NCL Method ITA-8

Chemotaxis 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 provides a protocol for rapid, quantitative measure of the chemoattractant capacity of a nanoparticulate material. Leukocyte recruitment is a central component of the inflammatory process, both in physiological host defense and in a range of prevalent disorders with an inflammatory component. In response to a complex network of pro-inflammatory signaling molecules (including cytokines, chemokines and prostaglandins), circulating leukocytes migrate from the bloodstream to the site of inflammation.

2. Principles

This assay represents an in vitro model in which promyelocytic leukemia cells (HL-60) are separated from control chemoattractant or test nanoparticles using a 3 μm filter. Cell migration through the filter is monitored by detection of cells in the bottom chamber of the culture plate using the fluorescencent dye Calcein AM.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. HL-60 promyelocytic cells (ATCC, CCL-240)
2. Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
3. Bovine serum albumin (BSA) (Sigma, A4503)
4. Fetal bovine serum (FBS) (GE Life Sciences, Hyclone , SH30070.03)
5. RPMI-1640 (Invitrogen, 11875-119)
6. Calcein AM (Molecular Probes, C3099)
7. Pen/Strep solution (Invitrogen, 15140-148)
8. Trypan Blue solution (Invitrogen, 15250-061)
9. L-glutamine (Hyclone, SH30034.01)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Multichannel pipettor

3. Multi-Screen-MIC 96-well filter plates with 3 μm membrane (Millipore, MAMIC 3S10)
4. Multi-Screen 96-well culture tray (feeding tray) (Millipore, MAMCS9610)
5. 96 microwell Nunclon optical bottom plates (Nunc, 165305)
6. Polypropylene tubes, 50 and 15 mL

3.3 Equipment

1. Centrifuge, 400x*g*
2. Refrigerator, 2-8ºC
3. Freezer, -20ºC
4. Cell culture incubator, 5% CO_2 and 95% humidity
5. Biohazard safety cabinet approved for level II handling of biological material
6. Inverted microscope
7. Vortex
8. Hemocytometer
9. Multiwell plate reader, fluorescence mode at 485 nm and 535 nm

4. Preparation of Reagents and Controls

4.1 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 five minutes. Single use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

4.2 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; and 100 μg/mL streptomycin sulfate. Store at 2-8ºC, protected from light for no longer than one month. Before use, warm in a 37ºC water bath.

4.3 Starvation Media (SM)

The starving RPMI medium should contain the following reagents: 0.2 % BSA, 4 mM L-glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin sulfate. Store at 2-8°C protected from light for no longer than 1 month. Before use, warm in a 37ºC water bath.

4.4 Positive control

On the day of the experiment, dilute heat-inactivated FBS in serum-free medium supplemented with 0.2% BSA to a final concentration of 20%.

4.5 Negative Control

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

4.6 Calcein AM (CAM) working solution

Calcein AM is supplied as a 1 mM solution. Dilute this stock solution in pre-warmed (37°C) 1X PBS to a final concentration of 4 μM (e.g., add 10 μL of stock Calcein AM to 2.49 mL of 1X PBS). The working dilution should be prepared just before use.

4.7 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 3.0 mL of nanoparticle solution dissolved/resuspended in starvation medium, at a concentration 1X the highest final test concentration. Test concentrations are based on the calculated 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 have been reviewed elsewhere [1] and are summarized in Box 1 below.

This assay evaluates four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two serial 1:5 dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 2 mg/mL is prepared. This sample is then diluted 10 fold (0.2 mg/mL), followed by two 1:5

serial dilutions (0.04 and 0.008 mg/mL). Use 150 µL of each sample dilution per well. Each nanoparticle concentration is plated three times.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

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

$$\text{human dose} = \frac{\text{mouse dose}}{12.3} = \frac{123 \text{ mg/kg}}{12.3} = 10 \text{ mg/kg}$$ (see reference [5])

The blood volume of a human is 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 a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg x } 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

6. HL-60 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 [2]. 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.

1. Expand cells until they are approximately 80-90% confluent (~3-5 days before experiment). Two days before the experiment, feed the cells following regular maintenance procedures.
2. One day before the experiment count cells using trypan blue. If the cell viability is at least 90%, pellet cells for 5 minutes at 400x*g* in a 15 mL tube.
3. Resuspend cells in Starvation Medium and incubate overnight (16–18 hr) at 37°C in a humidified incubator (95% air, 5% CO_2).
4. On the day of experiment, count cells using trypan blue and adjust concentration to $1x10^6$ **viable** cells/mL in Starvation Medium.

7. Experimental Procedure

The procedure described below is based on reference 3.

1. Insert filter plate into feeding tray and set aside.
2. Add 150 μL of starvation medium, positive control, negative control and test-nanomaterial in starvation medium into fresh feeding tray. Avoid generating bubbles while adding solutions to wells. Plate additional test-nanomaterial wells for use as cell-free controls (refer to plate map in Appendix).
3. Add 50 μL of cell suspension (prepared in step 6.4) per well of Multi-Screen filter plate (50,000 cells per well) from Step 7.1. Avoid generating bubbles while adding cells to wells. Add 50 μL of media to cell-free control wells.
4. Gently assemble Multi-Screen filter plate (step 7.3) and feeding tray containing controls and test particles (step 7.2). This is the "Assay Plate". An example of the assay plate template is shown in the Appendix. Avoid shaking or tilting plates as it will disturb the concentration gradient.
5. Cover the plate and incubate for 4 hr at 37°C in a humidified incubator (5% CO_2, 95% air). During incubation, pre-warm PBS to 37°C and equilibrate calcein AM to room temperature.
6. Prepare working solution of Calcein AM (CAM) as described in section 4.6.
7. After the 4 hr incubation, remove the chemotaxis assay plate from the incubator. As before, avoid shaking or tilting the plate.
8. Gently remove the Multi-Screen filter plate and discard.
9. Add 50 μL of 1X PBS and 50 μL of CAM working solution to appropriate wells, and 150 μL of 1X PBS plus 50 μL of CAM working solution to reagent background control wells on the feeding tray as outlined in the example template shown in the Appendix. This is the "Calcein Plate". Incubate the plate for 1 hr at 37°C.
10. Transfer 180 μL of solution from the Calcein Plate to corresponding wells on a Nunc optical bottom plate and read the Nunc plate on a fluorescent plate reader at 485 nm excitation/535 nm emission.

8. Calculations

8.1 A percent coefficient of variation (CV) should be calculated for each control or test according to the following formula:

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

8.2 Background Chemotaxis:

$$\text{Mean } FU_{\text{SM/CAM wells}} - \text{Mean } FU_{\text{SM/PBS wells}} - \text{Mean } FU_{\text{reagent background control wells}}$$

8.3 Sample Chemotaxis:

$$\text{Mean } FU_{\text{TS/CAM wells}} - \text{Mean } FU_{\text{SM/PBS}} - \text{Mean } FU_{\text{reagent background control wells}}$$

8.4 A comparison of sample chemotaxis to background chemotaxis is performed to evaluate chemotactic potential of test material. In general, fold chemotaxis induction ≥ 2 is considered positive. Statistical methods such as Student's t-test should be applied to evaluate the significance of differences between background chemotaxis and that of test samples.

9. Acceptance Criteria

1. The %CV for each control and test sample should less than 30%.
2. If two of three replicates of the positive or negative control fail to meet acceptance criterion described in 9.1, the assay should be repeated.
3. Within the acceptable assay, if two of three replicates of the unknown sample fail to meet acceptance criterion described in 9.1, this unknown sample should be re-analyzed.

10. References

1. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. *J Control Release*. 2013;172(2):456-66.
2. Gallagher R, et al. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood* 1979;54:713-733.
3. Kamath L. Multi-Screen-MIC Application note. Lit# AN1060EN00, Millipore Life Science Devision.

11. Abbreviations

BSA	bovine serum albumin
CAM	Calcein AM
CV	coefficient of variation
FBS	fetal bovine serum
FU	fluorescence units
PBS	phosphate buffered saline
RBC	Reagent background control
SD	standard deviation
SM	starvation media
VC	vehicle control

12. Appendix

Example Assay Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	SM	SM	SM	SM	SM	SM	NC	NC	NC	PC	PC	PC
B	SM	SM	SM	SM	SM	SM	NC	NC	NC	PC	PC	PC
C	SM	SM	SM	SM	SM	SM	NC	NC	NC	PC	PC	PC
D	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 2.0 mg/mL	TS 2.0 mg/mL	TS 2.0 mg/mL
E	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 2.0 mg/mL	TS 2.0 mg/mL	TS 2.0 mg/mL
F	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 2.0 mg/mL	TS 2.0 mg/mL	TS 2.0 mg/mL
G	VC	VC	VC									
H	NO CELLS TS 0.008 mg/mL	NO CELLS TS 0.008 mg/mL	NO CELLS TS 0.008 mg/mL	NO CELLS TS 0.04 mg/mL	NO CELLS TS 0.04 mg/mL	NO CELLS TS 0.04 mg/mL	NO CELLS TS 0.2 mg/mL	NO CELLS TS 0.2 mg/mL	NO CELLS TS 0.2 mg/mL	NO CELLS TS 2.0 mg/mL	NO CELLS TS 2.0 mg/mL	NO CELLS TS 2.0 mg/mL

This is the template for the feeding tray. Wells in rows A-F in the upper filter plate receive 50,000 cells in 50 µL.

Row H contains the cell-free test samples; they do not receive cells.

SM: Starvation Medium; NC: Negative Control; PC: Positive Control; TS: Test Sample; VC: vehicle control

Example Calcein Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM
B	PBS	PBS	PBS	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM
C	PBS	PBS	PBS	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM
D	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM
E	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM
F	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM
G	CAM	CAM	CAM	RBC	RBC	RBC	RBC	RBC	RBC	RBC	RBC	RBC
H	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM	CAM

PBS: 50 µL phosphate buffered saline; CAM: 50 µL Calcein AM;

RBC: Reagent Background Control, 150 µL PBS + 50 µL Calcein AM