Application Note

Testing of a Toxoid Vaccine with the PyroDetect System

Introduction

A vaccine is a preparation of a weakened or killed pathogen, such as a bacterium or virus, or of a part of the pathogen's structure. Following administration, antibody production or cellular immunity against the pathogen are stimulated without causing a severe infection.
There are different types of vaccines in use, which represent different strategies to reduce the risk of illness, while retaining the ability to induce a beneficial immune response, e.g. killed, attenuated, toxoid vaccines and others. In the present Application Note the test of a toxoid vaccine using the PyroDetect System is described.

Toxoid vaccines belong to the group of dead vaccines. They are composed of detoxified toxins (toxoids) which are the effective components of the vaccine. Due to particular procedures during the production- and purification process the protein-based toxins are rendered harmless (inactivated or killed toxins). However, their antigenic determinants are preserved and make the vaccine immunogenic, at the same time excluding any pathogenicity. To increase the immune response, the toxoid components are usually adsorbed to aluminum or calcium salts, which serve as adjuvants.
Vaccines are parenterally administered and have to be tested for pyrogenic contamination before their release (see the corresponding chapters of the EP).

Pyrogenic substances in pharmaceutical products can induce life-threatening fever-including reactions. Therefore, it is mandatory for the respective industry to ensure that the pyrogen concentrations in pharmaceutical products do not exceed the allowed limits. The two canonical procedures for pyrogen detection either require the use of animals (Rabbit Pyrogen Test, RPT) or are limited to the detection of lipopolysaccharides (LPS) from the cell walls of gram-negative bacteria (Bacterial Endotoxin Test, BET, or Limulus Amoebocyte Lysate Test, LAL Test). Furthermore, both tests do not fully reflect the biological potency of the different pyrogens in man. Such a test employing fresh or cryo-conserved human blood was described and validated as the in-vitro pyrogen test [1,2]. In April 2010 it was introduced in the European Pharmacopoeia (EP) as the Monocyte-Activation Test (MAT) [3].
The PyroDetect System from Merck Millipore uses the principle of the Monocyte-Activation Test for the detection of pyrogens in pharmaceutical products. In this Application Note the test of a toxoid vaccine for pyrogenic substances is discussed.

Principle of the PyroDetect System

The PyroDetect System uses cryo-preserved human blood as a source of monocytes. The monocytes activated by pyrogens produce cytokines, which are then detected in an immunological assay (ELISA) involving specific antibodies and an enzyme-mediated color change.

The following figure gives a schematic overview of the test procedure:

Cryoblood incubation

Samples or endotoxins are mixed with cryoblood in a cell culture microplate and kept in an incubator at 37℃ for 8-24 hours for IL-1ß production.

Interleukin-1ß ELISA

For the IL-1ß detection the cryoblood incubation mixture is transferred to an ELISA plate coated with a monoclonal antibody specific for interleukin-1β. Interleukin molecules present in the culture supernatant are bound by the immobilized antibody. After removing unbound components by several washing steps, an enzyme-linked polyclonal antibody specific for IL-1ß is added.

With the addition of the substrate a color reaction is started, which allows the detection of the bound IL-1ß in an ELISA reader.

Read-out & data analysis

The pyrogen concentration in the sample is then determined from the IL-1ß concentration via an endotoxin standard curve, and analyzed with the PyroDetect Data Analysis Tool.

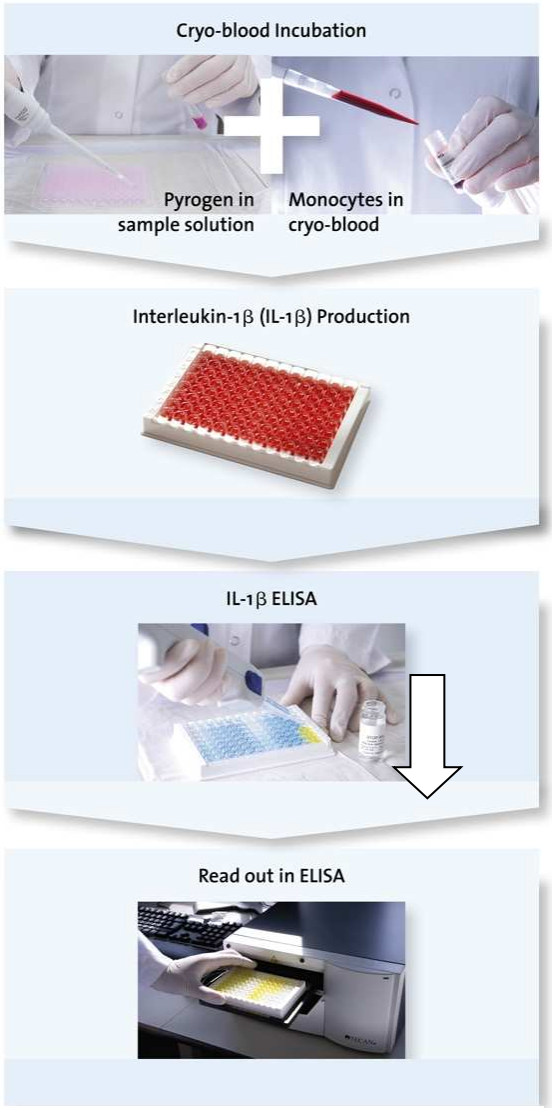


Fig.1: Overview of the test procedure with the PyroDetect System

Material and Methods

Material

The following test material was used:

- Vaccine:
    - Type: Toxoid vaccine
    - Origin: Bacteria (gram-positive, anaerobic)
- PyroDetect Kit (Cat. No. 1.44154.0001)
- PyroDetect Cryoblood (Cat. No. 1.44155.0001)
- PyroDetect Endotoxin standard Cat. No.1.44161.0001)

The additionally needed equipment (pipettes, instruments, and consumables) and the detailed test procedure are described in the user manual provided with the kit. The tests were performed according to this manual.

Methods

In the first step of the vaccine testing the test for interfering factors (TIF) was performed to discover whether the product contains components that influence the test results by inhibition or enhancement of the interleukin-1ß production.
For the test for interfering factors the following concentrations of the product were selected, each without and with the addition of a 0.5EU/mL endotoxin spike: 1:1 (undiluted), 1:2, 1:4, 1:8, 1:16. 1:32, 1:64, 1:128, 1:256; 1: 512, 1:1024, 1:2048, 1:4096, 1:8192 and 1:16384. The sample dilutions were prepared as quadruplicates with cell-culture medium (RPMI).

In parallel to the test for interfering factors, an endotoxin standard curve was generated to provide a tool for the quantification of the pyrogen amount or, respectively, for the determination of the spike recovery. For the standard curve the following endotoxin concentrations were used: 2EU/mL, 1EU/mL, 0.5EU/mL, 0.25EU/mL and 0.125EU/mL. The pure RPMI medium served as negative control.

With the test for interfering factors those concentrations of the product were determined that were free of interfering (enhancing or inhibiting) substances. Subsequently, the highest product concentration found to be free of interferences was taken as the initial concentration for the pyrogen detection with Method B (semi-quantitative method). This test comprised an endotoxin standard curve (as mentioned for the TIF) and a test of three different sample dilutions with and without addition of a 0.5EU/mL endotoxin spike. Each concentration was set up in quadruplicates. The dilution 1:512 was taken as the initial concentration of the tested vaccine. Additionally, the product was diluted 1:1024 and 1:2048.

The analysis of the test results was performed by using the PyroDetect Data Analysis Tool (Cat. No. 1.44299.0001).

Results and Discussion

Results

The test for interfering factors was performed to discover whether the toxoid vaccine contains any enhancing or inhibiting factors that might interfere with the subsequent detection of pyrogens in the product. As already mentioned the TIF was started with the undiluted product up to a dilution of 1:16384. The first nine dilution steps of the vaccine showed an enhancing effect, meaning that the recovery of the added endotoxin spike was not within the required range of 50% - 200%.

The 1:512 dilution was the first concentration of the sample that was free of interferences, just as all the following concentrations. Therefore, the pyrogen detection with Method B was started with the 1:512 dilution. Fig.2 shows the relevant test results of the TIF where the switch from the enhancing effect to the valid region of the spike recovery is demonstrated.

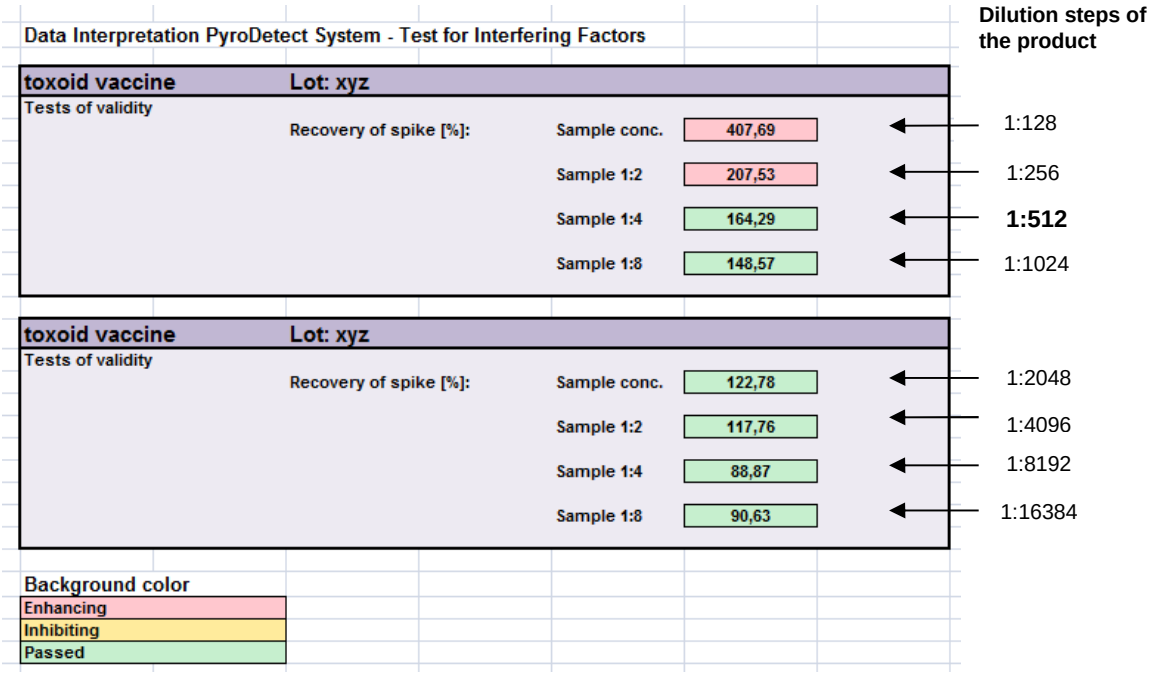
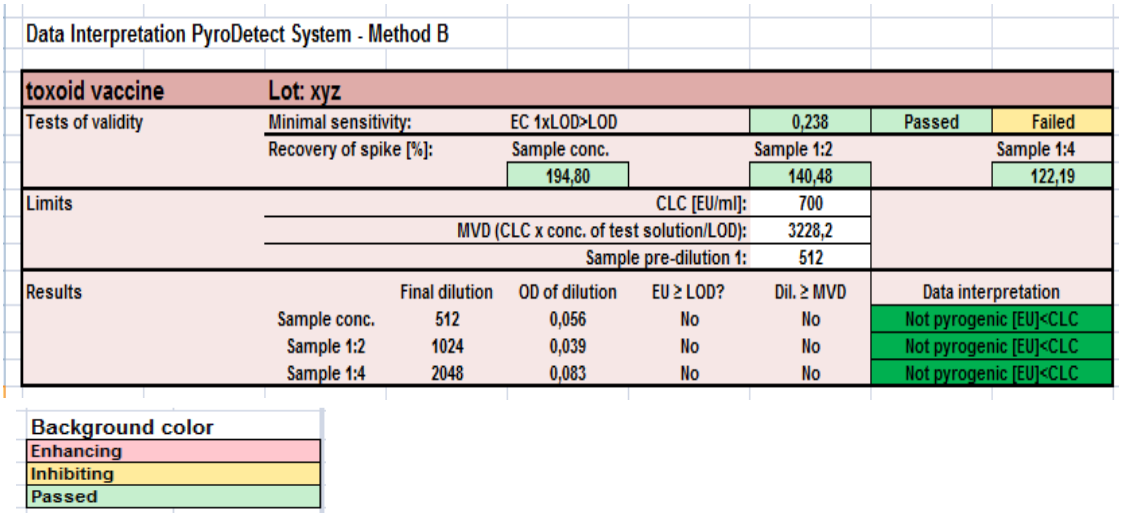


Fig.2: Results of the TIF, tested with a toxoid vaccine: Dilution steps 1:128 – 1:16384 are shown; 1:512 is the first concentration of the product that is free of interfering factors.

For the detection of pyrogenic substances in the toxoid vaccine, Method B (semi-quantitative test) was performed. Three dilutions of the product (1:512, 1:1024 and 1:2048) were tested without and with the addition of an endotoxin spike (0.5 EU/mL). The results are shown in Fig.3 and confirm the results obtained in the TIF. The spike recovery for the selected dilutions of the product was within the required range of 50% - 200%.

The Contaminant Limit Concentration (CLC) of 700 EU/mL for the tested vaccine was calculated according to the allowed contamination limit for parenterally administered pharmaceutical products (5EU per kg body weight and hour). The Maximum Valid Dilution (MVD) was calculated automatically by the PyroDetect interpretation software from the CLC value for the product and the Limit of Detection (LOD) for the test. The MVD determined for the toxoid vaccine in the current test was 3228, meaning that, all selected concentrations of the product were within the allowed dilution range (≤ MVD). The pyrogenic contamination of all dilutions was below the LOD. Therefore, the product was considered as “Not pyrogenic”.



Data Interpretation PyroDetect System - Method B

toxoid vaccine	Lot: xyz					
Tests of validity	Minimal sensitivity:	EC 1xLOD>LOD		0,238	Passed	Failed
	Recovery of spike [%]:	Sample conc.		Sample 1:2		Sample 1:4
		194,80		140,48		122,19
Limits			CLC [EU/ml]:	700		
			MVD (CLC x conc. of test solution/LOD):	3228,2		
			Sample pre-dilution 1:	512		

Results		Final dilution	OD of dilution	EU ≥ LOD?	Dil. ≥ MVD	Data interpretation
	Sample conc.	512	0,056	No	No	Not pyrogenic [EU]<CLC
	Sample 1:2	1024	0,039	No	No	Not pyrogenic [EU]<CLC
	Sample 1:4	2048	0,083	No	No	Not pyrogenic [EU]<CLC

Background color
Enhancing
Inhibiting
Passed

Fig.3: Results of Method B, tested with a toxoid vaccine: The three selected dilution steps 1:512, 1:1024 and 1:2048 have to be considered as “Not pyrogenic”.

Discussion

Vaccines are a highly heterogenic group of pharmaceutical products. They contain dead or inactivated organisms or purified products derived from them. There are several types of vaccines in use, eg. attenuated, toxoid, subunit or conjugate vaccines. The broad spectrum of vaccines gives reason to expect that their response to a certain test method such as the PyroDetect System is also diverse and cannot be generalized. Especially, in the case of vaccines that contain components of bacterial cell walls, a pyrogenic reaction can be caused by the product itself. Nevertheless, the testing of vaccines using the PyroDetect system is advantageous compared with the rabbit- and the LAL test because it exactly reflects the situation in the human body and thereby increases the predictive capacity. Furthermore, masking effects that can be induced by the used adjuvants, e.g. $Al(OH)_3$, are known to cause difficulties in the LAL test. They were not observed with the PyroDetect system (data not shown).

Apart from the diversity of the products, the general procedure for a vaccine test with the PyroDetect System can be described as follows:

- An extensive test for interfering factors has to be performed. It is advisable to test more than the common four concentrations of the product to be sure that the dilution that is free of interferences is covered in any case. Otherwise, a second TIF may be needed to reach the correct range of dilutions.
- The contaminant limit concentration (CLC) has to be calculated (e.g. from a special product monography or defined by individual requirements from the manufacturer).
- The test for pyrogenic contamination (Method A or B) has to performed. Here it is advisable to choose Method B, since in many cases the potential contamination of a product is unknown and might consist of endotoxins as well as non-endotoxins. It has to be ensured that the decisive concentrations of the product are within the allowed range (≤ MVD).
- For data interpretation the PyroDetect Data Analysis Tool is recommended.

References

[1] Hoffmann S, Peterbauer A, Schindler S et al. (2005) International validation of novel pyrogen tests based on human monocytoid cells. J Immunol Meth 298: 161-173
[2] Schindler S, Spreitzer I, Löschner B. et al. (2006) International validation of pyrogen tests based on cryopreserved human primary blood cells. J Immunol Meth316: 42-51
[3] Pharmacopoeia 6th Edition (6.7) (2010) Chapter 2.6.30 Monocyte- Activation Test

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