Institut für HIV Forschung
SOP#10-00 **(April 2015)**

CFSE Staining Protocol

Reagent	Manufacturer	Order Number
RPMI-1640	Sigma	R0883
Pen/Strep (5000 IU Pen/ 5000ug/mL Strep)	Cellgro	30-001-Cl
L-glutamine (200mM; 29.2 mg/mL)	Cellgro	25-002-Cl
HEPES (1M; 238.3mg/mL)	Cellgro	25-060-Cl
FBS, Heat-inactivated	Sigma	F4135 (we have tested lot for the lab)
PBS	Sigma	D8537
5(6)-CFDA, SE; **CFSE** (5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester), mixed isomers	Life Technologies	C1157

Media:

R10:

Reagent	Stock Concentration	Volume to Add	Final Concentration
RPMI 1640	-	500	-
FBS	100% (v/v)	55ml	10% (v/v)
Penicillin/Strep	5000 IU/mL Pen 5000ug/mL Strep	5.5mL	50 IU/mL Pen 50ug/mL Strep
L-glutamine	200mM	5.5mL	2mM
HEPES	1M	5.5ml	10mM

- Each new stock of CFSE should be diluted to a 10mM concentration and titrated for optimal staining time (between 5-10 min)
- 10mM aliquots of CFSE are found in the -20°C freezer.

Protocol:

1. Incubate PBMCs isolated either from Ficoll centrifugation or from a frozen aliquot them in R10 at 37°C for 3-6 hours prior to staining/stimulation. They can also be left to rest overnight.
2. Warm R10 and a 20mL aliquot of PBS to 37°C
3. Keep a 20mL aliquot of R10 at 4°C
4. Spin cells at 350xg for 5 min. Aspirate supernatant
5. Add 20mL warm PBS to cells and spin at 350xg, 5 min. Aspirate supernatant
6. Resuspend cells at $1x10^7$ cells/mL in warm PBS
7. Thaw CFSE (use aliquot only once, repeated freeze/thaw decreases staining and increases the background.) and dilute 10mM stock solution to 100µM in warm PBS. Vortex to mix solution.

8. Add enough of the 100µM CFSE dilution to bring the final concentration of the cell suspension to 0.5µM CFSE. Mix with either gentle pipetting or inverting the tube. DO NOT vortex.
9. Incubate 5-10min at 37°C, 5% CO_2 in incubator. Length of time depends on CFSE stock. Be sure to titrate new CFSE lots prior to use
10. Add at least 3 volumes cold FCS to quench reaction.
11. Add ≥3mL cold R10 to wash. Spin at 350xg, 5 min. Aspirate supernatant.
12. Wash cells with 2mL warm R10. Spin again 350xg, 5 min. Aspirate supernatant.
13. Resuspend in warm R10 (1-2 x 10^6 cells/mL) and aliquot.
14. In 96-well round bottom plate, aliquot 150,000-200,000 cells/well
    a. When aliquoting cells into plates, never use outside wells. These tend to do worse and outside wells should be filled instead with PBS to maintain humidity inside the plate.
15. Add antigens/stimulants and be sure to include a positive and negative control (stimulated with superantigen and unstimulated respectively).
16. You may add costim molecules (CD28/49d), but this may increase background.
17. Incubate 7 days at 37°C, 5% CO_2 in incubator
18. It is advised that you refresh the media on day 3 by carefully aspirating half the medium (without disturbing the cells on the bottom) and replacing it with fresh, warm media.
19. When performing flow cytometry, avoid using PE and PerCP as CFSE is hard to compensate with these channels.