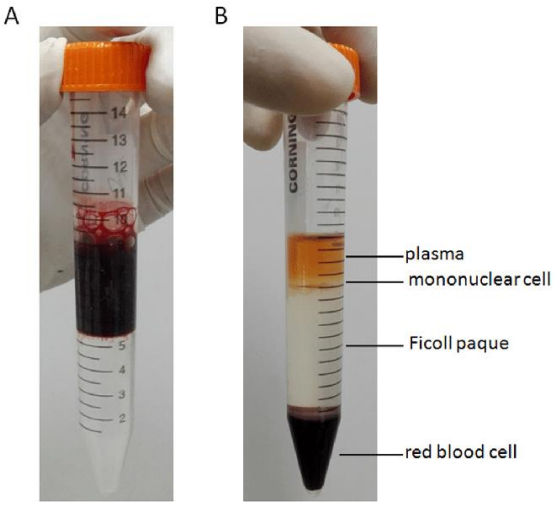
Processing cecal tonsils and spleen samples for Flow Cytometry- FACS sorting

1. Collect both cecal tonsils and spleen in 2 ml tubes with 1.5 ml RPMI and keep the tubes on ice.
2. Homogenize the tissues with a 5-ml syringe plunger through a 40 µm cell strainer after adding 3 ml of RPMI.
3. Layer the single cell suspension (3ml) on top of 3 ml of Ficoll (1.077 g/mL). make sure to cover ficoll tubes prior to use.
4. Centrifuge the overlay at 1200 x g for 10 min at 10ºC with centrifuge brakes off.
5. Collect the PBMC layer seen in the below figure



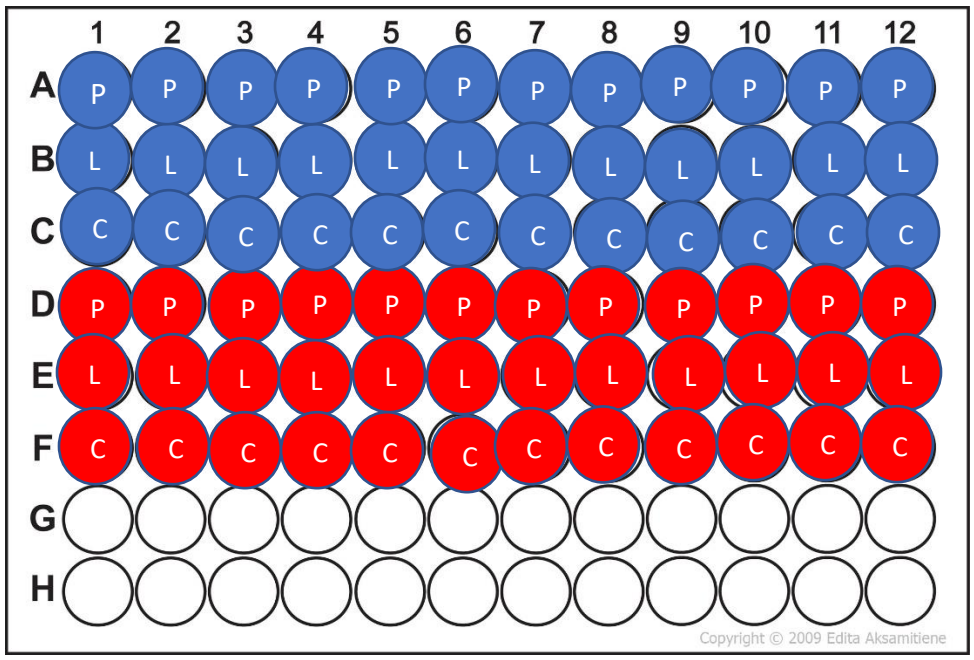
6. Wash the collected PBMCs with 5 ml of complete RPMI by centrifuging the cells at 300 x g for 10 min
7. Count the cells using hemocytometer: preferred 10:90 (Cells: Typan blue).
8. Use the excel sheet to get 0.5 ml of 10^6cells/ml.
9. Proceed to:
   - Flow cytometry
   - NO assay
   - FACS

Nitric Oxide Assay

1. Add 100 µl of 10^6 cells/ml in 96-well plate. Target cell density is 10^5 cells per well (use 6 wells per pen: 2 for LPS, 2 for *C. jejuni* and 2 for PBS)
2. Add 100 µl of LPS (10 µg/ml LPS or 1 µg/ml) to first 2 wells
3. Add 100 µl of *C. jejuni* in RPMI to 2 wells
4. Ass 100 µl of complete RPMI + PBS
5. LPS, C. jejuni or PBS should all be diluted in complete RPMI
6. Incubate for 48 hours at 37/42 ºC and 5% CO_2
7. Centrifuge plate at 2000RPM for 5 minutes
8. Remove supernatant
9. Determine nitrite concentration from a standard curve by plotting different concentration of sodium nitrite solutions against their optical density.
10. In a new flat bottom 96 well plate
    a. Add 100 µL of supernatant per well
    b. Add 100 µL of griess reagent to each well
    c. Allow the color to develop for 5 min
    d. Measure the absorbance at 540nm

11. **Preparation of nitrite standard curve**
    - Add 3.45 g of sodium nitrite to 50 ml of distilled water (1 M stock)
    - Diluted 10X in 3 series the 1M 1 ml in 9 ml of distilled water to get a 1mM or 1000 µM stock
    - Prepare the standards in duplicates as indicated below
    - Plate 100 µl of standards and 100 µl of Griess reagent allow color to develop for 5 min and then read absorbance at 540 nm
    - Plot the concentration Vs OD graph and take the formula (check for R2)

	Medium (µl)	Stock (µl)	Final concentration (µM)
1000	500	500	500
500	500	500	250
250	500	500	125
125	500	500	62.5
62.5	500	500	31.25
31.25	500	500	15.625
15.625	500	500	7.8125
0	500		0

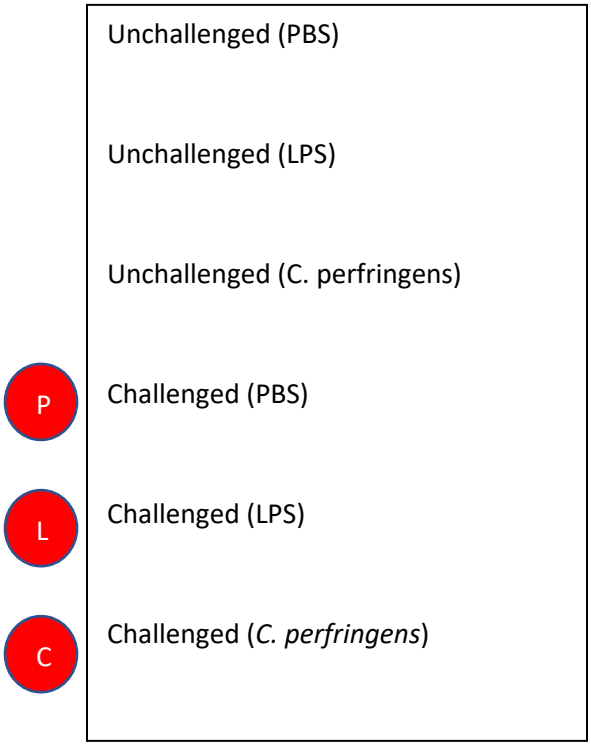


CD4+/CD8+ plan

p

L

C


Unchallenged (PBS)
Unchallenged (LPS)
Unchallenged (C. perfringens)
P
Challenged (PBS)
L
Challenged (LPS)
C
Challenged (C. perfringens)