NCL Method ITA-18

Human Leukocyte Proliferation Assay (HuLa)

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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

Immunosuppression is a common reason for drug withdrawal from the market. Traditional immune function tests used to estimate a material's immunosuppression is T-cell dependent antibody response (TDAR). This method involves a 28 day in vivo study evaluating an animal's antibody titer to a known antigen (KLH) with and without challenge. Due to the limited quantities of novel drug candidates, an in vitro method called Human Leukocyte activation (HuLa) has been developed as a substitute for the traditional TDAR assay during early preclinical evaluation. The method presented herein is based on the HuLa assay originally developed by Mark Collinge, et al at Pfizer [1]. The protocol presented in this document has been adapted for use with engineered nanomaterials.

2. Principles

Lymphocytes are isolated from human blood anti-coagulated with Li-heparin using Ficoll-Paque Plus solution. The blood is obtained from healthy donor volunteers vaccinated with the current season flu vaccine (Fluzone). The isolated cells are incubated with Fluzone in the presence or absence of nanoparticles for 72 hours. Following incubation, BrdU is added to the cells and the incubation continues for additional 24 hours. At the end of the incubation period the cells are fixed and incorporation of BrdU into the DNA of proliferating cells is analyzed by ELISA.

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 three prescreened donors, anti-coagulated with Li-heparin
2. Ficoll-Paque Plus (GE LifeSciences, 17-1440-02)
3. Phosphate buffered saline (PBS) (GE Life Sciences, SH 30256.01)
4. Fluzone (Sanofi Pasteur). Note, this is a prescription medication and may not be available to all research laboratories.

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, 24020-117)
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 (GE Life Sciences, Hyclone, SH30034.01)
12. Glycine (Sigma, G7403)
13. Sodium chloride (Sigma, S7653)
14. BrdU cell proliferation assay (Calbiochem, QIA58)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. 96-well flat bottom plates (for BrdU plate)
3. 96-well round bottom plates (for MTT plate)
4. Polypropylene tubes, 50 and 15 mL

3.3 Equipment

1. Centrifuge, 400x*g*, 700x*g*, 900x*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

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: 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 Negative Control

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

4.4 Positive control stock (Dexamethasone, DXM**)**

Clinical grade DXM is provided at a stock concentration of 4 mg/mL. Use from the commercial stock for a final concentration of 250 µg/mL.

4.5 BrdU Kit Reagents

1. After initial thaw of the commercially supplied material, divide into small aliquots and store at -20°C. On the day of experiment, thaw the required number of aliquots and dilute BrdU 1:2000 in fresh complete media. Prepare immediately before use.
2. BrdU-specific Antibody

   Dilute antibody 1:100 in antibody diluent. Prepare immediately before use. After initial thaw, divide into small aliquots and store at -20°C.
3. Peridoxase Goat Anti-Mouse IgG HRP Conjugate

   Reconstitute Peridoxase Goat Anti-Mouse IgG HRP Conjugate in 250 µL of PBS and incubate at RT for 10 minutes. Once reconstituted, divide into small aliquots and store at -20°C. For use, dilute Peridoxase Goat Anti-Mouse IgG HRP Conjugate in Conjugate Diluent according to the dilution instructions on the vial. The dilution factor is lot specific. Prepare immediately before use.
4. Allow the Fixative/Denaturing Solution to sit at room temperature for 4 hr prior to use.
5. Thaw the Conjugate Diluent, Substrate, Plate Wash Concentrate and Stop Solution overnight at 4°C. Once thawed, these components can be stored at 4°C.

6. Dilute Plate Wash Concentrate (20X) to 1X by adding 25 mL of concentrate to 475 mL of deionized water. Store at 4°C.

4.6 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).

4.7 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.

4.8 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.

4.9 Fluzone

Fluzone is supplied as a stock with a final concentration of 90 μg/mL of influenza hemagglutinin. Dilute this commercial stock 1:50 with complete culture media. This is a seasonal prescription medication and may not be available to all research laboratories. The vaccine is available as both single and multidose vials. Mutidose vials usually contain preservatives (e.g., mercury) which are contraindicated in this assay. Use only preservative-free versions.

4.10 Research Donor Blood

Blood from at least three donor volunteers vaccinated with the current season flu vaccine 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 0.5 mL of nanoparticle solution dissolved/resuspended in complete culture medium, at a concentration 16X 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 [2] 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 32 mg/mL is prepared. This sample is then diluted 10 fold (3.2 mg/mL), followed by two 1:5 serial dilutions (0.64 and 0.13 mg/mL). When 0.01 mL of each of these sample dilutions is added to the plate and mixed with 0.05 mL of Fluzone and 0.1 mL of cell suspension, the final nanoparticle concentrations tested in this assay are: 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

7.1 Cell Treatment with Nanoparticles and Controls

1. Adjust cell concentration to $1x10^6$ cells/mL with complete medium.
2. Aliquot 100 μL of cell suspension to the appropriate wells of two 96 well plates (see plate map). One plate will be labeled for BrdU and one plate will be labeled for

MTT. Repeat this step for each individual donor. Refer to the plate map for an example template. **Important**: Use flat bottom plates for BrdU and round bottom plates for MTT.

3. Add 10 µL of test nanoparticle, positive control and negative control to their respective wells. Prepare no-cell control wells containing nanoparticles only (refer to the plate map for an example template).
4. Incubate 1 hr at 37 °C.
5. Add 50 µL of media or Fluzone vaccine to appropriate wells. **Important**: Do not add Fluzone to "No Fluzone" wells.
6. Incubate at 37°C for 72 hours.
7. Add 20 µL of BrdU label to appropriate wells on BrdU plate, taking care not to add BrdU to "No BrdU" wells. **Important**: Do not add BrdU to the MTT plate. Add 20 µL of culture media to appropriate wells on the MTT plate.
8. Incubate 24 hours.
9. Spin BrdU plate for 4 minutes at 400x*g*.
10. Aspirate media from BrdU plate, add 200 µL of fixative per well, and incubate at room temperature for 30 min. Remove fixative and tap the plate on a paper towel. Proceed to the next step. **Important**: Do not fix the MTT plate. Process this plate according to the MTT procedure described in step 7.3 below.

    Note: At this step the plate may be stored at 4°C for up to seven days before proceeding to the next step.

7.2 BrdU Assay

1. Aspirate plate and add 100 µL of diluted BrdU-specific antibody to all wells.
2. Incubate at RT for 1 hour.
3. Aspirate plate.
4. Wash plate three times using 250 µL/well of 1X Wash Buffer. Blot the plate on a paper towel to remove excess buffer.
5. Add 100 µL of Peroxidase-conjugated Goat Anti Mouse IgG to all wells.
6. Incubate 30 minutes at RT.
7. Aspirate plate.

8. Wash plate three times using 250 µL/well of 1X Wash Buffer. Blot the plate on a paper towel to remove excess buffer.
9. Fill wells completely with distilled water.
10. Aspirate plate. Blot the plate on paper towels to remove excess buffer.
11. Add 100 µL of Substrate Solution to all wells.
12. Incubate at RT for 30 minutes in the dark.
13. Add 100 µL of Stop Solution.
14. Read plate at dual wavelengths of 450 (Lm1) and 540 nm (Lm2) within 30 minutes of addition of Stop Solution. The final result is OD = Lm1 - Lm2.

7.3 MTT Assay (Perform the same time as day two of BrdU assay.)

1. Centrifuge the plate for 5 min at 700x*g*, then aspirate the media leaving approximately 50 µL.
2. Add 200 µL of fresh complete media.
3. Add 50 µL of MTT solution.
4. Incubate at 37°C for 4 hr in the dark. Centrifuge the plate for 5 min at 700x*g*.
5. Aspirate plate.
6. Add 200 µL of DMSO.
7. Add 25 µL of glycine buffer. Pipette up and down several times to ensure all formazan crystals have been dissolved.
8. Shake on plate shaker for 5 minutes. Transfer 200 µL of the well contents to a fresh 96 well flat bottom plate.
9. Read on plate reader at 570 nm.

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 Stimulation Index (SI):

$$SI = \frac{\text{Mean OD}_{\text{testsample}} - \text{Mean OD}_{\text{cells no BrdU}}}{\text{Mean OD}_{\text{No Fluzone sample}} - \text{Mean OD}_{\text{cells no BrdU}}}$$

A good SI in the absence of immunosuppressive agents is ≥ 3. The preferable SI is ≥ 6-7.

9. Acceptance Criteria

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

2 If positive or negative control fails to meet the 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 the acceptance criterion described in 9.1, this unknown sample should be re-analyzed.

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

5 The positive control is considered positive if it results in at least a 2-fold reduction in SI when compared to the baseline sample.

6 A test sample is considered positive if it results in at least a 2-fold reduction in SI when compared to the baseline sample.

10. References

1. Collinge M, Cole SH, Schneider PA, Donovan CB, Kamperschroer C, Kawabata TT. Human lymphocyte activation assay: an in vitro method for predictive immunotoxicity testing. J Immunotoxicol. 2010;7(4):357-66.
2. 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 assay
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
SI	stimulation index

12. Appendix

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium (baseline)	NC	VC	PC		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 (0.2 mg/mL)
B	Medium (baseline)	NC	VC	PC		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 (0.2 mg/mL)
C	TS1 (0.2 mg/mL)	TS1 (0.2 mg/mL)	TS1 (2.0 mg/mL)	TS1 (2.0 mg/mL)	TS1 (2.0 mg/mL)	NC	VC	PC	Medium (baseline)	Cells No Fluzone	Cells No Fluzone	Cells No BrdU
D	TS1 (0.2 mg/mL)	TS1 (0.2 mg/mL)	TS1 (2.0 mg/mL)	TS1 (2.0 mg/mL)	TS1 (2.0 mg/mL)	NC	VC	PC	Medium (baseline)	Cells No Fluzone	Cells No Fluzone	Cells No BrdU
E	Cells No BrdU											
F	Cells No BrdU											
G	TS1 (0.008 mg/mL)	TS1 (0.04 mg/mL)	TS1 (0.2 mg/mL)	TS1 (2 mg/mL)								
H	TS1 (0.008 mg/mL)	TS1 (0.04 mg/mL)	TS1 (0.2 mg/mL)	TS1 (2 mg/mL)								

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