# NCL Method ITA-6

# 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.

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## 1. Introduction

This document describes a protocol for assessing the effect of a nanoparticle formulation on the basic immunologic function of human lymphocytes, i.e. measurement of lymphocyte proliferative responses [1, 2]. This assay will allow for measurement of a nanoparticles' ability to induce proliferative response of human lymphocytes or to suppress that induced by phytohemaglutinin (PHA-M).

## 2. Principles

Lymphocytes are isolated from 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 and analyzed spectrophotometrically via the MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay.

## 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. Human blood from at least 3 donors, anti-coagulated with Li-heparin
2. Ficoll-Paque Plus (GE Life Sciences, 17-1440-02)
3. Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
4. Phytohemaglutinin (PHA-M) (Sigma, L8902)
5. Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)
6. RPMI-1640 (Invitrogen, 11875-119)
7. Hank's balanced salt solution (HBSS) (Invitrogen, 14025-092)
8. Pen/Strep solution (Invitrogen, 15140-148)
9. Trypan Blue solution (Invitrogen, 15250-061)
10. MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma, M5655)
11. L-glutamine (Hyclone, SH30034.01)
12. Glycine (Sigma, G7403)

13. Sodium chloride (Sigma, S7653)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. 96-well round bottom plates
3. 96-well flat bottom plates
4. Polypropylene tubes, 50 and 15 mL

3.3 Equipment

1. Centrifuge, 700x*g*, 9000x*g*
2. Refrigerator, 2-8ºC
3. Freezer, -20ºC
4. Water bath, 37ºC, 56ºC
5. Cell culture incubator with 5% $CO_2$ and 95% humidity
6. Biohazard safety cabinet approved for level II handling of biological material
7. Inverted microscope
8. Vortex
9. Hemocytometer
10. Plate reader, 570 nm

## 4. Reagent and Control Preparation

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: 10% FBS (heat inactivated); 2 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 Phytohemaglutinin-M Stock Solution, 1 mg/mL (PHA-M Stock)

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

4.4 Positive Control

Dilute PHA-M stock in cell culture medium to a final concentration of 100 µg/mL and prepare working solutions at the following concentrations: PC1, 20 µg/mL; PC2, 10 µg/mL; and PC3, 5 µg/mL. The final concentrations of PC1, PC2 and PC3 in the well after addition of cell suspension will be 10, 5 and 2.5 µg/mL, respectively.

**Note**: The volume of PC1 needed for the assay depends on the number of samples. This control is used to prepare samples combining nanoparticle treatment with PC. When comparing leukocyte proliferation in nanoparticle+PC sample, use proliferation in PC2 as benchmark.

4.5 Negative Control

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

4.6 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.

4.7 MTT solution

Prepare MTT solution in PBS at a final concentration of 5 mg/mL. Store for up to one month at 4ºC in dark (e.g., wrap the storage bottle in foil).

4.8 Glycine Buffer

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

4.9 Research Donor Blood

The blood from at least three donor volunteers should be drawn in vacutainers containing Li-heparin as an anti-coagulant. The first 10 cc collected during phlebotomy should be discarded. Cells from each donor should be tested separately.

## 5. Preparation of Study Samples

This assay requires 3.0 mL of nanoparticle solution dissolved/resuspended in complete culture medium, at a concentration 4X 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 [3] 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 8 mg/mL is prepared. This sample is then diluted 2 fold (4 mg/mL). For the purpose of this document, these stocks are referred to as stock A (8 mg/mL or 4X the highest tested concentration) and stock B (4 mg/mL or 2X of the highest tested concentration). Both stock A and stock B will then be diluted 10-fold (0.8 mg/mL and 0.4 mg/mL, respectively). Each sample will then be diluted by two 1:5 serial dilutions (0.16 and 0.032 mg/mL, and 0.08 and 0.016 mg/mL, respectively). Stock A and its dilutions (group A) are used for the preparation of the nanoparticle + PC treatments. Stock B and its dilutions (group B) are used for the nanoparticle only treatments. When 0.05 mL of each sample from group A is added to the plate and mixed with 0.05 mL of positive control 1 (PC1) and 0.1 mL of cell suspension, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. When 0.1 mL of each sample from group B is mixed with 0.1 mL of cell suspension, the final nanoparticle concentrations in these samples will also be 2.0, 0.2, 0.04 and 0.008 mg/mL.

***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:

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

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. Isolation of Human Lymphocytes

1. Place freshly drawn blood into 15 or 50 mL conical centrifuge tubes. Add an equal volume of room-temperature PBS and mix well.
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, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, when using 50 mL conical tubes overlay 20 mL of diluted blood over 15 mL of Ficoll-Paque solution. To maintain Ficoll-blood interface it is helpful to hold the tube at a 45º angle.
3. Centrifuge 30 min at 900x*g*, 18-20ºC, without brake.
   **Note**: For certain types of centrifuges it may be advisable to set acceleration speed to minimum as well.
4. Using a sterile pipet, remove the upper layer containing plasma and platelets and discard.
5. Using a fresh sterile pipet, transfer the mononuclear cell layer into a fresh centrifuge tube.
6. Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400x*g*, 18-20ºC. The HBSS volume should be approximately three times the volume of mononuclear layer.

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

7. Discard supernatant and repeat wash step once more.
8. Resuspend cells in complete RPMI-1640 medium. Dilute cells 1:5 or 1:10 with trypan blue, count cells and determine viability using trypan blue exclusion. If viability is at least 90%, proceed to the next step.

## 7. Experimental Procedure

1. Adjust cell concentration to 1 x $10^6$ cells/mL using complete RPMI medium.
2. Dispense a) 100 µL of controls; b) 100 µL test samples from group B (i.e. at 2X the final test concentration) for nanoparticle treatment only; and c) 50 µL of test samples from group A (i.e. at 4X the final test concentration) for nanoparticle plus positive control treatment and 50 µL of PC1 per well on a 96-well round bottom plate. Each dilution is analyzed three times in duplicate. Always include cell-free controls as well (i.e., 100 µL nanoparticles (at 2X final test concentration concentration) and 100 µL of cell culture media). See Appendix for example plate map.
3. Dispense 100 µL of cell suspension (or cell culture medium for cell-free controls) per well. Gently shake the plate to allow components to mix. Repeat steps 1-3 for each individual donor.
4. Incubate 72 $\pm$ 2 hours in a humidified 37ºC, 5% $CO_2$ incubator.
5. Centrifuge plate for 5 minutes at 700x*g*. Aspirate medium, leaving cells and approximately 50 µL of medium behind. Add 150 µL of fresh medium to each well.
6. Add 50 µL of MTT to all wells.
7. Cover in aluminum foil and incubate in a humidified 37ºC, 5% $CO_2$ incubator for 4 hours.
8. Remove plate from incubator and spin at 700x*g* for 5 minutes.
9. Aspirate media and MTT.
10. Add 200 µL of DMSO to all wells.
11. Add 25 µL of glycine buffer to all wells.

**Note**: You may need to pipet the content of the plate up and down several times to ensure that all formazan crystals are solubilized.

12. Transfer 200 μL of the plate into a 96-well flat bottom plate. The transfer into flat bottom plate may be skipped if a plate reader can operate with round bottom plates.
13. Read at 570 nm on plate reader.

## 8. Calculations

8.1 Percent Coefficient of Variation (%CV)

The % CV should be calculated for each control and test sample:

$$\frac{\text{Standard Deviation}}{\text{Mean}} \times 100\ \%$$

8.2 Analyze the data as follows:

$$\%\ \text{Proliferation} = \frac{(\text{Mean OD}_{\text{test sample}} - \text{Mean OD}_{\text{Untreated cells}})}{\text{Mean OD}_{\text{Untreated cells}}} \times 100\ \%$$

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

**Notes**:

a. The calculations above are done with the assumption that an increase in the number of viable cells detected by this assay is due to proliferation and not due to an increase in the longevity of the individual cells in culture.
b. If percent inhibition is negative, this indicates the test compound increased proliferation rather than decreasing proliferation.
c. Percent proliferation values above negative control observed in the no cell control samples suggest that nanoparticles interfere with the assay, i.e., a false positive.
d. When comparing leukocyte proliferation in nanoparticle+PC sample, use proliferation in PC2 as benchmark.

## 9. Acceptance Criteria

1. The %CV for each control and test sample should be less than 30%.
2. When positive control (PC2) or negative control fails to meet acceptance criterion described in 9.1, the assay should be repeated.
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.
4. If two duplicates of the same study sample demonstrate results different by more than 30%, this sample should be reanalyzed.
5. If significant variability is observed in results obtained using leukocytes from three initial donors, the experiment should be repeated with additional donor cells.

## 10. References


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.
2. Standard practice for evaluation of immune responses in biocompatibility testing using ELISA tests, lymphocytes proliferation, and cell migration. ASTM F1906-98.
3. 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.


## 11. Abbreviations

| | |
|---|---|
| CV | coefficient of variation |
| DMSO | dimethyl sulfoxide |
| FBS | fetal bovine serum |
| HBSS | Hank’s balanced salt solution |
| MTT | 3-(4, 5-dimethyl-2-thiazolyl)-2.5-diphenyl-2H-tetrazolium bromide |
| OD | optical density |
| PBS | phosphate buffered saline |
| PHA-M | phytohemaglutinin |
| SD | standard deviation |

## 12. Appendix

Example Plate Map

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Untreated cells | NC | PC1 | PC2 | PC3 | VC | | | | | | |
| **B** | Untreated cells | NC | PC1 | PC2 | PC3 | VC | | | | | | |
| **C** | 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 (1.0 mg/mL) | TS (1.0 mg/mL) | TS (1.0 mg/mL) |
| **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 (1.0 mg/mL) | TS (1.0 mg/mL) | TS (1.0 mg/mL) |
| **E** | TS (0.008 mg/mL) + PC1 | TS (0.008 mg/mL) + PC1 | TS (0.008 mg/mL) + PC1 | TS (0.04 mg/mL) + PC1 | TS (0.04 mg/mL) + PC1 | TS (0.04 mg/mL) + PC1 | TS (0.2 mg/mL) + PC1 | TS (0.2 mg/mL) + PC1 | TS (0.2 mg/mL) + PC1 | TS (1.0 mg/mL) + PC1 | TS (1.0 mg/mL) + PC1 | TS (1.0 mg/mL) + PC1 |
| **F** | TS (0.008 mg/mL) + PC1 | TS (0.008 mg/mL) + PC1 | TS (0.008 mg/mL) + PC1 | TS (0.04 mg/mL) + PC1 | TS (0.04 mg/mL) + PC1 | TS (0.04 mg/mL) + PC1 | TS (0.2 mg/mL) + PC1 | TS (0.2 mg/mL) + PC1 | TS (0.2 mg/mL) + PC1 | TS (1.0 mg/mL) + PC1 | TS (1.0 mg/mL) + PC1 | TS (1.0 mg/mL) + PC1 |
| **G** | TS (0.008 mg/mL) | TS (0.04 mg/mL) | TS (0.2 mg/mL) | TS (1.0 mg/mL) | | | VC | Untreated cells | NC | PC1 | PC2 | PC3 |
| **H** | TS (0.008 mg/mL) | TS (0.04 mg/mL) | TS (0.2 mg/mL) | TS (1.0 mg/mL) | | | VC | Untreated cells | NC | PC1 | PC2 | PC3 |

Wells 1-4, in Rows G & H are the cell-free test samples; they do not receive cells.

NC: negative control; PC: positive control; TS: test sample; VC: vehicle control

**Note**: PC1 on this template refers to the working solution of the positive control with a concentration of 20 µg/mL. The final concentration of PHA-M in this sample is 5 µg/mL. Therefore, the data from nanoparticles+PC1 wells should be compared to the data in PC2 wells.