Intracellular Cytokine Staining

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1 Works for me Share dx.doi.org/10.17504/protocols.io.sqdeds6

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ABSTRACT

This protocol outlines the steps used for Intracellular cytokine staining.

In brief: Cells were treated with protein transport inhibitor containing Brefeldin A (GolgiPlug; cat: 555029, BD) and plated at 1e6 cells per well in a 96-well flat bottom plate. Cells were stimulated for 5 hours at 37°C with either T cell media, Phorbol 12-Myristate 13-Acetate (Sigma, cat: P8139) (used at 20ng/mL) and Ionomycin (Sigma, cat: I0634)(used at 1mg/mL), or 1mg/mL of peptide (as indicated). $Msln_{406-414}$ (GQKMNAQAI), OTI (SIINFEKL), $LCMV_{gp33-41}$ (KAVYNFATM), $MSLN_{20-28}$ (SLLFLLFSL), and $MSLN_{530-538}$ (VLPLTVAEV) peptides were ordered from ELIM peptide (>80% purity). The BD Fix/Perm kit (cat: 554714) was used for intracellular staining. Cells were fixed in 0.5% paraformaldehyde until data acquisition.

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MATERIALS TEXT

MATERIALS

Phorbol 12-myristate 13-acetate (PMA) **Sigma Aldrich Catalog #P8139**

Ionomycin calcium salt from Streptomyces conglobatus **Sigma Aldrich Catalog #I0634**

96-well Cell Culture Plate, Flat-Bottom with Lid, polystyrene **Corning Catalog #3599**

96-well Cell Culture Plate, round-bottom with lid **greiner bio-one Catalog #650 180**

1 Prepare a flat bottom 96 well plate. Resuspend cells at 10e6 cells/ml in T cell media. Transfer at least 350ul of cells per condition into a fresh tube (you want at least 3 replicate wells at 100ul/well for each condition).

2 Dilute Golgi Plug to 1:50 (2mg/ml) in cells for the experiment. (Once the treatment condition is added the final concentration will be 1:100 = 1mg/ml)

3 Plate 100ul of cells per well.

4 Dilute the PMA (prepare at 40ng/mL PMA so that the final concentration in the assay will be 20ng/mL) and Ionomycin (prepare at 2mg/mL Ionomycin so that the final concentration in the assay will be 1mg/mL) in one tube of media. Resuspend peptide at 2ug/100ul.

5 Add 100ul of media or PMA/I or peptide to each well. Incubate 4-6 hours in 37°C incubator.

6 Transfer cells to U-bottom 96 well plate.
- at this step, you can combine the replicate wells if you were low on cells.

7 Centrifuge (Cfg) plate at 4^{O}C for 2 min @809*xg* (with high brake) + dump supernatant.

8 Add 50ul Live/Dead stain per well (dilute 5ul of dye in 2.5ml 1x DPBS).

9 Incubate for 20-30 minutes in fridge at 4^{O}C (dark).

10 Add 100 ul of FACS Buffer per well to wash. Cfg plate at 4^{O}C for 2 min @809*xg* (with high brake) + dump supernatant.

11 Prepare cocktail of antibodies for cell surface stain. Add 50-75uL of surface stain mix per well. Resuspend pellet by pipetting up + down at least 5x.

12 Incubate plates 30 min in fridge at 4^{O}C (dark).

13 Add 100 ul of FACS Buffer per well to wash. Cfg plate at 4^{O}C for 2 min @809*xg* (with high brake) + dump supernatant.

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14 Add 150 ul of FACS Buffer per well to wash a second time. Cfg plate at 4^{O}C for 2 min @809*xg* (with high brake) + dump supernatant.

15 Add 150 ul of FACS Buffer per well to wash a third time. Cfg plate at 4^{O}C for 2 min @809*xg* (with high brake) + dump supernatant.

16 Add 100 ul of Fixation/Permeabilization solution per well. Resuspend pellet by pipetting up + down at least 5x. Incubate plate 20 minutes at 4^{O}C in fridge (dark).

17 Add 100 ul of Perm/Wash Buffer (PWB) per well to wash. CHANGE BREAK to SETTING 2 for all remaining centrifugation steps.

18 Cfg plate at 4^{O}C for 2 min @809*xg* (with brake = 2) + dump supernatant.

19 Add 150 ul of Perm/Wash Buffer per well to wash a second time. Cfg plate at 4^{O}C for 2 min @809*xg* (with brake = 2) + dump supernatant.

20 Add 150 ul of Perm/Wash Buffer per well to wash a third time. Cfg plate at 4^{O}C for 2 min @809*xg* (with brake = 2) + dump supernatant.

21 Prepare cocktail of antibodies in PWB buffer for Intracellular stain. Add 50-75uL of intracellular stain mix per well. Resuspend pellet by pipetting up + down at least 5x.

22 Incubate plate 30 minutes at 4^{O}C in fridge (dark).

23 Add 100 ul of Perm/Wash Buffer (PWB) per well to wash. Cfg plate at 4^{O}C for 2 min @809*xg* (with brake = 2) + dump supernatant.

24 Add 150 ul of Perm/Wash Buffer per well to wash a second time. Cfg plate at 4^{O}C for 2 min @809*xg* (with brake = 2) + dump supernatant.

25 Add 150 ul of Perm/Wash Buffer per well to wash a third time. Cfg plate at 4^{O}C for 2 min @809*xg* (with brake = 2) + dump supernatant.

26 Resuspend in 200 ul of 0.5% PFA (can leave overnight at this step). Store in fridge at 4^{O}C until acquisition. Run samples on the flow cytometer within 24 hours for best results.

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