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Screening Tips and Multi-well Plates for Bacterial Endotoxin Testing

Technical Tips

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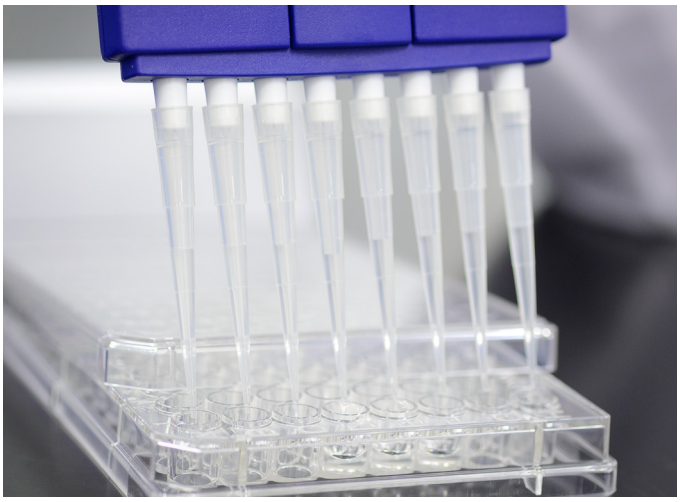
Plastic accessories

Regardless of which Bacterial Endotoxin Test (BET) method you are utilizing in the laboratory, you are probably using plastic accessories at some point in the procedure. For a gel clot assay, you may be using plastic pipette tips to dispense samples and reagent into the glass reaction tubes. If you are using a kinetic method to test for endotoxin, you are likely running the assay in a disposable plastic 96-well plate.

BET required testing for plasticware

Product inserts provided by BET manufacturers caution that care must be taken to avoid microbiological or endotoxin contamination of the sample to be tested and the BET reagent, which includes "accidental" contamination through accessories used in the test. Therefore, testing of plastic accessories is a requirement of the United States Pharmacopeia (USP), and the USP BET[2] states: "If employing plastic apparatus, such as microplates and pipet tips for automatic pipetters, use apparatus that is shown to be free of detectable endotoxin and does not interfere in the test." Portions of the USP BET were harmonized in 2000 with the European and/or Japanese Pharmacopeias, and all three Pharmacopeias are in agreement with this statement, making testing of plasticware a global requirement. This requirement is also reflected in Lonza's Kinetic-QCL®



Package Insert[1] that specifically states "All materials coming in contact with the specimen or test reagents must be endotoxin-free." Glassware is easily rendered endotoxin-free by heating to 250°C for 30 minutes, but how does one ensure the plastic accessories used are endotoxin-free? Suggestions for ensuring pipette tips and 96-well plates are endotoxin-free will be offered in this article.

One method for developing a testing protocol for pipette tips and 96-well plates is to borrow the USP formula for testing medical devices. USP Chapter <161>, Transfusion and Infusion Assemblies and Similar Medical Devices[3], describes a procedure for extracting endotoxin into a rinse or soak solution (typically Water for BET) and evaluating that solution for endotoxin. Please note that the volume of the solution may vary depending on the size of the device. For instance, more volume will be needed to soak ten 96-well plates than to soak ten 200 µL pipette tips. The soak solution should be in contact with the device sampling for 1 hour at room temperature. If plates or tips are soaked or rinsed individually, it is allowable to pool the solutions prior to testing with a BET method. The volume of the pooled solution will be taken into account when determining whether or not the lots of tips or 96-well plates pass the endotoxin test.

From USP Chapter <161>, the endotoxin limit for the solution is equal to

$$\text{Endotoxin Limit} = \frac{K \times N}{V}$$

Where K is the endotoxin limit per device; N is the number of devices tested; and V is the total solution volume. The standard acceptable endotoxin limit for a medical device is 20 EU/device, however, those devices coming into contact with the cerebrospinal fluid have an endotoxin limit of 2.15 EU/device. Applying either of these limits to a pipette tip or a microplate well would not be appropriate. Although the preferred endotoxin level is 0 EU/mL, it is not possible to test for a zero level. Therefore, you will have to decide the appropriate endotoxin limit for each tip or 96-well plate well, and it will be dependent on your application.

As you set the "device" endotoxin limit for plastic accessories, keep in mind that you may be testing a pooled extraction solution. If you set a limit too low, your solution volume may dilute the solution endotoxin limit below the detection level of any current BET method. If you set a limit too high, a passing lot may contain just enough endotoxin to cause positive BET reactions ("hot wells") during the routine testing of products. The following examples illustrate this.

When you use a pipette tip for adding Kinetic-QCL® Reagent to your reaction vessel, 100 µL (or 0.1 mL) of the reagent is dispensed. This reagent is sensitive to 0.005 EU/mL or 0.0005 EU/tip (calculated by 0.005 EU/mL x 0.1 mL/tip = 0.0005 EU/tip). If you set your tip endotoxin limit at 0.0005 EU/tip and test 10 tips by soaking in 100 mL of water, then the calculated endotoxin limit for the solution is (0.0005 EU/tip x 10 tips)/100 mL or 0.00005 EU/mL. Since this value is below the sensitivity of any current method, negative results will be meaningless.

Endotoxin levels that fall between 0.00005 EU/mL and the detection limit of 0.005 EU/mL for the Kinetic-QCL® Kinetic Chromogenic Assay will not be detected, but some tips may have detectable endotoxin levels that could cause issues during your routine product testing. Setting a higher limit might solve your detection problem. For example, if you develop a protocol to screen tips with 100 mL of rinse solution at 0.2 EU/tip, the limit for the rinse solution becomes 0.02 EU/mL and this level can be detected with a kinetic chromogenic assay. However, depending on the BET method you use for your routine product testing, 0.2 EU/tip may be too high.

Screening pipette tips used for endotoxin testing

If a low endotoxin limit per pipette tip is desired, as in the above example of 0.0005 EU/tip, testing conditions and parameters can be adapted to allow testing at this limit. A strategy for doing so is outlined below.

The most crucial portion of the pipette tip to screen, relative to endotoxin content, is the portion that comes into contact with the solution to be dispensed, i.e. the end and the internal surface. A pipette can be used to draw in and dispense Water for BET (rinse water), essentially mimicking how the tip is actually used in the BET. Instead of pooling the rinse water at the end of the rinsing, we suggest rinsing a number of tips with the same water. This will help lower the solution volume and allow the tips to be tested at the low endotoxin limit.

The use of a multichannel pipette will allow for quick rinsing of several pipette tips at the same time. With this in mind, the rinse volume should be set at a level that will be accommodated in a reagent reservoir and adequately fill each of the tips with every rinse cycle. Although only 100 µL is typically dispensed in a BET test, rinsing as much of the interior surface of the tip as possible is preferable for the endotoxin screening protocol.

For a 200 µL pipette tip, the rinse volume per tip should be 200 µL. When an 8-channel multi-pipette is used,

eight pipette tips rinsed by 200 µL per tip would need 1.6 mL of total rinse water. An additional amount should be added to help ensure sufficient liquid in the reagent reservoir fills the tips equally and still has some liquid remaining. A total of 2.5 mL should be acceptable for a typical reagent reservoir.

Next, an acceptable endotoxin limit that will be the pass/fail acceptance limit for the test should be chosen. If, for example, 0.02 EU/mL is chosen, this means that if the tip rinse solution contains greater than 0.02 EU/mL, then the lot of tips being tested fails.

With the endotoxin limit per tip (K), the total rinse solution volume (V) and an acceptable endotoxin limit for the rinse solution, there is one variable missing for the test: the number of tips to test (N). The formula listed above for medical device testing is (K x N) / V equals the solution release limit. Rearranging the equation to solve for N yields:

$$N = \frac{\text{Endotoxin Release Limit} \times V}{K}$$

Inserting the values discussed above, N equals (0.02 EU/mL x 2.5 mL) / 0.0005 EU/tip. The number of tips to be tested equals 100 tips.

Procedure

Step 1 Fill a reagent reservoir with 2.5 mL of Water for BET.

Step 2 Using an 8-channel multi-pipette, attach 8 tips and wash up and down with 200 µL per tip several times into the reservoir. Discard the used tips, obtain 8 fresh tips and wash them with the same 2.5 mL in the reservoir.

Step 3 Keep repeating this process until 100 tips have been rinsed into the reservoir (the last set will contain 4 tips).

Step 4 Test the solution in the reagent reservoir to determine the endotoxin content, including a positive product control.

Pass /Fail Results

If the endotoxin content is less than the 0.02 EU/mL limit, then the lot of tips passes. If the tested content is above 0.02 EU/mL, then the lot of tips fails. A passing test result indicates that the tips contain less than 0.0005 EU/tip.

Screening 96-well plates for endotoxin testing

Just as 0.0005 EU/tip is desired to avoid an apparent "hot well" due to a "hot" tip, a similar endotoxin limit should be established for the 96-well plates used in BET. However, rather than attempting to soak a number of plates, utilizing a procedure that mimics how the 96-well plates are used in the assay is preferred. If you are familiar with running a Uniformity Assay, the screening test is similar.

Rather than placing a single endotoxin standard in each well of the 96 wells and running the assay to determine the reaction time % CV across the plate for that standard, the screening test utilizes only Water for BET in a majority of the wells. A few wells are used for positive controls, containing the lowest standard detected by the BET of choice. The test nvolves determining whether or not the reaction times of the water-only wells are slower than an average of the positive control wells, thus indicating that the endotoxin content of the water-only wells are below that level. For a Kinetic-QCL® Assay, 0.005 EU/mL is the lowest standard. As 0.1 mL is tested, the resulting endotoxin level is 0.0005 EU/well, therefore, the overall test results may indicate that the endotoxin content of the tested 96-well plate is less than 0.0005 EU/well.

Procedure

Step 1 For each new lot of 96-well plates, a specified number of plates is chosen at random for testing.

Step 2 100 µL of Water for BET is placed in all of the wells on each plate.

Step 3 To four of the wells on each plate, a quantity of endotoxin should be added, and the locations should vary among the plates. For instance, Plate 1 may have the four positive control wells in positions A1, B2, C3 and D4, while Plate 2 has the control wells in E9, F10, G11, and H12. A 10 µL aliquot of a single endotoxin standard solution should be placed in the control wells. For the Kinetic-QCL® Test, 10 µL of the 0.05 EU/mL standard will be placed in the control wells, yielding 0.005 EU/mL in each. The 0.05 EU/mL standard is prepared by making dilutions of a concentrated endotoxin standard.

Step 4 The 96-well plate containing the Water for BET and the positive control wells is placed in the incubating 96-well plate reader. If using Lonza's WinKQCL® Software, a Uniformity Test can be started directly from the software. Once initiated, the plate will incubate at 37°C for 10 minutes. The software will prompt you as to when to add the LAL reagent and to start the assay.

Step 5 At the end of the assay, a Uniformity Test Report showing the reaction times for each well can be printed. The procedure will be repeated for all the sampled 96-well plates.

Reading the results

When reviewing the results, both the positive control wells and the water-only wells should be examined. Calculate the % CV for the positive control wells and verify that it is < 10%. Next, calculate the average reaction time for the four positive control wells on the plate. Compare this time with the reaction times for the 92-wells that only contained Water for BET. Any of the water-only wells with a shorter reaction time (lower number) than the positive control well average will be marked as positive.

After testing the 96-well plates, the number of positive wells (among the water-only wells) per 96-well plate and per batch of all the plates sampled should be examined. An acceptable number of positives should be established, based on a statistical confidence level, for example, 95% of a specified failure rate and the test results compared to that acceptable number. For instance, when testing a sample set of six 96-well plates, the per individual plate acceptable level could be set at "no more than 4" positive wells, while the per batch of six plates level could be set at "no more than 11". Any lot that meets the % CV specification for the positive controls and both the individual plate and the batch of plate levels can be passed for use in BET.

Routine Testing of New Lots vs. New Vendor Screening

The methods described here are suggestions for the routine testing of new lots of tips and 96-well plates. When screening items from a new vendor, a more extensive validation protocol may be required that is similar to what is done for a new product, for instance, the same procedure performed on 3 different production lots. It is important to remember that not all plastic is the same. There may be differences from vendor to vendor as well as lot to lot variability from a single vendor.

Setting endotoxin limits at 0.0005 EU/tip and 0.0005 EU/well can be testable. Lots that meet these limits will provide the end-user with confidence that the plasticware used for BET will not interfere with the routine testing of products. End-user must determine which procedures and endotoxin limits are acceptable to them.

Save time with pre-screened plasticware

An alternative to in-house testing of plasticware is to purchase tips and plates that are already tested for endotoxin content. Pre-screened pipette tips, reagent reservoirs and 96-well plates are available from Lonza (Table 1). Certificates indicate the lot was tested and shown to be less than a specific endotoxin content. For instance, the Certificate of Analysis (CoA) for a lot of LAL Reagent Grade Multi-well Plates lists the endotoxin content as less than 0.0005 EU/well.

Meeting the recommendation of the BET manufacturers and the requirement of the pharmacopeial BET chapters should not seem overwhelming. Testing procedures can be devised to test plasticware at the low levels of sensitivity available with the current BET methods. End-users can also purchase pre-screened plasticware that has been tested at low levels. Limiting "hot" tips or "hot" wells will help keep re-testing to a minimum, reducing the cost of QC testing and increasing your confidence in your routine testing results. Whether you test your plasticware or purchase them already pre-screened, you should be confident the plastic accessories being used are as endotoxin-free as can be determined.

References

1. Lonza, Limulus Amebocyte Lysate (LAL) Kinetic-QCL® Package Insert.
2. Bacterial Endotoxins Test, Chapter <85>, USP 36, NF 31, Supplement 1, May 1 to July 31, 2013, United States Pharmacopeia, Rockville, MD.
3. Transfusion and Infusion Assemblies and Similar Medical Devices, Chapter <161>, USP 36, NF 31, Supplement 1, May 1 to July 31, 2013, United States Pharmacopeia, Rockville, MD.

Table 1. Pre-screened plastic accessories available from Lonza

Sample	Cat. No.	Size	Quantity	Comments
Eppendorf Biopur® Pipette Tips	25-415	2–200 µL	For all 3 sizes: 5 trays per package 96 tips per tray	---
	25-416	2–300 µL		Only for multichannel pipettors
	25-417	50–1000 µL		---
Reagent Reservoir	00190035	60 mL	10 / pack	< 0.005 EU/mL
LAL Reagent Grade Multi-well Plates	25-340	---	50 plates per case	96-well plates, <0.0005 EU/well

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