Endotoxin Testing

Materials:

Pyrotell *Limulus* Amebocyte Lysate (LAL) -- stored at -20 °C
For gel-clot method; 0.25 EU/ml sensitivity
Multi-test 2 ml/vial for 20 tests
Cat # G2250
Associates of Cape Cod, Inc.

Control Standard Endotoxin (CSE) -- stored at 4 °C
Escherichia coli O113:H10
0.5 ug/vial
Cat # E0005
Associates of Cape Cod, Inc.

Pyrotubes -- stored at room temp
Depyrogenated tubes; 10x75 mm
For gel-clot method
Cat # TS050
Associates of Cape Cod

Reagent Water -- stored at 4 °C
LAL Reagent Water
20 mL/bottle, 10 bottles/pack
Cat # W020P
Associates of Cape Cod
-OR-
Water for Irrigation
1000 ml / bottle
Cat # 4142
UNMC Pharmacy Warehouse

Protocol:

Note: The assay should be performed in a biosafety cabinet

** An hour before the assay, reconstitute CSE (0.5 µg/vial) with 5 ml LAL Reagent Water (LRW). Mix gently & cover with parafilm.

** Throughout the assay, it is important to note that improperly performed procedures can lead to false negatives, and that false positives can occur due to introduction of endotoxin during the assay. Sterility and careful handling of reagents is crucial.

- **Note:** Use sterile yellow (P200) and blue (P1000) tips that have been autoclaved, but not opened.

1. Prepare materials by aliquoting 5 to 10 ml Reagent water into a sterile 15 ml conical tube, labelling Pyrotubes for standard curve and samples (see below), labelling Pyrotubes for dilutions (see below), bringing a vortex mixer into the biosafety cabinet, and finding appropriate test tube racks for the assay

2. In a test tube rack, set up the tubes for the standard curve:
   -Set up 5 pyrotubes for dilutions and label 1,2...5.
   -Set up 5 pyrotubes for reactions and label 2,3,4,5 and 0.
   -To the dilution tubes, add Reagent water. CHANGE pipet tip after each addition.

Tube 1 - add 990 ul
Tube 2 - add 900 ul
Tubes 3 to 5 - add 200 ul

-Vortex the CSE for 15 seconds.
-Remove parafilm and remove 10 ul from bottle with clean pipet tip.
-Add to Tube 1, and vortex. Wrap CSE with fresh parafilm.
-Remove 100 ul from Tube 1 and add to Tube 2. Vortex and change tips.
-Remove 200 ul from Tube 2 and add to Tube 3. Vortex and change tips.
-Remove 200 ul from Tube 3 and add to Tube 4. Vortex and change tips.
-Continue with two-fold serial dilutions in the same manner to Tube 5. Remove 200 ul from Tube 5 and discard.

-Add 100 ul of dilutions in Tubes 2,3,4,5 to corresponding reaction tubes. Vortex each dilution before removing sample and use a clean tip for each addition.
-Add 100 ul of Reagent water to the 0 tube.
-Standard Curve consists of:

Tube 2 = 100 pg/ml
Tube 3 = 50 pg/ml
Tube 4 = 25 pg/ml <--sensitivity of LAL assay
Tube 5 = 12.5 pg/ml
Tube 0 = 0 pg/ml

3. In the test tube rack, set up tubes for the samples:
   Note that the maximum number of reactions per assay is 18-19. If more samples need be run, decrease number of dilutions per sample.
   If testing ADA or Human Serum for the laboratory, 2 dilutions of each sample must be run.
   -For each sample, set up 2 dilution tubes.
   -For each sample, set up 3 reaction tubes, labelled “undiluted”, “1:10”, “1:100”.
   -In the dilution tubes, make a 1:10 and a 1:100 dilution of each sample with Reagent water.
   Note: Volume must be >100 ul, and each dilution must be vortexed.
   -Add 100 ul of sample to “undiluted” reaction tube, using a clean pipet tip.
   -Vortex 1:10 dilution and add 100 ul to “1:10” reaction tube, using a clean pipet tip.
   -Do the same for the 1:100.

4. Set dilution tubes aside and line up reaction tubes such that, when reading, the standard curve will be read first (positive to negative), and samples are read in the order of “undiluted”, “1:10”, “1:100”, followed by the next sample.

5. Remove the LAL from -20 °C and let sit at room temperature in the biosafety cabinet 1-2 minutes. Tap bottle on cabinet surface to get as much reagent at the bottom of the tube as possible.

6. Remove wrap, and gently lift stopper of bottle just until the vacuum is released. Then remove stopper and discard. Do not attempt to add any LAL on the stopper back to the bottle. Discard it.

7. Add 2 ml of Reagent water to LAL with strippette (glass, if available and sterile), and swirl gently. Vigorous mixing will cause unwanted foam. Swirl and incubate the LAL at room temp until solution is no longer turbid. If undissolved LAL is stuck on the sides, try to gently swirl until it is dissolved.

** DO NOT make up the LAL “in advance”. Reconstituted LAL left to sit can cause both false negatives and false positives.

8. Once the LAL is dissolved, set the repeat pipetter to dispense 100 ul. Bring a test tube rack into the biosafety cabinet that is open at the bottom, so the reaction can reach 37°C. Set (but do not start) a timer for 60 minutes.

9. To the first tube, dispense 100 ul LAL, vortex the tube and place in the rack used for incubation. Repeat for each tube, moving as quickly as possible. Once the tube is in the incubation rack, do not move it for any reason.

10. When all reactions are complete, transport the rack to the 37°C incubator with minimal disturbance to the tubes and start the timer.

11. Incubate 37 °C for 60 minutes. Any disturbance to the tubes during incubation will cause false negatives.

12. After 60 minutes, read the tubes. Do the reading at the incubator; do not pull out the rack to read somewhere else. Pull out one tube at a time (in the order described in step 4), and in one swift motion, turn it 90°.

13. As each tube is read, record the results (it is best to have your own worksheet already made up).
    - A positive (+) reaction will remain as a gel clot, even when the tube is upside down.
    - A pos/neg, or weakly positive (+/-) reaction will appear as a gel that breaks, or turbidity/flocculent precipitate in the reaction.
    - A negative (-) reaction occurs when there is no gel formation in the reaction. This will be evident when the tube is tilted 90°.

14. Discard tubes in the biohazardous glass waste container in the BSL2+ laboratory.

Results

1. An ideal standard curve will read as:

100 pg/ml	+
50 pg/ml	+
25 pg/ml	+/-
12.5 pg/ml	-
0 pg/ml	-

   - If the 25 pg/ml and 50 pg/ml standards consistently come up - or +/-, the batch sensitivity of the LAL may be low. Interpret the results accordingly.
   - If the 100 pg/ml standard reads - or +/- (or the 12.5 pg/ml reads +), the assay is invalid.

2. If an ADA or human serum tests positive at any dilution, you may want to run a titration assay on the sample to determine the end-point (check with *Lisa* if enough reagent is available). A sample that reads negative undiluted, but positive at a 1:10 or 1:100 dilution is positive. Ensure, however, that the diluent is not causing false positive results.

3. For negative samples, one may wish to run a control assay to determine if the sample is inhibiting the LAL reaction. Inhibition Controls consist of 100 ul of the sample spiked with 10 ul of Tube 3 in the standard curve dilutions (amount of CSE added cannot exceed 2x the batch sensitivity of LAL (in EU/ml)). (This is optional!)

Notes on Reagents:

- A bottle of CSE, reconstituted, is stable at 4 °C for 3 months. When ordering new CSE, it is necessary to know the lot number of the LAL currently in use.
- Conversely, it is necessary to know the lot number of the CSE in use when ordering LAL.
- A Certificate of Analysis will come with any CSE. Directions for reconstitution and data on endotoxin activity are contained in this certificate. It is specific to that bottle of CSE.
- Do not store any materials used in one assay, including sample and standard dilutions, for another assay. Storage leads to false results.
- When using plastics, ensure they are polystyrene. Polypropylene will lead to false results.