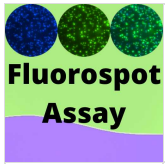

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Fluorospot Assay

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ABSTRACT

This protocol details the steps required to collect and assay mouse anti-human cells using fluorospot assay.

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MATERIALS TEXT

Reagents:

- IPFL for Fluorospot (Mabtech)
- Methanol
- Sterile water
- PBS (GIBCO BRL 10010-023)
- Culture media
- Peptide pools (1 and 2)
- PHA
- Stimuli
- BSA
- Fluoroescence enhancer-II
- Paper towels

Antibodies:

- mouse anti-human IFN-y antibody Clone 1-D1K (Mabtech)
- mouse anti-human IL-5 antibody TRFK5 (Mabtech)
- mouse anti-human IL-10 antibody

Detection Antibodies:

- IFNg: 7-B6-1-FS-BAM
- IL-5: 5A10-WASP
- IL-10: 12G8

Fluorophores:

- IFNg: anti-BAM-490
- IL-5: anti-WASP-640
- IL-10: SA-550 1:200

Equipment/Consumables:

- Pipettes
- Pipette tips
- Centrifuge
- Incubator
- 96-well plates
- Fluorospot reader

SAFETY WARNINGS

See the Safety Data Sheet (SDS) for all safety hazards and warnings.

Day 1

1 Coat plates with antibodies.

1.1 Perform all work in laminar flow hood.

1.2 Use IPFL for Fluorospot (Mabtech).

1.3 Prewet plates with **50 µl 70% MeOH** / well

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1.4

Discard MeOH and wash plate **3x** with **100 µl sterile water** .

1.5 Coat plate with **50 µl antibody diluted in PBS** per well (according to table below):

	[Starting]	[Final] Conc	Amount	Dilutions
mouse anti-human IFN-y antibody Clone 1-D1k Mabtech	1 mg/ml	5 ug/ml	25 uL	1:200
mouse anti-human IL-5 antibody TRFK5 Mabtech	1 mg/ml	5 ug/ml	25 uL	1:200
mouse anti-human IL-10 antibody	1 mg/ml	10 ug/ml	50 uL	1:100
PBS: GIBCO BRL 10010-023			5 mL	-

2

Leave plates **Overnight** at **4 °C** .

Day 2

3 Block plates:

3.1 Discard coating antibody and tap on paper.

3.2

Wash with **100 µl PBS** / well **3x**.

3.3 Add **100 µl culture media** .

3.4 1h

Incubate at **37 °C** for **01:00:00** .

4

Harvest cells from culture wells (14 day expansion) with pipette (pipetting up and down), centrifuge and count.

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#	Donor	Stimulation	Cells/mL	Vol (mL)	Total # Cells	% viability	Total Volume to get 2x10^6/mL (uL)
1							

5 Prepare stimuli dilutions:

Stimuli	Stock Conc.	Working Conc. (2X final conc.)	V stimuli (uL)	V media (uL)
Neg	100%	(to match max DMSO of pools)		
PHA	1 mg/ml	20 ug/mL		
Peptide pool		10 ug/mL		

6 Plate **50 µl** /well each of stimuli and PBMCs.

7 1d

Incubate plates for **24:00:00** at **37 °C , 5% CO2** .

8 Plate layout:

	(1-3)	(4-6)	(7-9)	(10-12)
A	Peptide pool 1	Peptide pool 2		
B	PHA	PHA		
C	Neg for peptide pool 1	Neg for peptide pool 2		
D				
E				
F				
G				
H				

Day 3 3h 15m

9

Remove the cells by emptying the plate and wash **5 times** with **200 µl PBS** /well (plate washer).

10 Dilute the detection antibodies, per full plate:
NEW SYSTEM

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Component	[Starting]	[Final] Conc.	Amount	Dilutions
IFNg: 7-B6-1-FS-BAM	-	-	50 ul	1:200
IL-5: 5A10-WASP	-	-	50 ul	1:200
IL-10: 12G8	1 mg/ml	2 ug/ml	20 ul	1:500
PBS-0.1% BSA			10 mL	

11 2h

Add **100 µl** /well and incubate for **02:00:00** at **Room temperature** in the dark.

12

Wash 5 times with **200 µl PBS** /well.

13 Dilute the fluorophores, per full plate:
NEW SYSTEM

Component	[Starting]	[Final] Conc.	Amount	Dilutions
IFNg: anti-BAM-490	-	-	50 ul	1:200
IL-5: anti-WASP-640	-	-	50 ul	1:200
IL-10: SA-550 1:200	-	-	50 ul	1:200
PBS-0.1% BSA			10 mL	

14 1h

Incubate at **Room temperature** in the dark for **01:00:00** .

15

Wash **5 times** with **200 µl PBS** /well.

16 15m

Empty the plate and add **50 µl Fluorescence enhancer-II** /well and leave the plate for **00:15:00** at **Room temperature** .

17 Empty the plate and remove residual Fluorescence enhancer by firmly tapping the plate against clean paper towels.

Do not wash in the sink.

Remove the underdrain (the soft plastic under the plate).

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18

19 Leave the plate in the dark to dry; plate should be completely dry before analysis.

20 Inspect and count spots in a Fluorospot reader.

21 Store plate in the dark at **Room temperature** .

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