ACCESSORIES FOR BET

Plastic accessories are part of almost every laboratory routine, mainly because they are inexpensive. The same applies to the Bacterial Endotoxin Test (BET). Although manufacturers generally recommend using glassware for endotoxin testing, plastic materials are used, when necessary, provided they have been verified to be free from interference[1].

Glass offers the advantage of being depyrogenable and chemically inert, although slight differences exist depending on the glass quality [for example, borosilicate glass is generally considered superior to soda-lime glass.] Glass accessories should be accompanied by a **certificate of quality** confirming both the residual endotoxin level and the absence of assay interference. However, when glassware is reused, several factors must be carefully controlled. Residual detergents may inhibit the reaction, while repeated cleaning cycles can cause ion leaching from the glass surface, also leading to inhibition. Visible halos or film residues can interfere with optical readings in kinetic assays using tube readers. Recycling glass pipettes can also be problematic, as they are difficult to dry completely, which increases the risk of contamination. For all reusable glassware, a validated depyrogenation cycle must be applied, monitored with appropriate endotoxin indicators. Proper handling and depyrogenation are therefore essential to maintain the integrity and reliability of BET results when glass materials are reused.

For **plastics**, depending on the manufacturer, material composition, and exposure conditions such as temperature, plastics can release substances that either inhibit or enhance the test reaction or interact with the endotoxin itself. In general, a certificate ensuring accessories *sterility* alone does not confirm the absence of endotoxin contamination. Similarly, a "*pyrogen-free*" certificate does not guarantee that the accessory will not interfere with the assay or that its residual endotoxin level is below the detection limit of the chosen method. For this reason, regulatory guidelines such as the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) BET Chapters state that ***"if employing plastic apparatus such as microtiter plates and pipette tips for automatic pipetters, use only that which has been shown to be free of detectable endotoxin and not to interfere with the test."***

In practice, laboratories have two options:

1. purchase BET accessories certified by the reagent supplier
2. perform in-house verification to confirm their suitability for endotoxin testing.

[1]USP<1085>: GUIDELINES FOR BACTERIAL ENDOTOXINS TESTING

THE IMPACT OF PLASTIC ON BET

The main issue currently observed when using certain accessories instead of others is the different adhesiveness of polymers. The potential interference of materials with the LAL test was first reported by the FDA in 1985[2], which described how both plastic and glass can affect endotoxin recovery.

Three major problems may arise from the use of single-use materials:

- Contamination by endotoxins and/or glucans
- Release of interfering substances/ions
- Adsorption of endotoxin by the polymer

Single use labware, typically made of **polystyrene** or **polypropylene**, is generally produced by injection molding of resin. During this process, the high temperature helps reduce the endotoxin content.
However, the addition of mold-release agents and packaging steps can introduce interfering substances. It is well known that the type of plastic used can influence the outcome of the Bacterial Endotoxin Test. Among the most critical materials, polypropylene is known to cause inhibition because it tends to adsorb endotoxin molecules, increasing the risk of false-negative results when used for sampling or storage. Polystyrene is generally considered less interfering, but its behavior can still vary depending on the manufacturer, the grade of the material, and the presence of mold-release agents or additives. In some cases, ionic leaching from the plastic matrix into the solution can also occur, potentially affecting assay performance.
For these reasons, it is essential to verify the compatibility of plastic materials with the BET method before use, either through supplier certification or in-house interference testing.

Pipettes are generally used to dispense water to resuspend reagents or CSE. While glass pipette are suggested, plastic pipette are widely used. Those containing cotton or cellulose filters are particularly concerned for LAL reagents, since they may release interfering particles, like beta-glucans, activating LAL reagents. For the same reason, **Micropipette tips** equipped with cotton filters should not be used**.** It's not the case with rFC which misses Factor G, glucan-specific pathway.

Microplates can be a source of "hot wells," wells that produce false-positive results. These artifacts often stem from contamination during production or packaging of the plates, accidental contamination by the operator, or the presence of air bubbles. Such anomalies lead to out-of-specification results that may require investigation. Thus, for microplates, certifications should confirm the endotoxin content/well, the absence of interferences and the expected frequency of hot wells.

Tubes and Containers are commonly used for sampling and storage. It is done for Water loops samples where high volumes are taken for both bioburden and BET. In this case, the risk is associated with inhibition due to a possible effect of absorption of endotoxins to surface. For this reason, as for any other plastic accessory, also containers should be certified for endotoxin content and absence of interferences. Polypropylene sample container should be not used for storage due to know compatibility issues with endotoxins), as required by USP<1085>: "*Likewise, some materials could contain extractable or leachable substances that could inhibit the BET. The PPC will indicate whether the normal, routine use of the plastic results in any leached inhibitory substance. If a laboratory uses any disposable plastic container, such as sterile polystyrene or polyethylene, it should be qualified to show that there are no inhibitory or enhancing factors that could affect the accuracy of the test (the recovery of endotoxin). [Note—Polypropylene sample containers should not be used for storage due to known compatibility issues with endotoxins[3].]*"

TESTING ACCESSORIES

Before testing accessories in-house, it is important to answer two preliminary questions:

- How should the endotoxin limit be selected?
- Which method should be used to test accessories?
- How many accessories and how many batches?

For the last question, it depends on the initial scope. Based on our experience, several approaches are used in the pharmaceutical industry:

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[2] Department of Health, Education, and Welfare, Public Health Service, Food and Drug Administration, ORA/ORO/DEIO/IB. (1985). Bacterial Endotoxins/Pyrogens. Inspectors Technical Guide, No. 40

[3] Novitsky TJ, Schmidt-Gengenbach J, Remillard JF. Factors affecting recovery of endotoxin adsorbed to container surfaces. PDA J Pharm Sci Technol. 1986;40(6):284–286

- one-time product qualification,
- testing of each incoming lot,
- annual verification,
- or testing together with operator qualification.

If a new type of accessory is being qualified (for example, from a manufacturer not previously used) it is good practice to request three different lots of the same accessory.
The number of accessories tested per batch should be determined through a reasonable risk evaluation.
Testing only two tips from a 96-tip box would clearly underestimate the risk, while testing two full boxes of 96 tips from a 10-box package might be excessive, considering that the manufacturing process is highly automated and typically free of human intervention from molding to packaging. For multiwell, for each new lot, it is advisable to test more than one multiwell plate, ideally selecting at least 3 plates from different packages as a good practice.

Endotoxin limit: unit of measurement

The endotoxin limit represents the maximum acceptable level of endotoxins for a given product or accessory.
For accessories, it is best expressed as EU per unit (for example, EU/tip, EU/tube, or EU/well in the case of 96-well plates).
Supplier certificates often report results in **EU/mL**, and failing to "translate" EU/mL into EU/device can lead to **false positives**.

USP <1085> (2025) provides clear examples of the impact of misinterpreting a supplier's certificate:

> *"If a laboratory accepts a vendor labware CoA for endotoxin content, there should be an understanding of the methods used to determine the reported test result. Two scenarios to consider are provided below:*
> *If a shipment of dilution tubes is received from a vendor and is labeled 'non-pyrogenic', what does this really mean? Was the lot tested for pyrogens using an appropriate in vivo or in vitro test? Was it tested using a BET, and if so, at what level of endotoxin does the vendor consider the material to be 'non-pyrogenic'? It is important to ask the vendor how the material was prepared and tested.*
> *If a shipment of 10-mL tubes is received from a vendor with the label '<0.5 EU/mL', what does that mean? With what volume was the tube extracted (1, 5, or 10 mL)? A 1-mL extraction would mean <0.5 EU/tube, whereas a 5-mL extraction would mean <2.5 EU/tube and a 10-mL extraction would mean <5 EU/tube. The laboratory should ask the vendor about the method of endotoxin extraction from the tube as well as the BET test conditions that were used to generate the result."*

In the last example, the 10-mL tube was released as **<0.5 EU/mL**. But it remains unclear if each tube actually contains <0,5 EU or 5 EU. Failing to convert from **EU/mL** to **EU/tube** can significantly affect the assessment of possible contamination in the tested product. For this reason, it is preferable to use accessories whose certificate expresses results in **EU/unit** rather than **EU/mL**, as this immediately clarifies the potential impact on the test performed at a given sensitivity.

The same reasoning applies to **pipette tips**. What does it mean when **200 µL tips** are released as **<0.03 EU/mL**? It clearly indicates that the manufacturer most probably performed the test using the Gel Clot method. But how many tips were actually tested, and in what way? Were the tips simply **immersed in water** so the endotoxin content is relative to internal and external lumen? or were they **used to pipette up and down** the water, and how much volume was used?

These details are critical, because the **extraction volume** directly influences the measured endotoxin level and the question if the lab can use such tips or not with their test sensitivity. As lab technician, you most probably want to know if the endotoxin content on 1 tip can be too high for your test sensitivity thus impacting the final result in your sample.

Regarding the Endotoxin Limit acceptable for an accessory, as mentioned in the previous paragraph, it should not exceed the sensitivity of the method used.

Method for testing accessories

Endotoxins are amphipathic molecules capable of forming complex structures. While extracted endotoxins (such as Control Standard Endotoxin, CSE) can create aggregated difficult to dissolve in water, Naturally Occurring Endotoxins (i.e. NOE, those that may contaminate samples or accessories and originate from Gram-negative bacteria previously present) are less prone to form aggregates[4] and are widely considered to easily dissolve when they come into contact with an aqueous solution.

[4] USP DEPYROGENATION, USP 39-NF 34, FIRST SUPPLEMENT, 2016:7807. ROCKVILLE, MD: US PHARMACOPEIAL CONVENTION.

From a technical standpoint, accessories can be regarded as medical devices, since they are non-liquid materials. As such, they can be handled according to the approaches described in the regulatory references commonly applied to BET testing of medical devices, primarily USP <161>, and secondarily the ANSI/AAMI ST72:2019 guideline. USP <161> specifies:

> *"The standard extracting fluid for extracting, rinsing, or soaking medical devices is Water for BET. If necessary, other solvents maybe used after it has been demonstrated that they do not interfere with the performance of the assay. […]*
> *The standard extraction method is to soak or immerse the device or flush the fluid pathway with extracting fluid that has been heated to 37 ± 1.0°, keeping the extracting fluid in contact with the relevant surface(s) for NLT 1 h at controlled room temperature. Alternate extraction or rinsing methods may be used but must be demonstrated to be equivalent to or better than the standard method. Extracts from individual medical devices are typically pooled for testing."*

These guidelines clearly describe the testing approach: a self-validated method that uses Water for BET or another buffer suitable for BET, pre-warmed to 37 ± 1.0°C, and kept in contact for at least 1 hour to extract endotoxin from the accessories.

Alternative methods can be used, provided they demonstrate the ability to recover endotoxin.
This can be shown, for example, by artificially contaminating the device with CSE and proving a recovery between 50% and 200% of the endotoxin.

POSSIBLE APPROACHES FOR IN-HOUSE TESTING

The following examples illustrate possible approaches to testing accessories. They are not prescriptive but provide a reference framework.

Micropipette Tips

- **RINSING OF THE INTERNAL LUMEN**: in this case 200mL tips are used to pipette up and down pre-warmed water. The same water is used to "rinse" several tips. The water is then tested as a normal sample, according to the chosen method. In the following example, the scope is to verify if the tips content is below 0,005 EU/tip.

1. Use a reservoir and a multichannel pipette to speed up the process.
2. Preheat Water for BET to 37 °C.
3. Open a new rack of tips, following the usual BET handling precautions.
4. Dispense a known volume (e.g., 5 mL) of pre-warmed BET water into the reservoir.
5. Pick up 8 tips with the multichannel pipette.
6. Pipette up and down several times with 200 µL per tip
7. Discard the tips.
8. Repeat the procedure with another 8 tips, using the same extraction water.
9. Discard the tips again.
10. Test the resulting solution according to the selected BET method.

Calculation example

To respond to the question "Which is the acceptable result to have tips certified as <0,005EU/tip endotoxin content?", use the following formula:

$$N = \frac{Endotoxin\ Limit\ *\ Volume}{K}$$

Where:
K = Acceptable Endotoxin Limit/tip (in this example <0,005 EU/tip)
V = Extraction volume used (in this example 5mL)
N = Number of tips tested (in this example 16 tips)

In the case:

$$16\ tips = \frac{x\ EU/mL\ *\ 5\ mL}{0{,}005\ EU/tip}$$

which gives:

$$x\frac{EU}{mL} = \frac{16tips * 0{,}005EU/tip}{5mL} = 0{,}016\ EU/mL$$

If the test result on the replicates is < 0.016 EU/mL, and the acceptance criteria are met (including Spike Recovery %, which indicates possible inhibition or enhancement effects), the tips can be certified as <0,005 EU/tip and non-interfering for BET.

- **IMMERSION:** Alternatively, the test can be executed by immersing a certain number of tips in water for BET pre-warmed at 37°C for 1 h, then testing water. This method is the one described in USP<161> for Medical Devices. The formulas above do not change.

96-well Microplates

- **HOT WELLS**: the procedure requires testing the plate under the same conditions in which it will be used, meaning with a maximum volume of 200 µL per well, including sample and reagent, even though the actual capacity per well is around 400 µL.
  For this test, all wells are filled with Water for BET. Some wells, selected at random, are spiked with the lowest standard of the calibration curve normally used in routine (for example 0,005 EU/mL). Then the reagent is added to the whole plate. The purpose of the test is to confirm that water-only wells reactivity does not exceed that of the spiked wells, demonstrating that their endotoxin concentration remains below the defined limit. Pre-warming and rinsing of the wells is not necessary, as the assay is performed directly in the wells and at 37 °C. The plates are tested without a curve, since the scope is not the quantification of endotoxins, but the confirmation of no reactivity of waterfilled wells.

For ex: considering 3 plates, 3*4 wells used as spike, 3*92 water-only wells are tested/plate; if less than 14 water-only well showed reactivity, the batch can be certified for having <5% of hot-wells.

- **INTERFERENCE TESTING**: When working with new suppliers or previously unused brands of microplates, it is important to verify that they do not exert inhibitory or enhancing effects. This test is much simpler, as it can be performed using a single plate on which the standard curve intended for routine use is built. The sample, along with the respective Positive Product Controls (PPCs), will consist of Water for BET, tested in duplicate or more. The absence of interference is confirmed when all test and curve criteria are met and the spike recovery percentage falls within the range of 50% to 200%.