

INTRACELLULAR CYTOKINE STAINING PROCEDURE

-Prepare Media from stocks.

Stocks:

- Ionomycin** - 2.5 mg/ml in DMSO - stored -80°C
10µl stock → 40µl Clone Media = diluted ionomycin (500µg/ml)
Calbiochem cat #542400
- PMA** - 2.5 mg/ml in DMSO - stored -80°C
10µl stock → 490µl Clone Media = diluted PMA (50µg/ml)
Calbiochem cat #407952
- Monensin** - 2mM in ETOH - stored -20°C - Sigma cat#M5273
- Good IX PBS** - stored room temperature - lab prepared
- FBS** - stored -20°C - Gemini Bio Products cat #100-106
- NaN₃** - 20% - stored room temperature - lab prepared
- CytoFix/CytoPerm Kit**: - stored 4°C - Pharmingen cat#2075KK
- DNase** - 1mg/ml in PBS - stored -20°C - Sigma cat#D5025
- EMA** - stored -20°C - Molecular Probes cat#E1374
- Para-formaldehyde** - 10% - stored -20°C - lab prepared

Clone Media:

- RPMI 1640 - w/o L-glutamine (500ml)
- 10% FCS (50ml)
- 2mM L-glutamine (5ml)
- 10mM Hepes (5ml)
- 25µg/ml gentamicin (1ml)
- 50µM 2-ME (0.5ml)
- 2U/ml IL-2 (0.85ml)

Stock Solutions

- FCS (HyClone)
- L-glutamine - 200mM
- Hepes buffer - 1M
- gentamicin sulfate - 12.5mg/ml
- 2-ME - 50mM
- IL-2 - 1200U/ml

Stimulating Media: 50µl diluted ionomycin (50µg/ml)
5µl diluted PMA (0.5µg/ml)
445µl Clone Media
0.5ml total volume - use immediately

(Hint: Add 485 µl Clone Media to ionomycin vial; add 490 µl Clone Media to PMA vial, mix and transfer 5µl of diluted PMA to ionomycin vial yielding the final solution.)

Stain Media: 1L Good 1X PBS
30ml FBS (3%)
2ml NaN₃ stock (0.04%)
approx 1L total volume - store 4°C - 6 months

PBS-A 1L Good 1X PBS
2ml NaN₃ stock (0.04%)
approx 1L total volume - store RT - 1 year

DNase Solution PBS-A containing 10% stock DNase (0.1mg/ml)
(1ml per sample - make an extra dose)
Must be made fresh.

EMA Staining Mix Stain Media + 0.04% EMA
(250µl per sample - make an extra dose)
Must be made fresh - protect from light at all times.

CytoFix/CytoPerm: Pharmingen:

Cytofix/Cytoperm: pre-prepared
Perm/Wash Buffer: dilute 1:10 in ddH₂O

Final Fixing Buffer: 1ml para-formaldehyde stock (1%)
9ml Stain Media
10ml total volume - use immediately - light sensitive

Isolation / Preparation of Spleen Cells

Collect spleens from mice and place each in a small petri dish containing 5ml Clone Media.

Isolate lymphocytes using the syringe puncture method.

Wash and lyse rbc's with ACT.

Wash with Clone Media and resuspend in 2ml Clone Media.

Place exactly 1ml in one well of a 12 well plate.

Add an additional 1ml of Clone Media to the well.

Can store spleen suspensions in 12 well plate overnight in refrigerator.

Use remaining spleen cells for phenotype FACS if desired - wash with Staining Buffer to remove Clone Media.

Stimulation of Spleen Cells

Prepare Stimulating Media.

-Add 10 μ l Stimulating Mix per 1ml of cell suspension (ie 20 μ l/well).

Note: final concentrations should be 5ng/ml PMA and 500ng/ml ionomycin.

-Incubate at 37°C for 3 hours.

-Add 1 μ l Monensin stock per 1ml cell suspension (ie 2 μ l/well).

Note: final concentraion should be 1 μ M Monensin.

-Incubate at 37°C for 2 hours.

Remember to thaw out Th1 and Th2 positive cells to treat along with spleen cells in the following steps.

-Place cells into FACS tubes containing 1ml Stain Media and centrifuge at 1200rpm for 5 min.

-Resuspend in 1ml DNase Solution.

-Incubate in 37°C water bath for 10 minutes.

-Wash 1x with Stain Media.

Remove approximately 10ul of cells from one sample's cell pellet to be used for unstained and single color controls

-Prepare EMA staining mix - protect from light.

-Resuspend each sample in 250 μ l EMA Staining Mix.

-Incubate on ice PROTECTED FROM LIGHT for 15 minutes.

-Expose to fluorescent light for 10 minutes - place on bench under bench-top fluorescent light - approx 12-18 inches away.

-Wash in 3ml Stain Media.

-Resuspend each pellet in 200 μ l CytoFix/CytoPerm solution.

-Incubate in refrigerator or on ice PROTECTED FROM LIGHT for 20 minutes.

-Wash 2 times in 1ml Perm/Wash Buffer.

-Resuspend cell pellet in 50 μ l Perm/Wash Buffer X number of rxns.

-Remove some cells for EMA single color control.

-Distribute 50 μ l into individual tubes or wells.

-Add antibody mixes to appropriate wells.

- Incubate in refrigerator for 30-60 minutes.
- Wash 2x with CytoPerm Wash Buffer..
- Resuspend in 50 μ l Final Fixing Buffer.
- Place cell suspension in labeled FACS tubes containing 100-200 μ l Stain Media prior to running on FACSCalibur.