use for a specific substance or product. → i.e. product-specific validation only.
  - *Replacement of BET method prescribed in monograph by an rFC assay is regarded as the use of an alternative method, as per the General Notices.*

rFC assays

The individual monograph for an API prescribes the use of an LAL assay. Can I use an rFC assay instead and if so, what are the requirements?

Alternative methods can be used, as per the General Notices. Requirements for the introduction of rFC assays have been clarified in the revised chapter 5.1.10 Guidelines for using the BET (to be published in Ph. Eur. Supplement 10.3).

2.6.30 Monocyte-Activation Test (MAT)

Monocyte-Activation Test

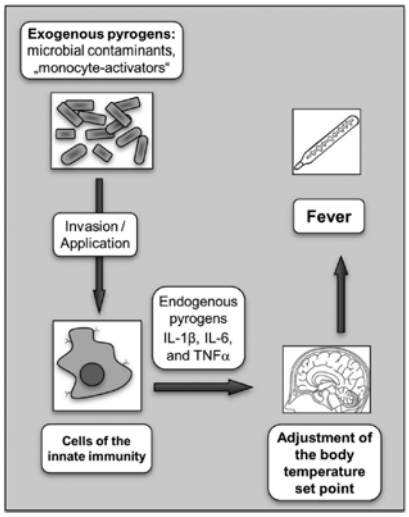

Figure: Human fever reaction.
Source: Hasiwa et al. ALTEX 30, 2/13 2013

- Principle: Upon activation by pyrogens, human monocytes release mediators such as pro-inflammatory cytokines (e.g. IL-6, IL-1β, TNF-α), which are detected in an immunoassay (ELISA).
- Can detect endotoxin and non-endotoxin pyrogens
- Based on the human fever response (better prediction of pyrogenic activity in humans)
- Non-animal test

Monocyte-Activation Test

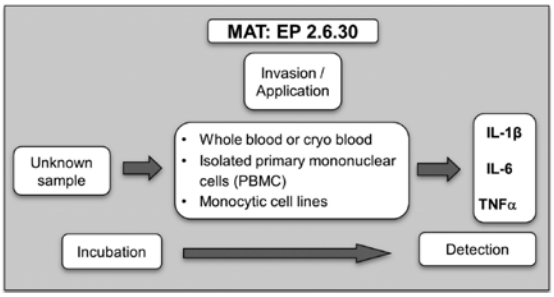

Figure: Principle of MAT.
Source: Hasiwa et al. ALTEX 30, 2/13 2013

- Different variants of MAT depending on:
  - Source of human monocyte: whole blood (fresh or cryopreserved), PBMCs (fresh or cryopreserved), human monocytic cell line
  - ELISA read-out: IL-6, IL-1β, TNF-α…
- 3 methods described in chapter 2.6.30:
  - Method A (Quantitative test): comparison of the preparation being examined with a standard endotoxin dose-response curve
  - Method B (Semi-quantitative test): comparison of the preparation being examined with standard endotoxin
  - Method C (Reference lot comparison test): comparison of the preparation being examined with a validated reference lot of that preparation

2.6.30 Monocyte-Activation Test

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6. PREPARATORY TESTING *6-1. ASSURANCE OF CRITERIA FOR THE ENDOTOXIN STANDARD CURVE* *6-2. TEST FOR INTERFERING FACTORS* *6-3. METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS* *6-4. INTERFERENCE IN THE DETECTION SYSTEM*

7. METHODS 7-1. METHOD A: QUANTITATIVE TEST 7-2. METHOD B. SEMI-QUANTITATIVE TEST 7-3. METHOD C: REFERENCE LOT COMPARISON TEST Test procedure, Calculation and interpretation, Pass/fail criteria of the preparation Guidance notes

Guidance notes at the end of chapter 2.6.30

Guidance note
1. INTRODUCTION
2. METHODS 2-1. INFORMATION REGARDING THE CHOICE OF METHODS 2-2. CALCULATION OF CONTAMINANT LIMIT CONCENTRATION 2-3. INFORMATION REGARDING CRYO-PROTECTANTS 2-4. INTERFERENCE TESTING 2-5. CROSS-VALIDATION
3. REPLACEMENT OF THE RABBIT PYROGEN TEST BY THE MAT
4. VALIDATION OF ALTERNATIVE METHODS

2.6.30 Monocyte-Activation Test

Recommendations of ECVAM Workshop 43 (2001)

First version published in 2010, (*Supplement 6.7*)

EDQM survey (2013) on implementation of MAT & applicability of 2.6.30

➢ **OUTCOME**:
- MAT uses: for product release, to rule out the presence of NEPs, for in-process testing, for trouble-shooting
- Chapter 2.6.30 is useful
- However some technical guidance for successful performance of the test are required

Elaboration of the revised chapter (meetings, drafting, data)

Pharmeuropa 27.4 (2015): > 80 comments

Improvement

Revised chapter published in 2017 (*Supplement 9.2*)

Implementation of MAT

- Despite the introduction of chapter 2.6.30, the uptake of MAT by Ph. Eur. users has been slow
- Barriers to broader MAT implementation (*based on comments received during the EDQM survey*): acceptance by competent authorities in all regions, lack of NEP standards, patent situation not always clear to users (e.g. licence to use cell lines), use of human whole blood/human blood cells…

Revised chapter 2.6.30 MAT

SUPPLEMENT 9.2

As a result of a survey distributed by the EDQM in 2013 to users of the Ph. Eur., the following improvements have been made.

Introduction: the wording 'very steep dose-response curves' has been changed to 'very steep or non-linear dose-response curves' as the latter better characterises the responses when non-endotoxin contaminants are present.

Definitions: clarification added that calculation of the maximum valid dilution (MVD) is based on the endotoxin reference standard; the possibility of using an estimated limit of detection (LOD) based on historical data when calculating MVD has been introduced.

- ✓ Qualification of cell sources: requirements according to the origin, preparation and intended use of cells; caution statement regarding the averaging effect when cells are pooled
- ✓ Methods improvement: validation of the system with non-endotoxin ligands for toll-like receptors; more detailed description of methods A, B and C including examples for calculation and interpretation of results;
- ✓ Guidance notes:
  - ✓ Choice of methods: further information on situations where method A is not appropriate
  - ✓ Use of MAT as part of validation exercise when replacing RPT by a BET (to rule out the presence of NEPs)

- Section 2-1. Information regarding the choice of methods. Further clarification is given on the inappropriateness of Method A if the dose-response curve for the preparation to be examined is not parallel to that of the standard endotoxin curve. In addition, a notice has been added regarding the product specific validation and capacity of the chosen method to identify non-responders along with low and high responders to a particular product/contaminant(s) combination(s).
- Section 2-5. Cross-validation has been added. Regarding the presence of non-endotoxin pyrogens in the product, a recommendation to perform cross-validation of the monocyte-activation test together with the bacterial endotoxins test has been introduced. In the context of the 3Rs, the rabbit pyrogen test can be performed for cross-validation purposes where the monocyte-activation test cannot be validated.
- A new entry has been included in Table 2.6.30.-4 for 'Parenteral formulations administered per square metre of body surface', in accordance with the recently revised general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins*.
- It is now specified that MAT is considered as a replacement for the rabbit pyrogen test.

MAT

I am considering setting up the MAT in my laboratory. How do I select the most appropriate MAT method (i.e. method A, B and C)?

Information regarding the choice of methods is provided under the section "Guidance notes", at the very end of chapter 2.6.30 on MAT.

Endotoxin and Pyrogen Testing Q&A session

Q&A

Thank you for your attention

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