Sugden Lab.

EBNA-1 Antibody purification from mouse Hybridoma Cells

A. Supplies to order:
   1. Disposable Bioreactors: IBS Integracelline 350 Bioreactors
      Cat#: 90010 5/case
      Argos Technologies Inc.
      1141 East Main Street
      Suite 104
      East Dundee, IL 60118
      1-847-783-0456
   2. Fisherbrand Sterile, plugged, shorty style10ml pipets
      Cat#: 13-678-36C

B. Media
   1. Complete Media-for inside cell compartment-
      500ml DMEM w/ HG
      5ml 100X Pen/Strep
      5ml 100X Sodium pyruvate
      75mls FCS
   2. Nutrient Media-for cell flask
      500ml DMEM w/ HG
      5ml 100X Pen/Strep
      5ml 100X Sodium Pyruvate
      25mls FCS

C. Starting Cells
   1. Thaw cryotube containing 500uls hybridoma cells at 37C.
   2. Resuspend cells by flicking tube with finger
   3. Wipe tube with 70% EtOH
   4. Plate 2-100mm dishes as follows: Add 200uls of cells to each dish containing 20mls complete media/dish. Doubling time is ~24hrs-Sarah Duellman starts new cells on Fridays, then amplifys to 4-5 100mm dishes on next Monday. Let cells go 1 week total and freeze down more cells and innoculate bioreactor on the next Thursday.

D. Freezing Down Cells
   1. Take 1-100mm dish started a week ago and put cells into 50ml conical tube. Take cell count using Trypan Blue Stain to check cells for viability. Remove 100uls cells and add 100uls trypan blue stain. Remember your dilution factor is 2 because of the dye. (# viable cells in16 squares of hemacytometer X 2 = viable cells X 10e4 cells/ml) Want 2-4 X10e5 cells/ml. Don't go over 5 X10e5. 2-3 X 10e5 is optimum, but 4 X 10e5 is acceptable.
   2. Spin down cells @ 1000RPM 5 min.-remove supe and save. This will have antibody in it. Store supe @ -20C.

3. Resuspend pellets in appropriate amount of 90/10.(90% FSC, heat shocked @ 56C for 30 min., store frozen. 10% DMSO from stock room, # D2650). Work quickly once you add this because cells are very unhappy. Aliquot 500uls/cryotube. Add a few drops of cells to a separate cryotube and use this for checking viabiltiy.
4. Put tubes in freezer boxes O/N @ -80C.
5. Next day move tubes to liquid Nitrogen. Start cells saved for viability check in 60mm dish + complete media. If they look good after a couple days, you know the cells frozen down should be good.

E. Inoculating Flasks

1. Combine 2-100mm dishes into 50ml conical. Since you have 4- 100mm dishes, you will use 2-50ml conicals. Be sure to resuspend cells well on dishes before transferring to conical tube. Spin @ 1000RPM's 5 min., remove supe and save as this will contain antibody that can be used. Freeze @ -20C.
2. Resuspend BOTH pellets in 5mls total complete media. Take cell count using trypan blue stain. RULE TO REMEMBER; 1.5 – 8 X10e6 cells/ml is the range of cell concentration needed to start flasks.
   NEVER exceed 8 X 10e6 cells/ml. If cell concentration is above 8 X 10e6 cells/ml, add another 5mls of complete media to cells(will now have 10mls in tube) and use only 5mls to inoculate bioreactor. Just dump extra 5mls of cells.
3. Inoculate bioreator as follows; ALWAYS USE THE 10ML PIPETS SPECIALLY ORDERED . The GREEN CAP is for the nutrient media port. This must be loosened when adding to the white cap port. The WHITE CAP is for the complete media port and where you grow the cells.
   a. Loosen both caps to start.
   b. Moisten membrane by placing ~10mls nutrient media into the nutrient media compartment(GREEN CAP)-leave cap loose.
   c. Pipet up 5mls of cell suspension w/ 10ml pipet (resuspend well) and inoculate 5mls of cell suspension into the cell chamber (WHITE CAP) by inserting the pipet into the black rubber compartment port. IMPORTANT STEP TO FOLLOW: pipet cells into port, STOPPING with ~1ml of cells remaining in pipet. Aspirate cells back up into pipet-this will remove the air bubbles present in the cell chamber. Contiue with this step(adding cells and pipetting back up), until all air has been removed from cell chamber. Once all the air is gone, inoculate cells by pipetting cells into chamber leaving ~0.5ml of cells in pipet. This will insure that you don't introduce air back into thechamber.
   d. Remove pipet and tighten down white cap.
   e. Add 340mls of warm nutrient media to nutrient chamber(GREEN CAP). Add media by pouring and watching vol. markings on flask.
   f. Once filled, tighten green cap.
   g. Wipe around white and green caps w/ paper towel containing EtOH.

h. Put in incubator containing 5% CO2 @ 37C.
i. Check cells @ day 5 and begin filling out a celline data sheet for each cell line.

F. Cell counts and media changes
1. Remove Nutrient Media by removing green cap and aspirating out media with a pasteur pipet. Remove as much as possible. Return green cap but leave it loose.
2. Remove cells from growth chamber using a10ml pipet. Pipet up and Down 3-4X to resuspend cells. DO NOT ADD AIR TO THE CHAMBER. Remove cells to a 15mls conical tube. Remove 100uls of cells for cell count and spin remaining @ 1000RPM 5 mins.
3. Take cell counts(as described above) and fill in data sheet.
4. After the spin, REMOVE SUPE TO NEW 15ml CONICAL(date,celline,name, day #) THIS CONTAINS YOUR ANTIBODY!!! Store @ -20C.
5. Cells are ready to split when viable cells are >80 x 10e6. If they are ready to split, resuspend pellet in 10 mls complete media, but only add back 5mls to the chamber.(1:2 split-you can make any split you feel appropriate,ie,1:3,1:4,etc). GREEN CAP SHOULD BE LOOSE WHEN ADDING TO THE GROWTH CHAMBER. If cells are not ready to split, resuspend pellet in 5.5mls complete media and add all back to the chamber leaving ~0.5ml in pipet so as not to add air to the chamber. Tighten white cap.
6. Add 350mls of nutrient media to nutrient chamber. Tighten green cap. EtOH off around both caps and return to incubators.
7. Take cell counts every 3-4 days or as necessary depending on cells. **Don't forget to save all supe after each cell count. This is your antibody.**

G. ELISA Assays: Checking antibody titers
1. Day1
a. Coat plates with 50uls antigen. Use 50ngs/well contained in 50uls 1X PBS. Coat at least 2 hours at room temp. with shaking or overnight @4C. When calculating the number of wells needed, don't forget to include 4 control wells, (2+ controls=Ab that works/2-controls=blotto) and make enough mix for 4 extra wells to account for pipetting errors.
b. Use the multichannel pipettor and aliquot 50uls/well. Parafilm plate well and let go 2 hrs. on shaker.
c. Remove Antigen by dumping in the sink and tapping plates on paper towels.
d. Block overnight in 1% blotto(1X PBS+dry milk) 200uls/well.
e. Thaw serum to be tested overnight in frig.
2. Day 2
a. Dump plates in sink and tap on paper towels
b. Add antibody (media) diluted in blotto as follows. Top row of plates should have 300uls blotto. All others below should have 150uls. Add 3uls of media to top row = 1:100 diln. Make dilns. down the row by pipetting 150uls from top row to next row, continuing down all 8 wells. At 8th well, throw out last 150uls. Remember

the positive control can be any antibody that works, and the negative control is just blotto. Do both in duplicate. Parafilm plates and put at RT 1.5hrs with shaking.

c. Dump plates in sink and tap on paper towel.
d. Wash plates 3X with PBST using the Burgess lab plate washer. Use plate washer as follows:Hook up one end to vacuum flask and the other to the PBST carboy. Push bar in back to release PBST into wells, push down on top to aspirate up liquid. Try to aspirate all the liquid each time as this will help eliminate high background signal.
e. Diluting secondary antibody: Goat anti-mouse peroxidase labeled, 1:1000 in blotto. Add 50uls/well and incubate 1hour at RT with shaking.
f. Dump and tap plates on paper towel. Wash plates 5X with plate washer. Fill on the 6th time, but let sit. Set up OPD subtrate as follows:
   10mg tablet OPD
   10mls ddH20
   10mls citrate buffer
   20uls H2O2
g. Finish washing 8 washes, add 100uls/well OPD mix. Watch for color change to occur.
h. Quench reaction with 1M H2SO4, 50uls/well.
i. Read on Burgess lab plate reader.

3. Ordering ELISA plates: order from Fisher. Cat# ICN7603105
   Microplate, Linbro, MP Biomedicals
   96-well, clear polystyrene
4. Summary of ELISA
   a. Materials needed:
      Antigen
      Antibody
      Lindbro 96-well tissue culture plate
      Goat anti-mouse IgG peroxidase labeled
      Substrate (OPD and H2O2)
      PBS
      PBST, PBS + 0.1% Tween 20
      1% Blotto, dry skim milk in PBS
      Quenching reagent, 1M H2SO4
   b. OPD Substrate (for 2 plates)
      10mg tablet OPD-in frig door room 808-Sigma p8287
      in 10mls ddH20
      10ml Citrate Buffer
      20uls H202
   c. Citrate Buffer, 0.1M, pH5
      0.1M citric acid-19.2g/liter
      0.1M sodium citrate(dihydrate)*2H20-29.4g/liter
      add acid to base

H. IgG1 mAb Purification

1. Day 1
   a. Calculate the amount of saturated ammonium sulfate required for a 45% cut. Volume ammonium sulfate=0.82(vol. of media)
   b. add ammoniun sulfate slowly and stir on ice 20 mins.
   c. Refrigerate O/N
2. Day 2
   a. centrifuge media 20 min. @ 7000RPMs 4C-decant off supe and save sample for SDS-PAGE gel.
   b. add 1/2 volume of 1X Ab Buffer to pellet at room temp.
   c. dialyze O/N in 1 liter 1X Ab buffer @ 4C.
   d. 10X Ab Buffer
      500mM Tris-HCl, pH 6.9
      250mM NaCl
3. Day 3
   a. Spin down 7K 20 mins. to remove any ppt.
   b. determine size of column needed-5mls for 20mls media, 3mls for smaller vols.
   c. prepare column resin: for 3ml column use 1.5g DE 52 resin
      For 5ml column use 2.5g DE52 resin. Mix resin with 8mls 1X Ab Buffer. Pour into 10ml Bio-Rad disposable column. Rinse tube with more 1X Ab Buffer and add to column. Once all Ab Buffer flows through- Add another 2mls of 1X Ab buffer and let flow through.
   d. Load spun down sample 1ml at a time- collect 1ml aliquots.
   e. Wash column with 1 column vol. of 0.5M NaCl to remove any nonspecifically bound antibody. Collect in 1ml aliquots.
   f. Save 2uls and 10uls of each aliquot. The 2uls will be loaded on a protein gel and the 10uls will be used for quantifying protein concentration.
   g. Run protein gel to determine which aliquots have antibody-toss out rest.
   h. Spec. 10ul sample- dilute 1/10 in 1X Ab buffer, at A280. Use following equation to determine antibody concentration.
      A280/1.38 x diln. factor(10) x vol=mg mAb
   i. Stored all good aliquots in –80C freezer in designated boxes.
4. Supplies
   a. Whatman DE52 Resin
      Order from Fisher; Cat# 05-720-5 500g