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.