

STANDARD OPERATING PROCEDURE

Title: Lymphocyte Proliferation Assay (LPA) Using ³ H-Thymidine Incorporation Assay	Core Name: Lloyd Mayer, Mount Sinai Medical Center	SOP # ITN2800
	Effective Date: 02/16/2012	
	Trial Number: ITN047AI	SOP Owner (Department): Biomarker and Discovery Research (BDR)

Approval

Name/Title	Signature	Date
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A. PURPOSE AND SCOPE

Described below is the Standard Operating Procedure (SOP) for Lymphocyte Proliferation Assay (LPA) using ^3H -thymidine incorporation.

Lymphocyte proliferation assay measures the ability of lymphocytes in short-term tissue culture to undergo proliferation when stimulated *in vitro* by a foreign antigen. Typically, CD4^+ lymphocytes proliferate in response to antigenic peptides in association with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). This proliferative response of lymphocytes to antigen *in vitro* occurs only if the patient has been immunized to that antigen, either by having recovered from an infection with the microorganism containing that antigen, or by having been vaccinated. The ^3H -thymidine incorporation assay utilizes a strategy wherein a radioactive nucleoside, ^3H -thymidine, is incorporated into new strands of chromosomal DNA during mitotic cell division. A scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division that has occurred in response to an antigen/mitogen. The amount of radioactivity incorporated into DNA in each well is measured in a scintillation counter and is proportional to the number of proliferating cells, which in turn is a function of the number of lymphocytes that were stimulated by a given antigen to undergo a proliferative response. The readout is counts per minute (cpm) per well.

Lymphocytes from normal healthy volunteers can be stimulated to proliferate non-specifically by stimulating them with mitogens e.g. phytohemagglutinin (PHA) or antibodies against T cell receptors CD3 and CD28. These reagents often serve as positive controls for the assay. This SOP will be used to test lymphocyte proliferation for both pilot (part A and part B) as well as for the main study (ITN047AI). In part A, the LPA assay will be used to determine whether Immucothel is immunogenic by itself or requires an adjuvant (montanide) to elicit a detectable immune response; in part B and in the main study, the data from LPA assay will help us address the question whether Biosyn native KLH can induce tolerance when administered orally. The peripheral blood mononuclear cells (PBMCs) from participants, immunized with Immucothel alone or depending on the response, potentially with adjuvant as well (Part A) or fed orally with two rounds of Biosyn native KLH, followed by SQ administration (twice - seven to 10 days apart) of Immucothel and potentially an adjuvant (Part B + main trial), will be stimulated briefly (5 days) *in vitro* with 2 different concentrations of Biosyn native KLH (100 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$) and lymphocyte proliferation measured.

A. RESPONSIBILITY

Dr. Lloyd Mayer is responsible for ensuring that the individual executing this procedure is appropriately trained.

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B. DEFINITIONS/ABBREVIATIONS

LPA	Lymphocyte Proliferation Assay
MHC	Major Histocompatibility Complex
APC	Antigen Presenting Cells
PHA	Phytohemagglutinin
TdR	Tritiated thymidine
KLH	Keyhole Limpet Hemocyanin
PBMC	Peripheral blood mononuclear cells

C. REAGENTS

For a defined study enough reagents will be ordered from the same lot/batch to test all samples, to avoid batch variations due to changes in serum etc. If the samples are to be collected over a study period longer than the shelf life of the reagents this will be discussed with the ITN before the study begins and a validation plan for new batches of reagents put in place.

1. KLH (Biosyn)
2. Human AB Serum – Fisher Scientific Cat # BP2525100
3. Culture medium: RPMI 1640 with L-glutamine – Gibco Life Technologies Cat # 11875
4. L-glutamine: 200 mM (100X), Penicillin-Streptomycin: 10,000 units of penicillin G (sodium salt) and 10,000 µg of streptomycin sulfate (yields a 100X stock solution) liquid - Gibco Life Technologies Cat # 10387
5. Dulbecco's Phosphate-Buffered Saline (D-PBS), without calcium and magnesium. - Gibco Life Technologies Cat # 10010
6. Tritiated thymidine ($[^3\text{H}]\text{TdR}$) MP Biochemicals. 5mCi/5 mL. Cat 2407005. Lot #34700401
7. Scintillation Fluid – Scintiverse BD cocktail Fisher Scientific Cat # SX18-4
8. Anti-CD3/CD28 beads – Dynabeads Human T cell activator CD3/CD28. Dynal Cat # 111.31D

D. EQUIPMENT AND MATERIALS

1. Cell harvester for quantitatively transferring and washing cells from 96-well plates onto glass fiber filter mats
2. Scintillation counter for measuring the incorporation of ^3H -thymidine
3. Centrifuge capable of spinning at 200 x g for washing cells
4. Microscope and hemocytometer for counting cells
5. 37°C, CO₂ humidified incubator
6. Calibrated single and multichannel pipettes (set capable of accurately dispensing from 1 µL to 1 mL)
7. 96-well, round-bottom sterile tissue culture plates with lids

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E. PROCEDURE

Specimen:

1. PBMCs from enrolled participants in ITN047AI (Pilot and Main study):
Peripheral blood mononuclear cells isolated from fresh heparinized blood according to ITN protocol [RUCDR-SOP-003]. Once PBMCs are isolated, they should be plated within 1 hour to avoid the loss of adherent APCs that may stick to the plasticware. Freshly isolated PBMCs from a healthy donor will be included in all assays as laboratory control (frozen PBMCs usually give a low ^3H thymidine response).

Reagents

1. Preparation of complete culture medium

Add human AB serum (20% final), and Penicillin-Streptomycin (100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin final) to RPMI 1640 + L-glutamine. Label with day of supplementation. Complete media can be stored at 4°C for up to 30 days.

2. Preparation of working solutions of KLH (Day 0)

Dilute KLH stock solutions to make 2X working solution in complete culture medium (200 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$). Final concentrations in culture will be 100 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$

3. Preparation of working solution of anti-CD3/anti-CD28 beads (Day 0). Directly add 2.5 μL of beads to 1 mL of complete medium to make the working solution

4. Preparation of [^3H]TdR (Day 5):

The [^3H]TdR is supplied by the manufacturer at a concentration of 1 $\mu\text{Ci}/\mu\text{L}$ no further dilution is necessary. The final concentration of [^3H]TdR should be 1 $\mu\text{Ci}/\text{well}$. Isotope is drawn up using a Hamilton syringe for precise measurement. Unused [^3H]TdR solution can be stored at 4°C for 1 year.

Assay Setup (Day 0):

1. Thaw KLH stock solution
2. Label the tissue culture plate with ITN barcode or patient identification (PID), date plated, date to be pulsed with isotope and data to be harvested/counted using water-resistant markers.
3. Dispense 100 μL of the PBMC suspension (2×10^6 cells/mL or 200,000 cells per well) into culture wells. Be certain to keep cells suspended, by gentle mixing, while the cells are being dispensed. Cells should be dispensed within 30 mins. to avoid the medium becoming alkaline. Each sample will be tested in triplicate.

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4. Dispense 100 μL of KLH working stocks or 100 μL diluted anti-CD3/CD28 beads into triplicate wells as appropriate and per plate map (Appendix 1). Note each PBMC sample is cultured with KLH at both 10 $\mu\text{g}/\text{mL}$ as well as 100 $\mu\text{g}/\text{mL}$. Add 100 μL of culture media alone for the negative control wells.
5. Add 200 μL of RPMI to any unused wells to prevent plate edge effects/drying out during incubation.
6. Allow the cells to proliferate for five days at 37°C in a humidified CO₂ tissue culture incubator.
7. On day five, add 1 μL /well of [³H]TdR (5mCi/5 mL) using Hamilton syringe. Incubate plates for another 18 hours during which tritiated thymidine is incorporated into the newly synthesized DNA of the dividing cells.

Harvesting and Counting

On the morning of day 6, harvest on to glass fiber filters using a cell harvester according to the manufacturer's instructions. After harvesting, allow filters to dry at room temperature on the bench top for 30 mins. Add scintillation fluid to fiber filter mats (inserted into plastic holders) and leave filters in scintillation fluid according to the manufacturer's directions prior to counting. Count vials using a Wallac beta scintillation counter (all 96 wells counted in the same run) at laboratory-determined settings to measure counts per minute (cpm).

F. CALCULATION OF STIMULATION INDEX (SI)

For determination of the primary end point, a stimulation index (SI) will be calculated using cpm values from the assessment day* (Day 16 for part A and Day 42 for part B and main trial). First, the average cpm's will be calculated for stimulated (KLH 100 $\mu\text{g}/\text{mL}$ or KLH 10 $\mu\text{g}/\text{mL}$ as determined per the acceptance criteria) and unstimulated (medium only) wells using the following criteria:

Within triplicate values, if any one value differs by >20% from the median value, then it will be excluded from the calculation of the SI. For example, for values 3278, 3196 & 2400 – the median value is 3196 and $\pm 20\%$ range is 2557 and 3835 respectively based on 20% difference from the median. In this example, 2400 value would be deleted from analysis as it is less than 2557 but 3278 will be included as it is less than 3835. In this case, average (mean) will be calculated using 3196 and 3278 only. In the unlikely event that all 3 values are separated by more than 20% (e.g. 2400, 3196, & 3850) then, the entire set will be discarded as both 2400 and 3850 fall outside of median $\pm 20\%$ rule and the assay will be repeated using the “back-up” banked frozen cells.

Once the average cpm is calculated, then, the SI for the primary end point will be calculated by taking the ratio of two numbers as follows:

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SI (on assessment day*) = average cpm on assessment day with stimulation (KLH 100 µg/mL or KLH 10 µg/mL) / average cpm on assessment day without stimulation (medium only)

**: assessment day differs based on the stage of ITN047AI. Therefore, for part A, assessment day = Day 16; for part B and for the main part of the trial; assessment day = Day 16 (unfed group) and Day 42 (fed group).*

G. ACCEPTANCE CRITERIA FOR ASSAY DATA

1. The average cpms of control/medium only wells must be below 1000 for the assay to be considered valid. If the control cpms are >1000, the assay is considered uninterpretable and must be repeated, even if the SI is >3. However if the SI is >7, medium only wells >1000 cpms are acceptable, and the assay is valid. Note this is not the same as a patient failing to respond to KLH, the patient is simply scored as giving uninterpretable data in the ex vivo KLH proliferation assay.
2. We expect wide ranging SI values due to natural variation in the immune response. A stimulation index >3 is considered positive. All SI values below 3 are scored as negative for proliferation
3. The assay is considered valid if the SI for the positive control i.e. anti-CD3/anti-CD28 is ≥ 3 . However, if SI for positive control is ≤ 3 , but the SI for any one patient sample is ≥ 3 , then, the assay data for those samples assayed at the same time will be also considered evaluable. Data at baseline, day 0, will be examined to control for the patient having a prior response to KLH. In the event of the SI for samples stimulated with 100 µg/mL KLH giving an SI ≥ 3 , then the stimulation index for cultures with 10 µg/mL KLH will also be calculated. If the latter SI is also greater than or equal to 3, that patient does not meet trial criteria and will be considered non-evaluable. If the latter value is <3, then that patient has only a mild initial cross-reactive response to KLH at a high dose and the results will be considered acceptable.

In summary, if at day 16 or 42 the average of medium alone wells is greater than 1000 cpm, then the data from patient/normal control that donated this sample will not be entered into the study unless the SI is >7. If BEFORE vaccination with KLH the individual has an SI response >3 at both doses of in vitro KLH, then the data from this individual cannot be evaluated. If the response of >3 occurs only at the high dose then the data from this patient/control can be entered into the study but will only be evaluated at the lower (in vitro) concentration of KLH.

