

Cell Fusion/Hybridoma Production Protocol

Wagner Lab – Cornell University – May 2007

Annotations and notes – Baldwin Lab, UMass, July 2008

Immunization

1. Use BALB/c female mice, 8-10 weeks of age, no preferred vendor
2. Immunize mouse three times (d0, d14, d21) i.p. with protein of interest and 100µl Gerbu adjuvant (total volume of 200µl). Use 1-100ug of protein - depending on purity, concentration, and availability. The protein amount for the first immunization is twice the amount of all subsequent immunizations.
3. Bleed mouse before each immunization for serum collection (~100ul).
4. Perform mouse serum ELISA after d21 sampling. The titer from day 0 to day 21 should increase by 100-1000 fold.
5. When the titer is sufficient (usually after three immunizations, occasionally more), boost with protein (no adjuvant, add 100µl 1xPBS instead) three times three days in a row (d28, d29, d30)
6. Perform fusion on d31

Fusion Protocol

-Prewarm all mediums and PEG 1500 to 37°C.

Typically need:

1-1.5L HAT medium (assuming 3-5 x 10⁸ splenocytes)

~100ml HYB-SFM + 10% FBS

~25ml HYB-SFM, no FBS

Myeloma cells

-Assess and count X63-Ag8.653 myeloma cells. Leave in Hyb+10%FCS in incubator throughout spleen cell recovery.

(X63-Ag8.653s grow best in 150mm tissue culture treated petri dishes, approx. 60 ml each. Aim for 8 plates per fusion. This usually yields twice as many cells as needed for the fusion.

X63s are thawed the week before the fusion, and are split out 1:2 each day until the day before the fusion (i.e. Monday through Wednesday) to keep in exponential growth.)

Spleen cell collection

1. Euthanize mouse via cervical dislocation.
2. Place entire mouse in a beaker containing ~200ml of 80%Betadine, 20% 70%-ethanol.
3. Remove spleen using aseptic techniques
(use 3 sets of tools (scissors, forceps), one each for skin, IP sheath, spleen)
4. Transfer spleen to Petri dish containing 5ml Hyb SFM + 10%FCS
(for transport only – not necessary if removing spleen in same area where fusion will be done)
5. In hood, move spleen to new Petri dish in 1ml Hyb-SFM+10%FCS.
6. Trim fat and connective tissue, then cut spleen into small pieces.

- (cut spleen into 5 sections longitudinally, then cut each section in half)*
7. Using two sterile watchmaker's forceps, secure a piece of spleen with one forceps while "milking" cells from the piece into the medium.
(Place capsule pieces aside in a pile in the petri dish.)
 8. Transfer cells to a 50ml tube, through several (~4) washes with 2-3ml medium
(Also wash capsule sections. Wash "main" spleen cell collection 4 or 5 times, and capsule collection 2 or 3 times. Transfer all material, including chunks and capsules, to 50ml tube)
 9. Pipette cell suspension a few times, then let sit (~1min) until larger tissue pieces have fallen to the bottom of the tube.
 10. Collect upper cell suspension and place in new 50ml tube. Centrifuge at 900-1000rpm for 5min.
 11. Pour off supernatant.
 12. Resuspend spleen cells in 20ml HYB-SFM + 10% FBS
 13. Count spleen cells (both undiluted and 1:10)
(Don't count obvious RBCs, but when in doubt, count)
 14. Calculate required number of X63s (i.e., 1/2 number of spleen cells). Combine X63s directly with spleen cells.
(if desired, place combined cells in 37C incubator and go have lunch)

Fusion

1. Centrifuge mixed cells 900-1000rpm 5 min.
2. Wash cells with 25ml Hyb-SFM medium (no additives) and centrifuge
(just do one wash only; do not resuspend)
3. Loosen pellet by finger-flicking. Aim for a slurry of cells; do not want chunks or pelleted cells.
4. Slowly add 1.5ml PEG per 3×10^8 mixed cells.
(Do at room temperature – do not need to keep in 37C waterbath. Dispense PEG along sides of tube. Does not need to be 1ml/min slow, just slow enough to keep from blasting cells. Do not touch cell/PEG slurry with pipette. After dispensing PEG, mix by swirling tube.)
5. Incubate for 1 minute at 37C
6. Add very slowly (slower than cells over Ficoll) a total of 20ml Hyb-SFM. (use two 10ml pipettes).
1ml in first minute
3ml in second minute
(Dispense as close to dropwise as possible. Times should be treated as bare minimums – if it takes longer, so be it.)
16ml in third minute
7. Centrifuge
8. Calculate total amount of HAT Fusion medium necessary for plating.
Use tissue-culture treated 24 well plates, 10^6 total cells/well.
For each well, calculate 2ml of medium
9. Subtract 200ml from total HAT medium volume and divide into workable volumes (<500ml) in wide-mouth Erlenmeyer flasks.

10. With the remaining 200ml HAT medium, slowly add 25ml at a time to the mixed cell pellet and gently pour even amounts into each HAT bulk volume (first 25ml into one flask, second 25ml into next flask, etc in an attempt to finish with 10^3 cells/ml/flask)

(Do not try to resuspend cells. Dispense 25ml along side of tube slowly, as if layering over Ficoll. Do not pick up media with a pipette – pour directly from 50ml tube into Erlenmeyer flask.

Assume that more cells will be picked up earlier, so when dispensing into flasks 1-4, dispense into 1, 2, 3, 4, 4, 3, 2, 1 in that order.)

11. Put all but one flask into incubator to maintain temperature while plating.
12. Prelabel plates
13. Gently swirl flask to keep cells evenly distributed
14. Using 25ml pipette, plate 2ml/well. Gently swirl flask on a regular basis throughout plating (ex. after each 1-2 plates)
15. Wrap plates in Saran Wrap, three to a stack, place in incubator (37°C, 5% CO₂)

Clone Testing

1. After 10-14 days when clones are visible by eye and medium in some wells just begins to turn yellowish, test all wells by ELISA.
2. Pick individual clones from ELISA positive wells
Transfer to round bottom, cell-treated 96 well plates, 1 clone/well
150ul medium/well
Use 30ul to pick clones
3. When 96-well clones are just visible by eye (usually 3 days later), test by ELISA. Test supernatants for IgG (or other fusion protein tag) and the protein of interest in parallel.
4. For clones that recognize the protein of interest and not the IgG or fusion protein tag, transfer to 24 well plates – 1ml/well
5. Transfer to 6well plates – 3-4ml/well. Collect the supernatants for additional testing depending on the protein: e.g. fusions for CD markers or surface proteins should be tested by flow cytometry. Monoclonal antibodies to an IgG isotype should be tested against various isotypes, etc to identify clones of highest interest.
6. When well-grown, fix an aliquot of cells for clonality testing by flow cytometry, freeze an aliquot, and collect supernatant for mouse isotype ELISA
7. After an additional 2 weeks in HAT (4 weeks total), wean cells into HT medium.
8. After 2 weeks in HT medium, wean cells into Hyb+10%FCS
9. Wean to Hyb-SFM for bulk culture for purification.

Supplies:

Gerbu Adjuvant MM, #3001A, 6x1ml, Accurate Chemical & Scientific Corp.

Hybridoma-SFM, #12045-076, 1000ml, Invitrogen

HAT, H0262-10VL, 10 vials, Sigma

HT, H0137-10VL, 10 vials, Sigma

Recombinant human IL6, RIL61, 20ug, Endogen/Pierce

PEG 1500, #783641, 10x4ml, Roche Diagnostics

Media recipes:

<u>HAT Fusion Medium (500ml)</u> 50ml FCS 10ml HAT (1 vial of Sigma H0262-10VL) 5ml Pen/Strep 500ul (25mg) Gentamicin 3000 U rhumIL6 (optional) in Hyb-SFM	<u>HT Fusion Medium (500ml)</u> 50ml FCS 10ml HT (1 vial of Sigma H0137-10VL) 5ml Pen/Strep 500ul (25mg) Gentamicin in Hyb-SFM
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