	POLICY ON LIMULUS AMEBOCYTE LYSATE (LAL) TEST

The Limulus Amebocyte Lysate (LAL) test, using the gel-clot, chromogenic or turbidimetric technique, can be utilised to demonstrate that a product is free of bacterial endotoxins (pyrogens). The LAL test can be used as an alternative testing method to the rabbit pyrogen test.

This document outlines the requirements which must be met by companies who intend using the LAL test. The information contained herein is modified from "Guideline on validation of the Limulus Amebocyte Lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices", published by US Department of Health and Human Services, Public Health Service, Food and Drug Administration Any LAL technique (gel-clot, chromogenic or turbidimetric) can be used in testing a product for bacterial endotoxin. The methodology for the gel-clot technique is described in the British Pharmacopoeia and should be utilised whenever possible.

The LAL test must be validated (as described below) for each end-use product.

1. Validation of the LAL test

Validation of the LAL test includes the following:

a. initial qualification of the laboratory
b. inhibition and enhancement tests.

a. Initial qualification of the laboratory

Various methodologies have been described for the detection of endotoxin, using limulus amebocyte Iysate. Currently, commercially available lysates use the gel clot, chromogenic, endpoint-turbidimetric or kinetic-turbidimetric techniques. Other methods which have been reported show potential for increasing further the sensitivity of the LAL method.

Companies should assess the inter- or intra-laboratory test variability of the laboratory before any official tests are performed. Appendix A gives the procedures and test criteria for the currently available techniques.

b. Inhibitor and enhancement testing

The degree of product inhibition or enhancement of the LAL procedure should be determined for each drug formulation before the LAL test is used to assess the endotoxin content of any drug. All validation tests should be performed on undiluted drug product or on an appropriate dilution. Dilutions should not exceed the Maximum Valid Dilution (MVD) (see Appendix B). At least three production batches of each finished product should be tested for inhibition and enhancement.

i. Gel-clot technique

The validation (inhibition/enhancement) method for this technique involves taking a drug concentration containing varying concentrations of a standard endotoxin that bracket the sensitivity of the Iysate and comparing it to a series of the same endotoxin concentrations in water alone. The drug product is "spiked" with endotoxin and then diluted with additional drug product (so that the drug concentration remains constant) to the same endotoxin concentrations in water. Results of endotoxin determination in water and the drug product should fall within plus/minus a twofold dilution of the labelled sensitivity. If the undiluted drug product shows inhibition, the drug product can be diluted, not to exceed the MVD (see Appendix B), with the same diluent that will be used in the release testing and the above procedure repeated. Negative controls (diluent plus Iysate) should be included in all inhibition/enhancement testing.

ii. Chromogenic and endpoint-turbidimetric techniques

In inhibition/enhancement testing by these techniques, a drug concentration containing 4 lambda concentration of the reference standard endotoxin (RSE) or control standard endotoxin (CSE) (lambda is equal to the lowest endotoxin concentration used to generate the standard curve) is tested in duplicate according to the Iysate manufacturer's methodology. The standard curve for these techniques shall consist of at least four RSE or CSE concentrations in water that extend over the desired range. If the desired range is greater than one log, additional standards concentrations should be included. The standard curve must meet the criteria for linearity as outlined in Appendix A(ii). The detected amount of endotoxin in the spiked drug must be within plus or minus 25% of the 4 lambda concentration for the drug concentration to be considered to neither enhance nor inhibit the assay. If the undiluted drug product shows inhibition, the drug product can be diluted, not to exceed the MVD (see Appendix B), and the test repeated. An alternate procedure may be performed as described above except the RSE/CSE standard curve is prepared in LAL negative drug product, i.e. no detectable endotoxin, instead of LAL negative water. The standard curve must meet the test for linearity, i.e. r equal to or greater than 0.980, and in addition the difference between the O.D. readings for the lowest and highest endotoxin concentrations must be greater than 0.4 and less than 1.5 O.D. units. If the standard curve does not meet these criteria, the drug product cannot be tested by the alternate procedure.

iii. Kinetic-turbidimetric technique

In inhibition/enhancement testing by this technique, a drug concentration containing 4 lambda concentration of the RSE or CSE (lambda is equal to the lowest endotoxin concentration used to generate the standard curve) is tested in duplicate according to the Iysate manufacturer's methodology. The standard curve shall consist of at least four RSE or CSE concentrations. If the desired range is greater than one log, additional standard concentrations should be included. The standard curve must meet the criteria outlined in Appendix A(iii). The calculated mean amount of endotoxin in the spiked drug product, when referenced to the standard curve, must be within plus or minus 25% to be considered to neither enhance nor inhibit the assay. If the undiluted drug product shows inhibition or enhancement, the drug product can be diluted, not to

exceed the MVD, and the test repeated. An alternate procedure may be performed whereby the RSE/CSE standard curve is prepared in drug product or product dilution instead of water. The drug product cannot have a background endotoxin concentration of more than 10% (estimated by extrapolation of the regression line) of the lambda concentration (lambda equals the lowest concentration used to generate the standard curve). The standard curve must meet the test for linearity, i.e. r equal to or less than -0.980, and in addition the slope of the regression must be less than -0.1 and greater than -1.0. If the standard curve does not meet these criteria, the drug product cannot be tested by the alternate procedure. In those instances when the drug is manufactured in various concentrations of active ingredient while the other components of the formulation remain constant, only the highest and lowest concentration need be tested. If there is a significant difference, i.e. greater than twofold, between the inhibition endpoints or if the drug concentration, per rnL, in the test solutions is different, then each remaining concentrations should be tested. If the drug product shows inhibition or enhancement at the MVD, when tested by the procedures in the above sections, and is amenable to rabbit testing, then the rabbit test will still be the appropriate test for that drug. If the inhibiting or enhancing substances can be neutralized without affecting the sensitivity of the test, or if the LAL test is more sensitive than the rabbit pyrogen test, the LAL test can be used. For those drugs not amenable to rabbit pyrogen testing, the manufacturer should determine the smallest quantity of endotoxin that can be detected. This data should be submitted to the NRA for review. The inhibition/enhancement tests must be repeated on one unit of the product if the Iysate manufacturer is changed. If the Iysate technique is changed, the inhibition and enhancement tests must be repeated using three batches. When the manufacturing process, the product formulation, the source of a particular ingredient of the drug formulation, or Iysate lot is changed, the positive product control can be used to test the validity of the LAL test for the product. Companies that are obtaining an ingredient from a new manufacturer are encouraged to include as part of their vendor qualification the rabbit pyrogen test to determine that the ingredient does not contain non-endotoxin pyrogens.

2 Routine Testing of Drugs by the LAL Test

End-use product testing is to be based on data from the inhibition/enhancement testing as outlined above. Samples, standards, positive product controls and negative controls should be tested at least in duplicate.

For the gel-clot technique, an endotoxin standard series does not have to be run with each set of tests if consistency of standard endpoints has been demonstrated in the test laboratory. It should be run at least once a day with the first set of tests and repeated if there is any change in Iysate lot, endotoxin lot or test conditions during the day. An endotoxin standard series should be run when confirming end-use product contamination. Positive product controls (two lambda concentration of standard endotoxin in product) must be positive. If your test protocols state that you are using the methodology described in the BP, a standard series is to be run with each test.

For the chromogenic and endpoint-turbidimetric techniques, an endotoxin standard series does not have to be run with each set of tests if consistency of standard curves has been demonstrated in the test laboratory. It should be run at least once a day with the first set of tests and repeated if there is any change in Iysate lot, endotoxin lot or

test conditions during the day. However, at least duplicates of a 4 lambda standard concentration in water and in each product (positive product control) must be included with each run of samples. The mean endotoxin concentration of the standard must be within plus/minus 25% of the actual concentration and the positive product control must meet the same criteria after subtraction of any endogenous endotoxin. An endotoxin standard series should be run when confirming end-product contamination. If the alternate procedure is used, a standard in product series must be conducted each time the product is tested.

For the kinetic-turbidimetric test, it is not necessary to run a standard curve each day or when confirming end-use product contamination if consistency of standard curves has been demonstrated in the test laboratory. However, at least duplicates of a 4 lambda standard concentration in water and in each product (positive product control) must be included with each run of samples. The mean endotoxin concentration of the standard when calculated using an standard curve (See Appendix C, contained in "Guideline on validation of the Limulus Amebocyte Lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices", published by US Department of Health and Human Services, Public Health Service, Food and Drug Administration), must be within plus/minus 25% of the actual concentration and the positive product control must meet the same criteria after subtraction of any endogenous endotoxin. If the alternate procedure is used, a standard in product series must be conducted each time the product is tested.
Before a new lot of Iysate is used, the labelled sensitivity of the Iysate or the performance criteria should be confirmed by the laboratory, using the procedures described in Appendix A.

The sampling technique selected and the number of units to be tested should be based on the manufacturing procedures and the batch size. A minimum of three units, representing the beginning, middle, and end, should be tested from a lot. These units can be run individually or pooled. If the units are pooled and any endotoxin is detected, repeat testing can be performed. The LAL test may be repeated no more than twice. The first repeat consists of twice the initial number of replicates of the sample in question to examine the possibility that extrinsic contamination occurred in the initial assay procedure. On pooled samples, if any endotoxin is detected in the first repeat, proceed to second repeat. The second repeat consists of an additional 10 units tested individually. None of the 10 units tested in the second repeat may contain endotoxin in excess of the limit concentration for the drug product.

APPENDIX A

QUALITY CONTROL PROCEDURE

The following procedures and criteria are used for initial qualification and requalification of analysts in the laboratory, and to test new lots of Iysate before use.

i. Gel-clot endpoint technique

For the gel-clot technique the procedures in the BP should be used for quality control testing.

ii. Chromogenic and endpoint-turbidimetric techniques

Each test should be conducted according to the specific manufacturer' s methodology.

Using the RSE or CSE whose potency is known, assay 4 replicates of a set of endotoxin concentrations which extend over the labelled linear range. The standard concentrations must include the stated lower and upper limits of the range. Linear regression analysis is performed on the absorbance values of the standards (y-axis) and their respective endotoxin concentrations (x-axis). The coefficient of correlation, r, shall be greater than or equal to 0.980. If r is less than 0.980 the cause of the non-linearity should be determined and the test repeated. This linearity limit is also used to judge the validity of standard curves used for inhibition/enhancement tests and sample tests. In addition to meeting these requirements, any other test or requirements specified by the Iysate manufacturer should also be met.

iii. Kinetic-turbidimetric technique

Each test should be conducted according to the manufacturer's instructions.

Using the RSE or CSE whose potency, in endotoxin units (See Appendix C, contained in "Guideline on validation of the Limulus Amebocyte Lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices", published by US Department of Health and Human Services, Public Health Service, Food and Drug Administration), is known, assay at least 6 concentrations in triplicate which extend over the range 0.03 - 1.0 EU/mL. If instrument configuration does not allow you to run all 6 concentrations at one time, the data can be obtained in multiple runs and combined. Perform regression-correlation analysis on the log of the Time of Reaction (T) versus the log of the endotoxin concentration (E). The coefficient of correlation, r, shall be less than or equal to -0.980. If r is greater than -0.980 the cause of the non-linearity should be determined and the test repeated. In addition to meeting these requirements, any other test or requirements specified by the Iysate manufacturer should also be met.

APPENDIX B

MAXIMUM VALID DILUTION

To determine how much the product can be diluted and still be able to detect the limit endotoxin concentration, the following two methods will determine the Maximum Valid Dilution (MVD):

i. Method 1

This method is used when the limits listed in Appendix E, contained in "Guideline on validation of the Limulus Amebocyte Lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices", published by US Department of Health and Human Services, Public Health Service, Food and Drug Administration, are used. MVD = Endotoxin Limit x Potency of Product For drugs administered on a weight per kilogram basis, the potency is expressed as mg or units/mL and for drugs administered on a volume-per-kilogram basis, the potency is equal to 1.0 mL/mL.

ii. Method 2

This method is used when there is no limit listed in Appendix E, contained in "Guideline on validation of the Limulus Amebocyte Lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices", published by US Department of Health and Human Services, Public Health Service, Food and Drug Administration.

Step 1: Minimum Valid Concentration (MVC)

MVC = λ M/K

Where:

λ = GEL CLOT: Labelled sensitivity-EU/mL
CHROMOGENIC, TURBIDIMETRIC and KINETIC-TURBIDIMETRIC:
The lowest point used in the standard curve.

M = Rabbit Dose or Maximum animal Dose/kg of body weight that would be administered in a single one hour period, whichever is larger.

K = 5.0 EU/kg for parenteral drugs.

Step 2. Maximum Valid Dilution (MVD)

MVD = Potency of Product/MVC

For drugs administered on a weight-per-kilogram basis, the potency is expressed as mg or units/mL and for drugs administered on a volume-per-kilogram, the potency is equal to 1.0mL/mL.