K. Ohmura / 03

Intracellular cytokine staining Protocol

- Prepare cells at a concentration of $1x10^6$ / ml in 24 well-plate
- Add 10 µl PMA/Ionomycin mix per 1ml of cells and incubate for 2 hrs. at 37°C
- Add 10 µl of Brefeldin A (1mg/ml) per ml of cells and incubate for 2 hrs. at 37°C
- Harvest cells on ice and centrifuge at 1200 rpm x 5 mins. at 4 °C
- Add ice-cold PBS and centrifuge at 1200 rpm x 5 mins. at 4 °C
- Suspend cells in 0.1 ml of 1x PBS and stained with CyChrome-CD4 (0.25 ul/$1x10^6$) for 20 min.
- Wash with 1xPBS once
- Suspend cells in 1 ml of 1x PBS, and prepare at 2 x 10^6 cells /ml in ice cold PBS
- Add an equal volume of 4 % Formaldehyde (in PBS) and mix well.
- Leave the cells at room temperature for > 20 min
- Add ice-cold PBS and centrifuge at 4 °C
- Suspend in PBS/BSA/Azide buffer (2-5 x 10^5 cells / 200µl).(Here you can store cells for up to 2 days)
- Plate out 2-5 x 10^5 cells / well in 96 well U bottom plate (Costar Cat # 3799)
- Centrifuge at room temperature at 1200 rpm x 5 min.
- Quickly flick out sup. once into sink and immediately blot plate dry and turn right side up.
- Add 150µl of Permeabilization Buffer per well and mix gently.
- Leave the plate at room temperature for 10 min.
- Centrifuge the plate and quick flick out sup……

 Add 25 µl of Permeabilization Buffer containing antibodies.

1 : no antibodies
2: FITC-isotype ctr, PE-isotype ctr
3: FITC-IFNg (0.25 ul), PE-IL-4 (0.25 ul)
4: FITC-IL-2 ( ), PE-TNFa ( )
5: FITC-IL-10 ( ), PE-IL-5 ( )

 incubate for 30 min at room temperature in the dark

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 Add 150 µl of Permeabilization Buffer / well

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Centrifuge

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 Flick out sup…… and add 150 µl of Permeabilization Buffer / well

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Centrifuge

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 Flick out sup…… and add 150 µl of PBS/BSA/Azide Buffer (no Saponin) / well

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Centrifuge

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 Flick out sup…… and add 150 µl of PBS/BSA/Azide Buffer / well

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FACS assay

Regents and solutions

PMA/Ionomycin mix

PMA: Sigma #P-8139
Ionomycin: Calbiochem #407952

PMA stock :5 mg/ml in DMSO, aliquots of 50 ul, store at –80C)
Ionomycin stock: 1mM in DMSO

Dilute 25 ul of PMA in 2.5 ml of culture media (10%FCS) to make a stock of 50 ug/ml (**diluted PMA**)
Mix 20 ul of diluted PMA (final 5 ug/ml)
13.5 ul of ionomycin (final 50 ug/ml)
166.5 ul of culture media

add 10 ul of PMA/Ionomycin mix per 1 ml of cells (Final concentrations of PMA and Ionomycin in media will be 50 ng/ml and 500 ng/ml, respectively).

Brefeldin A (BFA)

Dissolve 1 mg of BFA (Sigma #) in 1 ml of 100% EtOH, and store at –80C.
Add 10 ul of BFA per 1 ml of cells

PBS/BSA/Azide buffer

1 liter of PBS pH7.4
650 ul of 10% Azide
Layer 5g of BSA on top of the liquid mixture. Do not shake the bottle. Allow BSA to dissolve at room temperature, without stirring. Sterile filter the mixture. Store at 4C.

10 % Saponin:

Mix 5g Saponin (Sigma #S-7900)
50 ml PBS pH7.4
Place at 37C until the saponin has dissolved completely.
Filter the solution through a 0.22 micron filter
Store the solution at 4C.

Permeabilization Buffer

Mix 5 ml 10% Saponin
95 ml PBS/BSA/Azide buffer