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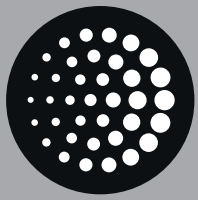
Protocol

FluoroSpot

General guideline for the analysis of analyte-secreting cells with FluoroSpot.

Please consult the analyte-specific datasheets for recommended concentrations and cell incubation times.



Protocol Introduction

This FluoroSpot protocol describes the steps for detection of cells secreting one or multiple analytes. When combining several analytes, cell numbers and incubation times need to be considered.

A Coating (sterile conditions)

1. In the same tube, dilute the capture antibody/antibodies in sterile PBS as specified in the datasheets.
2. Pre-wet the membrane of a FluoroSpot plate with 35% EtOH, 15 µl/well, for maximum 1 minute. It is essential that the membrane does not dry after the treatment.
3. Wash the plate 5 times with sterile water, 200 µl/well.
4. Add the capture antibody solution, 100 µl/well, and incubate overnight at 4-8 °C.

B Cell Incubation (sterile conditions)

Freshly prepared and cryopreserved cells may be used in the assay. Cryopreserved cells should be rested for ≥1 hour at 37 °C to allow removal of cell debris. Recommendations for cell numbers and incubation times are listed in the datasheet.

1. Wash the plate 5 times with sterile PBS, 200 µl/well.
2. Add 200 µl/well of cell incubation medium supplemented with e.g., 10% fetal calf serum to block/condition the plate. Alternatively, serum-free medium with a rich protein source may be used. Homologous serum is not recommended. Incubate ≥ 30 minutes at room temperature.
3. Remove the medium from the wells and add stimuli followed by the cell-suspension. Cells and stimuli can also be mixed before addition to the plate.

   When applicable, add a co-stimulatory anti-CD28 antibody together with the stimuli of interest.
4. Incubate the plate at 37 °C in a humidified incubator with 5% CO_2. Do not move the plate during incubation and avoid evaporation e.g., by wrapping the plate in aluminum foil.
5.

C Detection

For optimal results, filter the working dilutions of all antibodies and conjugates throughout the assay using a 0.2 µm low protein binding filter.

1. Remove the cells by emptying the plate and wash 5 times with PBS, 200 µl/well. An ELISA plate washer can be used, provided that the washer head has been adapted to FluoroSpot and ELISpot plates. Do not include Tween or other detergents in the buffer.
2. In the same tube, dilute the detection antibody/antibodies in PBS containing 0.1% BSA (PBS-0.1% BSA) to the concentrations specified in the datasheet. Add 100 µl/well and incubate for 2 hours at room temperature.
3. Wash the plate 5 times with PBS, 200 µl/well.
4. In the same tube, dilute the fluorophore-conjugated reagents in

PBS-0.1% BSA to the concentrations specified in the datasheet. Add 100 µl/well and incubate for 1 hour at room temperature.

5. Wash the plate 5 times with PBS, 200 µl/well.

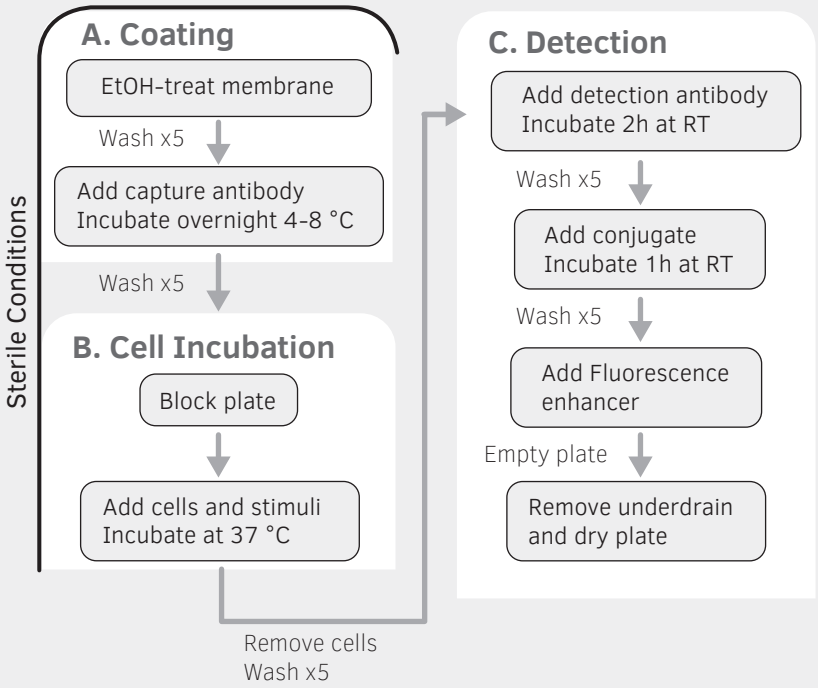
6. Empty the plate and add Fluorescence enhancer, 50 µl/well, and leave the plate for 5-15 minutes at room temperature.

7. Remove the Fluorescence enhancer by flicking the plate. Do not wash. It is important that the plate is not tapped against paper towels since dust particles may interfere with the assay.

8. Remove the underdrain (the soft plastic under the plate). Dry the plate protected from daylight. The plate should be completely dry before analysis. Store plate in the dark at room temperature.

9. Analyze spots with an automated FluoroSpot reader equipped with filters for the fluorophores used. Filters should have high specificity to avoid bleed-through artifacts. Spots from cells secreting multiple analytes are identified by coordinates i.e., based on spot position. Fluorescent spots may fade with time and exposure to light and it is recommended that the plate is analyzed within one week of development.

FluoroSpot Overview



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