Standard Operating Procedure

Title: Bacterial Endotoxin Testing - KCA Method

EHS Statement

Amoebocyte Lysate is a preparation of Horseshoe crab blood and as with all blood related products, appropriate care should be taken when handling both the freeze-dried powder and reconstituted solution.

The bacterial endotoxin used for KCA is a concentrated preparation of *E.coli* endotoxin. This preparation is not sterile and has the potential to cause infection and fever. Appropriate care should be taken when handling both the freeze-dried powder and reconstituted solution.

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Procedure

1. General

1.1. New operators must be adequately trained by a competent staff member and perform a satisfactory Operator Verification, (as per section 3.3), prior to performing routine testing. Routine testing may be performed once all training requirements are met and have been deemed to be satisfactory following review.

1.2. Bacterial endotoxin testing is carried out in order to determine whether endotoxin has been introduced into a product via the manufacturing process, raw materials or packaging, which may cause a pyrogenic reaction to the end user.

1.3 The KCA method utilises a co-lyophilised mixture of lysate and a synthetic colour producing substrate to detect endotoxin chromogenically. A sample is mixed with the lysate/substrate reagent, placed in the BioWhittaker KQCL reader and is automatically monitored over time for the development of a Yellow colour. The Yellow colour is caused by the cleaving of the Yellow substrate p-nitroaniline from a colourless peptide chain by an enzyme in the Limulus Amoebocyte lysate activated by bacterial endotoxin. The time required for the development of colour is directly proportional to the amount of endotoxin present (i.e. The more endotoxin present, the faster the colour development). The concentration of endotoxin in unknown samples can be calculated by comparing their reaction times to the reaction time of endotoxin standards from a standard curve.

2. Materials required

1. Depyrogenated Borosilicate test tubes wrapped in foil.
2. Pyrogen free disposable pipettes 1mL, 10mL.
3. Vial opener, Parafilm, vortex, timer
4. LAL grade WFI and Magnesium Chloride for dilutions

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4.1 The Maximum Valid Dilution (MVD) must be calculated **(Form 600).** The MVD is the degree to which a product can be diluted before the sensitivity of the assay method to detect the diluted endotoxin concentration is exceeded (i.e. When a product is diluted to overcome interference, any endotoxin present is also diluted).

4.2 The MVD is equal to the endotoxin limit divided by Lambda.

4.3 Lambda (λ) is equal to the label claim for the gel clot lysate or the lowest standard for the KCA.

4.4 The MVD is the maximum dilution that must not be exceeded for routine product testing.

4.5 **Examples of MVD Calculations:**

GEL-CLOT	KCA
Lysate sensitivity = 0.06 EU/mL	Lowest Standard = 0.005EU/mL
Endotoxin Limit for Product = 3.0EU/mL	Endotoxin Limit for Product = 3.0EU/mL
MVD = 3.0EU/mL / 0.06EU/mL	MVD = 3.0EU/mL / 0.005EU/mL
MVD = 50	MVD = 600

Please note that the higher sensitivity of the KCA method will give a larger MVD. This in turn will allow a greater dilution to overcome any inhibition that may be present.

The **Endotoxin Limit** is determined by:

1) Specific monograph for that product (BP, EP, USP or JP).
2) By regulatory requirement (local or export).
3) By corporate product specification or 4) by calculation.

See below for calculation.

The Endotoxin Limit = (K/D) x Potency

K = Maximum allowable endotoxin exposure

5EU/Kg/Hour for intramuscular

0.2 EU/Kg/Hour for intrathecal

D = Maximum human dose

Potency = drug concentration (this is not required if the dose is expressed in ml)

An average human weight for the purpose of MVD calculation is regarded as 70kg (or 60Kg for Japan).

Examples of Calculation for Endotoxin Limits:

Endotoxin Limits	
Example 1 – Product 1	Example 2 – Product 2
Dose = 2 mg/Kg	Dose = 10mL/Kg
Potency = 100mg/mL	Potency = not applicable
Endotoxin Limit = 5EU/Kg x 100mg/mL / 2mg/Kg = 250EU/mL	Endotoxin Limit = 5EU/Kg / 10mL/Kg = 0.5EU/mL

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7.5. Select the 'Template' button on the screen. This will show a representation of a microtitre plate with all test and standard positions labelled. For Validation, Diluent Verification and Routine testing assays, the products / diluents to be assayed must be entered. Delete existing records by double clicking on each of the entries and selecting 'delete'. When clear, select 'new', then 'products', scroll down until you find the desired product, highlight it then select 'copy'. This copies the product to the product sheet. Complete all the required batch details ensuring that the dilution details are correct, and include the Positive Product Control (PPC) when necessary (see respective sections for details). Select 'OK' to copy product details to template sheet.

7.6. As shown on the template sheet, add 0.1mL aliquots of the Blank, Standards and Products to be assayed to the appropriate wells of the plate. Spike the Positive Product controls as shown (10µl from 50 EU/mL standard for 5 EU/mL PPC, 10µl from 10 EU/mL standard for 1 EU/mL PPC).

7.7. Select 'Done', select 'Save', select 'Run'. If only one assay is to be run on the plate select 'OK'. (If more than one is to be run, select 'Add' then select the other assays to be run.)

7.8. Follow the instructions on screen to start the 10-minute incubation at 37°C.

7.9. Near the end of the incubation period rehydrate the required number of chromogenic lysate vials with LAL grade WFI as instructed on the vial. Swirl gently to fully dissolve the powder, avoiding air bubbles, and pour into reagent well.

7.10. At the end of the incubation period follow the software instructions to add 0.1mL of lysate, with the multi channel pipette vertically from left to right, to the wells used in the microtitre plate. Take care not to cross contaminate the pipette tips. Follow the on screen instructions to start the assay.

7.11. When the assay is complete, follow the instructions on screen to save and print the results.

7.12. **Interpreting results**

An acceptable assay has the following:

7.12.1 A Correlation Coefficient of greater than the absolute value of 0.980. This indicates the linearity of the points in the standard curve. For greatest accuracy, a coefficient of above 0.997 is recommended.

7.12.2 Coefficient of variances of <10% for standards, products and positive product control spikes. This is a measure of the correlation between reaction times of replicates. The closer to zero, the better.

7.12.3 Endotoxin standard recoveries of 100 ±25%. (Only applicable when a standard curve stored on the Win-KQCL system is used for multiple test runs.)

7.12.4 Endotoxin recoveries from positive product control spikes must be 100 ±50%. Less than 100% is showing inhibition, greater than 100% is showing enhancement.

7.12.5 Endotoxin content of diluents and products must conform to limits. These limits can be found in the front of the KCA Product Validation files. Results are printed in the product report section under "Results EU/ml". This is automatically calculated from the "Raw EU" result by multiplying by the dilution factor.

7.12.6 **Re-Test, Out-of-Specification Result and Test Failure Response**

Re-Test

A repeat test is permitted under any one or more of the following circumstances;

- The correlation coefficient is <0.980.

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Maximum Valid Dilution For Products Tested By LAL KCA Method

(Ref. MICLAB 085)

Product:	
Concentration:	mg/mL
Maximum Valid Dilution:	
Dilution used:	1 :
Dilutent used:	
pH adjustment necessary:	
Validation Requirements met:	
Reagent Supplier:	

Position:	Name:	Signature:	Date:
Senior Microbiology Staff			
Microbiology Manager			

Product:		
M = Maximum human dose/kg/hr		mg/Kg/hr
C = Concentration of Product		mg/ml
λ (Sensitivity of Lysate)		EU/mL
K = 5.0 EU/kg/hr for parenteral preparations (Intravenous)		
K =0.2 Eu/kg/hr for parenteral preparations (Intrathecal)		
K = Threshold pyrogenic dose of endotoxin per kilogram of body mass in a single hour period.		

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