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APPLICATION NOTE

Quantitative Endotoxin Assay of Ultrapure Water

Introduction

The cell wall components of gram-negative bacteria, such as *E. coli* and *Pseudomonads*, are designated as endotoxins. They have a hydrophilic polysaccharide and a lipophilic lipid component and, unlike the bacteria from which they originate, are highly heat and pH-stable. Endotoxins are pyrogens (*i.e.*, they can cause fever if they come in contact with mucous membranes or enter the bloodstream).^[1]

According to the prevalent pharmacopeias, the defined limit values for endotoxin content may not be exceeded during the manufacturing process of pharmaceuticals.

In mammalian cell cultures that are used to produce biopharmaceuticals, such as immunoglobulins, the presence of endotoxins can lead to cell death. For this reason, ultrapure media (*i.e.*, ultrapure water, with levels proven to be below the limits) must be used to manufacture biopharmaceuticals or to propagate cell lines or cell cultures.

The objective of this study is to demonstrate that the ultrapure water produced by the arium pro VF system exhibits an endotoxin content that is far below the prescribed limits and that can be used for the applications mentioned above.

Endotoxin Testing

A method for detection of endotoxins is the *Limulus* amoebocyte lysate (LAL) test which uses the clotting reaction of a lysate of amoebocytes isolated from the horseshoe crab (*Limulus polyphemus*). This reaction involves a primitive evolutionary coagulation cascade in which proteases are sequentially activated, thus resulting in



FIGURE 1. The arium pro VF ultrapure water system.

the formation of a clot.

The coagulation cascade (also called the *Limulus* enzyme cascade^[2,3]) can be triggered by endotoxins or alternatively by β -glucans, which are short-chain polysaccharides found in the cell walls of yeasts and molds. The β -glucan pathway may lead to false-positive results.^[1] Based on coagulation, the liquid, colorless lysate reacts by congealing to form a solid, milky gel; hence

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the term “gel-clot method.” Because the sensitivity of the *Limulus polyphemus* lysate is extremely high, it is imperative to rule out a false-positive reaction caused by contamination with endotoxins or β -glucan. For this reason, exceptionally high purity requirements must be placed on ultrapure water.

In addition to the gel-clot method, the endotoxin content can also be assayed using a quantitative chromogenic method where a synthetic chromogenic

substrate is added to the lysate. Whereas in the gel-clot method, a gel clot is formed by the clotting enzyme, the synthetic chromogenic substrate in the chromogenic method is split by the clotting enzyme, resulting in a color change to yellow. The intensity of the yellow coloration correlates directly with the endotoxin content of the sample (chromogenic test). Preparing the lysate for either the gel-clot or the chromogenic method must therefore be done using ultrapure water.

Ultrapure Water System Description

The arium® pro VF system (Figure 1) has been designed to produce ultrapure water from pretreated drinking water and removes any contaminants still present in this potable water. Ultrapure water production requires continuous recirculation and a constant water flow rate which is achieved using a pump system with controlled pressure. The conductivity of the water is measured at the feed water inlet and at the downstream port or product water outlet.

The arium pro VF system used in the tests described in this paper operates with two different cartridges. These are filled with a special active carbon adsorber and mixed-bed ion exchange resins to deliver ultrapure water with a

low total organic carbon (TOC) content. Moreover, the unit has an integrated UV lamp that has a bactericidal and oxidizing effect at wavelengths of 185 nm and 254 nm, respectively.

Furthermore, the arium ultrapure water system has a built-in ultrafilter module used as a crossflow filter. The ultrafilter membrane utilized in this filter retains colloids, microorganisms, endotoxins, RNA, and DNA. A 0.2 μ m final filter installed at the water outlet serves to remove particulates and bacteria during dispensing of the ultrapure water stream produced. The process is shown in Figure 2.

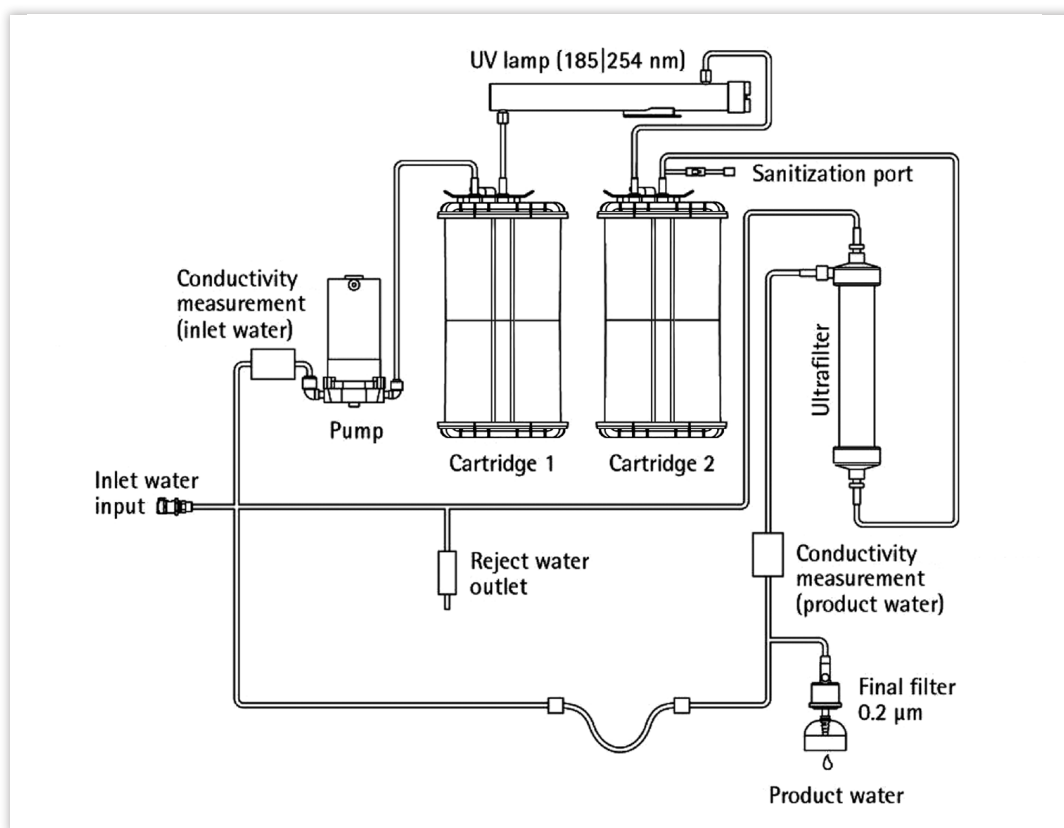


FIGURE 2. Schematic flow diagram for the arium pro VF ultrapure water system. (For enhanced clarity, the valves and their controllers are omitted.)

Assays Performed to Determine Endotoxin Content of Ultrapure Water Produced in the arium pro VF System

Gel-Clot Method

The lyophilized lysate ([Endosafe® 15006](#), Charles River) was dissolved in ultrapure water obtained from the arium pro VF system and frozen by portions in pyrogen-free test tubes. An endotoxin standard series consisting of lipopolysaccharides (LPS) [E. coli 055:B5](#) (Lonza N185) was prepared in concentrations of 0.001–25 EU/mL using ultrapure water from the arium pro VF system. The dissolved lysate was tested using the endotoxin standard prepared in order to verify that it had the sensitivity indicated (0.06 EU/mL). A sample of 100 µl was pipetted into 100 µl of the lysate and incubated in a water bath at 37°C. After one hour, each tube was individually removed from the water bath, turned by 180° and assessed for gel-clot formation.

Chromogenic Method

The chromogenic substrate ([Endosafe® KR1710K](#), Charles River) was dissolved in ultrapure water produced by the arium pro VF system. The chromogenic substrate and endotoxin standard samples were pipetted into 96-well plates (endotoxin-free) in a ratio of 1:1. The absorption of each sample was measured continuously at one-minute intervals over one hour at 405 nm using a [TECAN](#) Safire plate reader at 37°C.

Results

In the range of 0.001–0.05 EU/mL, the gel-clot method showed a negative result (no gel-clot formation); thus no endotoxins were detected (Table 1). A concentration of ≥ 0.1 EU/mL resulted in the formation of a gel clot. This result shows that within the detection sensitivity limits of the gel-clot method, no endotoxins were present in the ultrapure water filtered by arium pro VF.

TABLE 1. Assay of the endotoxin content in arium pro VF ultrapure water and endotoxin standard samples using the gel-clot and the chromogenic methods.

Sample	Endotoxin Standard Prepared (EU/mL)*	Gel-Clot	Chromogenic Assay (EU/mL)*
arium pro VF ultrapure water	0	No	0**
Endotoxin standard	0.001	No	0**
Endotoxin standard	0.005	No	0**
Endotoxin standard	0.01	No	0.009
Endotoxin standard	0.05	No	0.055
Endotoxin standard	0.1	Yes	0.083
Endotoxin standard	0.5	Yes	0.63
Endotoxin standard	1.0	Yes	1.2
Endotoxin standard	5.0	Yes	4.47
Endotoxin standard	10.0	Yes	8.4
Endotoxin standard	25.0	Yes	25.0
*EU/mL, endotoxin unit; 1 EU approximately corresponds to 100 pg of endotoxin (as a rule of thumb)			
**Below the quantifiable detection limit			

The 60 absorption readings at 405 nm (Figure 3) in the chromogenic method yielded curves with different slopes depending on the specific endotoxin content of the standard samples. The times that were needed to reach a specific absorption were determined using a special computer program and used as the basis for extrapolating the samples with an unknown endotoxin content (values are shown in Table 1). As the standard samples with the lowest concentrations (0.005 and 0.001 EU/mL) and

the Arium water did not attain the extinction value of 1 required to calculate the endotoxin content, they were assessed as below the detection limit.

The values attained with arium pro VF ultrapure water were considerably lower than those plotted for the curve of 0.001 EU/mL.

Figure 4 shows the data collected on endotoxin concentrations in tap water, deionized (DI) water, and arium water in comparison with two selected endotoxin

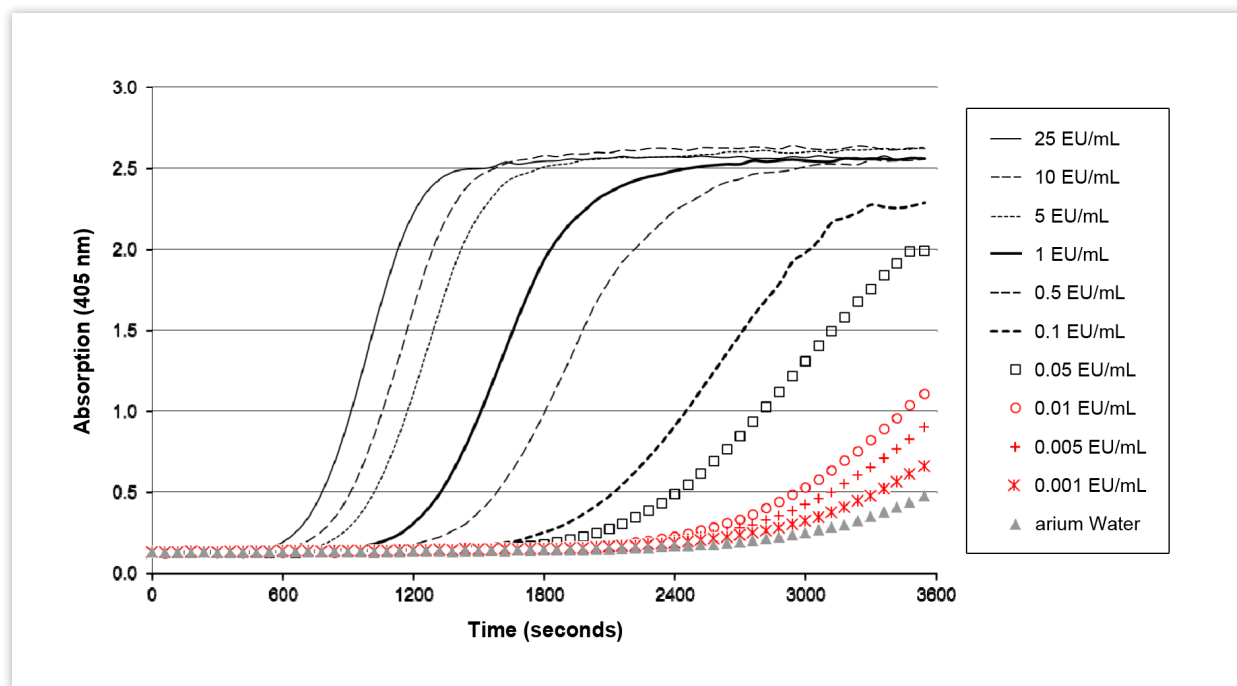


FIGURE 3. Absorption readings for endotoxin standard samples over a 60-minute period using the chromogenic method.

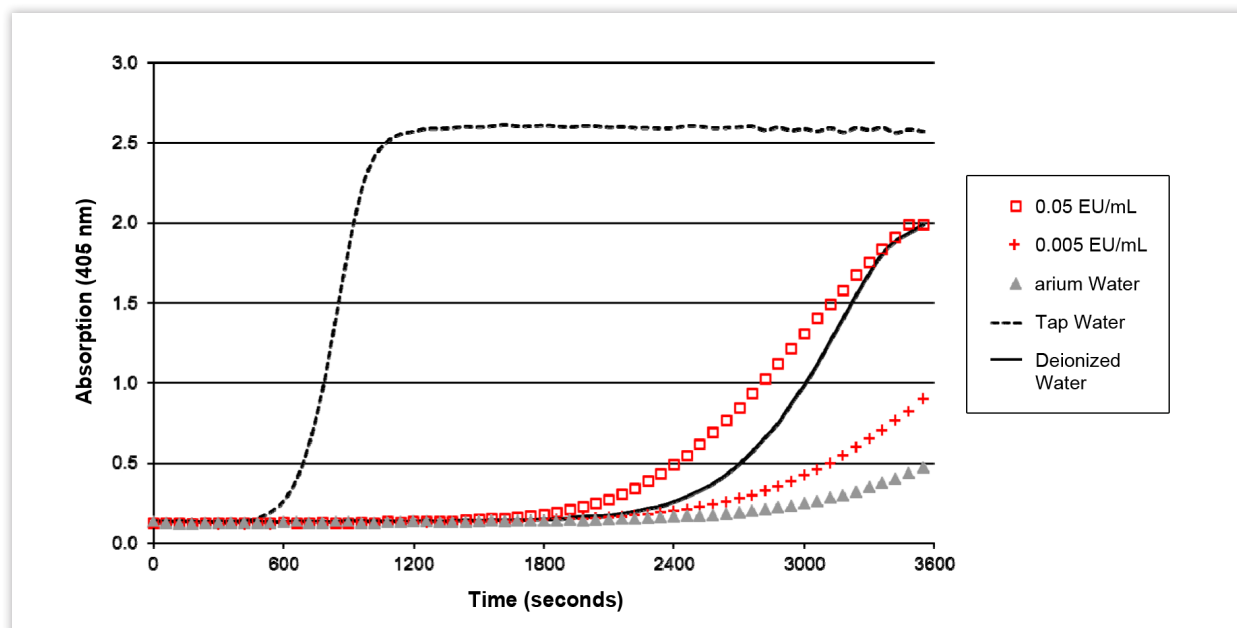


FIGURE 4. Endotoxin assay of water samples.

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standards (0.05 EU/mL and 0.005 EU/mL).

The endotoxin content in water samples was calculated from the values measured using the endotoxin standards and is shown in Table 2.

In the DI water sample, endotoxins of 0.02 EU/mL were detected, which was below the currently valid limits for WFI (water for injection). In tap water, with 25 EU/mL an exceptionally high endotoxin load was found. This finding was not further analyzed. By contrast, an exceptionally low value of < 0.001 EU/mL was measured in ultrapure water obtained from the arium pro VF system, which is far below the usual limits encountered.

TABLE 2. The endotoxin content in different water samples.

Sample	Concentration of Endotoxin (EU/mL)
arium pro VF ultrapure water	<0.001
Deionized water	0.02
Tap water	25.0

Discussion

The test results show that ultrapure water produced by the arium pro VF system can be readily used as an affordable alternative for preparing samples to be assayed in endotoxin tests as the detectable concentrations of endotoxin in the ultrapure water produced are exceptionally low (< 0.001 EU/mL). The results obtained confirm earlier tests in which an endotoxin load of < 0.001 EU/mL was found in Arium ultrapure water.^[4]

The endotoxin concentration in arium pro VF ultrapure water is far below the limits required by the United States Pharmacopeia which therefore makes this water theoretically suitable for use in the manufacture/endotoxin monitoring of pharmaceutical products. Examples of such uses are product formulations, diafiltration solutions, chromatography buffers and water for extraction, rinsing and sanitization steps, as well as for cell culture solutions.

Precisely within the scope of cell culture applications one has to pay attention to contaminations in the various steps of these processes. To maintain control over the effects of endotoxins on such cultures and on the sensitivity of cells, particularly with respect to such endotoxins, the media for cell growth must be demonstrably free of detectable endotoxins.^[5]

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NOTE

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