



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. The traditional immune function test used to estimate materials' immunosuppression is T cell dependent antibody response (TDAR). This method involves a 28 day, in vivo, study evaluating animal antibody titers 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 to the traditional TDAR assay in early preclinical development. 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 Premium 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. After a 72 hour incubation, BrdU is added to cells and the incubation continues for additional 24 hours. At the end of incubation, the cells are fixed and incorporation of BrdU into 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; 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 Healthcare, 17-5442-02)

- 3.1.3 Phosphate buffered saline (PBS) (GE Life Sciences, Hyclone, SH30256.01)
- 3.1.4 Fluzone (Sanofi Pasteur); this is prescription medication and may not be available to all research laboratories. Other preservative-free influenza vaccines may be used, although screening of vaccines and donors is recommended prior to committing test material.
- 3.1.5 Fetal bovine serum (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 (Gibco, 15250-061)
- 3.1.10 MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma-Aldrich, M5655)
- 3.1.11 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)
- 3.1.12 Glycine (Sigma-Aldrich, G7403)
- 3.1.13 Sodium chloride (Sigma-Aldrich, S7653)
- 3.1.14 BrdU cell proliferation assay (Sigma-Aldrich, QIA58)
- 3.2 Materials
 - 3.2.1 Pipettes covering a range of 0.05 to 10 mL
 - 3.2.2 96-well flat bottom plates (for BrdU plate)
 - 3.2.3 96-well round bottom plates (for MTT plate)
 - 3.2.4 Polypropylene tubes, 50 and 15 mL
- 3.3 Equipment
 - 3.3.1 Centrifuge
 - 3.3.2 Refrigerator, 2-8°C
 - 3.3.3 Freezer, -20°C
 - 3.3.4 Cell culture incubator with 5% CO₂ 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

4. Preparation of Reagents and Controls

4.1 Heat-Inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8°C and allow to equilibrate to room temperature. Incubate 30 minutes at 56°C in a water bath mixing every five 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.

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 2-8°C, protected from light for no longer than one month. Before use, warm in a 37°C water bath.

4.3 Negative Control

Use PBS as a 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 the stock concentration of 4 mg/mL. Dilute in PBS or media from the commercial stock for a final concentration of 250 µg/mL.

4.5 BrdU Kit Reagents

4.5.1 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.

4.5.2 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.

4.5.3 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.

4.5.4 Allow the Fixative/Denaturing Solution to sit at room temperature for 4 hr prior to use.

4.5.5 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.

4.5.6 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 4°C 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. Multidose 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 nanoparticles dissolved/resuspended in complete culture medium to a concentration of 16X 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 “theoretical plasma concentration”. Considerations for estimating theoretical plasma concentration were reviewed elsewhere [2] and are summarized in Box 1 below.

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 32 mg/mL will be prepared and diluted 10-fold (3.2 mg/mL), followed by two 1:5 serial dilutions (0.64 and 0.13 mg/mL, respectively). When 0.01 mL of each sample is added to the plate and mixed with 0.05mL of Fluzone and 0.1mL of cell suspension, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

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:

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

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.

$$\text{in vitro concentration}_{\text{human matrix}} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

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 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 45° angle.
- 6.3 Centrifuge 30 min at 900xg, 18-20°C, 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.
- 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, 18-20°C. 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 once more.
- 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 Cell Treatment with Nanoparticles and Controls

- 7.1.1 Adjust cell concentration to 1×10^6 /mL with complete medium.
- 7.1.2 Aliquot 100 μ L of cell suspension to the appropriate wells of two 96-well plates (see plate map in Appendix). One plate will be labeled for BrdU and one plate will be labeled for MTT. Repeat this step for each individual donor (see example plate map in Appendix). **Important: Use flat bottom plate for BrdU and round bottom plate for MTT.**
- 7.1.3 Add 10 μ L of test nanoparticle, positive control and negative control to the respective wells. Prepare no-cell control wells containing nanoparticles only (refer to the Appendix for an example plate map).
- 7.1.4 Incubate 1 hour at 37°C.
- 7.1.5 Add 50 μ L of media or Fluzone vaccine to appropriate wells. **Important: Do not add Fluzone to “No Fluzone” wells.**
- 7.1.6 Incubate at 37°C for 72 hours.
- 7.1.7 Add 20 μ L 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 μ L of culture media to well on the MTT plate.**
- 7.1.8 Incubate 24 hours.
- 7.1.9 Spin BrdU plate for 4 minutes at 400xg.

7.1.10 Aspirate media from BrdU plate, add 200 μ L of fixative per well and incubate at room temperature for 30 min. Remove fixative and tap the plate on paper towel. Proceed to 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 7 days before proceeding to the next step.

7.2 BrdU Assay

7.2.1. Aspirate plate and add 100 μ L of diluted BrdU-specific antibody to all wells.

7.2.2 Incubate at RT for 1 hour.

7.2.3 Aspirate plate.

7.2.4 Wash plate 3 times using 250 μ L/well of 1X Wash Buffer. Blot the plate on paper towels to remove excess buffer.

7.2.5 Add 100 μ L of Peroxidase-conjugated Goat Anti Mouse IgG to all wells.

7.2.6 Incubate 30 minutes at RT.

7.2.7 Aspirate plate.

7.2.8 Wash plate 3 times using 250 μ L/well of 1X Wash Buffer. Blot the plate on paper towels to remove excess buffer.

7.2.9 Fill wells completely with distilled water.

7.2.10 Aspirate plate. Blot the plate on paper towels to remove excess buffer.

7.2.11 Add 100 μ L of Substrate Solution to all wells.

7.2.12 Incubate at RT for 30 minutes in the dark.

7.2.13 Add 100 μ L of Stop Solution.

7.2.14 Read plate at dual wavelengths of 450 nm (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 at the same time as day two of BrdU assay.)

7.3.1 Centrifuge the plate for 5 min at 700xg, then aspirate the medium leaving ~50 μ L behind.

7.3.2 Add 150 μ L of fresh complete media.

- 7.3.3 Add 50 µL of MTT solution.
- 7.3.4 Incubate at 37°C for 4 hours in the dark. Centrifuge the plate for 5 min at 700xg.
- 7.3.5 Aspirate plate.
- 7.3.6 Add 200 µL of DMSO.
- 7.3.7 Add 25 µL of glycine buffer. Pipet up and down several times to ensure all formazan crystals have been dissolved.
- 7.3.8 Shake on plate shaker for 5 minutes. Transfer 200 µL of the well content to a fresh 96-well flat bottom plate. **Note:** Transfer of well contents to a flat-bottomed plate is not necessary if the plate reader can read round-bottom plates.
- 7.3.9 Read on plate reader at 570 nm.

8. Calculations

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

8.2 Stimulation Index (SI):

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

A good SI in the absence of immunosuppressive agent is ≥ 3 . Preferable SI is $\geq 6-7$.

9. Acceptance Criteria

- 9.1 The %CV for each control and test sample should less than 30%.
- 9.2 If positive control 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 significant variability is observed in results obtained using leukocytes from three initial donors, the experiment needs to be repeated with additional donor cells.

- 9.5 Positive control is considered positive if it results in at least 2-fold reduction in SI when compared to baseline sample.
- 9.6 A test sample is considered positive if it results in at least 2-fold reduction in SI when compared to 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 Oct-Dec;7(4):357-66.3.
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 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
SD	standard deviation
RT	room temperature
SI	stimulation index

12. Appendix

Example Plate Map

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