**Antibody Staining of Surface Antigens in a 96 Well Plate in Flow Cytometry**

Written by: Anni Skovbo

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This guideline works in connection with the FACS Core Facility Guidelines

* Panel Design in Flow Cytometry (1)
* Titration in Flow Cytometry (2)
* Controls in Flow Cytometry (3)
* Compensation in Flow Cytometry (4)

**Notes**

This is a general guideline based on staining mononuclear cells from human peripheral blood.

It does not concern specific antibodies; however, it takes into consideration: Incubation, washing and fixation.

For other cell types you may need to adjust the procedure.

For some antibodies/stains you may need to adjust the procedure.

If you work with V bottom plate, be aware if you need to use smaller volumes and increase the amount of washes.

We strongly recommend that you evaluate if blocking is essential for your experiment staining (5). It depends on the cell type you are working with. Different subpopulations of human blood cells have Fc receptors, which can cause unspecific staining. If in doubt, make a parallel experiment with and without blocking.

In this protocol the antibody incubation is performed at room temperature (RT). If you need to incubate on ice, the incubation time should be expanded to 30-60 minutes and the centrifuge should be cold (+4°C).

In each well there will be approximately 15µl left after pouring off the supernatants from a round bottom plate.

Be aware of safety rules concerning human material and the fixation buffer.

**Materials**

1. Stain buffer/wash buffer: PBS pH 7.4 with 0.5% BSA or 2% FBS.
Na-azide can be added (0.09% ) to prevent capping and shedding of the antibodies when you do not incubate cold.
2. Cells at 1-10x106/ml in stain buffer.
3. Blocking Reagent: Human Ig or commercial Fc Block (5).
4. Fixable viability stain.
5. Fluorochrome conjugated antibodies.
6. A round bottom 96 well plate.
7. Fixation buffer: PBS pH 7.4 with 0.9% formaldehyde.
8. PBS pH 7.4.

**Procedure**

Be cautious at any time not to mix fluid from one well to another when pouring fluid off.

Even a tiny bit of antibody can stain your cells.

1. Make a scheme showing which antibodies in which wells.
2. Adjust your cell suspension to 1-10x106/ml in stain buffer.
3. Blocking can be performed now: Add blocking reagent. E.g human Ig, 100ug/ml cell suspension, mix and incubate at +4 °C for at least 15 minutes.
4. Add antibodies to the wells.
5. Transfer 100 µl cell suspension to the blank well(s) before you add viability stain to the rest of the cell suspension.
6. Add viability stain to the rest of the cell suspension and whirl mix immediately (to avoid absorbtion of the viability dye to proteins in the buffer).
7. Transfer 100 µl cell suspension to each well and pipette up and down 3 times to mix cells and antibodies.
8. Incubate in the dark 15-30 min at RT.
9. Add 100 µl wash buffer to each well if there is room for this volume in the wells. Otherwise you can skip this point.
10. Centrifuge the plate at 350xg for 2 min at RT (or cold).
11. Place a thick paper tissue flat on the table next to the sink.
12. Pour off the supernatant in one sliding movement into the sink and press the plate gently, firmly and briefly against the paper tissue before turning the plate bottom downwards again.
13. Loosen the cell pellets by gentle knocking on the side of the plate.
14. Add 200 µl wash buffer to each well using a multichannel pipette, resuspend cells cautiously using the pipette.
15. Repeat 10-14.
16. Then repeat from 10-13 (makes a total of 3 washes ending up with pellets).
17. Fix and resuspend the cells by adding 200 µl fixation buffer to each well and pipette up and down 10 times immediately. Avoid bubbles.
18. Incubate for 30 min. at RT in the dark.
19. Centrifuge the plate at 400xg for 2 min.
20. Place a thick paper tissue flat on the table next to a wide container in the fume hood. Place som paper tissues in the container to avoid splashing.
21. Pour off the supernatant on the paper tissues in the container in the fume hood in one sliding movement and press the plate gently, firmly and briefly against the paper tissue next to the container before turning the plate bottom downwards again.
22. Add 100-250 µl PBS and resuspend using the pipette.
23. Place a lid on the plate and keep it cold and dark until analysis.
24. The use of tandem dyes will limit the storage time.
We strongly recommend analysing as quickly as possible.

**References:**

1. [FACS Core Facility Guidelines, Panel Design in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
2. [FACS Core Facility Guidelines, Titration in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
3. [FACS Core Facility Guidelines, Controls in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
4. [FACS Core Facility Guidelines, Compensation in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
5. Blocking: Elimination of erroneous results in flow cytometry caused by antibody binding to Fc receptors on human monocytes and macrophages by Andersen et al 2016, [Cytometry A.](https://www.ncbi.nlm.nih.gov/pubmed/27731950) 2016 Nov;89(11):1001-1009

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