

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. CO₂ (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 50 μ l spleen cell suspension, and add 450 μ l 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 0.2 μ m 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% CO₂ 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 ddH₂O

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 10⁸. 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 1/6th the number of spleen cells. For example: if the spleen cell count is 1.2 x 10⁸ then 2 x 10⁸ SP2/0 cells are needed. Or more generally, one average spleen to 60 ml of SP2/0 cells at a density of 2 x 10⁸ is about 1/6th.
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 150 μ l. If 150 μ l 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.