EBV Transform B-Cells out of Frozen PBMCs

Prepare EBV Supernatant

1. Thaw a vial of B95-8 cells rapidly-10 million cells/vial-add to 50 mL RPMI warmed to 37C.
2. Centrifuge 5 min x 750 rpm at room temp. Carefully remove supernatant.
3. Resuspend cells in 10 mL RPMI/15% FCS and add to T75 flask. Incubate at 37 C 5% CO_2
4. Next day carefully remove media without disturbing cells as the bottom of the flask-add back 20 mL RPMI/15% FCS.
5. Incubate at 37 C/5% CO_2 for 5-7 days.
6. Add the 20 mL of B95-8 cells to 200 mL RPMI/15% CO_2 in T175 flask.
7. Incubate at 37 C/5% CO_2 for 7 days.
8. Harvest the supernatant and sterile filter through 0.22um filter.
9. Aliquot 3 mLs into 15 ml conical tubes.
10. Store at -80 C.

Prepare Conditioned Media (CM-PMA)

1. Thaw a vial of EBV-transformed B-cells (There are several vials of JH EBV transformed B-cells in liquid N_2) and grow in RPMI/15% FCS.
2. Once there are enough cells, seed at 400,000/ml in 150-200 mL RPMI/15% FCS + 4 ng/mL PMA.
3. Incubate 48 hr at 37 C 5% CO_2
4. Pellet cells and harvest supernatant.
5. Sterile filter supernatant through 0.22 uM filter.
6. Aliquot 5 mL in 15 mL conical tubes.
7. Store at 4 C.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1453235/pdf/immunology00171-0037.pdf

Prepare Feeder Cells

1. Draw 20 mL blood from healthy donor
2. Isolate PBMCs using ficoll-hypaque discontinuous gradient.
3. Remove buffy coat and wash cells 3x with serum free RPMI.
4. Resuspend cells at 5 million/mL
5. Irradiate cells-4000 Rad (email Rendy Strawbridge to schedule a time to irradiate)
6. Keep irradiated cells on ice until needed.

EBV Transform

1. Thaw a vial of PBMCs-add to pre-warmed 50 mL RPMI.
2. Centrifuge and resuspend in 1-2 mL RPMI/15% FCS. Determine cell count.
3. Pellet cells again and resuspend at 2 million/mL in RPMI/15% FCS. 50 ul of these cells will be used per well of a 96-well round bottom plate. At this point determine how many wells the cells will cover.
4. For every 50,000 PBMCs combine the following:

   50,000 PBMC (50uL of cells at 2 million/mL)

200 ng/mL Cyclosporin A (1:10,000 dilution of stock)
2.5 ug/mL CpG (0.1 uL of 500 uM ODN2006 from Invivogen)
50,000 feeder cells (10 uL of 5 million/mL feeders)
50 uL EBV supernatant

5. Add 100 uL to 96-well round bottom plate.
6. Incubate at 37 C/5% CO2.
7. At two weeks, add 25 uL of CM-PMA.
8. Check wells for cells – media turns yellow or can see a group of cells at the bottom of the wells.
9. Expand cells into new 96-well round bottom plate-50 uL of original transferred to clean well and 50 uL of RPMI/15% FCS with 50% CM-PMA is added.
10. Incubate 24-72 hrs at 37 C/5% CO2.
11. Transfer 50 ul to 48 well plate and add 250 ul of RPMI/15% FCA/25% CM-PMA
12. Incubate 48 hrs at 37 C/5% CO2..
13. Remove 50 uL to stain for CD19 and IgG for flow cytometry.
14. If flow confirms B-cells, expand into 48-well and then 24-well plate in RPMI/15% FCS/25% CM-PMA.
15. Subclone some cells in 96-well round bottom at 0.3, 3, and 30 cells/well in RPMI/15% FCS/25% CM-PMA.

EBV Transform Memory B-Cells

Isolate Memory B-cells

1. Obtain whole blood and dilute 1:1 with serum free RPMI.
2. Carefully layer 25-30 ml of diluted blood over 15 mL ficoll-hypaque in 50 mL conical tube.
3. Centrifuge 30 min at 1400 rpm at room temp.
4. Carefully remove layer of cells at interface (PBMCs) and transfer to clean 50 mL tube. Top off tube with serum free RPMI.
5. Centrifuge 15 min at 1400 rpm at room temp-discard supernatant.
6. Resuspend in 50 mL serum free RPMI.
7. Centrifuge 10 min at 1400 rpm at room temp-discard supernatant.
8. Resuspend in 50 mL serum free RPMI-determine number of cells using ethidium bromide/acridine orange stain and hemocytometer.
9. Centrifuge cells 5 min at 1400 rpm at room temp.
10. Resuspend in appropriate volume of B-Cell isolation Buffer (PBS/2% FCS/2 mM EDTA-sterile filtered through 0.22 micrometer filter)
11. Isolate B-cells using Dynabeads Untouched Human B-cells kit. See kit for protocol or find online at https://tools.thermofisher.com/content/sfs/manuals/Dyna_untouch_human_bcell_man.pdf
12. Stain purified B-cells with APC-CD19, PE-IgM and FITC-IgD (make sure to use sterile reagents and wash buffers)
13. Sort cells on Aria (contact Gary Ward to schedule sorting time). Collect cells that are CD19+ (usually > 90% are CD19+ after B-cell isolation), IgM- and IgD-. Presumably the CD19+, IgM-, IgD- are positive for IgG or IgA and are largely memory B-cells.

14. Pellet the B-cells and resuspend at 50 cells/50 uL of RPMI/15% FCS containing 2.5 ng/uL CpG and 1000 cells/ul irradiated feeder cells.
15. Plate 100 ul into 96-well round bottom plates.
16. Incubate at 37C/5% CO2. Check plates at 12-14 days.
17. Add 25 ul of CM-PMA to each well and return plates to incubator.
18. Have patience-check plates for signs of cell growing up-media turns yellow or you can see cells growing. This could take a month-just make sure volume in well stays around 125 uL.
19. Once clones have grown up-take a few and stain for CD19 and IgG to verify they are memory B-cells.
20. Cells may now be expanded in RPMI/15% FCS and 25% CM-PMA until established.