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- c. Wash by adding 100µl of *Wash/Perm* buffer and spin plates for 5min at 1300rpm at 4°C, flick off supernatant and vortex plates shortly
- d. Wash a second time by adding 200µl of *Wash/Perm* buffer and spin plates for 5min at 1300rpm at 4°C, flick off supernatant and vortex plates shortly
- e. Dilute antibody (check dilutions on fridge door!) for intracellular staining in Wash/Perm buffer and add 100µl of antibody solution per well. Do NOT include single stain and unstained controls! Instead, add *Wash/Perm* buffer alone to those wells!

Example: 2ml Wash/Perm buffer 20µl IFNg-FITC 20µl IL-17-PE 20µl IL-4 APC

add 100µl per well (enough for 20 wells)

- f. Incubate for 30-60min at 4°C in the fridge
- g. Wash by adding 100µl of *Wash/Perm* buffer and spin plates for 5min at 1300rpm at 4° C
- h. Flick off supernatant and vortex plates shortly
- i. Wash a second time by adding 200 μ l of *Wash/Perm* buffer and spin plates for 5min at 1300rpm at 4°C
- j. Flick off supernatant and vortex plates shortly
- k. Resuspend cells in 200µl **FACS buffer** and transfer to 1.2ml library tubes
- l. Add additional 200 μ l FACS buffer to library tubes to achieve a total volume of 400 μ l.
- m. The cells are now ready for acquisition on the FACSCalibur.

Notes:

Cell counting and adjusting the concentration is the exact method. With a little bit of experience one doesn't need to count all the sample and still gets the same quality of data and FACS plots. Here are a few rules of thumb:

Spleen: If one spleen is smashed in resuspended in 10ml, use ca 100-150µl per well.

MLN: If the MLNs from one mouse are liberase digested and resuspended in about 2-3ml, use ca $300\mu l$ per well.

LP: The more you can spare the better...