

Monday May 5 and 6, 2008

Lab Practical

Endotoxin and Mycoplasma Testing

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Objectives

1. To observe and understand the rationale behind product purity testing
 - a. Focus on endotoxin testing by kinetic-QCL method (Lonza)
 - b. Gel cloth assay (Lonza)
 - c. Rapid endotoxin testing by Endosafe PTS (Charles Rivers)
2. Observe an selected steps in the Mycoplasma testing
 - a. DNA hybridization method
 - b. Mycoplasma by PCR
3. Understating the differences in methods.

Contents

1. Introduction to CPL
2. Types of testing performed in CPL
3. SOP-CPL-0243 Procedure for Endotoxin testing by Kinetic-QCL method
4. SOP-CPL-0019 Endotoxin Testing by LAL Assay
5. SOP-CPL-0051 Procedure for Mycoplasma Detection Assay
6. Endotoxin assay run example

The Cellular Product Laboratory (CPL)

The CPL is dedicated to generation and preparation of quality products for cellular and gene therapy of cancer. This includes autologous cells activated and cultured in the presence of cytokines, tissue or blood cells variously modified in vitro for adoptive transfers. More recently, the CPL has focused on the generation and culture of dendritic cells (DC) for anti-cancer vaccines. Both cleanrooms are used exclusively for culture/processing of human cellular products for therapy. No animal cell or any other cell products are generated in this laboratory. All operational guidelines follow the GMP requirements.

The cleanrooms have ceiling-mounted HEPA filters and are positive pressure with respect to the outer lab. Each cleanroom is entered via a “locker room” that provides a change of shoes and gowning. The cleanrooms have hard ceilings, one-piece floors and stainless steel casework. They are joined to the outer lab with an interlocked anteroom (such that the door to the outer lab and the cleanroom cannot be simultaneously opened).

The GTL offers specialized services in gene transfer technology (extensive experience in the culture of human cells for transfection with adenoviral or retroviral vectors) and selection of stable transfectants and their culture and expansion for transfer to patients. Methodology is established for culture, transduction and selection of genetically modified human fibroblasts that can be used as components of vaccines.

- Both cleanrooms are fully equipped for cell culture. Non-transfected cells are usually cultured in the CPL, but once genetically modified; these cells are segregated to the GTL and never return to the CPL.
- Services are provided to a variety of different users, and technologists servicing cleanrooms are experienced with multi-user service formats.

Quality Control of the final product as performed in CPL

1. Products generated in IMCPL are evaluated for viability, purity, identity (phenotype by flow) and potency (IL-12 production assay) prior to release for administration.
2. As well as all product are sampled for Gram stain, 14-day sterility, mycoplasma and endotoxin testing.
3. The CPL laboratory performs the testing on culture (48-72hr prior to harvest) as well as on the final product:
 - a. Endotoxin by Limulus Amebocyte Lysate Assay: SOP-CPL -0019 This assay is performed using the Limulus Amoebocyte Lysate (LAL) Pyrogen Plus assay (Lonza). The acceptable level of endotoxin is < 5EU/kg of body weight per dose. Cultures containing endotoxin levels higher than 5EU/kg of body weight per dose are not released for administration. Endotoxin results are always available at the time of final product release.

- b. Endotoxin testing by Kinetic-QCL method: SOP-CPL-0243. KineticQCL is a quantitative, kinetic assay for detecting of Gram-negative bacterial endotoxin. This test is performed using the Limulus Amoebocyte Lysate in combination with an incubating photometer and appropriate software, to detect endotoxin photometrically.
- c. Endotoxin testing using the EndoSafe PTS cartilages: SOP-CPL-00235. This method is used for testing product with very short stability e.g. Stem cells.
- d. Mycoplasma detection assay: SOP-CPL-0051 GEN-PROBE[®] Mycoplasma T. C. Rapid Detection System Kit (GEN-PROBE[®] Inc. San Diego, CA). This is a commercially available test kit that utilizes DNA hybridization techniques and it is based on hybridization between rRNA of the target organism and a complementary DNA probe. In the current assay, the probe is labeled with a chemiluminescent label.
- e. Mycoplasma detection by PCR (e-Myco[™] Mycoplasma PCR Detection, SOP-CPL-0064). The e-Myco[™] kit is designed to allow for detection of a broad range of *Mycoplasma* species with high specificity and sensitivity by using a primer set that is specific to a highly conserved region within the 16S rRNA *Mycoplasma* gene. Eukaryotic and bacterial DNA are not amplified. Each tube of the e-Myco kit contains all components for PCR (*i*-StarTaq[™], DNA Polymerase, dNTPs, 1x buffer, and primers).

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IMMUNOLOGIC MONITORING AND CELLULAR PRODUCTS LABORATORY

ENDOTOXIN TESTING BY KINETIC-QCL METHOD

1. Purpose and scope

- 1.1. This assay is used in the laboratory to test for the presence of endotoxin. If present, could alter the results of the cellular assays performed in this lab. To detect the presence of endotoxin, Kinetic-QCL utilizes a preparation of Limulus Amebocyte Lysate (LAL), in combination with an incubating photometer and appropriate software, to detect endotoxin photometrically.
- 1.2. The principle of this test is that LAL reacts with endotoxin and releases p-nitroniline from a synthetic substrate, producing a yellow color. Kinetic-QCL is a quantitative, kinetic assay for the detection of Gram-negative bacterial endotoxin. A sample is mixed with the LAL/SUBSTRATE reagent, place in an incubating plate reader, and automatically monitored over time for the appearance of a yellow color. The time required before the appearance of yellow color (Reaction Time) is inversely proportional to the amount of endotoxin present. That is, in the presence of a large amount of endotoxin the reaction occurs rapidly; in the presence of a small amount of endotoxin the reaction time is increased. The concentration of endotoxin in unknown samples can be calculated from a standard curve.

2. Responsibility

- 2.1. Laboratory staff is responsible for following this procedure and for completing all documentation which is then forwarded along with results to the Laboratory Supervisor.
- 2.2. The Laboratory Supervisor is responsible for reviewing all results and documentation for accuracy and completeness.
- 2.3. The QA Manager is responsible for reviewing and approving this procedure.
- 2.4. The Laboratory Director is responsible for reviewing and approving this procedure. The Laboratory Director is also responsible for annual review of this procedure.

3. Safety and Precautions

ALL HUMAN SAMPLES ARE POTENTIALLY BIOHAZARDOUS! All equipment, supplies, and reagents in contact with human blood, body fluids and/or tissues should be handled and disposed of as if biohazardous. **HANDLE ALL MATERIALS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.** When performing this procedure, follow the UPCI IMCPL Biohazard Precautions as outlined in IMCPL safety manual.

Review and Approved by	Signature	Date
Laboratory Supervisor		
QA/QC Manager		
Laboratory Director		

3.1. Sample Acceptability

- 3.1.1. This assay requires $\geq 500\mu\text{L}$ of the sample to be tested. Sample must be free of particulate material. If sample is cloudy or visibly contains solid material, the sample must be centrifuged for 10 minutes at 1,200 rpm (300 x g).
- 3.1.2. It may be necessary to adjust the pH of the sample to within the range 6.0-8.0 using endotoxin-free sodium hydroxide or hydrochloric acid.
- 3.1.3. Samples should be stored at 2 - 8°C for less than 24 hours; samples stored longer than 24 hours should be frozen.

4. Definitions

- 4.1. LAL - Limulus Amebocyte Lysate
- 4.2. PPC- Positive Product Control
- 4.3. CSE- Control Standard Endotoxin
- 4.4. RSE- Reference Standard Endotoxin

5. Equipment, Materials and Reagents

5.1. Equipment

- 5.1.1. Pipetman P10, P100, P200 and P1000 (Rainin, Cat # P100, P200, P1000, or equivalent)
- 5.1.2. 70°C heat block (Thermolyne Type 17600, or equivalent)
- 5.1.3. Polypropylene test tube rack (Fisher, Cat # 14-809A, or equivalent)
- 5.1.4. Timer (Fisher, Cat # 06-662-5, or equivalent)
- 5.1.5. Eight-Channel multi-pipettor (Labnet Biopette E, or equivalent)
- 5.1.6. Centrifuge (Sorval T-6000, or equivalent)
- 5.1.7. Vortex (Fisher Scientific)
- 5.1.8. Microplate reader (ELx808 IU Reader, Cambrex, #25-315; or equivalent)
- 5.1.9. WinkQCL Software (Cambrex, #25-300, #25-301, 25-348 or current version)

5.2. Materials

- 5.2.1. Sterile pipets 1 mL, 5 mL, 10 mL (Fisher, Cat # 7521, 7543, 7751, or equivalent)
- 5.2.2. Sterile pipet tips (Biorad #223-9035, or equivalent)
- 5.2.3. Disposable endotoxin-free 13 x 100 mm glass dilution tubes (Cambrex, #N207 or equivalent)

5.2.4. Sterile 96-well, flat bottom microplates, (**Recommend Costar Brand, Cat # 3595**) NOTE: If 96-well microplates other than those recommended by Lonza are used, they **MUST BE** pre-qualified. This must be done for each lysate lot by having no less than 3 endotoxin standards, spanning the desired concentration range (50-0.005 EU/mL) run in the microplate. The absolute value of the correlation (r) of the calculated standard curve should be ≥ 0.980 .

5.2.5. Reagent reservoirs (Cambrex, #25-268 or equivalent)

5.2.6. Lab Tape (Fisher Scientific, Cat # 1590110R or equivalent)

5.3. Reagents

5.3.1. Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit, (Lonza, Cat# 50-650H or equivalent)

5.3.2. E. coli 059:B5 Endotoxin (CSE) included with kit above

5.3.3. Sodium hydroxide, 0.1N, or Hydrochloric acid, 0.1N, dissolved in LAL Reagent Water, for pH adjustment of samples if necessary

5.3.4. LAL Reagent Water (Lonza, Cat# W50-100 or recommended water from Lonza)

NOTE: Do not substitute LAL water with water from other vendors.

5.4. Reagent Preparation

5.4.1. Preparation of Limulus Amebocyte Lysate **PERFORMED IMMEDIATELY PRIOR TO ADDING TO THE ASSAY PLATE!! (Step 6.3.12)**

5.4.1.1. Immediately before use reconstitute lyophilized lysate per label directions (2.6ml/vial) with LAL reagent water. **Swirl gently** but thoroughly for at least 30 seconds. **Do not shake or vortex** as contents will foam.

5.4.1.2. Reconstituted Kinetic-QCL Reagent should be used promptly. Protect from long term exposure to light. It is stable for 8 hours at 2 - 8°C without loss of sensitivity. Reconstituted lysate can be divided into more convenient volumes and stored below -10°C for up to 2 weeks. Frozen liquid lysate should be thawed immediately before use and only once.

5.4.2. Preparation of E. coli 059:B5 Endotoxin (CSE)

5.4.2.1. Reconstitute the CSE as per label instructions or as per the Certificate of Quality, included with each kit to yield a solution containing 50EU/ml.

5.4.2.2. Vortex vigorously for at least 15 minutes at high speed. Prior to each future use, the solution must be warmed to room temperature and vigorously vortexed for 15 minutes.

5.4.2.3. The reconstituted stock is stable for 4 weeks at 2 - 8°C.

6. Test Procedure

6.1. If a new lot number of the Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit has been received an INITIAL QUALIFICATION ASSAY **MUST** be run prior to using the new lot. See Step 6.4 to proceed.

6.2. Preparation of Standard Curve Dilution Series

6.2.1. Prepare the Standard Curve dilutions using the 50 EU/mL stock CSE solution as follows in Table 1 below. Vortex each dilution for 1 minute before continuing to the next dilution. In order to have adequate volume to spike all samples, make 2x volume of the 5EU/mL stock as shown below.

TABLE 1

Endotoxin Concentration	LAL Reagent Water	Volume added to LAL Reagent Water
5 EU/ml	1.8 ml	0.2 ml of 50 EU/ml
0.5 EU/ml	0.9 ml	0.1 ml of 5 EU/ml
0.05 EU/ml	0.9 ml	0.1 ml of 0.5 EU/ml
0.005 EU/ml	0.9 ml	0.1 ml of 0.05 EU/ml

NOTE: In order to have a valid assay run, no fewer than 3 endotoxin standard concentrations along with an LAL Reagent Water blank assayed in triplicate should be run.

6.3. Running the Assay using the WinkQCL Software

6.3.1. Double click on the WinkQCL Software icon located on the desktop.

6.3.2. Log into software using assigned username and password.

6.3.3. Select TEMPLATE from the main menu

6.3.3.1. Select desired template, check parameters and verify plate set-up, value, etc. Select RUN to begin assay.

6.3.3.2. To create a new template:

6.3.3.2.1. Select NEW

6.3.3.2.2. Enter template NAME

6.3.3.2.3. Select ROUTINE from the drop down menu for “Test” type.

6.3.3.2.4. Select KQCL as “Type of Assay”

6.3.3.2.5. Select LOT NO./ EXP DATES and the corresponding lots and expiration dates for all related components. Click OK.

- 6.3.3.2.6. Enter the number of STANDARDS to be run (must be 3-5). Select STANDARD SETUP and enter the standard values. Click OK.
- 6.3.3.2.7. Select “Plate Layout” as STANDARD, enter the “Number of Replicates” (minimum of 3), “Replicate direction” is HORIZONTAL and “Fill Up Direction” is VERTICAL. Click OK
- 6.3.3.2.8. Select “ASSAY PLATE”
- 6.3.3.2.9. Select NEW and enter sample product description, lot#, release limit (if applicable), dilution (if applicable), click INCLUDE PPC box, add comments (optional). Click OK. Repeat this step for each product sample. See example of a TEMPLATE including the blank, 5 standards, and 2 samples below. Click “Exit”.

Table 2. Sample Assay Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	BL	02-	02-	02-						
B	S1	S1	S1	02+	02+	02+						
C	S2	S2	S2									
D	S3	S3	S3									
E	S4	S4	S4									
F	S5	S5	S5									
G	01-	01-	01-									
H	01+	01+	01+									

- 6.3.3.2.10. Clicking on any of the samples listed in the ASSAY PLATE template allows user to see the description of what should be placed in that well. This template can also be printed as an aid in placement of samples into the 96-well plate.
 - 6.3.3.2.11. Select SAVE in the “Template Manager” window to complete the template.
 - 6.3.3.2.12. From the main TEMPLATE window highlight the template created and select RUN to begin assay.
- 6.3.4. Load 100µL of the blank and standards into the designated wells as indicated on the correct template in the following order:
- 6.3.4.1. BLANK
 - 6.3.4.2. Standard Concentration Dilutions; beginning with the least concentrated to the most concentrated.
- 6.3.5. Add 10µL of the 5 EU/mL Standard Dilution to all wells indicating a spiked sample (positive product control, or PPC). (This results in a 1:10 dilution of the standard and should therefore yield a PPC recovery value of 0.5 EU/mL).

6.3.6. To prepare sample, use pre-determined dilution and/or denaturation (most common is 1:10 dilution of sample).

NOTE: Denaturation must be performed on any sample known to include serum, HSA, etc. This can be done by making the determined dilution of the sample (typically 1:10) and then heating the sample at 70°C for 10 minutes.

6.3.7. Add 100µL of sample to each of the wells including the PPC wells.

6.3.8. The “Configure Assay Plate for Run” window appears. Verify that selected template is highlighted in left box, select NEXT.

6.3.9. The reader will perform a self-test and verify a temperature of 37± 1°C. A window will appear.

6.3.10. Place filled plate into microplate reader, close lid and click OK.

6.3.11. Pre-incubate the plate for ≥ 10 minutes at $37 \pm 1^\circ\text{C}$.

6.3.12. Near the end of the pre-incubation period, reconstitute each of the appropriate number of LAL vials with 2.6 ml LAL Reagent Water per vial. Mix gently but thoroughly. See Step 5.4.1.

6.3.13. Pool the reagents into a reagent reservoir and mix by gently rocking the reservoir from side to side.

6.3.14. When the pre-incubation is completed, using an 8-channel multi-pipettor, dispense 100µl of the LAL Lysate into all wells of the microplate beginning with the first column (A1-H1) and proceeding in sequence to the last column used. Add reagent as quickly as possible. Avoid causing bubbles!

6.3.15. Immediately click OK to initiate the test. The run time is ~ 60 minutes.

NOTE: The Kinetic-QCL assay is performed with the microplate cover removed.

6.3.16. When completed ASSAY COMPLETE will appear, check box to use POWERCURVE and then OK.

6.3.17. Select to VIEW RESULTS. Observe all values and check for run acceptability. Apply necessary e-signatures by selecting appropriate designations.

6.4. Initial Qualification Assay

6.4.1. If using a new lot of the Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit an INITIAL QUALIFICATION assay **MUST** be performed. This assay is required as part of the validation of the LAL assay requirements by the FDA.

6.4.1.1. Prepare a Standard Series Dilution, vortexing 1 minute between each dilution, using Table 3.

TABLE 3

Endotoxin Concentration	LAL Reagent Water	Volume added to LAL Reagent Water
5 EU/ml	0.9 ml	0.1 ml of 50 EU/ml
0.5 EU/ml	0.9 ml	0.1 ml of 5 EU/ml
0.05 EU/ml	0.9 ml	0.1 ml of 0.5 EU/ml
0.005 EU/ml	0.9 ml	0.1 ml of 0.05 EU/ml

- 6.4.1.2. Double click on the WinkQCL Software icon located on the desktop.
- 6.4.1.3. Log into software using assigned username and password.
- 6.4.1.4. Select TEMPLATE from the main menu
 - 6.4.1.4.1. Select NEW
 - 6.4.1.4.2. Enter template NAME (Initial Lot Qualification Lot # xxxxxxxx)
 - 6.4.1.4.3. Select INITIAL QUALIFICATION from the drop down menu for “Test” type.
 - 6.4.1.4.4. Select KQCL as “Type of Assay”
 - 6.4.1.4.5. Select LOT NO./ EXP DATES and the corresponding lots and expiration dates for all related components. Click OK.
 - 6.4.1.4.6. Enter the number of STANDARDS to be run (must be 5). Select STANDARD SETUP and enter the standard values. Click OK.
 - 6.4.1.4.7. Select “Plate Layout” as STANDARD, enter the “Number of Replicates” as 4, “Replicate direction” is HORIZONTAL and “Fill Up Direction” is VERTICAL. Click OK
 - 6.4.1.4.8. Select “ASSAY PLATE” and verify that the blank and 5 standards appear correctly within the plate.
 - 6.4.1.4.9. Select SAVE in the “Template Manager” window to complete the template.
 - 6.4.1.4.10. From the main TEMPLATE window highlight the template created and select RUN to begin assay.
- 6.4.2. Load 100µL of the corresponding blank and standards into the designated wells as indicated on the correct template.
- 6.4.3. The “Configure Assay Plate for Run” window appears. Verify that selected template is highlighted in left box, select NEXT.
- 6.4.4. The reader will perform a self-test and verify a temperature of 37± 1°C. A window will appear.
- 6.4.5. Place filled plate into microplate reader, close lid and click OK.

- 6.4.6. Pre-incubate the plate for ≥ 10 minutes at $37 \pm 1^\circ\text{C}$.
- 6.4.7. Near the end of the pre-incubation period, reconstitute each of the appropriate number of LAL vials with 2.6 ml LAL Reagent Water per vial. Mix gently but thoroughly. See Step 5.4.1.
- 6.4.8. Pool the reagents into a reagent reservoir and mix by gently rocking the reservoir from side to side.
- 6.4.9. When the pre-incubation is completed, using an 8-channel multi-pipettor, dispense 100 μl of the LAL Lysate into all wells of the microplate beginning with the first column (A1-H1) and proceeding in sequence to the last column used. Add reagent as quickly as possible. Avoid causing bubbles!
- 6.4.10. Immediately click OK to initiate the test. The run time is ~ 60 minutes.
NOTE: The Kinetic-QCL assay is performed with the microplate cover removed.
- 6.4.11. When completed ASSAY COMPLETE will appear, select to VIEW RESULTS. Observe all values and check for run acceptability. Apply necessary e-signatures by selecting appropriate designations.
- 6.4.12. After a successful run, the new lot is validated and ready for use.

6.5. Types of Kinetic-QCL Assays

- 6.5.1. ROUTINE: This assay calculates the concentration of endotoxin unknowns by comparison to the performance of a series of endotoxin standards. A PPC is used to monitor product inhibition or enhancement. (A PPC is a sample to which a known amount of endotoxin spike has been added). The WinkQCL Software automatically calculates the amount of endotoxin recovered in the PPC, allowing for the comparison to the known amount of endotoxin spike.
- 6.5.2. INHIBITION/ENHANCEMENT: This Assay is designed to determine what level of product dilution overcomes inhibition or enhancement. Each product dilution must be accompanied by a PPC. The WinkQCL Software calculates the amount of endotoxin recovered in the PPC for comparison to the known amount of endotoxin spike. Therefore, it can determine which product dilutions are non-inhibitory. (It is recommended that the endotoxin spike result in a final endotoxin concentration in the sample equal to 0.5EU/ml. The endotoxin recovered should equal the known concentration of the spike within 50 – 200%. Refer to WinkQCL Software Manual for detailed instructions on how to run this assay.
- 6.5.3. RSE/CSE: This assay is designed to determine the potency of a Control Standard Endotoxin (CSE) in terms of the concentration units of the Reference Standard Endotoxin (RSE). The assay requires a single series of RSE dilutions and one or more sets of dilutions of the CSE. Depending on the concentration units of the CSE, the WinkQCL Software automatically computes mean potency values in terms of EU/ng or EU/ml. Refer to the WinkQCL Software Manual for detailed instructions on how to run this assay.

6.5.4. INITIAL QUALIFICATION: This assay is required as part of the validation of the LAL assay requirements by the FDA. It also is performed with each new lot of Kinetic-QCL.

6.6. Analysis

6.6.1. The WinkQCL Software monitors the absorbance at 405 nm of each well of the microplate. Using the initial absorbance reading of each well as its own blank, the reader determines the time required for the absorbance to increase 0.200 absorbance units. This time is termed Reaction Time. The WinkQCL Software automatically performs a log/log linear correlation of the Reaction Time of each standard with its corresponding endotoxin concentration. If the correlation coefficient (r) is ≥ 0.980 , a polynomial model (PowerCurve) can be used to construct a standard curve and in turn predict endotoxin concentrations of test samples. The PowerCurve improves the accuracy of predicting endotoxin concentrations over the entire endotoxin range.

7. Documentation

7.1. RESULT ACCEPTABILITY

7.1.1. Do not report patient results if any part of the run is invalid. **Repeat assay if run is unacceptable.**

7.2. RESULT REPORTING:

7.2.1. Any positive patient result will be handled as follows:

7.2.1.1. **Supervisor notified immediately.**

7.2.1.2. Testing repeated on the same sample to rule out testing error.

7.2.1.3. If repeat is still positive, **supervisor is notified immediately** and then the appropriate investigator (i.e., IMCPL staff or physician) is notified.

7.3. Acceptable data is filed in corresponding binders and/or patient files.

8. Attachments

8.1. AW-0084, CPL Mycoplasma/ Endotoxin Request for Testing and Results Worksheet

9. References

9.1. Lonza Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit Manual (included in every assay kit)

9.2. WinKQCL Software Manual, Version 3.1 (Lonza, supplied with WinKQCL software)

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ENDOTOXIN TESTING BY KINETIC-QCL METHOD

10. Revision History

Version	Reason for change	Author	Date approved
1	New SOP	Vicki Elborne, Jennifer Sprague	04/30/08

Annual Review and Approval

2009

No revision necessary

Revision necessary, SOP revised & archived

Lab Supervisor _____ Date _____

Lab Supervisor _____ Date _____

QA Manager _____ Date _____

QA Manager _____ Date _____

Lab Director _____ Date _____

Lab Director _____ Date _____

In document control Staff review initiated

2010

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Lab Supervisor _____ Date _____

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QA Manager _____ Date _____

Lab Director _____ Date _____

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2011

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Lab Supervisor _____ Date _____

QA Manager _____ Date _____

QA Manager _____ Date _____

Lab Director _____ Date _____

Lab Director _____ Date _____

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2012

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Revision necessary, SOP revised & archived

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Lab Supervisor _____ Date _____

QA Manager _____ Date _____

QA Manager _____ Date _____

Lab Director _____ Date _____

Lab Director _____ Date _____

In document control Staff review initiated

UNIVERSITY OF PITTSBURGH CANCER INSTITUTE
IMMUNOLOGIC MONITORING AND CELLULAR PRODUCTS LABORATORY

ENDOTOXIN TESTING BY LAL ASSAY

1. Introduction

- 1.1. This assay is used in this laboratory to test for the presence of endotoxin. If present, endotoxin could alter the results of the cellular assays performed in this lab. To detect the presence of endotoxin, the Limulus Amebocyte Lysate (LAL) test is utilized.
- 1.2. The principle of this test is that gram negative bacterial endotoxin catalyzes the activation of a pro-enzyme in Limulus lysate. The activated enzyme (coagulase) hydrolyses chemical bonds within the clotting protein (coagulogen) and causes the formation of a gelatinous clot.

2. Responsibility

- 2.1. Laboratory staff is responsible for following this procedure and for completing all documentation which is then forwarded along with results to the Laboratory Supervisor.
- 2.2. The Laboratory Supervisor is responsible for reviewing all results and documentation for accuracy and completeness.
- 2.3. The QA/QC Director is responsible for reviewing and approving this procedure.
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3.1. Sample Acceptability

- 3.1.1. This assay requires one mL of the sample to be tested. Sample must be free of particulate material. If sample is cloudy or visibly contains solid material, the sample must be centrifuged for 10 minutes at 1,200 rpm (300 x g).

4. Definitions

Review and Approved by	Signature	Date
Laboratory Supervisor		
QA Manager		
Laboratory Director		

- 4.1.CSE - Control Standard Endotoxin
- 4.2.LAL - Limulus Amebocyte Lysate

5. Equipment, Materials and Reagents

5.1. Equipment

- 5.1.1. Pipetman P100, P200 and P1000 (Rainin, Cat # P100, P200, P1000, or equivalent)
- 5.1.2. 37°C water bath (Napco Model, Cat # 210A, or equivalent)
- 5.1.3. Polypropylene test tube rack (Fisher, Cat # 14-809A, or equivalent)
- 5.1.4. Timer (Fisher, Cat # 06-662-5, or equivalent)
- 5.1.5. Eppendorf repeater (Fisher Scientific)
- 5.1.6. Centrifuge (Sorval T-6000, or equivalent)
- 5.1.7. Vortex (Fisher Scientific)

5.2. Materials and Reagents

- 5.2.1. Sterile pipets 1 mL, 5 mL, 10 mL (Fisher, Cat # 7521, 7543, 7751, or equivalent)
- 5.2.2. Sterile pipet tips (Biorad #223-9035, or equivalent)
- 5.2.3. 12 x75 mm sterile snap-cap polystyrene tubes (Falcon, Cat # 2054 or equivalent)
- 5.2.4. 10 x 75 mm pyrogen-free glass reaction tubes (Lonza, Cat # N201)
- 5.2.5. Sterile combitips 5ml (Eppendorf Biopur, Cat # 2249610-7 or equivalent)
- 5.2.6. Lab Tape (Fisher Scientific)
- 5.2.7. Parafilm (Biostockroom)
- 5.2.8. Limulus Amebocyte Lysate Pyrogen Plus (0.06EU/mL sensitivity) (Lonza, Cat #: N284-06)
- 5.2.9. LAL Reagent Water (Lonza, Cat# W50-100)

5.3. Reagent Preparation

5.3.1. Preparation of Limulus Amebocyte Lysate

5.3.1.1.Reconstitute lyophilized lysate per label directions with LAL reagent water. **Swirl gently** but thoroughly for at least 30 seconds. **Do not shake or vortex** as contents will foam.

5.3.1.2.Reconstituted lysate can be stored for up to 24 hours at 2-8°C without loss of sensitivity. Reconstituted lysate can be divided into more convenient volumes and stored below -10°C for up to four weeks. Frozen liquid lysate should be thawed immediately before use.

5.3.2. Preparation of Control Standard Endotoxin (CSE)

5.3.2.1.Reconstitute CSE per label directions with LAL reagent water.

5.3.2.2.Vortex the vial of CSE for **at least 15 min.** (Do not exceed 20 min.)

5.3.2.3.Dilute the CSE with sterile, LAL Reagent water to a concentration of 1 EU/mL (Refer to the certificate of quality for the CSE reconstituted potency). Refer to Table 1 for initial dilution examples.

Table 1: S₀ Working Concentration Dilution Table

Initial standard concentration	Dilution for S ₀ working concentration (1 EU/mL)
For 10 EU/mL	100 µL (10 EU/mL STD) + 900µL H ₂ O
For 16 EU/mL	100 µL (16 EU/mL STD) + 1.5 mL H ₂ O
For 20 EU/mL	50 µL (20 EU/mL STD) + 950µL H ₂ O
For 50 EU/mL	20 µL (50 EU/mL STD) + 980µL H ₂ O

5.3.2.4.Each dilution should be vortexed for 60 seconds prior to proceeding to the next dilution.

5.3.2.5.Using the 1 EU/mL endotoxin solution (S₀), prepare a serial two-fold dilution series as shown on Table 2.

Note: When diluting the CSE, the series of dilution tubes must include a two-fold dilution greater than the labeled lysate sensitivity and also a two-fold dilution less than the labeled lysate sensitivity. For example, if the labeled lysate sensitivity is 0.06 EU/mL, then the series of dilution tubes must contain 0.25 EU/mL, 0.12 EU/mL, 0.06 EU/mL, 0.03 EU/mL and 0.015 EU/mL.

Table 2: Standard Dilutions

Tube #	LAL Reagent H ₂ O	Volume added to H ₂ O	Endotoxin Concentration
S ₁	500µL	500µL from S ₀	0.5 EU/mL
S ₂	500µL	500µL from S ₁	0.25 EU/mL
S ₃	500µL	500µL from S ₂	0.12 EU/mL
S ₄	500µL	500µL from S ₃	0.06 EU/mL
S ₅	500µL	500µL from S ₄	0.03 EU/mL
S ₆	500µL	500µL from S ₅	0.015 EU/mL

6. Procedure

- 6.1. Prepare the *LAL Endotoxin Assay Worksheet* (AW-052). Determine the number of patients that are to be assayed. **Note:** The control and unknown patient samples are to be run in duplicate, the CSE and water blank are to be run in triplicate.
- 6.2. Label the 10x75mm pyrogen-free reaction tubes according to following schematic, each block represents (1) endotoxin reaction tube:

H ₂ O	S6	S6	S6	C6	C6	X6	X6
H ₂ O	S5	S5	S5	C5	C5	X5	X5
H ₂ O	S4	S4	S4	C4	C4	X4	X4
	S3	S3	S3	C3	C3	X3	X3
	S2	S2	S2	C2	C2	X2	X2
	S1	S1	S1	C1	C1	X1	X1
Water Blanks	Standards (CSE Dilutions)			Controls (In- house Positive)		Samples (Unknowns)	

- 6.3. Label the sterile 12x75mm polystyrene snap-cap tubes for serial dilutions according to the following schematic, each block represents (1) polystyrene snap-cap tube:

S0	S1	S2	S3	S4	S5	S6
C0	C1	C2	C3	C4	C5	C6
X0* (Sample)	X1	X2	X3	X4	X5	X6

S0 is the CSE diluted to 1 EU/MI

C0 is the frozen control diluted to 1 EU/mL (follow the same dilution table as the CSE above)

X0* is the specimen with no particulate matter (*X denotes the specimen number on the worksheet)

- 6.4. Centrifuge specimens for 10 min. at 1200 rpm (300 g).
- 6.5. Add 500 µL of the LAL reagent water to all of the dilution tubes.

- 6.6. Initiate the serial dilution by removing 500 μ L from X0 and add to X1. Repeat this process and remove 500 μ L from X1 and add to X2. Continue to serial dilute through X6 in the same manner. **NOTE:** Each sample is run in parallel with the CSE and must include the same series of two-fold dilutions as the CSE.
- 6.7. Transfer 100 μ L of LAL reagent water to the Blank tubes and 100 μ L of each sample dilution to the appropriately marked 10 x 75 mm pyrogen-free reaction tubes.
- 6.8. After vortexing the CSE for 15 min., remove the appropriate amount of CSE to make a concentration of 1 EU/mL. (Refer to Preparation of Control Standard Endotoxin). Vortex S0 for one minute.
- 6.9. Remove 500 μ L from S0 and add to S1, which contains 500 μ L of LAL reagent water.
- 6.10. Vortex S1 for one minute, then aliquot 100 μ L of this concentration of standard into the three corresponding pyrogen-free reaction tubes. Remove 500 μ L from S1 and add to S2.
- 6.11. Vortex S2 for one minute, then aliquot 100 μ L of this concentration of standard into the three corresponding pyrogen-free reaction tubes. Continue this process until all serial dilutions (S6) have been made and aliquoted into the corresponding reaction tubes.
- 6.12. Once all of the sample and CSE dilutions have been aliquoted into the reaction tubes, add 100 μ L of the reconstituted lysate to each tube beginning with the negative control water tubes, then the highest concentration of endotoxin for the CSE. Immediately following the addition of the lysate to each tube, the contents should be mixed gently and the tube placed in a 37°C water or dry heat bath. This procedure should be followed for each dilution of the endotoxin. Change pipette tip between controls and patient samples.
- 6.13. Incubate all tubes for exactly one hour. The incubation time should be determined from the time each tube is placed in the 37°C bath. Do this by starting a timer at the beginning of the addition of lysate and start a second timer when the addition of lysate is complete.
- 6.14. After exactly one hour of incubation, each reaction tube is examined for gel clotting. Slowly invert each reaction tube 180° and observe whether or not there is gel formation. A positive reaction is indicated by a firm gel that remains intact momentarily when the tube is inverted 180°. Record the reaction as either positive or negative on the worksheet *LAL Endotoxin Test Results Worksheet* (AW-053).

NOTE: Reaction tubes **should not be removed from incubation or disturbed** prior to the time specified for reading the test.

7. Calculations

7.1. To determine the endotoxin concentration of an unknown solution, test serial two-fold dilutions of sample until an endpoint is reached. Calculate the geometric mean dilution and multiply by the lysate sensitivity. Using the worksheet *LAL Endotoxin Test Results Worksheet* (AW-053). See the examples below:

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)
Standard 1	+	+	+	+	-	-	0.06	-1.222
Standard 2	+	+	+	-	-	-	0.12	-0.921
Standard 3	+	+	+	+	-	-	0.06	-1.222

Mean: -1.121

Antilog10 (Mean) = *ALS = 0.08

*ALS = Calculated Lysate

SAMPLE X

Sensitivity

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)
	-	-	-	-	-	-	>0.50	>-0.301
	-	-	-	-	-	-	>0.50	>-0.301

Mean: >-0.301

Antilog10 (Mean) = >0.50

Endotoxin Conc = ALS/Antilog (Mean) = <0.16 EU/mL

SAMPLE Y

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)
	+	-	-	-	-	-	0.50	-0.301
	+	-	-	-	-	-	0.50	-0.301

Mean: -0.301

Antilog10 (Mean) = 0.50

Endotoxin Conc = ALS/Antilog (Mean) = 0.16 EU/mL

8. Quality Control

- 8.1. Each vial of LAL is labeled with the lysate sensitivity obtained using the FDA Reference Standard Endotoxin, and is expressed in Endotoxin Units.
- 8.2. As part of the initial quality control each user should re-verify the labeled lysate sensitivity using an endotoxin standard whose potency is known. Prepare serial, two-fold dilutions of the endotoxin standard which bracket the labeled lysate sensitivity. Each dilution of the CSE, as well as a negative water control, should be assayed in triplicate. After the one hour incubation period, the positive and negative results are recorded. The endpoint dilution is determined as the last dilution of endotoxin, which still yields a positive result (Figure 1).

FIGURE 1:

Assay Results - Gel-Clot Method							
Replicate	Endotoxin Dilution (EU/mL)					H ₂ O	Endpoint
	0.50	0.25	0.12	0.06	0.03		
1	+	+	+	-	-	-	0.12
2	+	+	+	-	-	-	0.12
3	+	+	+	+	-	-	0.06
4	+	+	+	-	-	-	0.12

- 8.3. The lysate sensitivity is calculated by determining the geometric mean of the endpoint. Each endpoint value is converted to log 10. The individual log 10 values are averaged and the lysate sensitivity is taken as the antilog 10 of this average log value (Figure 2).

FIGURE 2:

Calculation of Geometric Mean Endpoint	
Endpoint (EU/mL)	Log10 Endpoint
0.12	-0.921
0.12	-0.921
0.06	-1.222
0.12	-0.921

$$\text{Mean} = 0.996$$

$$\text{Antilog}_{10}(\text{Mean}) = 0.10 \text{ EU/mL}$$

*Acceptable variation is 1/2 to two times the lysate sensitivity (± 1 dilution)
 Ex: Labeled Lysate Sensitivity = 0.06 EU; endpoint can be between 0.12 – 0.03*

8.4. RESULT ACCEPTABILITY

8.4.1. Do not report patient results if:

8.4.1.1. The calculated lysate sensitivity is less than one-third or greater than three times the labeled lysate sensitivity. (Example: if the labeled lysate sensitivity is 0.06 EU/mL and the calculated lysate sensitivity is 0.50, then patient results should not be reported.)

8.4.1.2. Any one of the negative control water tubes is positive.

8.4.1.3. The replicates on standards or patient samples give inconsistent results over a 3-fold range of dilutions.

NOTE: Repeat assay if any of the above conditions occur.

8.5. RESULT REPORTING:

8.5.1. Any positive patient result will be handled as follows:

8.5.1.1. Supervisor notified immediately.

8.5.1.2. Testing repeated on the same sample to rule out testing error.

8.5.1.3. If repeat is still positive, **supervisor is notified immediately** and then the appropriate investigator (i.e., IMCPL staff or physician) is notified.

9. Appendix

9.1. LAL Endotoxin Assay Worksheet, AW-052

9.2. LAL Endotoxin Test Results Worksheet, AW-053

9.3. Mycoplasma/Endotoxin Request for Testing and Results Report Form, AW-084

10. References

10.1. Lonza (Cambrex) Multi Test Limulus Amebocyte Lysate package insert. Aug 2006.

10.2. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration "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." 1987.

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ENDOTOXIN TESTING BY LAL ASSAY

11. Revision History

Version	Reason for change	Author	Date approved
1	New SOP	Theresa L. Whiteside, PhD	02/04/2003
2	Modifications	Cathy Brown	04/09/2004
3	Update, modifications	Jennifer Sprague	11/30/2007

Annual Review and Approval

2008

No revision necessary
 Lab Supervisor _____ Date _____
 QA Manager _____ Date _____
 Lab Director _____ Date _____

Revision necessary, SOP revised & archived
 Lab Supervisor _____ Date _____
 QA Manager _____ Date _____
 Lab Director _____ Date _____
 In document control Staff review initiated

2009

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2010

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2011

No revision necessary
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 Lab Director _____ Date _____

Revision necessary, SOP revised & archived
 Lab Supervisor _____ Date _____
 QA Manager _____ Date _____
 Lab Director _____ Date _____
 In document control Staff review initiated

LAL ENDOTOXIN ASSAY WORKSHEET

Test Date: _____

Assay Tech: _____

Kit Lot #: _____

Labeled

Lysate Sensitivity: _____ Eu/ml

Data checked by: _____

Calculated

Lysate Sensitivity: _____ Eu/ml

Data checked

Exp. Date: _____

by Supervisor: _____

Specimen #	Lab Number	Sample Information	Protocol Number	Fresh/Frozen	Sample Type	Sup Generation Date	Results Eu/ml
Contro 1				Frozen	FNC		
1.							
2.							
3.							
4.							
5.							
6.							
7.							
8.							
9.							
10.							
11.							
12.							

COMMENTS:

Specimen #	Lab Number	Sample Information	Protocol Number	Fresh/Frozen	Sample Type	Supp Generation Date	Results Eu/ml
13.							
14.							
15.							
16.							
17.							
18.							
19.							
20.							
21.							
22.							
23.							
24.							
25.							
26.							
27.							
28.							
29.							
30.							
31.							
32.							

COMMENTS:

LAL ENDOTOXIN TEST RESULTS WORKSHEET

Test Date: _____ Endotoxin Lot #: _____ Lysate Lot #: _____
 Tech: _____ Aliquot Date: _____ Labeled Lys.: _____
 Kit Lot #: _____ Potency of STD: _____ Eu/ml Sensitivity: _____
 Calculated Sensitivity: _____

1Eu/ml= _____ μl STD + _____ μl H2O

H2O			
-----	--	--	--

CALCULATION OF GEOMETRIC MEAN

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)
Standard 1								
Standard 2								
Standard 3								

Mean: _____
 Antilog10 (Mean) = *ALS = _____
 *ALS = Calculated Lysate Sensitivity

Control

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)

Mean: _____
 Antilog10 (Mean) = _____
 Endotoxin Conc = ALS/Antilog (Mean) = _____

Sample #:

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)

Mean: _____
 Antilog10 (Mean) = _____
 Endotoxin Conc = ALS/Antilog (Mean) = _____

LAL ENDOTOXIN TEST RESULTS WORKSHEET

Sample #: _____

Assay Date: _____

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)

Mean: _____

Antilog10 (Mean) = _____

Sample #: _____

Endotoxin Conc = ALS/Antilog (Mean) = _____

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)

Mean: _____

Antilog10 (Mean) = _____

Sample #: _____

Endotoxin Conc = ALS/Antilog (Mean) = _____

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)

Mean: _____

Antilog10 (Mean) = _____

Sample #: _____

Endotoxin Conc = ALS/Antilog (Mean) = _____

DILUTIONS:	0.50	0.25	0.12	0.06	0.03	0.015	ENDPT	LOG10 (ENDPT)

Mean: _____

Antilog10 (Mean) = _____

Endotoxin Conc = ALS/Antilog (Mean) = _____

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 HCC, 5117 Centre Ave. Ste. 1.27, Pittsburgh, PA 15213 ph: (412)624-0080 fax: (412)624-0264**

CPL MYCOPLASMA / ENDOTOXIN REQUEST FOR TESTING AND RESULTS WORKSHEET

Test (circle): **Mycoplasma Genprobe/ Endotoxin**

Results Needed by: _____

Mycobatch Number	Endo. Batch Number	CPL Log-In #	Protocol #	Description	Date Gen.	mL Rec'd	Sample Type	Mycogenprobe Result	Endotoxin Result EU/mL	Endotoxin Result EU/kg of body weight
MYC-	End-									
MYC	End-									
MYC	End-									

Calculation of Endotoxin Result

Patient Weight in Pounds _____ / 2.205 (conversion to kg) = _____ weight in kg

Number of Flasks _____ X _____ mL per Flask = _____ mL Total Volume of Culture(s) for Vaccine

Endotoxin Result _____ EU/mL X _____ mL Total Volume = _____ EU

_____ EU / _____ kg of body weight = _____ EU/kg of body weight

Endotoxin Acceptable Result (<5EU/kg of body weight/dose): YES / NO

Mycoplasma Acceptable Result (**Negative**): YES / NO

Submitted by: _____ Date: _____ Test(s) Performed by: _____ Date: _____

Edit Checked by: _____ Date: _____ Supervisor Review: _____ Date: _____

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PROCEDURE FOR MYCOPLASMA DETECTION ASSAY

1. Purpose and Scope

- 1.1. A commercially available test kit that utilizes DNA hybridization techniques is used in the IMCPL for the detection of mycoplasma in cell cultures. All cell lines are tested monthly for possible mycoplasma contamination.
- 1.2. Human serum pools and cellular products prepared for human administration are also routinely tested for mycoplasma contamination. Until February, 2000, the IMCPL used the GEN-PROBE[®] Mycoplasma T. C. Rapid Detection System Kit, which used a radioisotopic (³H) label for detection.
- 1.3. The methodology was replaced with a non-isotopic system (NI), and the IMCPL has converted to the use of the new methodology. Both assays are based on hybridization between rRNA of the target organism and a complementary DNA probe. In the current assay, the probe is labeled with a chemiluminescent label.

2. Responsibility

- 2.1. Laboratory staff is responsible for following this procedure and for completing all documentation which is then forwarded along with results to the Laboratory Supervisor.
- 2.2. The Laboratory Supervisor is responsible for reviewing all results and documentation for accuracy and completeness.
- 2.3. The QA/QC Director is responsible for reviewing and approving this procedure.
- 2.4. The Laboratory Director is responsible for reviewing and approving this procedure. The Laboratory Director is also responsible for annual review of this procedure.

3. Safety and Precautions

ALL HUMAN SAMPLES ARE POTENTIALLY BIOHAZARDOUS. All equipment, supplies, and reagents in contact with human blood, body fluids and/or tissues should be handled and disposed of as if biohazardous. **HANDLE ALL MATERIALS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.** When performing this procedure, follow the UPCI IMCPL Biohazard Precautions as outlined in the safety section of this manual.

Warning: Avoid Contact of Detection Reagents I and II with skin and mucous membranes. Wash with water if these reagents come into contact with skin. Dilute reagent spills with water before wiping dry.

Reviewed and Approved by:	Signature	Date
Laboratory Supervisor		
QA Manager		
Laboratory Director		

4. Definitions

- 4.1. QA: Quality Assurance
- 4.2. QC: Quality Control
- 4.3. SOP: Standard Operating Procedure

5. Reagents, Materials and Equipment

5.1. Reagents

- 5.1.1. Mycoplasma Tissue Culture NI (MTC-NI) Rapid Detection System (GEN-PROBE® Inc., Cat. No. 4573)
 - 5.1.1.1. Reagent 1 (Probe Reagent)
 - 5.1.1.2. Positive Control
 - 5.1.1.3. Reagent 2 (Hybridization Reagent)
 - 5.1.1.4. Reagent 3 (Selection Reagent)
- 5.1.2. GEN-PROBE® Detection Reagent Kit (GEN-PROBE, Inc., Cat. No. 1791)
 - 5.1.2.1. Detection Reagent I: 0.1% hydrogen peroxide in 0.001 N nitric acid
 - 5.1.2.2. Detection Reagent II: 1N sodium hydroxide

5.2. Equipment and Materials

- 5.2.1. Pipetman P100, P200 and P1000 (Rainin, Cat # P100, P200, P1000, or equivalent)
- 5.2.2. Sterile pipet tips
- 5.2.3. Timer (Fisher, Cat # 06-662-5, or equivalent)
- 5.2.4. Eppendorf repeater (Fisher Scientific)
- 5.2.5. Sterile combitips 5ml (Eppendorf Biopur, Cat # 2249610-7 or equivalent)
- 5.2.6. Centrifuge (Sorval T-6000, or equivalent)
- 5.2.7. Microcentrifuge (12,000-15,000 x g) (Sorval M12 or equivalent)
- 5.2.8. 60°C water bath (Napco Model 210A)
- 5.2.9. 2 mL conical microcentrifuge tube
- 5.2.10. GEN-PROBE® Luminometer (LEADER® 50)
- 5.2.11. Vortex
- 5.2.12. Vacuum source and trap containing 10% bleach for decontamination
- 5.2.13. Black foam float rack

6. Procedures

6.1. Sample Acceptability:

- 6.1.1. Each patient cell culture suspensions, including the cells shall be tested for mycoplasma contamination.

Reviewed and Approved by:	Signature	Date
Laboratory Supervisor		
QA Manager		
Laboratory Director		

- 6.1.2. Supernatants of cell lines kept in continuous culture shall be tested for mycoplasma contamination.
- 6.1.3. Cell suspensions shall be removed from the culture NO SOONER than 72 hours after the addition of fresh medium. If cells are re-seeded in fresh medium and then the culture is sampled the next day for testing, the chances of detecting mycoplasma are less since fresh sterile medium will dilute the organisms.
- 6.1.4. For maximum sensitivity, samples should be prepared within 4 hours of collection. If this is not possible the media may be stored at 4°C for no longer than 3 days before sample preparation.
- 6.1.5. Samples, which have been stored at 4°C for longer than 3 days, will not give reliable results. Once the samples have been processed following the steps for sample preparation, they may be stored at 5-20°C for one month.
- 6.1.6. Patient samples are processed STAT if required for therapy.
- 6.1.7. Any sample, which shows evidence of bacterial contamination, will be rejected.

6.2. Preparation of Reagents and Equipment Prior to Sample Testing:

- 6.2.1. Assemble all of the equipment and reagents required to perform the GEN PROBE MTC-NI Rapid Detection System Assay.
- 6.2.2. Remove kit from 4°C storage, and allow reagents to equilibrate to room temperature (30-60 min.) prior to use. Check and make sure that the water bath temperature is at 60°C±1°C. Check the water level that it is appropriate for testing.
- 6.2.3. The Hybridization Reagent, Detection Reagent II, and the positive control may precipitate. Warm at 60°C for two minutes and swirl gently to assure homogeneity before pipetting.
- 6.2.4. Label microcentrifuge tubes with sample identification numbers.
- 6.2.5. Prepare the luminometer for operation. Turn on the instrument 20 minutes prior to use and allow to warm up. Check the optics and the reagent dispensing system. Refer to the procedure for the GEN-PROBE® LEADER® 50 luminometer (Attachment #4 or SOP: CPL-0233).

6.3. Sample Preparation

- 6.3.1. Prepare aliquot of final cellular product in tissue culture medium. The product has to be collected after the reagent, cytokine was added and after the last manipulation step.

6.3.1.1. Patient therapy

6.3.1.1.1. When preparing samples obtained from a patient therapy culture, verify that test sample contains cells. Expect $\sim 3.3 \times 10^4$ cells per test.

6.3.2. Pipette 1.5 mL of cell suspension in culture medium into a microcentrifuge tube. Identify and mark the side of the tube on which the pellet will form with the mycoplasma log number.

6.3.2.1. Tissue culture

6.3.2.1.1. For tissue culture (non-patient samples) centrifuge the sample for 5 min at 1200rpm, remove 1.5ml of the supernatant into a microfuge tube. Continue with step 6.3.3.

6.3.3. Keep any remaining (unused) samples until all tests are completed and signed off by supervisor.

6.3.4. Centrifuge the microfuge tubes at 12,000-15,000 x g for 10 minutes.

6.3.5. Remove the supernatant by vacuum aspiration using a trap containing bleach outfitted with a 2cc pipette and a disposable yellow tip.

6.3.6. Use a different pipette tip for each sample and discard.

6.3.7. The pellet may not be visible and care must be taken to avoid any loss of the pellet when removing the supernatant.

6.3.8. In order to reduce false positive assay results, remove as much of the supernatant as possible.

6.3.9. Do not allow samples to sit for any period of time after microcentrifugation. They must be aspirated immediately to avoid false negative or low result values.

6.3.10. Add 100 μ L of Hybridization Reagent to the microcentrifuge tube using an Eppendorf repeating pipettor with a 5mL combitip.

6.3.11. Vortex thoroughly for 1-3 seconds. If samples cannot be processed immediately, they can be stored at -20°C for one month prior to testing.

6.3.12. Frozen samples should be heated at 60 \pm 1°C for 2 min. and vortexed to achieve homogeneity prior to use in the assay.

6.4. Hybridization

- 6.4.1. Use Probe Reagent Tubes supplied with the kit.
- 6.4.2. The tubes are supplied in a vacuum sealed pouch (10/pouch).
- 6.4.3. Open the foil pouch by cutting evenly across the top of the pouch.
- 6.4.4. Remove a sufficient number of Reagent Tubes to test the samples and controls.
Remove and retain the caps.
- 6.4.5. Re-seal the pouch by folding the opened edge over several times and securing with a clip. Leave the desiccant pillow in the pouch.
- 6.4.6. Once opened, the tubes have an expiration of 30 days. Label the pouch sealed with the date opened and the expiration date.
- 6.4.7. Pipette 100µL of Hybridization Reagent into the bottom of the Negative Control Tubes.
- 6.4.8. Pipette 100µL of Positive Control in the bottom of the Positive Control Tubes.
- 6.4.9. Pipette 100µL of each test sample from the microfuge tubes into the bottom of the appropriate sample tube.
- 6.4.10. Check the tubes to be sure that all of the liquid test material is in the bottom of the tube.
- 6.4.11. Re-cap all tubes and mix the contents by vortexing at medium speed setting ≤ 3 seconds.
- 6.4.12. Incubate for a minimum of 45 min at $60^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 6.4.13. Make sure that the water level is high enough to assure that the reaction mixture in the tubes is completely submerged at all times.
- 6.4.14. It is critical that the hybridization step be done at $60^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 6.4.15. Check the water bath temperature occasionally to be sure that the temperature is maintained at $60^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

6.5. Selection

- 6.5.1. Remove the Probe Reagent Tubes from the water bath.
- 6.5.2. Remove and retain the caps.
- 6.5.3. Immediately pipette 300 µL of the Selection Reagent into each tube.

- 6.5.4. Use an Eppendorf repeating pipettor with a 5mL combitip.
- 6.5.5. Re-cap the tubes and vortex on medium speed for 1-3 seconds.
- 6.5.6. Check the tubes to be ensure that all liquid is in the bottom of the tubes.
- 6.5.7. Incubate the Probe Reagent tubes for 10 min. at $60^{\circ}\text{C}\pm 1^{\circ}\text{C}$ in a water bath.
- 6.5.8. Incubate at least 10 min but no more than 12 min.
- 6.5.9. Remove the Probe Reagent tubes from the water bath and leave them at room temperature for at least 5 min.
- 6.5.10. Remove and discard the caps.
- 6.5.11. Read the results in the luminometer within 30 minutes after removing the tubes from the water bath at the end of the selection step.

6.6. Detection

- 6.6.1. Adjust the luminometer according to the specifications provided by the manufacturer and Attachment #4 or SOP -0233.
- 6.6.2. Using a kimwipe, wipe each tube to ensure that there is no water residue present and insert the tube as per the instructions in this SOP attachment #4 or SOP-0233.
- 6.6.3. Read the Negative tube first. When the analysis is complete, remove the tube from the luminometer. Hold the tubes until the results have been printed. Check that the assay controls are within the acceptable range.
- 6.6.4. Discard the tubes in the biohazard trash.

6.7. Analysis of Results

- 6.7.1. The results of the GEN-PROBE® MYCOPLASMA TISSUE CULTURE NI (MTC-NI) RAPID DETECTION SYSTEM are based on Relative Light Unit (RLU) cut-off values.
- 6.7.2. Samples producing greater than or equal to the positive cut-off are considered positive, $\geq 5,000$ RLU.
- 6.7.3. Signals less than the negative cut-off value are considered negative, $< 3,000$ RLU.
- 6.7.4. Results in the repeat range should be repeated, 3,000 to 4,999RLU.

6.7.5. Samples continuing to give values within the repeat range may represent very low-level contamination and may require re-testing. If possible, process a larger sample volume to increase assay sensitivity.

6.8. Luminometer Quality Control and Acceptability of Results:

6.8.1. Ensure that the luminometer is calibrated and functioning properly (See Gen-Probe Luminometer Leader 50 equipment SOP-051).

6.8.2. Negative Control and Positive Control should be within the following ranges:

Negative Control: <3,000 RLU

Positive Control: 35,000-85,000 RLU.

6.9. Limitations:

6.16.1 The assay should only be used for the detection of bacterial contamination in tissue culture samples.

6.16.2 A positive result indicates contamination of the tissue culture sample.

6.16.3 Bacteria other than mycoplasma will vary in positivity based on their lysis during the sample preparation.

6.16.4 The test does not distinguish among bacterial species and a negative result does not exclude the possibility that mycoplasma contamination is present at a level below the sensitivity of the test, which is approximately 100,000 mycoplasma per mL of culture media.

6.16.5 This test will detect certain other bacterial species if they are lysed by the sample preparation procedures.

6.16.6 Refer to the product insert of the kit for a list of bacteria that can be detected in this assay.

6.17 Reporting Results:

6.17.1 Check control values to ensure that they fall within the acceptable range.

6.17.2 Record RLU values and result (+ or -) on the Mycoplasma Testing MTC-NI worksheet (see attachment 1). A **test sample** result will be reported as:

6.17.2.1 **Negative** when the RLU value < 3,000.

6.17.2.2 **Positive** when the RLU value \geq 5,000.

6.17.3 A sample with RLU between 3,000 and 4,999 is considered uninterpretable and is repeated.

7 Assay Quality Control

7.16.1 The Mycoplasma Detection Kit from GEN-PROBE® contains controls provided by the manufacturer. Kit controls must react in accordance with kit specifications.

7.16.2 The defined limits for the positive and negative may be changed by the manufacturer, so the package insert must be carefully checked. To date, the control values are listed below:

7.16.2.1 Positive control values should be 35,000 – 85,000 RLU

7.16.2.2 Negative control values should be < 3,000 RLU.

7.16.3 If the control values do not fall within the specified ranges, notify the supervisor immediately. Troubleshooting may involve consulting the Problem Solving section of the package insert or the Technical Service Department at **1-800-523-5001**.

7.16.4 If the assay is rejected and/or has to be repeated, then the person/lab group providing the sample and requesting testing must be notified so that another sample can be submitted for testing if possible.

7.17 Critical Parameters:

7.17.1 Growth of cultures in antibiotics may reduce mycoplasma levels.

7.17.2 It is recommended that cells be passaged twice in antibiotic-free media before performing the assay.

7.17.3 Medium to be tested must be in contact with tissue culture cells for a minimum of three days.

7.17.4 Contamination of tubes or reagents with Rnase may affect test results. Use aseptic technique when handling all tubes and reagents.

7.17.5 When preparing samples, aspirate as much supernatant as possible from the centrifuged samples with a Pasteur pipette. Be careful not to disturb the mycoplasma pellet.

7.17.6 The Hybridization and Selection Reagents must be thoroughly suspended to provide a homogenous suspension before pipetting into each vial.

7.18 Trouble-shooting:

7.18.1 Low Positive Control (Below 35,000 RLU)

7.18.2 Confirm that the positive control tube contains approximately 800 µL. Low readings may indicate that less than 100 µL of Positive Control was added to the

Positive Control probe tube. Check that the temperature of the water bath is $60^{\circ}\pm 1^{\circ}\text{C}$.

7.18.3 Ensure that the probe tube is immersed in water bath or heat block so as to completely cover the liquid contents during the reaction. Do not allow any water or other non-sterile solutions to enter the probe tubes during the assay.

7.18.4 Check luminometer settings to confirm a reading time of 2 seconds.

7.18.5 Check accuracy of pipetting instruments.

7.19 High Positive Control (Above 85,000 RLU)

7.19.1 Confirm that the positive control tube contains approximately 800 μl . High reading may indicate that 300 μl of the selection reagent was not added.

7.19.2 Check that the temperature of the water bath or heat block is $60^{\circ}\pm 1^{\circ}\text{C}$ and that the luminometer settings are correct.

7.19.3 Ensure that the tubes are incubated for 10 minutes during the Selection step

7.20 High Negative Control (Above 3,000 RLU)

7.20.1 Confirm that the positive control tube contains approximately 800 μl . High readings may indicate that 300 μl of the selection reagent was not added.

7.20.2 Check that the temperature of the water bath or heat block is $60^{\circ}\pm 1^{\circ}\text{C}$ and that the luminometer settings are correct.

7.20.3 Ensure that the tubes are incubated for 10 minutes during the Selection step.

7.20.4 Do not interchange the caps of positive and negative controls.

7.20.5 Eliminate static charge by wiping tubes with a damp cloth or tissue just prior to placing in the luminometer.

7.21 Samples in the Repeat Range (3,000 to 5,000 RLU)

7.21.1 Check that the temperature of the incubations is $60^{\circ}\pm 1^{\circ}\text{C}$. A calibrated thermometer must be used.

7.21.2 Check that the sample is homogeneous and that no drops remain above the surface of the liquid during either incubation.

7.21.3 Cultures may be contaminated with a very low level of bacteria. Increase the sensitivity of the test as follows:

7.21.3.1 Growth of cultures in antibiotics may reduce Mycoplasma levels.

7.21.3.2 It is recommended that cells be passed twice in the antibiotic-free media before performing the assay.

7.21.3.3 Media to be tested must be in contact with the tissue culture cells for a minimum of three days.

7.21.3.4 Culture media cannot be frozen before centrifugation.

7.22 Variable Readings

- 7.22.1 Check accuracy of pipetting instruments.
- 7.22.2 Eliminate static charge and any residue by wiping tubes with a damp cloth or tissue just prior to placing them in the luminometer.
- 7.22.3 Ensure that the entire sample pellet is homogeneously resuspended and that all of it is transferred to the probe tube.
- 7.22.4 Check the function of luminometer pumps as directed in the instrument manual. Check instrument calibration.
- 7.22.5 Contamination of tubes or reagents with RNase or bacteria may affect test results. Use antiseptic technique when handling all tubes and reagents.

8 Documentation

- 7.1 Complete the mycoplasma worksheet and attach the original printout from the luminometer and a photocopy to the worksheet. Always make and attach a photocopy since the image on the thermal paper will fade over time and become unreadable.

8 Attachments

- 8.1 Mycoplasma Testing Worksheet (AW-054)
- 8.2 CPL Mycoplasma-Endotoxin Request for Testing Form (AW-04-084)
- 8.3 Mycoplasma-Endotoxin Request for Testing Form (AW-05-085)
- 8.4 GEN-PROBE® LUMINOMETER (LEADER® 50): QUALITY CONTROL AND MAINTENANCE

9 References

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UNIVERSITY OF PITTSBURGH CANCER INSTITUTE
 IMMUNOLOGIC MONITORING AND CELLULAR PRODUCTS LABORATORY

PROCEDURE FOR MYCOPLASMA DETECTION ASSAY

Revision History

Version	Reason for change	Author	Date Approved
1	New SOP	C. Brown	2/1/00
2	Updated procedure and format	C. Brown and J.Giron	1/14/04
3	Updated format and worksheets	V. Sutherland, T. Temples, J. Stanson	3/17/05
4	Reformatted & revised section 6.1	Joanna Stanson	10/30/06
5	Attachment 4 added	J. Stanson	07/05/07
6	Attachments updated. Paragraph 6.3.2.1 & 6.3.2.2 added	J. Stanson	04/07/08

12. Annual Review and Approval

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