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Proliferation of Human Mononuclear Cells with ^{3}H-Thymidine

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Changes from last version			
Date of version	***Paragraphs***	***Date of version***	***Paragraphs***

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Content

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1. Subject

This Standard Operation Procedure (SOP) describes a method to assess cell proliferation by uptake of radioactive ^{3}H-Thymidine.

2. Application

Studying proliferative T-cell responses to stimulation with specific antigens or mitogens.

3. Definitions and Abbreviations

FBS	= foetal bovine serum (≈ foetal calf serum)
RT	= room temperature
P/S	= Penicillin-Streptomycin
Glu	= L-glutamine
v/v	= volume/volume
rpm	= rotations per minute
ml	= milliliter
DNA	= desoxynucleicacid
SI	= Stimulation Index
cpm	= counts per minute
RA	= radioactive
µCi	= micro Curie

4. Principle

When an antigen or mitogen is presented by antigen presenting cell (APC), specific T-cells can recognize and respond to these antigens or mitogens. They will start dividing (proliferation) and can also release specific cytokines and chemokines. The proliferation can be detected by adding ^{3}H thymidine to the dividing cells. This ^{3}H is incorporated into the DNA only during replication of a cell (1). The uptake is thus proportional to the number of cell divisions. When cells are lysed and the DNA is trapped in a filter, the amount of radioactive ^{3}H can be detected in this filter. The amount of ^{3}H uptake is a measurement for proliferation and is expressed as Stimulation Index (SI). A SI can be calculated by dividing the counts per minutes (cpm) of a condition with the cpm of resting cells (cells stimulated with only the culture matrix).

5. Safety precautions

Treat every sample containing human material such as AB serum and the lymphocyte samples as infectious material. Wear disposable gloves. ^{3}H thymidine is **radioactive** (β- emitter).

Suma-Tab contains sodium-di-chloro-isocynate	 Xn, Harmful
Trypan blue (0.4%)	 T, Toxic
Dry-Ice	 Xn, Harmful
Methyl- ^{3}H-Thymidine	 Radioactive

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Ethanol	F, Flammable
Scintillation fluid	F, Flammable

6. Reagents

6.1 *Chemicals*

Reagents	Formula	Supplier	order number	Store at (°C)
RPMI 1640	-	Invitrogen	52400-025	4
L-glutamine	-	Invitrogen	25030-024	-20
Penicillin-Streptomycin	-	Invitrogen	15140-122	-20
Foetal Bovine Serum	-	Invitrogen	10270-106	-20
Turk solution	-	Merck	1092770100	RT
Trypan Blue (0.4%)	$C_{34}H_{24}N_6O_{14}S_4Na_4$	Invitrogen	15250-061	RT
Human AB serum	-	Sanquin Bloodbank	-	-20
^{3}H-Thymidine	-	ICN	-	4
Ethanol	CH_3OH	Pharmacy (WKZ)	-	RT
Scintillation fluid (Betaplate Scint)	-	Perkin Elmer (former Wallac)	1205-440	RT

6.2 *Basic Culture Medium*

RPMI 1640 supplemented with 1% v/v P/S (= 5ml) and 1% v/v glu (=5ml)
Store at 4°C up to 1 month

6.3 *Wash Medium*

RPMI 1640 supplemented with 1 v/v% P/S (= 5ml), 1 v/v% glu (=5ml) and 2% v/v FBS (=10 ml)
FBS should be heat inactivated (1 hr at 56°C) and filtered through a 0.20 µm sterile filter using a 10 ml sterile syringe before usage. Store at 4°C up to 1 month.

6.4 *20% AB medium*

Thaw 5 ml heat inactivated (1 hr at 56°C) human serum and filter through a 0.20 µm sterile filter that is placed on top of a sterile 50 ml tube using a 10 ml sterile syringe. Add 20 ml basic culture medium with a sterile 25 ml pipet and mix. Store at 4°C up to 1 week.

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7. Equipment and Accessories tools

7.1 Equipment

- Centrifuge Hettich Rotanta 46 (UMC# 99-000-2142)
- Reichert brightline hemacytometer (Hausser Scientific Company, Horsham PA, USA)
- Easypet pipet (Eppendorf, Germany, 4006173)
- Pipets 10-1000 µl (Gilson, The Hague, The Netherlands)
- Multistep pipet (Eppendorf, Germany)
- Harvester (Mach II M, Tomtec)
- Beta counter (Wallac, Turku, Finland)
- Incubator at 60°C
- Incubator at 37°C, 5% CO_2
- Bagging support station (Wallac, Turku, Finland)
- Betaplate scintillation counter (Wallac, Turku, Finland)

7.2 Accessories

- 50 ml sterile polypropylene conical tubes (Falcon/ Becton Dickinson, Erembodegem, Belgium, 352070)
- Minisart 0.20 µm single use sterile filter (Sartorius, Hanover, Germany, 16534)
- Sterile 10 ml syringe (Becton Dickinson, Erembodegem, Belgium)
- 2 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357507)
- 5 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357543)
- 10 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357551)
- 25 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357525)
- Disposable gloves (Kimberly Clark, Zaventem, Belgium)
- Easyload 200 µl (741035) and 1000 µl (741000) sterile pipet tips (Greiner Bio-one, Germany)
- 96-Wells round bottom sterile culture plates (Nalge Nunc, Roskilde, Denmark, 163320)
- 5 ml Multistep pipet tips sterile (Eppendorf, Germany, 0030 069.455)
- Plastic foil (Fresh Cling)
- ^{3}H filters, filtermat A (Wallac, Turku, Finland,1205-401)
- Seal bags
- Scintillation fluid, betaplate scint (Wallac, Turku, Finland, 1205-440)

8. Samples

Sample Processing

All handlings of the sample should be done in a biohazard safety cabinet

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9. Procedure

1 Remove Cells from the liquid nitrogen tank and transport them on dry ice. Keep the cells as short as possible on dry ice (cells can be taken out from the liquid nitrogen one day prior use and placed in at –80°C).
2 Thaw cells as described in UCAN-U 0004
3 Resuspend the cells in 2 ml 20% AB medium

4 Count the cells using Trypan Blue (0.4% solution) using a Reichert hemacytometer as described in protocol UCAN-U 0001. Calculate the number of cells as described at §10.1 calculation of the counted cells
5 Adjust the volume of the cell suspension to a cell concentration of $2*10^{6}$ per ml using 20% AB medium
6 Seed cells in a 96-wells round bottom sterile culture plate by adding 100 µl cell suspension using a multistepper pipet
7 Take the desired peptides (antigens) and controls (mitogens) from –20°C and from +4°C
8 Place all vials on ice
9 Calculate a 2 times concentrated solution of the peptides as described at §10.2 calculation of peptide solutions (run each condition in triplicates)
10 Calculate the amount volume of the positive control as described at §10.3 calculation of the positive controls
11 Dissolve the aliquot of peptide stock solution or positive control in basic medium
12 Add 100 µl of the peptide solution, control solution or basic medium to the appropriate wells which already contain the cells using a multistepper pipet (run each condition in triplicates
13 Wrap the culture plate with plastic foil and place it in an incubator (37°C, 5% CO_2)
14 Incubate the cells for 96 hours

15 Bring cells to RA-laboratory
16 At the end of the day (± 17.00 hrs) add 1 µCu ^{3}H-thymidine per well (= 25 µl of pre made stock of ^{3}H-thymidine in basic medium, that is stored at 4°C at the ^{3}H-RA laboratory, according to the RA guidelines as stated at SOP P-ASL23 (in Dutch)
17 Wrap the culture plate in plastic foil and place it in an incubator (37°C, 5% CO_2) that is placed in the RA-laboratory
18 Incubate for 16-18 hours
19 Harvest the cells using the harvester in the RA-laboratory
20 Dry filters at 60°C for at least 90 minutes
21 Wrap filters in a plastic seal using the support station
22 Count the ^{3}H thymidine with the beta counter at the AZU
Calculate the ^{3}H thymidine incoporation stated as SI
23 Log the result

10. Processing of the Results

10.1 Calculation of the counted cells

Number of cells per ml =
Counted cells per mm^2 (25 squares) * dilution (10) * 10.000 =
Counted cells * 10^{5}

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10.2 Calculation of peptide solutions

Calculate the volume needed for the number of wells that needs to be stimulated with a petide (number of wells * 100 µl)
Calculate the double concentration of the desired end concentration in the well.

[µl peptide stock] * [peptide stock concentration] = [total volume] * [2x concentration per well]

Example for 3 wells
peptide stock concentration = 2.0 mg/ml
2x concentration per well = 20 µg/ml (so final concentration in the well = 10 µg/ml)
= 20 µg/ml → 0.020 mg/ml
total volume = 100 µl * number of wells → 3*100µl = 300 µl

[µl peptide stock] * 2.0 mg/ml = 300 µl * 0.020 mg/ml

[µl peptide stock] = (300 µl * 0.020 mg/ml) / 2.0 mg/ml

[µl peptide stock] = 3 µl (in 300 µl basic medium)

10.3 Calculation of positive controls

Concavalin A
Add 5 µl stock solution Concavalin A to 4.2 ml basic medium. Next make a 1:10 dilution with basic medium adjusted to the volume needed for the culture.

Example for 3 wells
Total volume 3 x 100 µl = 300 µl
1) 4.2 ml + 5 µl Concavalin A
2) mix 30 µl of solution 1) with 270 µl basic medium

Tetanus and Diphtheria Toxoid
Calculate the volume needed for the number of wells that needs to be stimulated with this antigen (number of wells * 100 µl) (a).
The stock solution is a 30 times stock. So divide the total volume with 30 (b). Take the volume of the stock solution and fill up to the calculated total volume (c).

Example for 3 wells
a) total volume: 3 x 100 µl = 300 µl
b) volume toxoid: 300 µl / 30 = 10 µl
c) take 10 µl toxoid and fill up to 300 µl with basic medium (= 290 µl)

11. Documentation

Document each sample used in your notebook. Remove the used vial with cells from the cellstorage
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12. Accuracies and Precision

Accuracy is hard to describe. Precision can be taken from the standard deviation of triplicates of samples. The counts of each data point of the triplicate samples should be within a range of 40-50%.

13. Quality Control

Samples should react on Concavalin A and on TT and/of DT when the patient is boosted with these antigens. Recognition of the peptides is depending on the patient. Please note that after 120 hours of incubation with Concavalin A cells can already be dead due to limited nutrient resources in the well. Thus a low SI with Concavalin A can be seen as positive control only when in the culture well the indicator of the RPMI 1640 (phenol red) has not turned to yellow.

14. Remarks

Cryovials are submerged in liquid nitrogen. So when a vial is not properly closed it will fill partly with nitrogen. When the vials are placed directly in warm water without letting the nitrogen evaporate by removing the lid of the vial, the nitrogen will expand and the vial will explode!

15. Literature

(1) Cave MD. Incorporation of tritium-labeled thymidine and lysine into chromosomes of cultured human leukocytes. J Cell Biol 1966; 29(2):209-222

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