CFSE/CFDA-Treg Suppression Assay
(M. D'Alise, J. Hill 2013)

1. **Prepare fresh "complete" RPMI media**
   500 ml RPMI-1640
   50ml FCS Heat inactivated (10% final)
   50 uM 2-mercaptoethanol
   5 ml L-Glutamine (Hyclone Cat. No. SH30034.01)
   Antibiotic cocktail (Pen/Strep, 5ml)

2. **Harvest & stain Splenocytes/Lymphocytes**
   - harvest fresh spleen and prepare single cell suspension by filtering through 40uM mesh filter or using glass slides then centrifuging 1500rpm for 5min
   - Resuspend cell pellet in 500uL ACK lysis buffer for 5min on ice to remove RBCS
   - wash 1x with complete RPMI media then centrifuging 1500rpm for 5min
   - Fluorescent-label using the following antigen targets (using Foxp3-reporter mice is an improved method to collect Tregs vs. Tconvs and the mAb stain must be adjusted accordingly)
     - CD4
     - CD25
     - CD90.2
     - CD8a/B220/CD11b/CD11c ("dump" channel)
   - incubate 20min on ice then wash 2x in complete RPMI
   - filter cells prior to cell sorting

3. **Cell Sorting**
   - (optional) use MACS depletion with beads to remove the "dump" channel
   - Sort Treg and Tconv cells as B220-CD8-CD11b-CD11c-CD4+ and either **CD25hi (Treg)** or **CD25-(Tconv)** (Moflo) or as CD4+GFP+Treg and CD4+GFP-Tconv cells from Foxp3-reporter mice. APC cells will be sorted as **CD90.2 negative** cells. Sorted cells will be collected in complete media.
   - Based on sorted cell s#, resuspend cells at **10^6 cells/ml**

4. **CFSE/CFDA Labeling of cells (Tconv)**
   CFSE/CFDA should be prepared in DMSO and stored in small aliquots at –20 (or -80). Cells which incorporate too much CFSE, will have reduced viabilty, so it is important to wash well.
   Reagent :Molecular probes Cat # V12883 VybrantR CFDA SE Cell Tracer Kit
   - Dilute CFDA stock (10mM) to 1:500 in media to 20uM e.g. 4uL stock to 2mL media
   - Add working dilution to cells you desire to label at final concentration of 10uM
   - incubate 37*C for 15min
   - centrifuge 1500rpm for 5min
   - resuspend pellet in 100uL 35% BSA
   - add 1mL cRPMI
   - centrifuge and wash 3x then resuspend at **10^6 cells/ml**

5. **Plating & Incubation**
   - Titrate Treg carefully in a round bottom 96 well plate .Use for the higher ratio (1:1 Treg:Tonv) at least 40-50k cells of Treg cells.
   - Activation of T conventional cells can be performed in presence of CD90.2 negative cells (APC) plus anti-CD3 Ab **(METHOD 1)** or in presence of anti-CD3/28 coated beads (**METHOD 2**)**.**

   METHOD 1:
   - Mix T conv and APC (CD90.2 -) cells at ratio 1-1. Before plating your cells, add the anti CD3 antibody (Anti-mouse CD3e, Clone 145-2C11) at a final concentration of 1.5 ug/ml.
   - Add the mixed Tconv-APC cells (100ul each well, which contains **50K** T conv, **50K** APC) to the wells in which Treg have been plated. As control of activation, remember to plate Tconv-APC cells in absence of Treg.Total volume in each well should be 150-200ul.

METHOD 2:

- Dynabeads® Mouse T-Activator CD3/CD28 (Invitrogen) can be used for T cell activation. Add 4-6 ul beads**per 10^6 cells and then plate T conv cells to the wells in which Treg have been plated. As control of activation, remember to plate Tconv cells presence of anti CD3/28 beads and in absence of Treg and.Total volume in each well should be 150-200ul.

  **The appropriate beads volume for bead-to-cell ratio = 1:1 would be determined by a titration experiment testing the optimal T cell activation, indications are also provided by Invitrogen).
- Incubate the cells at 37*C for 3 days then analyze the degree of Tconv proliferation using FACS analysis.