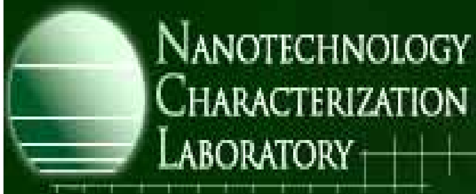

NCL Method ITA-6

Version 1.2

Leukocyte Proliferation 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.

Method is written by: ______________________ 8-10-07

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Method validation was conducted on: 8/12/2005-10/17/2005.

Date

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

1. Introduction

This document describes a protocol for assessing an effect of nanoparticle formulation on the basic immunologic function of human lymphocytes, i.e. measurement of lymphocytes proliferative responses. Lymphocytes are isolated from pooled human blood anti-coagulated with Li-heparin using Ficoll-Paque Plus solution. The isolated cells are incubated with or without phytohemaglutinin (PHA-M) in the presence or absence of nanoparticles. The assay therefore allows for measurement of nanoparticles ability to induce proliferative response of human lymphocytes or to suppress that induced by PHA-M. The assay requires 1800 μL of a test-nanomaterial.

2. Reagents

2.1. Human blood from at least 3 donors anti-coagulated with Li-heparin
2.2. Ficoll-Paque Plus, Amersham Biosciences, cat# 17-1440-02
2.3. PBS, HyClone, cat# AQB 22934
2.4. PHA-M, Sigma, cat# L8902
2.5. Fetal bovine serum, Hyclone.
2.6. RPMI-1640, Invitrogen, cat# 11875-119
2.7. Hanks balanced salt solution (HBSS), Invitrogen, cat# 24020-117
2.8. Pen/Strep solution, Invitrogen, cat#15140-148
2.9. β-mercaptoethanol, Sigma cat#M7522
2.10. Trypan Blue solution, Invitrogen, cat# 15250-061
2.11. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma # M5655)

Note: Equivalent reagents from other vendor can be used

3. Equipment

3.1. Pipettes covering range from 0.05 to 10 mL
3.2. 96-well 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

4. Reagent and Control Preparation.

5.1. 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. Warm the media in a water bath prior to use.

5.2. Phytohemaglutinin, 1mg/mL (PHA-M, Stock)

Add 1 mL of sterile PBS or cell culture medium per 1mg of PHA-M to the vial and gently rotate to mix. Store daily use aliquots at a nominal temperature of -20 °C. Avoid repeated freezing/thawing.

5.3. Positive control

Dilute stock PHA-M solution in cell culture medium to a final concentration of 100 μg/mL and use it to prepare three 1:2 serial working dilutions (1:5, 1:10 and 1:20) of the positive control. Working dilution are prepared in complete RPMI1640 medium.

5.4. Negative Control

Use PBS as a negative control.

5.5. Heat-inactivated fetal bovine serum

Thaw a bottle with FBS at room temperature or overnight at 2-8 °C, and allow to equilibrate to 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 indefinitely.

5.6. MTT solution.

Prepare MTT solution in PBS at a final concentration of 5mg/mL. Store for up to one month at 4°C in dark.

5.7. Glycine Buffer.

Prepare buffer by dissolving glycine and NaCl in water to final concentration of 0.1M for glycine (MW 75.07), and 0.1 M for NaCl (MW 58.44).
Adjust pH to 10.5. Store at room temperature.

5. Preparation of Study Samples.

This assay requires 1800 μL of nanoparticles dissolved/resuspended in complete culture medium, i.e three 100 μL replicates per sample analyzed in duplicate, 600 μL per cells from one donor. 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 the test concentration is selected based on results from general toxicity assays. A nanomaterial, which revealed toxicity in general toxicity assays, is tested at two concentrations selected at the low and the high end of the dose response curve. A nanomaterial, which did not reveal toxicity in a general toxicity assays is tested at one concentration equal to highest dose tested in general toxicity assay.

6. Isolation of human lymphocytes.

6.1. Place freshly drawn blood into 15 or 50 mL conical centrifuge tube, add equal volume of room-temperature PBS and mix well.

6.2. Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively blood/PBS mixture may be slowly layered over Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture.

Note: to maintain Ficoll-blood interface it is helpful to hold tube at 45° angle.

6.3. Centrifuge 30 minutes at 900g, 18-20 °C without brake.

6.4. Using sterile pipet, remove upper layer containing plasma and platelets and discard it.

6.5. Using fresh sterile pipet transfer mononuclear cell layer into another centrifuge tube.

6.6. Wash cells by adding excess of HBSS and centrifuging for 10 min at 400g, 18-20 °C. The HBSS volume should be ~3 times the volume of mononuclear layer.

Note: usually 4mL of blood/PBS mixture results in ~2mL of mononuclear layer and requires at least 6mL of HBSS for wash step. We use 10mL of HBSS per each 2mL of cells.

6.7. Discard supernatant and repeat wash step one more time.

6.8. Resuspend cells in complete RPMI1640 medium. Count cells and determine viability using trypan blue exclusion. If viability is at least 90%, proceed to the next step.

7. Experimental Procedure.

7.1. Adjust cell concentration to 1 x 10^6 cells/mL using complete RPMI medium

7.2. Dispense 100 µL of controls and test samples per well on a 96 well round bottom plate. Prepare duplicate wells for each sample.

7.3. Dispense 100 µL of cell suspension per well. Gently shake the plate to allow all components to mix.

Repeat steps 7.1-7.3 for cells obtained from each individual donor. There is no limit to the number of donors used in this test. It is advised to test each nanoparticle formulation using cells derived from at least three donors.

7.4. Incubate 3 days in a humidified 37C, 5% CO2 incubator.

7.5. Centrifuge plate for 5 minutes at 700g. Aspirate medium leaving cells and approximately 50 µL of medium behind and add 150 µL of fresh medium to each well.

7.6. Add 50 µL of MTT to all wells.

7.6. Cover in aluminum foil and incubate in a humidified 37C, 5% CO2 incubator for 4 hours.

7.7. Remove plate from incubator and spin at 700g for 5 minutes.

7.8. Aspirate media and MTT.

7.9. Add 200 µL of DMSO to all wells.

7.10. Add 25 µL of glycine buffer to all wells

7.11. Read at 570nm on plate reader

8. Calculations

A Percent Coefficient of Variation should be calculated for each control or test according to the following formula**:** %CV=SD/Mean x 100%

A percent viability is calculated as follows:

$$\% \text{ Cell Proliferation} = (\text{Mean OD}_{\text{sample}} - \text{Mean OD}_{\text{Negative Control}}) * 100$$

$$\% \text{ Proliferation Inhibition} = \frac{\text{Mean OD}_{\text{Positive Control}} - \text{Mean OD Positive}_{\text{Control+Nanoparticles}}}{\text{Mean OD}_{\text{Positive Control}}} \times 100\%$$

9. **Acceptance Criteria**

9.1. %CV for each control and test sample should less than 30%

9.2. If positive control or negative control fail to meet acceptance criterion described in 9.1 the assay should be repeated.

9.3. Within the acceptable assay if two of three replicates of unknown sample fail to meet acceptance criterion described in 9.1 this unknown sample should be re-analyzed.

9.4. If two duplicates of the same study sample demonstrated results different more then 30%, this sample should be reanalyzed.

9.5. If significant variability is observed in results obtained using leukocytes from three initial donors, the experiment need to be repeated with additional donor cells.

10. References.

10.1. Current Protocols in Immunology. Edited by: John E. Coligan (NIAID, NIH); Barbara Bierer (Brigham & Women's Hospital); David H. Margulies (NIAID, NIH); Ethan M. Shevach (NIAID, NIH); Warren Strober (NIAID, NIH); Richard Coico (Weill Medical College of Cornell University); John Wiley & Sons, Inc., 2005

10.2. Standard practice for evaluation of immune responses in biocompatibility testing using ELISA tests, lymphocytes proliferation, and cell migration. ASTM F1906-98.