SOP: AB103.7
Modified: 7/13/22 KE

Monoclonal Antibody Fusion

Materials and Reagents:

1. SP2/0 murine myeloma B cell fusion partner growing at mid-log phase
2. IMDM medium with L-glutamine, with sodium bicarbonate, with HEPES, suitable for hybridomas (Sigma 16529-500ML) ***OR*** DMEM/F12 + Glutamax (ThermoFisher/Fisher Scientific, Gibco, Cat #10565018) (Note 1)
    - Also called "***INCOMPLETE*****"** IMDM media ***OR*** DMEM/F12 media, ***need both cold (4℃) and warm (37℃)***
2. ***"COMPLETE"*** IMDM media ***OR*** DMEM/F12 (Note 2), ***need both cold (4℃) and warm (37℃)***
3. HAT supplement, 50X (Fisher Scientific Cat# 21060017, 100 mL)
4. Polyethylene glycol (PEG) solution Hybri-Max (Sigma Cat# C979L26, 5 mL x 5 ampules, 50% weight/volume in PBS)
5. Hemocytometer or EVE™ Automatic Cell Counter (See SOP: SP067.2 or SP078.1, respectively)
6. Petri Dish (35mm small, sterile, Corning Cat#351008)
7. Sterile forceps and surgical scissors
8. 1 cc syringe
9. 22-gauge needle
10. 70 µm sterile nylon cell strainer (Fisher Sci (Cat #08-771-2), Corning Falcon Cat# 352350)
11. ACK Lysing Buffer (ThermoFisher Cat# A1049201, 100 mL, sterile)
12. Sterile reagent reservoirs
13. Multichannel pipette
14. 200 µL wide-orifice filter tips (ThermoFisher Cat# 2069GPK, sterile)
15. Sterile 96-well, flat bottom tissue culture treated microplates
    - We have used different brands/vendors for microplates – as long as they are flat and tissue culture treated
16. 5 mL syringe with rubber stopper (sterile)
17. BD Microtainer tubes (Z-no additives, red) for blood collection (Fisher Scientific, Cat# 365963)
18. 0.22 µm Millipore Steriflip vacuum filter unit, PES membrane (Fisher Scientific, Cat# SCGP00525)
19. 0.22 µm 250mL filter unit, PES membrane (Fisher Scientific, Cat# 09-741-05)
20. 0.65 mL Eppendorf tubes
21. 50 mL Falcon centrifuge tubes
22. 15 mL Falcon centrifuge tubes
23. Sterile serological pipettes
24. Glass beaker, 400 mL
25. Tabletop centrifuges (one at 4°C and one at 37°C)
    - ***It may be helpful to keep the 37℃ centrifuge spinning for ALL OF fusion day as to keep temperature steady***
26. Water bath 37°C
27. Water bath room temperature/cool (placed in biosafety cabinet when needed)
    - Can use glass beaker filled with room temperature/cool water for ease of placing in biosafety cabinet
28. CO_2 (5%) Humidified Tissue Culture Incubator at 37°C
29. Biosafety cabinet (BSC)
30. Timer
31. Wypalls
32. 2.5% vesphene
33. 70% ethanol

Protocol:

Week before Fusion

1. _____Reserve necropsy room (for CO_2 asphyxiation) with LAR (basement of Foothills campus). Some CO_2 asphyxiation devices are in the operating rooms.
2. _____Reserve operating room BSC for splenectomy.

3. _____Reserve BSC for fusion process (D142 tissue culture room at Foothills campus). *Weekends are best for fusion days as there is less traffic in the labs with shared space*.
4. _____Make sure the BSC in the tissue culture room and incubator (in D142 tissue culture room at Foothills campus) are deep cleaned (Note 3).
5. _____Make ***complete*** IMDM media ***OR*** DMEM/F12 media with 10% FBS and ***complete*** IMDM media ***OR*** DMEM/F12 + Glutamax media with 20% FBS +1X HAT (Note 2).
6. _____Filter ***incomplete*** IMDM media ***OR*** DMEM/F12 media through a 0.22µm 250mL filter unit and store at 4°C.
7. _____Thaw one vial of SP2/0 murine cancer cells in ***complete*** IMDM media ***OR*** DMEM/F12 media from Step 5, ***without HAT supplement*** (Note 4).

Day before Fusion

8. _____Surface decontaminate tissue culture (D142) BSC with 2.5% vesphene (with contact time) followed by 70% ethanol.
9. _____Filter 50% PEG solution Hybri-Max through a 0.22µm Millipore Sterifilip vacuum filter unit. Store at 4°C overnight.
10. _____Repeat step 8 to decontaminate BSC for next day's use.
11. _____Collect all supplies needed for fusion day (put together in a transfer tub).
12. _____Place decontaminated UV light ballast in the BSC ***OR*** use the UV light already fixed in BSC, and leave on overnight with the BSC blower ON.

Fusion Day

13. _____ On the day of fusion, remove UV light ballast ***OR*** turn off UV light already fixed in BSC and allow BSC to stay on for ~30 minutes so that the airflow is corrected.
14. _____ Decontaminate the BSC with 2.5% vesphene (with contact time) and 70% ethanol.
15. _____ Place ***complete*** IMDM media ***OR*** DMEM/F12 media ***with 10%FBS*** AND ***complete*** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HAT*** in 37°C water bath.
16. _____ Aliquot 2 x 10 mL of ***sterile, cold incomplete*** IMDM media ***OR*** DMEM/F12 media and store at 4°C until step 23. ***Make sure to take these aliquots with you (in rack) for spleen collection***.
17. _____ Wipe down the ~20 T75 flasks of SP2/0 cells with a Wypall sprayed with 70% ethanol and transfer to BSC.
18. _____ Collect/combine the SP2/0 cells into 3 x T75 flasks.
19. _____ Return flasks to the 37°C, 5% CO_2 humidified incubator (***standing up*** as to not leak through filter) until collection in step 41.
20. _____ Decontaminate BSC with 2.5% vesphene (with contact time) and 70% ethanol.
21. _____ Decontaminate all supplies that will be used in the first part of the fusion (SP2/0 cell collection, straining of the spleen, etc.) with a Wypall sprayed with 70% ethanol and place them in BSC.
22. _____In LAR operating room (basement of Foothills campus), sacrifice two hyper-immunized mice 2-3 days after final antigen boost ("Fusion Booster") via CO_2 asphyxiation, followed by cervical dislocation (Note 5).
23. _____ Decontaminate and set up BSC in operating room. *Contact LAR for appropriate setup and take down of BSC as well as if vesphene can be used in their BSCs (they may use different disinfecting products).*
24. _____ Remove spleens aseptically in BSC and place each spleen into a separate 15 mL conical tube containing 10 mL of ***sterile, cold incomplete*** IMDM media ***OR*** DMEM/F12 media. *Make sure to label mouse spleen number on conical tube.*
25. _____Decontaminate and break down BSC in operating room properly. *Contact LAR for appropriate setup and take down of BSC as well as if vesphene can be used in their BSCs (they may use different disinfecting products).*
26. _____Transfer spleens to tissue culture BSC (room D142 at Foothills campus).

Steps 27-45 should use COLD IMDM media OR DMEM/F12 media.

27. _____ Decontaminate conical tubes (with a Wypall sprayed with 70% ethanol) containing spleens and place in a rack in the BSC.
28. _____ Place a sterile 70 µm nylon cell strainer in a sterile 35 mm sterile Petri dish containing 3 mL of ***sterile, cold incomplete*** IMDM media ***OR*** DMEM/F12 media.

29. _____ Cut up spleen into 3-4 smaller pieces with sterile dissection scissors and place in cell strainer.
30. _____ Mush the spleen with the rubber end of the 5 mL syringe stopper for 2-3 minutes, using a ***gentle, circular motion*** (swirl/squish) (Note 6).
31. _____ Place strainer on top of a sterile 50 mL conical tube.
32. _____ Pour media and cells (*from Petri dish)* through strainer into the conical tube to wash strainer.
33. _____ Mush spleen again **gently** while strainer is on top of conical tube.
34. _____ Wash cell strainer again with 5 mL of ***sterile, cold incomplete*** IMDM media ***OR*** DMEM/F12 media to collect residual spleen cells.
35. _____ Pellet cells in conical tubes at 1,200 rpm, 5 min at 4°C.
36. _____ Discard supernatant and resuspend cell pellet ***gently*** with 3 mL of ***sterile, cold incomplete*** IMDM media ***OR*** DMEM/F12 media and then bring the volume up to 10 mL with ***sterile, cold incomplete*** IMDM media ***OR*** DMEM/F12 media.
37. _____ Repeat steps 35 and 36, for a final wash of the cells (Note 7).
38. _____ Remove 50 µL of spleen cell suspension and add to a 0.65 mL tube.
39. _____ Add 450 µL of ACK red blood cell lysis solution to the cell suspension tube (*this will give a 1:10 dilution*).
40. _____Count the spleen cells (Note 8).
41. _____Collect all SP2/0 cells into 50 mL conical tubes from the 3 x T75 flasks. Tap the flasks to release cells from bottom of flasks.
42. _____Repeat steps 35 and 36 with the SP2/0 cells.
43. _____Count the SP2/0 cells (Note 8).
44. _____Combine the SP2/0 cells and the spleen cells in 1 x 50 mL conical tube at a ***ratio of 1:1*** (Note 9).
45. _____Repeat centrifugation from step 35 and gently remove supernatant.

Steps 46-51 should use WARMED (37°C) IMDM media OR DMEM/F12 media (with 10% FBS) and the centrifuge warmed at 37℃

46. _____ Wash the ***combined*** SP2/0 cells and spleen cells one more time with 40 mL of ***fresh, warm incomplete*** IMDM media ***OR*** DMEM/F12 media and centrifuge, as in step 35 (Note 10).
    - *Use centrifuge kept at 37℃ (here and for all subsequent steps)*
47. _____ While the cells are spinning, place filtered 50% PEG solution in 37°C water bath.
48. _____ After final wash of SP2/0/spleen cell mixture, remove supernatant via decanting once into a waste container, *careful not to disturb pellet.*
49. _____ Centrifuge the pellet at 1,200 rpm, 1 min at 37°C and remove any additional supernatant via decanting, *careful not to disturb the pellet.*
50. _____ Tap the tube briskly on a hard surface to break up the cell pellet.
51. _____ Smear cells evenly across the 50 mL conical tube as the cell pellet is broken up.
52. _____ Remove the warmed 50% PEG solution and the ***complete*** IMDM media **OR** DMEM/F12 media ***with 10% FBS*** from the 37°C water bath, wipe both with a Wypall sprayed with 70% ethanol, and then place both in BSC.
53. _____ Draw up 1.2 mL of the 50% PEG solution into a sterile 5 mL serological pipet (Note 11).
54. _____ Begin the 6-minute fusion process ***without stoppage***. Use timer.
    a. _____ Minute 1: Add the 50% PEG solution slowly to the smeared cells in the 50 mL conical tube (Note 12).
    b. _____ Minute 2: Continue to gently swirl/roll the cells and 50% PEG solution together.
    c. _____ Minute 3: Slowly add 1 mL of ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 10% FBS*** and continue to swirl/roll gently.
    d. _____ Minute 4: Slowly add another 1 mL of ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 10% FBS*** and continue to swirl/roll gently.
    e. _____ Minute 5: Add 5 mL of ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 10% FBS*** with gentle swirling/rolling.
    f. _____ Minute 6: Gently add ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 10% FBS*** for a final volume of 40 mL while swirling/rolling (Note 13).
55. _____ Pellet the cells at 800 rpm, 5 min at 37°C. Discard supernatant via decanting, *careful not to disrupt the pellet.*

56. _____ **Gently** re-suspend cell pellet in 3 mL of ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS+ 1X HAT*** and then bring the volume up to 50 mL with ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HAT*** (Note 14).
57. _____ Split the re-suspended cells into 2 x 50 mL conical tubes (25 mL each), then bring the volume up to 50 mL ***in each*** conical tube with ***warm, complete*** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HAT***.
58. _____ Transfer the 50 mL conical tubes with cell suspension via sterile serological pipet into sterile reservoirs. **Rock gently** to mix.
59. _____ Plate 100 µL of cell suspension into each well of a *flat bottom* 96-well microplate (tissue culture treated) using the **200 µL wide orifice filter tips**. Repeat for second 50 mL conical tube with cell suspension. The 100 mL cell suspension will plate approximately ten flat bottom 96 well plates (Notes 15 & 16).
60. _____ Place all 96-well microplates into a 5% CO2, 37°C humidified tissue culture incubator.
61. _____ Two days after fusion, add 100 µL of fresh, warm, complete IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HAT*** to all the wells in all 96-well microplates.
62. _____ Every other day, remove ~100 µL of supernatant from each well and add 100 µL of fresh, warm, complete IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HAT*** to all microplates (Note 17).
63. _____ See SOP: AB104.6 for Screening and Subcloning of Hybridomas.

Notes:

1. Check to make sure what media SP2/0 cells were grown in. Some SP2/0 cells were grown in IMDM and some in DMEM/F12 + Glutamax. The correct corresponding media *must* be used for fusions.
2. See SOP:M028.3 for complete IMDM media and SOP:M027.2 for complete DMEM/F12 +Glutamax media. The "Notes" section for each complete media includes the appropriate amount of HAT supplement to add for fusions.
3. See CSU Biosafety Operating Procedures, "How to Clean under the work area of a Biological Safety Cabinet (BSC)".
4. The SP2/0 myeloma B cell is the fusion partner for the spleen cells. For thawing and maintenance of SP2/0 cells see SOP: AB100.6. There should be ~20 x T75 flasks of SP2/0 cells growing for several days in mid-log phase for fusion day. These cells should be healthy and highly viable, >95%. The growth media for the SP2/0 cells should **NOT** contain HAT supplement.
5. The mouse must be sacrificed via CO_2 asphyxiation followed by cervical dislocation in accordance with IACUC certified protocols. ***PLEASE REFER TO APPROPRIATE IACUC PROTOCOLS FOR COMPLIANCE. PROPER TRAINING MUST BE COMPLETED BEFOREHAND.*** Collect as much blood as possible via cardiac puncture after sacrificing the mouse and save the polyclonal sera in a red Microtainer Z tube (Z = no additives) for a positive control. Store serum at -70°C. Normal yield is ~0.75 mL of blood collected.
6. A gentle force must be applied for thorough mushing of the spleen.
7. After final wash, spleen cells must stay in cool water bath as to not activate the spleen cells.
8. Normal yields for total spleen cells are usually $8x10^7$-2.0 x 10^8 cells. Normal yields for total SP2/0 cells are 1-$2.0x10^9$ cells. See SOP: SP067.2 for counting with a hemocytometer and SOP: SP078.1 for counting with EVE™ Automatic Cell Counter.
9. The optimal ratio for SP2/0 cells to spleen cells should be 1:1.
10. At this point, both cell types will have been centrifuged four times, twice separately, twice together. ***The cells must remain at 37°C for the rest of the fusion process.***
11. The fusion process takes a total of six minutes and should be done very carefully and accurately.
12. 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 onto the cells throughout the first minute. Continue to swirl/roll cells.
13. The cells will appear quite clumpy during the fusion process and after the addition of the IMDM ***OR*** DMEM/F12 media. *Do not attempt* to break up the clumps by rough pipetting during the fusion process.
14. Using a sterile 5mL serological pipette, very gently re-suspend 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* as this may break up lightly fused cells.
15. 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 24-48 hours as a result of the HAT selecting agent.

16. **Flat bottom** 96-well microplates (tissue culture treated) should be used for ***parental hybridomas,*** and **round bottom** 96-well microplates should be used for ***subcloning*** (see SOP: AB104.6 for subcloning).
17. It is important to not completely dry out the wells or perturbing the cells when attempting to take off 100µl. If 100 µL cannot be taken off, take as much as possible without drying out the well or perturbing the cells. 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 5-8, and the supernatants are harvested and tested generally between day 10-15 days following the fusion.

References:

1. **Antibodies Production Manual (Dobos). Version 1. August 26, 2005.**