# NCL Method ITA-9.1

# Phagocytosis Assay

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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.

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## 1. Introduction

This document describes a protocol for evaluation of nanoparticle internalization by phagocytic cells, where the foreign substances are isolated via a cell membrane enclosure and degraded by the phagolysosome. Phagocytosis is a receptor mediated endocytosis specific to the phagocytic cells, e.g. cells of the mononuclear phagocytic system (MPS). Phagocytosis is an active process and requires actin polymerization. There are four main receptors which mediate phagocytic uptake. Phagocytosis via three of these receptors (complement receptor (CR), FcγR receptor, and mannose receptor (MR)) is accompanied by inflammatory reactions (cytokine secretion). Phagocytosis via the fourth receptor (scavenger receptor (SR)) is not accompanied by inflammatory responses [1-5].

## 2. Principles and Limitation

This assay utilizes a luminescent-based approach in which nanoparticles are incubated with HL-60 promyelocytic cells, and the phagocytic activity is visualized with luminol. Luminol is a dye which is not luminescent unless exposed to the low pH of the phagolysosome.

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 changes in detection dye may be required for particles that demonstrate interference with luminol-dependent chemiluminescence.

## 3. Reagents, Materials, Cell Lines, and Equipment

*Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.*

3.1 Reagents

3.1.1 Phosphate buffered saline (PBS) (GE Life Sciences, Hyclone, SH30256.01)

3.1.2 Zymosan A (Sigma-Aldrich, Z4250)

3.1.3 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)

3.1.4 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)

3.1.5 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.6 Trypan Blue solution (Gibco, 15250-061)

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

3.1.8 Luminol (Sigma-Aldrich, 123072)

3.2 Materials

3.2.1 Pipettes covering the range 0.05 to 10 mL

3.2.2 Flat bottom 96-well white luminescence plates

3.2.3 Polypropylene tubes, 50 and 15 mL

3.3 Cell Lines

3.3.1 HL-60 promyelocytic cells (ATCC, CCL-240)

3.4 Equipment

3.4.1 Centrifuge capable of operating at 400xg and 2000xg

3.4.2 Refrigerator, 2-8ºC

3.4.3 Freezer, -20ºC

3.4.4 Cell culture incubator with 5% $CO_2$ and 95% humidity

3.4.5 Biohazard safety cabinet approved for level II handling of biological material

3.4.6 Inverted microscope

3.4.7 Vortex

3.4.8 Hemocytometer

3.4.9 Plate reader capable of working in luminescence mode

*Note: The plates used for this assay have a solid white bottom; therefore, the plate should be read from the top. Depending on the type of the plate reader, one may need to use a plate adaptor to provide optimal conditions for top read.*

3.4.10 Warm gel-pack

*Note: This material is optional and may be omitted. It is used to keep the plate warm for optimal phagocytosis. However, if it takes longer than 2 minutes to transfer the plate to the plate reader after addition of all reagents, the phagocytosis process will begin before one starts to analyze the plate on the plate reader.*

## 4. Reagent and Control Preparation

4.1 Complete RPMI-1640 Medium

The complete RPMI medium should contain the following reagents:

20% FBS (heat inactivated)

4 mM L-glutamine

100 U/mL penicillin

100 μg/mL streptomycin sulfate

Store at 2-8ºC 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 (read section 5 to determine which approach to use)

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 0.5 mL of original zymosan stock and a centrifuge setting of 2000xg for 2 min) and resuspend 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 2 mg/mL.

4.4 Negative Control

Use PBS as a negative control. Process this control the same way as test nanoparticle.

4.5 Heat-Inactivated Fetal Bovine Serum

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

4.6 Luminol Stock (10 mM 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.

4.7 Luminol Working Solution (250 µM in PBS).

On the day of the experiment, thaw an aliquot of luminol stock solution and dilute with PBS to a final concentration of 250 µM, e.g. add 250 µL of 10 mM stock into 9.750 mL of PBS. Protect from light. Discard unused portion.

4.8 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

## 5. Preparation of Study Samples

This assay requires 2 mL of nanoparticles at 3x the highest test concentration dissolved/resuspended in PBS. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called “theoretical plasma concentration”. Considerations for estimating theoretical plasma concentration were reviewed elsewhere [6] and are summarized in Box 1 below.

***Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests***

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$\text{human dose} = \frac{\text{mouse dose}}{12.3} = \frac{123 \text{ mg/kg}}{12.3} = 10 \text{ mg/kg}$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

$$\text{in vitro concentration}_{\text{human matrix}} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg x } 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

The assay will evaluate 4 concentrations: 10X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 6 mg/mL will be prepared and diluted 10-fold (0.6 mg/mL), followed by two 1: 5 serial dilutions (0.12 and 0.024 mg/mL). When 100 µL of each of these samples are combined in a culture plate well with 100 µL of luminol and 100 µL of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, 2 mg/mL. Each nanoparticle concentration is plated three times.

The human AB serum or plasma opsonization procedure used for the positive control is also used to opsonize the nanoparticles. The nanoparticle to serum/plasma volume ratio and incubation conditions are the same as described for the positive control in section 4.3.

If centrifugation to separate nanoparticles from bulk plasma is possible, Approach A (described in section 4.3) is used to prepare both the positive control and nanoparticles. When centrifugation is not applicable to the nanoparticles, both particles and positive control are prepared according to Approach B (described in section 4.3).

*Note: If approach B is used, nanoparticle opsonization can be performed either using the stock nanoparticle or each tested concentration. When nanoparticle stock is used for opsonization, further dilution of this stock should be performed in the medium containing serum or plasma.*

## 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 [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 the 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, allowing it to warm up to 37°C. Place an empty white 96-well test plate inside the plate reader chamber, allowing it to warm to 37°C as well. Set up the instrumental parameters.

7.2 Adjust cell concentration to 1 x $10^7$ cells/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 of the pre-warmed test plate. 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 (no cells); and 3) nanoparticles plus luminol (no cells). See Appendix for an example plate map.*

7.4 Add 100 µL working luminol solution in PBS to each sample- containing well. Do not forget to prepare two "luminol only" control wells.
*Note: Keeping the plate warm during sample aliquoting (e.g. using a plate warmer or warm gel pack) may be helpful to achieve optimal assay performance and reproducibility.*

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

7.6 Start kinetic reading on a luminescence plate reader immediately.
*Note: Plate readers capable of both top and bottom reading may require a plate adaptor for top reads. Check user manuals before proceeding with the plate analysis on the plate reader.*

## 8. Calculations

8.1 Using Excel or other relevant software, compare area under the curve (AUC) for all samples. An increase in the AUC at least 2-fold above the negative control (baseline) is considered a positive response. Use relevant statistical analysis to compare AUC values for test samples to that of the baseline.

8.2 A percent coefficient of variation is used to control precision and calculated for each control or test sample according to the following formula:

$$\%CV = (SD/Mean) \times 100\%$$

## 9. Acceptance Criteria

9.1 %CV for each control and test sample should be $< 30\%$.

9.2 Samples demonstrating higher variability should be re-analyzed.

## 10. References


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## 11. Abbreviations

| | |
|---|---|
| AUC | area under the curve |
| CV | coefficient of variation |
| DMSO | dimethyl sulfoxide |
| FBS | fetal bovine serum |
| PBS | phosphate buffered saline |
| RPMI | Roswell Park Memorial Institute |
| SD | standard deviation |
| VC | vehicle control |

## 12. Appendix

### Example Plate Map

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Blank | PC | NC | **TS1 (0.008 mg/mL)** | **TS1 (0.008 mg/mL)** | **TS1 (0.008 mg/mL)** | **TS1 (0.04 mg/mL)** | **TS1 (0.04 mg/mL)** | **TS1 (0.04 mg/mL)** | **TS1 (0.02 mg/mL)** | **TS1 (0.02 mg/mL)** | **TS1 (0.02 mg/mL)** |
| **B** | Blank | PC | NC | **TS1 (0.008 mg/mL)** | **TS1 (0.008 mg/mL)** | **TS1 (0.008 mg/mL)** | **TS1 (0.04 mg/mL)** | **TS1 (0.04 mg/mL)** | **TS1 (0.04 mg/mL)** | **TS1 (0.02 mg/mL)** | **TS1 (0.02 mg/mL)** | **TS1 (0.02 mg/mL)** |
| **C** | **TS1 (1 mg/mL)** | **TS1 (1 mg/mL)** | **TS1 (1 mg/mL)** | Blank | PC | NC | VC | VC | | | | |
| **D** | **TS1 (1 mg/mL)** | **TS1 (1 mg/mL)** | **TS1 (1 mg/mL)** | Blank | PC | NC | VC | VC | | | | |
| **E** | | | | | | | | | | | | |
| **F** | | | | | | | | | | | | |
| **G** | Luminol | TS (0.008 mg/mL) + Luminol | TS (0.04 mg/mL) + Luminol | TS (0.2 mg/mL) + Luminol | TS (1.0 mg/mL) + Luminol | TS (0.008 mg/mL) NO LUMINOL | TS (0.04 mg/mL) NO LUMINOL | TS (0.2 mg/mL) NO LUMINOL | TS (1.0 mg/mL) NO LUMINOL | | | |
| **H** | Luminol | TS (0.008 mg/mL) + Luminol | TS (0.04 mg/mL) + Luminol | TS (0.2 mg/mL) + Luminol | TS (1.0 mg/mL) + Luminol | TS (0.008 mg/mL) NO LUMINOL | TS (0.04 mg/mL) NO LUMINOL | TS (0.2 mg/mL) NO LUMINOL | TS (1.0 mg/mL) NO LUMINOL | | | |

PC: Positive Control; NC: Negative Control; TS: Test Sample; VC: Vehicle Control

No cells