NANOTECHNOLOGY CHARACTERIZATION LABORATORY

# NCL Method ITA-9
**Version 1.0**

# Phagocytosis Assay

**Nanotechnology Characterization Laboratory**
**National Cancer Institute at Frederick**
**SAIC-Frederick, Inc.**
**Frederick, MD 21702**
**(301)-846-6939**
**ncl@ncifcrf.gov**

**December 2005**

**This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.**

Method is written by: _____ 3/01/2006

Marina A. Dobrovolskaia, Ph.D. Date

_____ 2/1/06

Timothy M. Potter, B.S. Date

Method performance qualification* was conducted on: 12/14/05-12/23/05 .

Date

* Full validation was not conducted for this assay. Performance qualification included analysis of intra-assay and inter-assay variability, qualification of positive and negative controls, determination of assay threshold and qualification of opsonisation medium.

Testing facility: NCI-Frederick, Bldg/Rm# 469/250 .

## 1. Introduction

This document describes a protocol for evaluation of nanoparticle internalization by phagocytic cells. The assay requires 600 μL (concentration 2mg/mL) of a test nanomaterial. This protocol may not be applicable for certain types of nanomaterials. For example, nanoparticles with fluorescent capabilities such as quantum dots may be studied using confocal microscopy or flow cytometry. Modification(s) of the current procedure and or change in detection dye may be required for particles that demonstrate interference with luminol-dependent chemiluminescence.

## 2. Reagents

2.1. PBS, HyClone, cat# AQB 22934

2.2. Zymosan, Sigma, cat# Z4250

2.3. Fetal bovine serum, Hyclone.

2.4. RPMI-1640, Invitrogen, cat# 11875-119

2.5. Pen/Strep solution, Invitrogen, cat#15140-148

2.6. β-mercaptoethanol, Sigma cat# M7522

2.7. Trypan Blue solution, Invitrogen, cat# 15250-061

2.8. Human AB serum or plasma pooled from at least three donors.

*Note: Equivalent reagents from other vendor can be used*

## 3. Equipment

3.1. Pipettors covering range from 0.05 to 10 mL

3.2. Flat bottom 96 well white luminescence plates

3.3. Polypropylene tubes 5 and 15 mL

3.4. Centrifuge

3.5. Refrigerator, 2-8°C

3.6. Freezer, -20°C

3.7. Cell culture incubator with 5% $CO_2$ and 95% humidity.

3.8. Biohazard safety cabinet approved for level II handling of biological material

3.9. Inverted microscope

3.10. Vortex

3.11. Hemacytometer

3.12. Plate reader capable of working in luminescence mode.

## 4. Reagent and Controls Preparation

4.1. Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

2 mM L-glutamine

50 μM β-mercaptoethanol

100U/mL penicillin

100 μg/mL streptomycin sulfate

Store at 2-8°C protected from light for no longer than 1 month. Before use warm in a water bath.

4.2. Zymosan A Stock

Prepare Zymosan A stock at final concentration of 2 mg/mL in PBS. Use freshly prepared.

4.3. Positive control

Approach A - Combine Zymosan A stock and human AB serum or plasma. Use 1 mL of serum/plasma per each 0.5 mL of zymosan A stock. Incubate Zymosan A with serum/plasma for 30 minutes at 37°C. Wash Zymosan A particles two times with PBS (use 1 mL of PBS per each 0.5mL of original zymosan stock and a centrifuge setting of 2000xg for 2min) and re-suspend in PBS to a final concentration of 2 mg/mL.

Approach B – Reconstitute Zymosan A in 20% human AB serum/plasma in PBS to a final concentration of 2mg/mL.

4.4. Inhibition/Enhancement Control

To test ability of nanoparticles to interfere with phagocytosis of zymosan, the second set of the zymosan positive control is prepared. In this second set the zymosan obsonized according to Approach A in section 4.3 is reconstituted in nanoparticles solution/suspension to a final concentration of 2 mg/mL.

4.5. Negative Control

Use PBS as a negative control.

4.6. Heat-inactivated fetal bovine serum

Thaw a bottle with FBS at room temperature or overnight at 2-8°C, and allow to equilibrate top room temperature. Incubate 30 minutes at 56°C in a water bath mixing every 5 minutes. Single use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C.

4.7. Luminol Stock (10mM in DMSO).

Dissolve luminol in DMSO to a final concentration of 10 mM, e.g. dissolve 17.7 mg of luminal in 10 mL of DMSO. Prepare single use aliquots and store at -20°C; protect from light.

4.8. Luminol Working solution (250 μM in PBS).

On the day of experiment thaw an aliquot of luminol stock solution and dilute with PBS to a final concentration of 250 μM, e.g. spike 250 μL of 10mM stock into 10mL of PBS. Protect from light. Discard unused portion.

## 5. Preparation of Study Samples

This assay requires 600 μL of nanoparticles dissolved/resuspended in PBS, i.e. three 100 μL duplicates per sample. The following questions have to be considered when selecting the concentration: i) solubility of nanoparticles in a biocompatible buffer; ii) pH within physiological range; iii) availability of nanomaterial, and iv) stability. For the initial screen 1 mg of nanoparticles dissolved in 0.5 mL of PBS will be used. Human AB serum or plasma used for opsonisation of the positive control is used to opsonize the particles. Nanoparticles: Serum/Plasma volume ratio and incubation conditions are the same as described in positive control preparation section. Opsonization of nanoparticles should be done according to the same procedure used for the opsonization of the positive control, i.e. wherever it is possible to apply centrifugation to separate nanoparticles from bulk plasma, Approach A described in section 4.3 above is used to prepare positive control and nanoparticles; when centrifugation is not applicable to nanoparticles, both particles and positive control are prepared according to the Approach B described in section 4.3. above.

## 6. Cell preparation

HL-60 is a non-adherent promyelocytic cell line derived by S.J. Collins, et al. from a patient with acute promyelocytic leukemia (10.5). Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at $1x10^5$ viable cells/ml. **Do not allow cell concentration to exceed $1x10^6$ cells/mL.** Maintain cell density between $1x10^5$ and $1x10^6$ viable cells/ml. On the day of experiment count cells using trypan blue. If the cell viability is ≥90% proceed to the next step.

## 7. Experimental Procedure

7.1. Turn on plate reader, warm it up to 37°C, place empty white 96 well test plate inside the reader chamber to warm the plate up and set-up assay template.

7.2. Adjust cell concentration to 1 x $10^7$ per mL by spinning cell suspension down and reconstituting in complete medium (refer to section 4.1 for details). Keep at room temperature.

7.3. Add 100 μL of controls and test-nanoparticles in PBS to appropriate wells. Prepare three duplicate wells for each sample and two duplicate wells for positive and negative control.

***Note:*** *Always leave duplicate wells for each of the following controls: 1) luminol only control (no cells); 2) nanoparticles only, and 4) nanoparticles plus luminol (no cells). See Appendix 1 for an example.*

7.4. Add 100 μL working luminol solution in PBS to each well containing sample. Do not forget to add luminol to two "luminol only" control wells.

***Important: keep the plate warm during sample aliquoting (e.g. plate warmer or warm gel pack may be used).***

7.5. Plate 100 μL of cell suspension per well on 96 well white plate.

7.6. Start kinetic reading on a luminescence plate reader immediately.

## 8. Calculations

A Percent Coefficient of Variation is used to control precision and calculated for each control or test sample according to the following formula**:**

**%CV=**SD/Mean x 100%.

Fold Phagocytosis Induction (FPI) = Mean $RLU_{sample}$/Mean$RLU_{negative\ control}$

FPI of the positive control observed during assay qualification is ≥ 400.

Use statistical analysis such as for example Student t-test to evaluate the results.

## 9. Assay Acceptance Criteria

9.1. %CV of the positive control should less than 30%

9.2. If two of three replicates of positive control fail to meet acceptance criterion described in 9.1 the assay should be repeated.

9.3. Two of three replicates of the study sample should demonstrate comparable results (%CV within 20%) in order for sample to be acceptable. Otherwise analysis of this study sample should be repeated.

9.4. The negative control is considered negative if RLU is ≤ 2000.

## 10. References


10.1. Antonini JM., van Dyke K., ye Z., DeMatteo M., Reasor MJ. Introduction of luminol-dependent chemiluminescence as a method to study silica inflammation in the tissue and phagocytic cells of rat lung. Environ. Health Perspect., 1994, 102(suppl10), 37-42.

10.2. Gref R., Luck M., Quellec P., et al. Stealth corona-core nanoparticles surface modified by PEG: influences of corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein absorption. Colloids and surfaces B: Biointerfaces, 2000, 18: 301-313.

10.3. Leroux JC., Gravel P., Balant L., et al. Internalization of poly(D,L,-lactic acid) nanoparticles by isolated human leukocytes and analysis of plasma proteins absorbed onto particles. J.Biomed.Materials Res., 1994, 28:471-481.

10.4. Mold C., Gresham HD., DuClos TW. Serum Amyloid P component and C-reactive protein mediate phagocytosis through murine FcγRs. J.Immunol., 2001, 166:1200-1205.

10.5. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci U S A. 1978 May;75(5):2458-62.

Appendix 1.

Example of assay template.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Blank | PC | NC | TS1 | TS1 | TS1 | TS2 | TS2 | TS2 | Blank | PC | NC |
| Blank | PC | NC | TS1 | TS1 | TS1 | TS2 | TS2 | TS2 | Blank | PC | NC |
| LML | TS1<br>LML | TS2<br>LML | TS1 | TS2 | | | | | | | |
| LML | TS1<br>LML | TS2<br>LML | TS1 | TS2 | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

Cells; No cells