Project:

Leukocyte Proliferation Assay

Measuring leukocyte proliferation by 3H-thymidine incorporation

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1.0	08.02.2016	All	Initial Document	Neill Liptrott
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Table of Content

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]. 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 Principle of the Method

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 for 48 hours. Lymphocyte proliferation is then measured by the incorporation of 3H-thymidine. The amount of incorporated 3H-thymidine is directly proportional to the number of cells present.

3 Applicability and Limitations (Scope)

This assay is for the general measurement of lymphocyte proliferation; specific immune cell subsets are not analysed. It is recommended that, if proliferation is identified in response to test material, further studies in specific immune cell subsets are carried out. This may be conducted by multiparametric immunophenotyping in concert with CFSE staining or by repeating the incorporation of 3H-thymidine in isolated immune cells populations. Additionally, if cytotoxic concentrations of test materials are used then results may be affected by the loss of viable cells.

4 Related Documents

Table 1:

Document ID	Document Title

5 Equipment and Reagents

5.1 Equipment

5.1.1 Centrifuge

5.1.2 Refrigerator, 2-8ºC

5.1.3 Freezer, -20ºC

5.1.4 Cell culture incubator with 5% CO2 and 95% humidity.

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

5.1.6 Inverted microscope

5.1.7 Vortex

5.1.8 Cell counting equipment

5.2 Reagents

5.2.1 RPMI 1640 (Sigma; R0883)

5.2.2 Heat-inactivated human AB serum (Sigma H6914)

5.2.3 HEPES buffer (H0887)

5.2.4 L-glutamine (G7513)

5.2.5 Transferrin (T0665)

5.2.6 Phytohaemagglutinin (PHA-P) (Sigma; L1668)

5.2.7 Hank's balanced salt solution (HBSS) (Invitrogen, 14025-092)

5.2.8 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

5.2.9 Ficoll-Paque Plus (GE Life Sciences, 17-1440-02)

5.2.10 3H-Thymidine (Moravek Biochemicals; MT6031)

5.2.11 Human blood from at least 3 donors, anti-coagulated with Li-heparin

5.3 Reagent Preparation

5.3.1 **Complete RPMI-1640 medium**

Complete RPMI-1640 medium should contain the following;

10% heat-inactivated human AB serum

25 mM HEPES buffer

2 mM L-glutamine

25 µg/ml Transferrin

Store at 2-8ºC, protected from light for no longer than 1 month. Before use, warm in a water bath.

5.3.2 **Phytohemaglutinin-P Stock solution, 1 mg/mL (PHA-M Stock)**

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

5.3.3 **Positive control**

Dilute PHA-P 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.

5.3.4 **Negative Control**

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

5.3.5 **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.

5.3.6 **Heat-inactivated serum**

Thaw a bottle of serum 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.

5.3.7 **Research donor blood**

The blood from at least 3 donor volunteers should be drawn in vacutainers containing Li-heparin as anti-coagulant. First 6mL collected during phlebotomy should be discarded. Cells from each donors should be tested separately.

5.4 Preparation of study samples

This assay requires 3.0 mL of nanoparticles dissolved/resuspended in complete culture medium to a concentration of 4x 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.

Box 1. Example Calculation of Nanoparticle concentration for In Vitro Test

In this example, we assume the mouse dose is known to be 123 mg/kg.

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

Blood volume constitutes approximately 8% of body weight, (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows us to get a very rough estimation of what the maximum blood concentration may be.

$$in\ vitro\ concentration_{human\ maxtrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg\ \times 10\frac{mg}{kg}}{5.6\ L}$$

$$= \frac{700\ mg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10 X (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 8 mg/mL will be prepared and diluted 2-fold (4mg/mL). For the purpose of this document these stocks are referred to as stock A (8mg/mL or 4x of the highest tested concentration) and stock B (4mg/mL or 2x of the highest tested concentration). Both stock will then be diluted 10-fold (0.8mg/mL and 0.4 mg/mL for stock A and stock B, respectively), followed by two 1:5 serial dilutions (stock A - 0.16 and 0.032mg/mL, and stock B - 0.08 and 0.016 mg/mL). 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 0.05mL of positive control 1 (PC 1) 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. When 0.1mL of each sample

from group B is mixed with 0.1mL of cell suspension the final nanoparticle concentrations in these samples will also be 2.0, 0.2, 0.04 and 0.008 mg/mL.

6 Procedure

6.1 Isolation of human lymphocytes

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

6.1.2 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 50mL conical tube overlay 20mL of diluted blood over 15mL of Ficoll-Paque solution.

Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45º angle.

6.1.3 Centrifuge 30 min at 900 x g, 18-20ºC, without brake.

Note: for certain types of centrifuges it may be advisable to set acceleration speed to minimum as well

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

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

6.1.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400 x g, 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.

6.1.7 Discard supernatant and repeat wash step one more time.

6.1.8 Resuspend cells in complete RPMI-1640 medium. Count cells using an appropriate cell counting method. If viability is at least 90%, proceed to the next step.

6.2 Experimental procedure

6.2.1 Adjust cell concentration to 2.5 million cells/ml and plate out at 100 µl/well in a 96 well flat-bottomed plate

6.2.2 Add 50 µl of 20µg/ml PHA-P per well

6.2.3 Add 50 µl of either medium or medium containing drug to the wells in triplicate (all drugs should be added at 4 x the desired final concentrations to allow for the dilution that occurs when they are added to the cells).

6.2.4 Plates are cultured for 48 hours (37°C; 5% CO2), the final 16 h with 1µCi [3H]-thymidine per well

6.2.5 Harvest cells onto a filtermat using a tomtec harvester 96 and seal in a sample bag with melt on scint

6.2.6 Count incorporated radioactivity on a Perkin-Elmer MicroBeta detector.

6.3 Flow chart

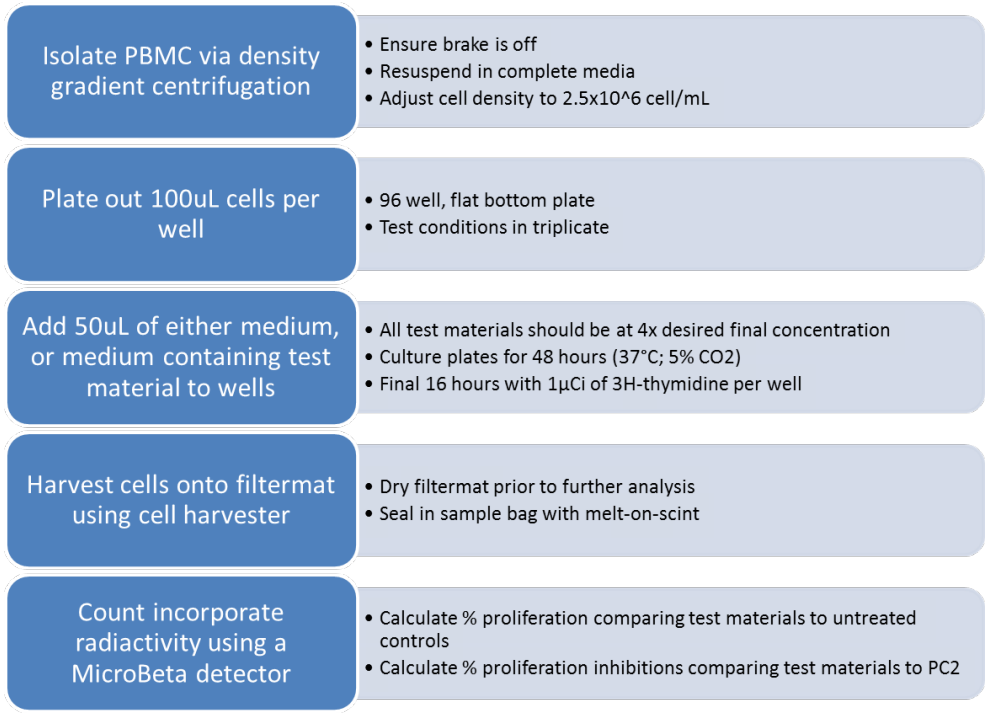

Figure 1: Brief outline of the workflow.

6.4 Calculation of percentage proliferation and proliferation inhibition

% Proliferation= (Mean $OD_{testsample}$- Mean $OD_{Untreated\ cells}$)/Mean $OD_{Untreated\ cells}$ x 100 %

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

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 Quality control/assurance

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

7.1.2 Percentage CV for each control and test sample should be less than 30%

7.2 Acceptance Criteria

7.2.1 When positive control (PC2) or negative control fails to meet acceptance criterion described in 7.1.2, the assay should be repeated.

7.2.2 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.

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

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

7.3 Data Interpretation

7.3.1 The calculations above are done with the assumption that an increase in the number of viable cells detected by this assay is due to the proliferation and not due to the increase in the longevity of the individual cells in culture

7.3.2 If percent inhibition is negative, it indicates that the test compound increased proliferation instead of decreasing it

7.3.4 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

7.3.5 When comparing leukocyte proliferation in nanoparticle+PC sample use proliferation in PC2 as benchmark

8 Health and Safety Warnings, Cautions and Waste Treatment

8.1 Universal precautions must be used when handling human peripheral blood.

- No sharps are to be used in the preparation of cell isolates of plasma from human healthy volunteer blood.
- All liquid waste must be discarded into 1% Virkon solution and left overnight (18 hours) in a class II BSC with the lid loose to allow for venting.

8.2 All work with radioisotopes should be conducted on spill trays. Local rules for working with radioactive sources must be observed at all times.

8.3 Monitoring for radioactivity should be conducted before and after experimental work. Representative swabs of work areas and equipment should be taken using deionised water and cotton wool swabs. Liquid scintillation counting should be used for monitoring of 3H and 14C labelled materials.

Abbreviations

CV	coefficient of variation
DMSO	dimethyl sulfoxide
HBSS	Hank's balanced salt solution
PBS	phosphate buffered saline
PHA-P	phytohemaglutinin
RPMI	Roswell Park Memorial Institute
SD	standard deviation

9 Appendix (example plate layout)

	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	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)	TS1 (0.2 mg/mL)	TS1 (0.2 mg/mL)	TS1 (1.0 mg/mL)	TS1 (1.0 mg/mL)	TS1 (1.0 mg/mL)
D	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)	TS1 (0.2 mg/mL)	TS1 (0.2 mg/mL)	TS1 (1.0 mg/mL)	TS1 (1.0 mg/mL)	TS1 (1.0 mg/mL)
E	TS1 (0.008 mg/mL) + PC1	TS1 (0.008 mg/mL) + PC1	TS1 (0.008 mg/mL) + PC1	TS1 (0.04 mg/mL) + PC1	TS1 (0.04 mg/mL) + PC1	TS1 (0.04 mg/mL) + PC1	TS1 (0.2 mg/mL) + PC1	TS1 (0.2 mg/mL) + PC1	TS1 (0.2 mg/mL) + PC1	TS1 (1.0 mg/mL) + PC1	TS1 (1.0 mg/mL) + PC1	TS1 (1.0 mg/mL) + PC1
F	TS1 (0.008 mg/mL) + PC1	TS1 (0.008 mg/mL) + PC1	TS1 (0.008 mg/mL) + PC1	TS1 (0.04 mg/mL) + PC1	TS1 (0.04 mg/mL) + PC1	TS1 (0.04 mg/mL) + PC1	TS1 (0.2 mg/mL) + PC1	TS1 (0.2 mg/mL) + PC1	TS1 (0.2 mg/mL) + PC1	TS1 (1.0 mg/mL) + PC1	TS1 (1.0 mg/mL) + PC1	TS1 (1.0 mg/mL) + PC1
G	TS1 (0.008 mg/mL)	TS1 (0.04 mg/mL)	TS1 (0.2 mg/mL)	TS1 (1.0 mg/mL)			VC	Untreated cells	NC	PC1	PC2	PC3
H	TS1 (0.008 mg/mL)	TS1 (0.04 mg/mL)	TS1 (0.2 mg/mL)	TS1 (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 concentration 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

10 References

1. Muul, L.M., et al., *Measurement of Proliferative Responses of Cultured Lymphocytes*, in *Current Protocols in Immunology*. 2011, John Wiley & Sons, Inc.
2. Dobrovolskaia, M.A. and S.E. McNeil, *Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines.* J Control Release, 2013. **172**(2): p. 456-66.