Antibodies Production Manual

Version 1

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Table to Contents:

- AB100 Cell line maintenance 2
- AB101 IgG purification of monoclonal antibodies 4
- AB102 QC of antibodies 6
- AB103 Monoclonal antibody fusion 7
- AB104 Screening and subcloning of monoclonal hybridomas 11
- AB105 Freezing down cell lines 14
- M012 Preparation of complete RPMI media 16
- SP039 ELISA assay 18
- SP040 Capture ELISA assay 20

SOP: AB100

Cell Line Maintenance

Materials and Reagents:

1. Complete RPMI (note 1)
2. Fetal Calf Serum (FCS)
3. Water Bath 37˚C
4. CO_2(5%) Humidified Tissue Culture Incubator 37˚C
5. 0.2 um, 25mm Acrodisc syringe filters
6. 50 ml falcon centrifuge tubes
7. 15 ml falcon centrifuge tubes
8. 225 ml falcon tubes
9. Nalgene 250ml filter unit, 0.2um
10. Hemocytometer
11. Pasteur pipettes
12. Serological pipettes
13. Tissue culture/biosafety hood
14. Tabletop centrifuge
15. Tissue culture flasks (T25,T75, T125)
16. Rollerbottle
17. Rollerbottle apparatus
18. Inverted microscope

Protocol:

1. _____ Remove cell line that is to be used from liquid Nitrogen tank and note in LN2 log book.

2. _____ Immediately transfer cell line to 37°C waterbath. It takes about 2-4 minutes to defrost.

3. _____ While cells are thawing, in a tissue culture hood, place 6 ml of complete RPMI media (note 1) in a 15 ml falcon tube and underlay with 1 ml of FCS (note 2).

4. _____ When the cells are thawed, aseptically transfer the cells to the RPMI layer in the 15 ml conical. Reserve 10µl of cell suspension (note 3).

5. _____ Centrifuge cells at 1000-1200 rpm at room temperature for 6-10 minutes.

6. _____ While cells are spinning, transfer the reserved cells (from step 4) to a hemocytometer and look at cells using an inverted microscope (note 4).

7. _____ If viability is good, then prepare a T25 flask containing 10ml of complete RPMI and label flask with all the information that is on the cryovial (note 5).

8. _____ Retrieve cells from centrifuge, decant supernatant and discard.

9. _____ Carefully resuspend cells in 1 ml of RPMI complete media and transfer to T25 (note 6).

10. _____ Transfer cells to 37°C incubator with 5% CO_2.

11. _____ Check cells daily, to see when media is beginning to turn acidic. For hybridomas, the cells usually need to be fed every 2-3 days.

12. _____ To check if the cells need to be fed, counts cells under a hemocytometer (note 7).

13. _____ Cells that are being refed are usually split 1:10 or 1:20 (note 8).

14. _____ Make note on flask that it has been fed and return cells to 37°C incubator with 5% CO_2.

15. _____ When media begins to turn acidic, upscale from a T25 to two T75 containing a total volume of 50 ml RPMI complete media plus cells (note 9).

16. _____ Refeed the original T25 and make note on flask that it has been fed.

17. _____ Return cells to 37°C incubator with 5% CO_2.

18. _____ When the media begins to turn acidic again, upscale to two T125 each containing a total volume of 150-200 ml of media plus cells (note 10).

19. _____ Return cells to 37°C incubator with 5% CO_2.

20. _____ In two days, the cells should be upscaled to rollerbottles. One T125 in each rollerbottle and bring the total volume up to 1L-1.2L.

21. _____ Gas the rollerbottles for at least 4 hours in the CO_2 incubator with the caps loosened (note 11).

22. _____ Close the caps on the rollerbottles very tight and seal with several layers of parafilm.

23. _____ Place on the rollerbottle apparatus at 37°C for 5-6 days (note 12).

24. _____ When the media is very acidic (yellow), harvest the supernatant by centrifuging the supernatant at 3200 rpm for 10 minutes. Repeat as needed and discard the pellets.

25. _____ Transfer the new supernatant to a new rollerbottle.

26. _____ Adjust pH to 7.4 (note 13).

27. _____ Store at -20°C until needed.

Notes:
1. See SOP:M012 for Complete RPMI.
2. The FCS acts as a barrier to separate the cells from the DMSO that is in the Freezing Media.
3. The reserved amount of cell suspension is to check for viability using a hemocytometer.
4. If cell viability looks low it is best to start cells in a 24 well tissue culture plate or the amount of FCS can be increased to 20%.
5. Check the vial in which the cells were frozen to see which media they were frozen in. If the vials says 'HAT' the cells must be grown in HAT media. HAT media is 600 ml RPMI complete media with 1 vial of HAT supplement added. Otherwise, RPMI complete should be used.
6. If using vented cap flasks: screw the lid on firmly, if not using vented cap, make sure the cap is loose to allow CO_2 exchange in the incubator.
7. Add 10µl of cell suspension to hemocytometer and count the number of cells in 16 squares. Multiple this number by 1×10^4 to obtain how many cells are in each ml of media. The cells will grow best when they are between 4×10^5 and 7×10^5. Cell greater than this density should be expanded to new flasks.
8. This means that the cell density must be adjusted to be between 5×10^3 and 2×10^4 cells per ml. The fewer the cells the longer they will take to recover, but usually the density will be back up in 2-4 days.
9. Do this by adding 5 ml of slightly acidic cells to each T75 with 45 ml of media.
10. Transfer the entire contents of one T75 into one T125 and add 100-150 ml media depending on the density of the T75. Do this for the other T75 flask.
11. The rollerbottles can be gassed overnight if necessary.
12. Check for leaks. If not tightly sealed, the bottles will leak or ambient air will replace the CO2 inside the flask and this will make the media alkaline (pink).
13. It is not necessary to use a pH meter. The media is a pH indicator and the color should be red at this pH (not pink or yellow).

SOP: AB101

IgG Purification of Monoclonal Antibodies

Materials and Reagents:

1. Serological pipettes
2. Disposable poly-prep Chromatography Columns
3. Protein G-sepharose in 20% ethanol suspension (P-3296 Sigma)
4. Phosphate Buffered Saline (PBS) pH 7.4 or 10mM Ammonium Bicarbonate (Ambic)
5. Pasteur pipettes
6. Clean rollerbottle
7. 2L culture supernatant of antibody to purify
8. 4°C Cold Room
9. 0.2M Glycine
10. Slide-A-Lyzer Dialysis Cassette 3500 MWCO (Pierce 66110)
11. Lyophilizer
12. Thin rubber tubing
13. 5 ml Falcon collection tubes
14. Western blot supplies
15. Silver Staining supplies
16. SDS-PAGE supplies

Protocol:

1. _____ Obtain 2L culture supernatant of antibody that is being purified (note1).

2. _____ Set up column by filling the column with PBS.

3. _____ Add 0.6-0.8ml of protein G-sepharose slurry (note 2).

4. _____ Using a Pasteur pipette flush the slurry until the bubbles disappear.

5. _____ Continue to add PBS so the column doesn't dry out.

6. _____ In a 4°C cold room, place the culture supernatant above the column (note 3).

7. _____ Place an empty clean 2L roller bottle below the column.

8. _____ Fill the column with culture supernatant.

9. _____ Connect the rubber tubing from the culture supernatant roller bottle to the top of the column.

10. _____ Connect another section of rubber tubing from the bottom of the column to the empty roller bottle.

11. _____ The proper flow rate should be ~ 1 drop every 4-6 seconds (note 4).

12. _____ Check on the flow rate every couple of hours.

13. _____ Repeat steps 7-12 two more times (note 5).

14. _____ Wash the column with 50 ml of PBS (note 6).

15. _____ Set up 6 falcon collection tubes, and add 0.75 ml of sterile 0.1M PBS to each tube (note 7).

16. _____ Elute with 0.5 ml of 0.2µm filtered, 0.2M Glycine HCL, pH 3.0 per collection tube.

17. _____ Run 1-2 ul of all the fractions on a SDS-PAGE, and Silver Stain (note 8).

18. _____ Pool the fractions that contain purified antibody and dialyze against PBS or Ambic, depending how the antibodies will be stored, using the Slide-A-Lyzer Cassette for 24 hours (note 9).

19. _____ Run a BCA on the dialyzed sample to determine antibody concentration (note 10).

20. _____ Run a SDS-PAGE gel of the purified samples and a western blot of the target antigen. Develop the western blot with dilutions of 1:500, 1:1000, and 1:2500 of the purified antibody (note 11).

21. _____ Aliquot the antibodies in 0.5mg and 1.0mg aliquots as determined by a BCA assay (note 12). Leave a 100ul sample of the purified antibody to perform QC.

22. _____ Freeze dry by lyophilization if needed (note 13).

23. _____ See SOP: AB102 for QC of antibodies to continue.

Notes:

1. See SOP: AB100 for cell line maintenance.
2. Protein G- sepharose is for IgG1 antibodies. Protein A sepharose can be used if the antibodies isotype is not IgG1 but most monoclonal antibodies are IgG1.
3. The culture supernatant must be above the column for gravity to drive the supernatant through the column.
4. It might be necessary to suck on the tubing connected to the culture supernatant to start the flow.
5. Be sure to not let the column run dry. When the top 2L culture supernatant bottle is empty, just switch bottles, so that the full one (with the flow though) is now above the column and the bottle that was originally full is now below the column.
6. This step can be done at room temperature. Discard the wash.
7. 10mM Ambic may also be used to neutralize the purified antibodies that are coming off the column. The buffer depends on how the antibodies will be stored. Use PBS if the antibodies will be freeze dried by lyophilization and use Ambic if they will be stored in solution.
8. See SOP: SP007 running polyacrylamide gels and SP012 performing a Silver Stain.
9. If there is more than 12 ml of solution use 3500 MWCO dialysis membrane.
10. See SOP: SP003 on BCA protein assay.
11. See SOP: SP011 running a western blot. Load 1-2 ug of pure protein or 5-10ug of subcellular fractions per well. It is best to make multiple wells containing the protein, so this can be used to titer out the antibody. If the titer doesn't fall into this range repeat the western blot until the proper titer is determined. For purified IgG antibodies the titer shouldn't be lower than 1:500. If it is, the purification should be repeated with new culture supernatant or a western can run be on the flow through to see if the antibodies did not bind to the column.
12. Default quantity for purified antibodies in 0.5mg.
13. See SOP: SP004 for use of lyophilizer.

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SOP: AB102

QC of Antibodies

Materials and Reagents:

1. Serological pipettes
2. Western blot assay supplies
3. Cryovials
4. Cryovial storage container

Protocol:

1. _____ Obtain a small aliquot of culture supernatant or IgG purified antibody (see SOP:AB100 or AB101).

2. _____ Run SDS-PAGE with 1ug of target protein (note 1).

3. _____ Perform western blot assay and vary the dilution of the antibody. For culture supernatant: make 1:20, 1:50, 1:100 dilutions. For IgG purified: make 1:500, 1:1000, 1:2500 dilutions (note 2).

4. _____ Once the titer is determined, run another SDS-PAGE with 1μg of the protein of interest and 5ug each of CFP, cytosol, cell wall and cell membrane.

5. _____ Run a western blot the determined titer of antibody (note 3).

6. _____ If performing QC on IgG purified antibody, run a gel of 1 μg of antibody and silver stain (note 4).

7. _____ Aliquot antibody in cryovials and store at -80˚C (note 5).

8. _____ Scan western blot and Silver stain (if applicable).

9. _____ Fill out QC sheet titled: TB Contract Antibody QC Sheet.

10. _____ See SOP:RM001 for Adding Material to Inventory for further direction.

Notes:

1. Run several lanes of the same protein, this will be used to determine the titer of the antibody. See SOP: SP007 on running polyacrylamide gels.
2. See SOP: SP011 for running a western blot. Cut the nitrocellulose into individual strips for each dilution. If the titer does not fall into these ranges adjust accordingly and repeat. If testing a monoclonal be sure to use anti-mouse IgG secondary, if testing a polyclonal be sure to use anti-rabbit IgG secondary.
3. For polyclonal antibodies, if titer is determined using a recombinant protein back the titer off by one dilution for the QC on subcellular fractions. For example: Titer for anti-ESAT6 tested against recombinant ESAT6 was determined to be 1:10000, the QC for this antibody would be done at 1:5000.
4. See SOP:SP012 for performing a Silver stain.
5. For culture supernatant: if titer is >1:50 make 0.5 ml aliquots (default), and if <1:50 make 1.0 ml aliquots (default). For IgG purified: make 0.5 mg (default) and 1.0 mg aliquots. IgG purified antibody should be freeze dried by lyophilization see SOP: SP004.

SOP: AB103

Monoclonal Antibody Fusion

Materials and Reagents:

1. RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Invitrogen 11875-093)
   *Also called Incomplete RPMI
2. Complete RPMI media (note 1)
3. HAT supplement, 50X (Sigma H-0262)
4. Polyethylene glycol, tissue culture grade (VWR EM-9727-2)
5. Water Bath 37˚C
6. CO_2 (5%) Humidified Tissue Culture Incubator 37˚C
7. 0.2µm, 25mm Acrodisc syringe filters
8. 50 ml falcon centrifuge tubes
9. 15 ml falcon centrifuge tubes
10. Nalgene 250ml filter unit, 0.2µm
11. Hemocytometer
12. Pasteur pipettes
13. Serological pipettes
14. Tissue culture/biosafety hood
15. Metofane or Ether
16. Petri Dish
17. Sterile forceps
18. 3 cc syringe
19. 22 gauge needle
20. 70µm sterile nylon cell strainer (Falcon 2350)
21. 1 cc syringe
22. Tabletop centrifuge
23. Gey's red blood lysis solution (note 2)
24. SP2/0 myeloma B cell fusion partner growing at mid-log phase
25. Beaker, 400ml
26. Sterile reagent reservoirs
27. Multichannel pipette
28. Sterile 96 well tissue culture plates

Protocol:

1. _____ Sacrifice hyper-immunized mouse 5 days after final antigen boost via cardiac puncture (note 3).

2. _____ Remove spleen aseptically and place into a 15 ml conical tube containing 10 ml of sterile complete RPMI. Keep at room temperature.

3. _____ In a biosafety hood, transfer the spleen to a 60mm sterile Petri dish containing 3 ml of complete RPMI.

4. _____ Using sterile forceps to hold the spleen, gently inject media from the Petri dish using a 3cc syringe and 22 gauge needle (note 4).

5. _____ Gently draw up the suspension and transfer to a 15 ml conical tube.

6. _____ Repeat the spleen wash two more times, keeping at room temperature (note 5).

7. _____ Pass the remainder of the spleen through a 70µm nylon syringe strainer (note 6).

8. _____ Wash the cells through the strainer twice more with 2 ml of medium. Pool suspensions in the 15 ml conical from step 5.

9. _____ Pellet the cells at 1600 rpm for 10 minutes.

10. _____ Discard supernatant and resuspend cell pellet gently with 3 ml of incomplete RPMI and then bring the volume up to 10 ml with incomplete RPMI (note 7).

11. _____ Repeat steps 9 and 10.

12. _____ Remove 50µl spleen cell suspension, and add 450µl of Gey's red blood cell lysis solution (this will give a 1:10 dilution).

13. _____ Count the spleen cells with a hemocytometer (note 8).

14. _____ Count the SP2/0 cells with a hemocytometer (note 9).

15. _____ Collect the appropriate amount of growing SP2/0 cells into two 50 ml conical tube (note 10).

16. _____ Prepare the SP2/0 cells in the same manner as the spleen cells, using 40 ml of incomplete RPMI to wash (step 9-11).

17. _____ Combine the SP2/0 cells and the spleen cells in one 50 ml conical tube.

18. _____ Repeat centrifugation from step 9.

19. _____ Wash the SP2/0 cells and spleen cells one more time with 40 ml incomplete RPMI and centrifuge (note 11).

20. _____ While the cells are spinning, prepare a 50% w/vol solution of polyethylene glycol (PEG) in a 15 ml conical tube and filter through a 0.2µm filter (note 12).

21. _____ After final wash of SP2/0/spleen cell mixture, remove the supernatant without disturbing the cell pellet.

22. _____ Centrifuge the pellet at 1500 rpm for 1 min and remove any additional supernatant with a Pasteur pipette.

23. _____ Tap the tube briskly on a hard surface to break up the cell pellet, and place the tube in a 400 ml beaker containing 37°C water (note 13).

24. _____ Place the PEG solution and the complete RPMI in the 37°C water and draw up 1.2 ml of the PEG solution into a sterile serological pipet (note 14).

25. _____ Begin the 6 minute fusion process without stoppage.

26. _____ Minute 1: Add the 50% PEG solution to the cells in the 50 ml falcon tube (note 15).

27. _____ Minute 2: Continue to gently swirl the cells and PEG solution together.

28. _____ Minute 3: Slowly add 1 ml of complete RPMI continue to swirl gently.

29. _____ Minute 4: Slowly add another 1 ml of complete RPMI media and continue to swirl gently.

30. _____ Minute 5: Add 5 ml of complete RPMI media with gentle swirling.

31. _____ Minute 6: Gently add complete RPMI up to 40 ml while swirling (note 16).

32. _____ Pellet the cells at 800 rpm for 5 minutes. Discard supernatant.

33. _____ Gently resuspend cell pellet in 3 ml of warm complete RPMI medium (note 17) and then bring the volume up to 50 ml with warm RPMI medium.

34. _____ Transfer 20 ml of the fusion solution to a sterile reagent reservoir then add 20 ml of warm complete RPMI medium (1:2 dilution). Using a multi-channel pipettor, aliquot 100µl per well into 96 well plates.

35. _____ Repeat step 34 until the entire 40 ml of the fusion solution (from step 33) is transferred to 96 well plates (note 18).

36. _____ Place all 96 well plates into a 5% CO_2 37°C humidified tissue culture incubator.

37. _____ Two days after fusion, add 175µl of complete RPMI medium with HAT supplement to all the wells in the 96 well plates (note 19).

38. _____ Five-six days after the addition of HAT, remove 150µl of supernatant from each well and add 200µl of complete RPMI with HAT to the plates (note 20).

39. _____ See SOP:AB104 for Screening Monoclonal Hybridomas and Subcloning.

Notes:

1. See SOP: M012 for Complete RPMI media.
2. Gey's RBC Lysis Solution is prepared by adding:
   4.15g NH4Cl
   0.5g KHCO3
   500 ml ddH20

Add all the ingredients together and filter through a 0.2µm filter. Store at 4°C. This solution will last an extremely long time as long as it is kept as sterile as possible.
3. The mouse must be anesthetized with either ether or Metofane by exsanguinations. Collect as much blood as possible and save the sera for positive control. Normal yield is 0.75ml of blood collected via cardiac puncture.
4. Move the needle around inside the spleen to release cells. Be very gentle and careful.
5. After initial 3 ml suspension is transferred to the 15 ml conical, add 3 ml more to the Petri dish and flush again. Repeat this one more time. The spleen may be manipulated while injecting the media to force out more cells. Pool all the spleen cells into the 15 ml conical tube.
6. After the final wash the spleen should look opaque in most areas. Use the rubber end of a 1 ml syringe to force the spleen through the strainer.
7. This is to wash the FCS from the suspension before the fusion. Resuspend the cells very carefully.
8. Normal yields are usually 1.2-2.0 x 10^8. To calculate the number of spleen cells take the number of cells in 4 squares x 4 (for 16 squares) x dilution factor x # ml in suspension.
9. The SP2/0 myeloma B cell is the fusion partner for the spleen cells. These cells should be growing for several days and be at mid-log phase growth and highly viable, >95%. See SOP:AB100 for cell line maintenance.
10. The number of SP2/0 cells needed for the fusion is $1/6^{th}$ the number of spleen cells. For example: if the spleen cell count is 1.2 x 10^8 then 2 x 10^8 SP2/0 cells are needed. Or more generally, one average spleen to 60 ml of SP2/0 cells at a density of 2 x 10^8 is about $1/6^{th}$.
11. At this point, both cell types will have been centrifuged four times, twice separately, twice together.
12. Add 1.5g of PEG, then 1.5 ml of incomplete RMPI to the tube. Microwave on high power for 10 seconds with cap loosened, then microwave for short 5 second bursts with mixing after each round until the solution is well dissolved. Allow the PEG to sit in a 37°C water bath. At the same time, place a 50 ml conical tube with complete RPMI medium in the water bath for step 24.
13. The cells must remain at 37°C for the rest of the fusion process.
14. The fusion process takes a total of six minutes and should be done very carefully and accurately.
15. Add the 50% PEG slowly at first using the tip of the pipet to gently swirl the cells and PEG together. Gradually add the rest of the PEG solution into the cells throughout the first minute. Continue to swirl cells.

16. The cells will appear quite clumpy during the fusion process and after the addition of the RPMI. Do not attempt to break up the clumps by rough pipeting during the fusion process.
17. Using a sterile Pasteur pipette, very gently resuspend the cell pellet until most of the larger clumps are broken up. Do not attempt to break up all the small clumps with more vigorous pipetting this may break up lightly fused cells.
18. There should be enough to cover ten 96 well plates.
19. HAT media: 1 vial (sigma #H-0262) HAT supplement per 600 ml of complete RPMI medium. The wells should appear mildly confluent due to growth of SP2/0. The SP2/0 cells that have not fused with spleen cells will die off within 24 hours as a result of the HAT selecting agent.
20. It is important to not completely dry out the wells when attempting to take off 150µl. If 150µl cannot be taken off, take as much as possible with out drying out the well. When sucking off old media do not touch the bottom off the wells rather suck from the top. This will prevent disturbance of any clones growing. It is okay to use the same pipet tips to suck off old media as long as the bottoms of the wells are not disturbed. The fresh medium should be added just 2-3 days prior to testing the first supernatants of wells in which clones grow up. The hybridomas usually become visible colonies at day 7-8, and the supernatants are harvested and tested generally between day 10-15 days following the fusion.

SOP: AB104

Screening and Subcloning of Monoclonal Hybridomas

Materials and Reagents:

1. Complete RPMI Medium (note 1)
2. HAT supplement, 50X (Sigma H-0262)
3. HT supplement, 50X (Sigma H-0137)
4. Polyethylene glycol, tissue culture grade (VWR EM-9727-2)
5. Water Bath 37˚C
6. CO_2 (5%) Humidified Tissue Culture Incubator 37˚C
7. pH meter
8. 50 ml falcon centrifuge tubes
9. 15 ml falcon centrifuge tubes
10. Nalgene 250ml filter unit, 0.2µm
11. Hemocytometer
12. Serological pipettes (1,5 and 10 ml)
13. Tissue culture/biosafety hood
14. SP2/0 myeloma B cell fusion partner growing at mid-log phase
15. Sterile reservoirs
16. Multichannel pipettor and pipet tips
17. Sterile 96 well tissue culture plates
18. ELISA 96 well plates
19. ELISA assay materials
20. KPL *p*-NPP developer kit (cat# 508000)
21. Western blot assay materials
22. 24 well tissue culture plates
23. T25 10 ml tissue culture flasks
24. Cryovials
25. Cryovial storage container

Protocol:

1. _____ Nine or ten days after the fusion process, the individual hybridomas will begin to make the media slightly acidic. Remove 200µl of culture supernantant from wells with growth and transfer them to a 96-well tissue culture sterile plate with lid (note 2).

2. _____ From the number of wells with growth, prepare an ELISA plate (note 3).

3. _____ For the wells that are ELISA positive, immediately transfer to a 24 well plate with 1 ml of HAT media and also re-feed the 96 well plate (note 4).

4. _____ Stong positive ELISA should also be tested by western blot (note 5).

5. _____ Remove 750-1000µl of culture supernatant from each well that will be tested by western blot (note 6) and replace with fresh HAT media.

6. _____ Hybridomas that test positive for western blot are subcloned directly from the 24 well plate stage (note 7).

7. _____ The day before subcloning, count the SP2/0 cells and plate them at a density of 2×10^4 cells per well in 100µl of complete RPMI. Make two 96 well plates for every hybridoma that will be subcloned and place these plates in a 37°C incubator (note 8).

8. _____ To subclone, count the cells in the 24 well plate using a hemocytometer.

9. _____ Calculate the number the cells that need to be added to 5ml of RPMI with HAT to achieve a density of 1×10^3 cells per ml (note 9).

10. _____ From this stock, add 100µl to row A, plate 1. This row represents 100 cells/well.

11. _____ Into a sterile reagent reservoir, add 1.8 ml of RPMI with HAT and 0.2 ml of the cell suspension from step 10. This will make a 2 ml stock of a 1:10 dilution.

12. _____ Mix the cell suspension and plate 100µl/well of this stock to row B, plate1. This row represents 10 cells/well.

13. _____ To the remaining 0.8ml in the reservoir, add 7.2ml of RPMI with HAT to make a second 1:10 dilution.

14. _____ From this stock, plate out 100µl/well into row C and D of plate 1. This represents 1 cell/well.

15. _____ To the remaining 5.6 ml in the reservoir add 9.5 ml of RPMI with HAT, and mix.

16. _____ Using a multi-channel pipettor, put 100µl/ well on the rest of plate 1 and all of plate 2. This represents 0.3 cell/well (note 10).

17. _____ Place the subcloned plates in a 37°C incubator.

18. _____ Upscale the rest of the cells in the 24 well plate to a T25 flask containing 10ml of RPMI with HAT.

19. _____ Freeze down at least two lines of each parental (note 11).

20. _____ 6-7 days later, remove 150µl of medium from the each well of the subcloned plates and add back 200µl of fresh RPMI with HAT.

21. _____ By day 9 or 10 individual wells should be growing well in the 0.3 cell/well rows. Harvest these growing supernatants in a 96 well plate to be tested by ELISA.

22. _____ Repeat ELISA Assay as before in step 2 (note 12).

23. _____ From the positive wells, pick 6 with the strongest signal and transfer these to a 24 well plate. The cells from this point on will be grown in complete RPMI media with HT (no aminopterin) (note 13).

24. _____ Expand these cells to T25 10 ml flasks containing RPMI with HT and freeze down at least two lines of each subclone.

25. _____ If desired, further upscale the cells to T75 cells (note 14).

Notes:
1. See SOP:M012 for Complete RPMI Medium.
2. If necessary, the culture supernatants may be pulled the night before and stored at 4°C. There is usually a total of 4 days worth of picking clones. This protocol is to be done for each day. It is best to circle the selected clones on the original 96 well plate with a different colored marker everyday. This will help prevent confusion and mistakes. Re-feed well with HAT media. HAT media is made by adding 1 vial of HAT supplement to 600 ml of complete RPMI.
3. See SOP: SP039 for ELISA assay. Prepare a stock solution of antigen for screening by add 100µg of protein to 10 ml of PBS. Mix well and coat 100µl of antigen per well. This antigen can be reused several times. Store at -20°C between each use. Be sure to include a positive (polyclonal sera) and negative (BSA) control.

4. When transfering to 24 well plate be sure to pipet up and down a few times to free hybridomas from the bottom of the 96 well plate, but do this gently as to not damage the hybridomas.
5. See SOP: SP006 for running SDS-PAGE and SOP: SP0012 for running a western blot assay. It is easiest to make strips out the nitrocellulose membrane containing 1µg of the pure protein. Make several strips at this stage so this step does not have to be repeated in the future. Hybridomas are also checked against western blot to determine if the epitope is conformational or linear. Only linear epitopes (positive for western blot) are selected for subcloning unless otherwise directed.
6. The culture supernatants should be allowed to grow for at least 24 hours before testing via western blot, the bottom of the well in the 24 well plate should have a moderately confluent layer. Be sure to not touch the bottom of the well or to mix the supernatant when pulling it off to be tested. This is to be avoided so the cells are not lost during this screening process.
7. These hybridomas are the parental lines. It is very crucial to not lose any of the positive clones before subcloning or freezing down at least one line from each parental.
8. SP2/0 cells are used as feeder cells. The cells are grown overnight so they will release factors into the medium which will promote the growth of single cells to a clone of cells that can be expanded.
9. The cells should be highly viable and have a density between 2×10^5 and 8×10^5. Never add less than 25µl or more than 200µl of cells to be subcloned.
10. The goal behind subcloning to get a single hybridoma in a single well. So if everything goes as planned, $1/3^{rd}$ of the 0.3 cells/well wells will grow up and each of these hybridomas will have been derived from a single B-cell.
11. See SOP:AB105 for Freeze Cell Lines.
12. Usually all the wells are positive for ELISA but if they are not this means that hybridoma is not clonal.
13. For HT Media: Add 1 vial of HT supplement to 600 ml of complete RPMI media.
14. See SOP AB100: Maintenance of cell lines.

SOP: AB105

Freezing Down Cell Lines

Materials and Reagents:
1. RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Invitrogen 11875-093)
2. Fetal Calf Serum (FCS)
3. DMSO tissue culture grade
4. CO_2(5%) Humidified Tissue Culture Incubator 37˚C
5. 50 ml falcon centrifuge tubes
6. 15 ml falcon centrifuge tubes
7. Hemocytometer
8. Serological pipettes (1 and 5 ml)
9. Tissue culture/biosafety hood
10. Cryovials
11. Cryovial storage container
12. -80°C Freezer
13. Liquid Nitrogen storage
14. Ice bucket
15. Table top centrifuge
16. 0.2 µm filter (Nalgene)
17. Inverted Microscope

Protocol:

1. _____ Look at cells using an inverted microscope (note 1).

2. _____ Count cells with hemocytometer (note 2).

3. _____ If cells are in normal density range, pellet them in a sterile centrifuge tube by spinning between 1200 – 1500 rpm for 10 minutes.

4. _____ While cells are spinning, label cryovials with appropriate information and place in ice bucket (note 3).

5. _____ In a tissue culture hood, remove supernatant carefully with a pipet and discard.

6. _____ Tap bottom of tube to break up cells and resuspend cells in appropriate amount of cold Freezing Media to obtain necessary density (note 4 and 5).

7. _____ Immediately transfer cell suspension to appropriately labeled cryovial and put on ice until it can be frozen (note 6).

8. _____ As quickly as possible, transfer cells to -80°C freezer. Let sit overnight (note 7).

9. _____ After 24 hours, the cell line may be transferred to liquid nitrogen storage. Make note of location in LN2 log book and in project notebook (note 8).

10. _____ After at least a day, thaw one vial from each lot of frozen cells to check viability (note 9).

Notes:
1. Cells should appear >90% viable. Any lower and the cells may not be able to recover from the freezing process.
2. For suspension cultures, cell density should be between 3×10^5 and 7×10^5 cells/ml.
3. Label:

Cell Line	*Date*
Specificity	*Media (if other than complete RPMI)*
Cell count	*Initials*

4. Freezing Media: for 200 ml

    120 ml RPMI 1640 medium
    60 ml of fetal calf serum (FCS) (final concentration is 30%)
    20 ml dimethylsulfoxide (DMSO) (final concentration is 10%)

Filter sterilize the medium, then aliquot to 15 ml conical tubes. Store frozen at -20°C. The media should be defrosted and placed in the ice bucket covered with ice for at least 10 minutes to ensure it is cold before use.

Do not add DMSO directly to FCS as a precipitate may occur.

5. Cells should be resuspended in 1 ml of media per cryovial, and should have a density between 3×10^6 and 7×10^6 cells/ml. As an example, if in 10ml, the cell count is 4×10^5 cells/ml, then the cell count is actually 4×10^6 cells total in the flask. So after pelleting, the cells should be resuspended in 1 ml of Freezing Media to maintain this density.

6. The cells should stay on ice for as little time as possible.

7. The cells should never be stored at -20°C for any period of time. Do not transfer cells directly to LN2 tank, as this will be too great of a temperature change and the viability will be low, or the difference in temperature will cause the lids to pop off and the line will have to be discarded.

8. Cell viability decreases from 1-10% per month if the cells are kept at -80°C.

9. See SOP:AB100 for Cell Line Maintenance.

SOP:M012

Preparation of Complete RPMI Media

Materials and Reagents:
1. RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Invitrogen 11875-093)
2. Fetal Calf Serum (FCS)
3. 2-mercaptoethanol
4. Dextrose (Sigma G-7021)
5. Essential Amino Acids 50X (Invitrogen 11130-051)
6. Non-essential Amino Acids 100X (Invitrogen 11140-050)
7. Sodium pyruvate 100X (Invitrogen 11360-070)
8. Sodium bicarbonate (Sigma S-5761)
9. Streptomycin/Penicillin 100X (Invitrogen 15140-122)
10. Nalgene filter unit, 0.2µm
11. 50ml falcon centrifuge tubes
12. Sterile roller bottle
13. 10N NaOH
14. Tissue culture hood
15. 15ml conical tubes
16. 50 ml conical tubes
17. Serological pipets

Protocol:

Tumor Coctail

1. _____ Prepare Tumor Cocktail in a tissue culture hood in a sterile roller bottle.

2. _____ Add 560ml of RPMI medium 1X, with L-glutamine to the roller bottle.

3. _____ Add 7.5g Dextrose.

4. _____ Add 75ml of Essential amino acids 50X and 140ml of Non-Essential amino acids 100X.

5. _____ Finally add 100ml of sodium pyruvate 100X.

6. _____ Mix ingredients together by swirling.

7. _____ Adjust pH to 7 with 10N NaOH (note 1).

8. _____ After adjusting the pH, add 8.5g of sodium bicarbonate.

9. _____ Then add 100ml of Penicillin/Streptomycin 100X.

10. _____ Bring the volume up to 1L with RPMI.

11. _____ Filter the tumor cocktail through a Nalgene filter unit, 0.2µm.

12. _____ Make 47 ml aliquots of tumor coctail in 50 ml conical tubes.

13. _____ Store at -20°C.

Complete RPMI

14. _____ In a tissue culture hood, add a 47ml aliquot of tumor cocktail to a new bottle of RPMI medium 1X, with L-glutamine.

15. _____ Add 6.5ml of 100X 2-mercaptoethanol (note 2).

16. _____ Finally add 50 ml of FCS (note 3).

17. _____ Mix the media with a 50ml serological pipet by pipetting up and down several times.

18. _____ Store at 4°C (note 4).

Notes:
1. Do not use 10N NaOH from the general lab stock. Make a separate stock and keep it as sterile as possible for future use. Also it is not necessary to use a pH meter, there is a pH indicator in the media. When the media is acidic it is yellow and when it is basic it is red. Adjust the pH so that the color of the media is a bright orangish-red, it will then be at a pH of 7.
2. 100X 2-mercaptoethanol is made by adding 35µl of 2-mercaptoethanol per 100ml RPMI medium 1X, with L-glutamine. This solution is mixed and filtered through 0.2µm filter. Aliquot this solution into 15ml falcon tubes and store at -20°C.
3. FCS is added last because it causes bubbles to form in the media and this should be minimized.
4. This media should be used within 2 months. After 2 months discard and make fresh media.

SOP: SP039

ELISA Assay

Materials and Reagents:
1. 96 well ELISA plate
2. Multi-channel pipettor
3. 1-200µl pipet tips
4. TBST (note 1)
5. TBS (note 2)
6. Blocking solution (note 3)
7. KPL *p*-NPP developer kit (cat# 508000)
8. Primary antibody being tested
9. Antigen testing against
10. Secondary antibody
11. 4°C cold room or fridge
12. Plate sealer

Protocol:

1. _____ Coat 96 well ELISA plate with appropriate antigen or sample (note 4).

2. _____ Incubate ELISA plate overnight at 4°C.

3. _____ Remove antigen and save for another assay if needed.

4. _____ Block ELISA plate with 200µl of blocking solution for 1 hour.

5. _____ Dump off blocking solution into sink.

6. _____ Transfer primary antibody to the ELISA plate, 100µl/ well (note 5).

7. _____ Incubate for 1½ -2 hours at room temperature.

8. _____ Discard the samples into sink or save primary antibody if needed.

9. _____ Wash the plates with 100-200µl of TBST five times and on the fifth wash let stand for ten minutes.

10. _____ Prepare secondary antibody: use anti-mouse alkaline phosphatase conjugated antibody at 1:2500 in TBS (note 6).

11. _____ Plate 100ul of the secondary antibody and incubate for 1½ -2 hours.

12. _____ Discard secondary in sink.

13. _____ Wash the plate with TBS fives times and on the fifth wash let stand for ten minutes.

14. _____ Prepare 10 ml of KPL *p*NPP developer per 96 well plate: 2 ml of Diethanolamine Buffer in 8 ml of ddH20 and add 1 *p*NPP tablet.

15. _____ Add 100µl of developer to each well and incubate at 37°C until reaction occurs (note 7).

16. _____ Read at 405nm on a microplate reader.

17. _____ Allow the developer to dry in a chemical hood before discarding the ELISA plate.

Notes:

1. TBST is prepared with 1.21g Tris, 8.77 g NaCl, pH 7.4, 2.5 ml 20% Tween 80 or 0.5 ml Tween 80, QS to 1L with ddH2O.
2. TBS is prepared with 1.21g Tris, 8.77 g NaCl, pH 7.4, QS to 1L with ddH2O.
3. 1-2% BSA in TBST.
4. Prepare a stock solution of antigen by add 100µg of protein to 10 ml of PBS. Mix well and coat 100µl of antigen per well, if using pure protein. The concentration of antigen can increase or decrease depending on individual assays. This antigen can be reused several times. Store at -20°C between each use. Be sure to include a positive and negative control.
5. If needed dilute the primary antibody to proper titer. Primary antibodies can be used more than once. Store at -20°C between each use.
6. If a mouse monoclonal antibody is not used for primary be sure to use the appropriate secondary. The secondary must be alkaline phosphatase conjugated to use this developing kit.
7. The development usually takes between 10-30 minutes.

SOP: SP040

Capture ELISA Assay

Materials and Reagents:

1. 96 well ELISA plate
2. Multi-channel pipettor
3. 1-200µl pipet tips
4. TBST (note 1)
5. 1% BSA-TBST
6. 0.1% BSA-TBST
7. Biotinylated detection antibody
8. Streptavidin-HRP antibody (Zymed)
9. Capture antibody
10. TMB+ Substrate-Chromogen developer (Dako #S1599)
11. Samples to be tested
12. 4°C cold room or fridge
13. Plate sealer

Protocol:

1. _____ Coat 96 well ELISA plate with 100µl capture antibody as per manufacturer's recommended concentration (note 2).

2. _____ Incubate ELISA plate overnight at 4°C.

3. _____ Discard the capture antibody in the sink.

4. _____ Block ELISA plate with 200µl per well of 1% BSA-TBST solution for 1 hour.

5. _____ Discard the blocking solution into sink.

6. _____ Transfer sample, positive and negative controls to the ELISA plate, 100µl/ well.

7. _____ Incubate for 1 ½ -2 hours at room temperature.

8. _____ Discard the samples into sink.

9. _____ Wash the plates with 100-200µl of TBST five times and on the fifth wash let stand for ten minutes.

10. _____ Prepare detection biotinylated antibody as per manufacture's recommended concentration in 0.1% BSA in TBST.

11. _____ Plate 100µl of the detection antibody and incubate for 1½ -2 hours.

12. _____ Discard secondary in sink.

13. _____ Wash the plate with TBST fives times and on the fifth wash let stand for ten minutes.

14. _____ Prepare the streptavidin-HRP conjugated antibody at a dilution of 1:2500 in 0.1% BSA in TBST, add 100 µl per well.

15. _____ Incubate at room temperature for 1 hour.

16. _____ Bring 10ml of TMB substrate to room temperature per ELISA plate.

17. _____ Add 100µl of developer to each well and watch for color change.

18. _____ After development, stop the reaction with 100µl of .18M H_2SO_4.

19. _____ Read at 450nm on a microplate reader.

20. _____ Allow the developer to dry in a chemical hood before discarding the ELISA plate.

Notes:
1. TBST is prepared 1.21g Tris, 8.77 g NaCl, pH 7.4, 2.5 ml 20% Tween 80 or 0.5 ml Tween 80, QS to 1L with ddH2O.
2. Be sure to coat enough wells for all samples, and a positive and negative control.