NCL Method ITA-6.2

Leukocyte Proliferation Assay

(Immunostimulation)

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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. This document is identical to part A of the NCL protocol ITA-6.1 (formerly NCL ITA-6).

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; 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 Human blood anti-coagulated with Li-heparin and obtained from at least 3 healthy donors
- 3.1.2 Ficoll-Paque Premium (GE Life Sciences, 17-5442-02)
- 3.1.3 Phosphate Buffered Saline (PBS) (GE Life Sciences, SH30256.01)
- 3.1.4 Phytohemaglutinin (PHA-M) (Sigma, L8902)
- 3.1.5 Fetal Bovine Serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)
- 3.1.6 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)
- 3.1.7 Hank's balanced salt solution (HBSS) (Gibco, 14175-095)
- 3.1.8 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.9 Trypan Blue solution (Invitrogen, 15250-061)

3.1.10 MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma, M5655)

3.1.11 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.12 Glycine (Sigma, G7403)

3.1.13 Sodium chloride (Sigma, S7653)

3.2 Materials

3.2.1 Pipettes covering a range of 0.05 to 10 mL

3.2.2 96-well round bottom plates

3.2.3 96-well flat bottom plates

3.2.4 Polypropylene tubes, 50 and 15 mL

3.3 Equipment

3.3.1 Centrifuge

3.3.2 Refrigerator, 2-8ºC

3.3.3 Freezer, -20ºC

3.3.4 Cell culture incubator with 5% CO_2 and 95% humidity

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

3.3.6 Inverted microscope

3.3.7 Vortex

3.3.8 Hemocytometer

3.3.9 Plate reader capable of operating at 570nm

4. Reagent and Control Preparation

4.1 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

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 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.

4.3 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. Final concentration 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, as this control will be used to prepare samples combining nanoparticle treatment with PC. When comparing leukocyte proliferation in nanoparticle+PC sample use proliferation in PC2 as benchmark.

4.4 Negative Control

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

4.5 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.

4.6 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.

4.7 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 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 nanoparticles dissolved/resuspended in complete culture medium to a concentration 2X the highest tested concentration. 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 the "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere [3] 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:

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

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.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10X (or when feasible 100X, 30X or 5X) of 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 4 mg/mL will be prepared. The stock will then be diluted 10-fold (0.4 mg/mL), followed by two 1:5 serial dilutions (0.08 and 0.016 mg/mL). The stock and its dilutions are used for the nanoparticle-only treatments. When 0.1 mL of each sample (stock and its dilutions) is added to the plate and mixed with 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.

6. Isolation of Human Lymphocytes

6.1 Place freshly drawn blood into 15 or 50 mL conical centrifuge tubes, add an 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, 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 a 50 mL conical tube overlay 20 mL of diluted blood over 15 mL of Ficoll-Paque solution.
Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45º angle.

6.3 Centrifuge 30 min at 900xg, 18-20ºC, without brake.
Note: For certain types of centrifuges it may be advisable to set acceleration speed to minimum as well.

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

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

6.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20ºC. The HBSS volume should be ~3 times the volume of mononuclear layer. *Note: Usually 4 mL of blood/PBS mixture results in ~ 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.*

6.7 Discard supernatant and repeat wash step one more time.

6.8 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

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

7.2 Dispense a) 100 µL of controls and b) 100 µL test 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.

7.3 Dispense 100 µL of cell suspension (or cell culture medium for cell-free controls) per well. Gently shake the plate to allow all components to mix. Repeat steps 7.1-7.3 for each individual donor.

7.4 Incubate 72±2 hours in a humidified 37ºC, 5% CO_2 incubator.

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

7.6 Add 50 µL of MTT to all wells.

7.7 Cover in aluminum foil and incubate in a humidified 37ºC, 5% CO_2 incubator for 4 hours.

7.8 Remove plate from incubator and spin at 700xg for 5 minutes.

7.9 Aspirate media and MTT.

7.10 Add 200 µL of DMSO to all wells.

7.11 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.

7.12 Transfer 200 µL of the plate into a 96-well flat bottom plate.

Note: The transfer into flat bottom plate may be skipped if plate reader can operate with round bottom plates.

7.12 Read at 570 nm on plate reader.

8. Calculations

8.1 A Percent Coefficient of Variation should be calculated for each control or test according to the following formula:

$$\%CV = \frac{standard\ deviation}{mean} * 100\%$$

8.2 Analyze the data as follows:

$$\%\ Proliferation = \frac{(Mean\ OD_{Test\ sample} - Mean\ OD_{Untreated\ cells})}{Mean\ OD_{Untreated\ cells}} * 100\%$$

Note:

- *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 the increase in the longevity of the individual cells in culture.*
- *Percent proliferation values above negative control observed in the no cell control samples suggest that nanoparticles interfere with the assay in the false positive way.*

9. Acceptance Criteria

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

9.2 When positive control (PC2) or negative control fails 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 by more than 30%, this sample should be reanalyzed.

9.5. If significant variability is observed in results obtained using leukocytes from three initial donors, the experiment needs to 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
RPMI	Roswell Park Memorial Institute
SD	standard deviation
TS	test sample
VC	vehicle control

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												
F												
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 20 µg/mL; the final concentration of PHA-M in this sample is 5 µg/mL.