Protocol for antigen labeling in eukaryotic cells and quantification by flow cytometry

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Abstract

Goal:

This document aims to standardize the protocol used for labeling intracellular or extracellular antigens in eukaryotic cells, using antibodies already associated with fluorochromes, and quantifying them by flow cytometry.

General considerations:

1. It is fundamental to clean the environment and to be careful when handling samples. Wear long-sleeved lab coats and use nitrile gloves in all steps of the protocol.
2. All centrifugations mentioned in this SOP should be performed at 1,400 RPM, which is equivalent to approximately 400 x g in the Centrifuge Beckman GS-6R Centrifuge with the GH 3.8 rotor (plate adapter; max radius approximately 163mm), for 5 minutes.
3. All incubations and centrifugations should be performed at 4ºC. It is important to protect samples from light after addition of antibodies conjugated to fluorescent molecules.
4. Previous to the assay, you should determine whether the cytometer is ideal for the combination of the desired fluorochromes, given the different fluorescence channels and lasers that the equipment has.
5. Each fluorochrome that is used in the experiment should be submitted to fluorescence compensation. Compensation is the correction of the overlap of fluorescence emitted by a fluorochrome, which ensures that the fluorescence detected by the equipment is derived from the fluorochrome being analyzed. In order to achieve that, a cell suspension labeled with only one fluorochrome is used at a time. If the target molecule to be labeled by the fluorochrome is poorly expressed by the cell, it is advisable to look for another target. For compensation you should choose highly expressed cell targets and preferably have a positive and a negative population.

Procedures:

1. Prepare the solutions to be used:

1.1 PBS 1X;

1.2 inactivated AB human plasma;

1.3 Washing Solution: 1% BSA; 1mM EDTA in PBS 1X;

1.4 Blocking Solution: 1% BSA; 5% human inactivated AB plasma in PBS1x;

1.5 Permeabilization Buffer: 0.1% of Saponin in Wash Solution;

1.6 Fixing Buffer: 2% Paraformaldehyde in PBS.

2. Depending on the cell type, follow one of the three steps:

2.1 If the labeling will be performed in previously non-stimulated PBMC, immediately after processing, the required number of cells must be transferred to 1.1 ml microdilution tubes (new Axygen Mini Tubes).

2.2 In the case of a non-adherent cell culture, the cells should be transferred to the microdilution tubes.

2.3 In the case of adherent cells or PBMCs in culture, the cells should be released adding EDTA 5mM at 4 °C to the culture plate and pippeting using uninterrupted movements, for 5-6 times per well. This method does not require inactivation of the cell lifting solution. Do not forget to check the efficiency of the process under the microscope. Transfer the suspension to 1.1 ml microdilution tubes, keeping it at 4 °C.

3. Centrifuge at 4 °C and discard the supernatant manually by pouring the tube (always taking care not to lose the pellet), so that about 10 - 50 µL of supernatant remains in the tube (check that the pellet is still in the tube). After centrifugation, store the supernatants for future analysis in 0.2 ml microtubes.

4. If you are using viability dye, proceed to step 5, otherwise proceed to step 9;

5. Add 400 µL of PBS1x, centrifuge and discard the supernatant as in step 3. Repeat this step;

6. Vortex the samples until the pellet is no longer visible;

7. Add 1µL of the Live/Dead® viability dye to every $1x10^6$ cells, add 200uL of PBS1x, vortex the samples, and then add 800 µL of PBS1x. Incubate for 30min at 4 °C;

8. Centrifuge and discard the supernatant, add 400 µL of PBS1x and centrifuge again, discarding the supernatant at the end. Repeat this step;

9. Add 400 µL of wash solution, centrifuge and discard the supernatant;

10. Add 200 µL of blocking solution to the cell suspension and incubate for 30 minutes at 4 °C.

During the 30 minutes incubation procedure, you could start the preparation of the antibodies mix. The user can put together a "cocktail", which is a solution with all the antibodies to be used.

An example follows below for labeling 10 tubes with 3 different antibodies:

Antibody to be used	Quantity for each 10^6 cells	Number of tubes to be labeled + error	Volume of antibody to be added to the cocktail	
CD3-FITC	1 uL		= 12 uL	
CD4-PE	2 uL	x 12	= 24 uL	
CD14-PECy7	0.5 uL		= 6 uL	
	Volume to be added in each tube (sum of the column above)	Volume to be effectively used of the cocktail	Cocktail final volume (sum of the column above)	Excess volume added due to pipetting error
	3.5 uL	3.5 uL x 10 = 35uL	42 uL	42 – 35 = 7uL

The user should not forget to prepare the fluorescence compensation controls. Prior to labeling, about 500,000 cells are added in each compensation tube so that each single-labeled tube has a final volume of 50 µL. Each compensation tube should be prepared with only one fluorochrome. Reserve a tube with no labeling to set up the cytometer. For example, using the tags from the example above, we would have one tube labeled with only CD3-FITC, one with CD4-PE only, one with CD14-PECy7 and one with no labeling.

11. In the remaining volume in the tubes, add the cocktail of antibodies to extracellular antigens to each tube. The dilution or optimal concentration of the antibodies should be prepared according to the manufacturer's recommendations or determined empirically. These tubes containing the antibody mixture and stock reagents should all be on ice or on an icy surface, with as little exposure to light as possible;

12. Vortex until the pellet is no longer visible (approximately 10 seconds) and incubate for 30 minutes at 4 °C in the dark;

13. Add 400 µL of washing solution to the cells, centrifuge and discard the supernatant as described in step 3.

14. Vortex the cells in the remaining volume. Check that there are no lumps in the tube before adding the fixing buffer;

15. Add 200 µl of buffer. Incubate for 20 minutes in the dark;

16. After this time, add 400 µL of washing solution, centrifuge and discard the supernatant.

Vortex;

17. Add 200 µL of wash solution to the cell suspension and read immediately on the flow cytometer (for samples with no intracellular antigen labeling) or store the samples in wash solution for up to 2 days at 4 °C in the dark until analysis with the flow cytometer (or to initiate the intracellular antigen labeling).

OBS: If the user is to perform intracellular antigen labeling, proceed to step 18. Otherwise, the user should stop at step 17.

Labeling for Intracellular Antigens using antibodies conjugated to fluorochromes.

18. Add 400 µL of permeabilization buffer. Centrifuge and discard the supernatant as in step 3;

19. Vortex for 30 seconds until dissolution of the pellet;

20. Add 400 µL of permeabilization buffer to the cell suspension. Incubate for 10-15 minutes at room temperature in the dark;

21. Centrifuge and discard as in step 3;

22. Add the desired cocktail of fluorochromes-conjugated antibodies to intracellular antigens to each tube. Vortex until the pellet is no longer visible;

23. Incubate for 30 minutes at room temperature in the dark;

24. Add 400 µL of permeabilization buffer to the cell suspension. Centrifuge and discard the supernatant as mentioned above;

25. Add 200 µL of washing solution;

26. Analyze immediately or within 18 hours on the flow cytometer.

OBS1: All remaining solutions should be stored at 4 °C for further use.

References:

This protocol was adapted from: Reis, S.R.N.I. et al; An in vitro model for dengue virus infection that exhibits human monocyte infection, multiple cytokine production and dexamethasone immunomodulation; Mem Inst Oswaldo Cruz; Rio De Janeiro; Vol 102(8): 983-990; 2007

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