

Murine Splenocyte Class-Switch Recombination

I. Sample Collection & Class-Switch Recombination

MATERIALS –

DPBS	Trypan-blue dye [Gibco, Cat. No. 15250-061]
Sterile pipette tips (uL & mL)	α -mouse CD40 (HM40-3)
Sterile glass pipettes for vacuum pump	[BD Biosciences, Cat. No. 553721]
Petri dishes (small)	IL4 (-20°C) [Peprotech, Ca. No. 2014-14]
12 well plates	└ once thawed goes in 4°C but only good for 7 days maximum
15mL conical tubes	
Sterile Cell Strainer (100uM)	
[Fisherrbrand, Cat. No. 22363549]	
RBC Lysis Buffer [Sigma, Cat. No. R7757]	

Murine B-cell culture media - filter with bottle top 0.22uM filter in hood [Corning, Cat. No. 28199-989]

500mL	RPMI 1640 1x with L-glutamine (heat inactivated) [Cellgro, Cat. No. 10-040-CV]
50mL	Fetal Bovine Serum [Atlanta Biologicals, Cat. No. S11150]
10mL	1M HEPES [Thermo Scientific, Cat. No. SH30237.01]
5mL	100x Sodium Pyruvate [Gibco, Cat. No. 1136-070]
5mL	100x MEM Non-essential amino acids [Gibco, Cat. No. 11140-076]
5mL	100x Penicillin/Streptomycin [Thermo Scientific, Cat. No. SV30010]
5mL	100x L-Glutamate (200mM) [Thermo Scientific, Cat. No. SH30034.01]
2-5uL	β -Mecaptoethanol (TC grade)

A. Sample Preparation - Day 0: *Bring B-cell media to room temp

1. Collect spleen samples using sterile technique and place on ice in PBS; keep separated and appropriately labeled in small petri dishes to prevent contamination or mixing up samples
2. Serially wash off spleens in 1xDPBS (4-6 times)
3. Crush up spleen in filter in any amount of cell culture media in a petri dish & sift to remove debris
4. Move cells from the petri dish into a 15mL conical tube and adjust volume to 15mL with cell culture media
 - a. Centrifuge for 5 minutes at 1000 rpms
 - b. Remove media with vacuum pump & sterile glass pipette
5. Flick cells once media is removed and add 1mL RBC lysis buffer/spleen
 - a. Incubate at RT for 5 minutes
 - b. Adjust volume to 15mL with PBS
 - c. Centrifuge for 10 minutes @ 1000 rpms
6. Remove supernatant and resuspend cells in cell culture media
7. Count cells: Add 10^6 cells/mL in each well, and amounts of stimulating reagents **FOR EACH STRAIN (or experimental treatment)**:
 - α CD40: 2ug/mL of anti-CD40 clone HM40-3 (Pharmingen or eBiosciences)
 - IL-4: 50 ng of IL4 (Peprotech, cat# 214-14)
8. Grow plates for the following in 37°C Tissue culture incubator:

Day 0	Day 1	Day 2	Day 3	Day 4
Set-up plates; stimulate & Time 0 Flow cytometry	N/A	Re-stimulate	N/A	Flow cytometry

B. Re-stimulate – Day 2:

1. Count cells:

Total cells/mL = # cells (in 5 x 5 boxes) • 2 (dye dilution factor) • 10⁴ (hemocytometer factor)

2. Adjust volume to 10⁶ cells/mL
3. Add appropriate amounts of α CD40 (1uL/mL) & IL4 (1.25uL/mL)

C. Flow Cytometry – Day 4:

1. Count cells as above (doesn't need to be done before flow analysis)
2. Flow cytometry analysis (see next page)

II. Flow cytometry Set-up

MATERIALS –

12 x 75mm polypropylene culture tubes (RNase & DNase free) [USA Scientific, Cat. No. 535-B-1]

IgG1 goat α -mouse

- PE conjugated

B220 α -mouse/human CD45R

- PE-cy5 conjugated

IgM rat α -mouse

- FITC conjugated

MACHINE ETIQUETTE

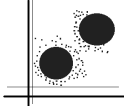

- * Clean off FACs with bleach (open 30s & closed 1 min) then water (open 30s & closed 1min)
- * If the end of the day increase the times above to 1 min & 5 min, respectively

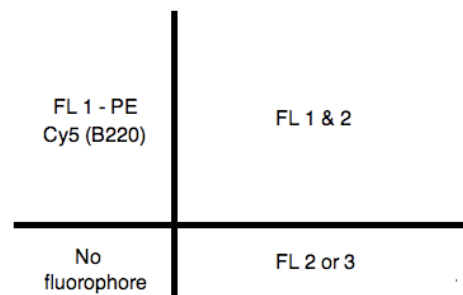
Steps 2-6 need be done on ice and in DARK

2. Spin down 1mL of each sample (need 2 tubes for each) in FACs tube for 5mins @ 1000 rpms
 - a. Decant media
 - b. Flick/drag across tube rack

- NOW READY TO STAIN
3. Mix fluorophore cocktails in FACs buffer (2% Fetal Bovine Serum in PBS):
 - IgG & B220 [1:50 – 5uL/mL & 1:100 – 10uL/1mL, respectively]
 - IgM & B220 [1:100 each – 10uL/1mL]
4. Add 100uL of fluorophore cocktails to each sample & incubate 15-30mins @ 4°C
5. Add 1mL FACs buffer to each sample & spin down for 5mins @ 1000 rpms
 - Decant supernatant
6. Add 200uL to remaining
7. Bring to FACs Caliber on ice in ice bucket with cap (see next page)

III. BD FACSCaliber

1. Check buffers: Sheath (should be full) & Waste (should not be full)
2. Turn cytometer on (if not already) and restart computer
3. Sign-in to Mills, Kevin
4. Wash with bleach solution on high for 30s (open) and then 1 min (closed)
5. Hit RUN & Wash with water for 30s (open) and then 1 min (closed)
6. Open Cell Quest Pro
7. Apple B
8. Apple 1,2,3,4
9. **Making a template:**
 - a. On the Untitled worksheet add a dot plot
 - b. Set R1 gate
 - i. SSC & FSC (Change *Analysis* to *acq* -> *Analysis* under Basic Plot on Ins: 
 - c. Duplicate
 - i. Set gates on new plots: G1 = R1
 - ii. Set fluorophores; FL 1 vs. FL 2; FL 2 vs. FL 3; FL 1 vs. FL3
 - d. Label *Untitled Parameter Settings*- fluorophores (P1-4: use sheet)
 - i. Save as settings
 - e. Under *Cytometer* at the very top choose *Instrument settings* [Use Muneers CSR settings] & click done
 - f. Set up standards: Set controls as close as possible to the middle of one another & negative control, use **voltage** or amps under the *Detectors/Amps* tab: **[B6 splenic**



cells- No fluorophore; B220; IgG; IgM].

- i. Use to set axes
 - g. Save as template
10. OR Instead of #9 open by double-clicking on actual template file
11. Load settings: *Cytometer – Instrument settings...*
 - a. Open; **SET**; Done
12. Run samples:
 - a. On *Browser* tab choose a new folder (**year-monthday, descriptor e.g. 10-0401, training**) for data being acquired under *Data export*
 - b. Set *File count & Sample ID*
 - c. Set *Counters* (under *Acquire*)
 - i. Under *Source* – change *Accept* to **Collect G1**
 - d. Put samples on and close arm immediately
 - e. Acquire each sample (make sure setup isn't checked)
13. Wash with bleach solution on high for 30s (open) and then 1 min (closed); or 1 min (open) and 5 min (closed) if the end of the day
14. Wash with water for 30s (open) and then 1 min (closed); or 1 min (open) and 5 min (closed) if the end of the day