Protocol

Polyethylene Glycol Fusion for Hybridoma Production

Edward A. Greenfield

Once a good immune response has developed in an animal and an appropriate screening procedure has been developed, the construction of hybridomas is ready to begin. Polyethylene glycol (PEG) is the fusing agent of choice for hybridoma production, allowing the rapid and manageable fusion of mammalian cells. PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve before mitosis. In this protocol, antibody-secreting cells are isolated from the appropriate lymphoid tissue (mouse spleen and lymph nodes), mixed with myeloma cells, centrifuged to generate good cell-to-cell contacts, and fused with PEG. The fused cells are then diluted into selective medium and plated in multiwell tissue culture dishes. Beginning ~1 wk later, samples of the tissue culture supernatants are removed from wells that contain growing hybridomas and tested for the presence of the appropriate antibodies. Cells from positive wells are grown, single-cell-cloned, and frozen. A procedure for screening batches of PEG for efficacy before hybridoma fusion is also included.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Culture medium (with and without 10% fetal bovine serum [FBS]) (for screening PEG samples)

D-10 medium for hybridomas <R>

DMEM

HAT fusion medium <R>

HT medium (HAT medium without aminopterin)

Mouse spleen or lymph nodes, fresh (see Step 14)

*Harvest tissues according to Protocol: **Harvesting Tissue from Immunized Mice, Rats, and Hamsters** (Greenfield 2017).*

MRC-5 cells

Myeloma cells (for screening PEG samples)

PBS (50 mL/fusion)

Polyethylene glycol (PEG) 1450, including samples for screening if needed

Fusions require very little PEG, and thus good batches of this reagent will last a long time. Bad batches contain trace amounts of toxic chemicals. For PEG screening, see Steps 1–7. However, only the occasional batch of PEG is

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E.A. Greenfield

unusable, and differences in batches are normally small. Most researchers do not bother to test different lots of PEG, but, as a precaution, buy the highest grade of PEG that is available. The American Type Culture Collection (ATCC), Invitrogen, and Roche supply high-quality PEG that does not need to be tested before use.

SP 2/0 cells

Trypan Blue

Equipment

Cell strainer (70 μ m)
Centrifuge tubes (50 and 15 mL)
CO₂ incubator
Countess Cell Counter or hemocytometer
Falcon tubes
Multichannel pipette
Petri dishes or culture dishes
Syringe piston
T25 flasks
Tissue culture dishes (96-well) (flat-bottomed)
Vacuum manifold
Water bath at 50°C (for screening PEG samples)
Water baths at 60°C and 37°C

METHOD

Most PEG fusion techniques are based on the work of Galfre et al. (1977).

Screening for Good Batches of PEG

1. Add 100 μ L of medium containing 10% serum to each well of a 96-well tissue culture dish (one dish per batch of PEG to be tested).
2. Wash myeloma cells by centrifuging at 400g for 5 min (use $\sim 10^6$ cells per assay). Resuspend the cells in medium without serum and centrifuge again.
3. While washing the myeloma cells, melt one vial (0.5 g) of each of the PEG samples to be tested in a water bath set at 50°C. Add 0.5 mL of medium without serum to each vial, and place it in a water bath set at 37°C.
4. Resuspend the cells in medium without serum, and aliquot samples containing 10^6 cells into fresh centrifuge tubes (one tube per batch of PEG). Centrifuge at 800g for 5 min. Carefully remove the supernatant from the cell pellet.
5. Resuspend each cell pellet with a different batch of PEG solution by pipetting up and down. Include one control that is resuspended in medium without serum or PEG. Incubate for 2 min at room temperature. Add 10 mL of medium containing 10% FBS. Take 100 μ L of this suspension, and dilute it into a second 10 mL of medium containing 10% FBS. Centrifuge the final dilution at 400g for 5 min.
6. Aspirate the supernatant, and resuspend the myeloma cells in 1 mL of medium with serum. Transfer 100 μ L of the cell suspension into each of the eight wells on the left-hand side of the 96-well tissue culture dish. Using an eight-well multichannel pipette, perform serial 1-in-2 dilutions across the plate. Return to the CO₂ incubator.
7. Check the plates after 14 d. Good batches of PEG will only slightly inhibit growth and will resemble the “no PEG” controls. Other batches should be discarded.

PEG Fusion

The Day before Fusion

Use D-10 medium for both the MRC-5 and SP 2/0 cultures.

8. Make sure that there are enough SP 2/0 cells. Split them at 1:1 so that they will be in log phase at the time of fusion.
9. Plate MRC-5 cells in flat-bottomed 96-well plates, 100 μ L/well (one frozen vial of MRC-5 cells is good for eight plates).
10. Wash the MRC-5 cells in D-10 medium once at 1200 rpm for 5 min and then plate.

Sample Preparation

11. Thaw the PEG 1450 by incubating it for 5–10 min at $\sim 60^{\circ}\text{C}$. Once thawed, add 2 mL of PBS, and leave the vial at 37°C until further use. Take 50 mL of PBS in a Falcon tube, and leave it in the 37°C water bath. Warm the Dulbecco's modified Eagle's medium (DMEM) and other medium ingredients to room temperature.
12. Collect the SP 2/0 cells (split 1:1 the previous day to keep the cells in log phase). Wash once in DMEM (with no additives such as serum or antibiotics) at 1200 rpm for 5 min at room temperature.

For a typical 10-plate fusion, at least 15 T7 5 flasks of SP 2/0 cells are required. They need to be split 1:1 the previous day to keep the cells in log phase.

13. Resuspend the cells in 25–50 mL of DMEM. Keep the cells on ice until further use.
14. Aseptically remove the spleen and/or lymph nodes from the freshly killed mouse. Place the tissues in DMEM with no additives in a 50-mL tube until further use.
15. Place the spleen in a Petri dish containing ~ 5 mL of DMEM. Mash it using the back of the syringe piston. Make sure that the entire spleen is thoroughly mashed.
16. Collect spleen cells from the Petri dish, and pass them through a 70- μ m strainer into a 50-mL conical tube.

Skip Steps 14 and 15 if using frozen splenocytes.

17. Wash the cells in DMEM once at 1400 rpm for 7 min, resuspend them in 10–25 mL of DMEM, depending on the size of the spleen, and place them on ice.

Splenocytes can be frozen at this stage if they are not used for the fusion.

18. Count both SP 2/0 cells and splenocytes using a Countess Cell Counter. For cell counting, mix 10 μ L of cell suspension + 10 μ L of Trypan Blue in a tube or a well of a 96-well plate. Add 10 μ L of this mixture to the counting slide (or a hemocytometer). Insert the slide in the Countess, adjust the magnification after zooming, and then start counting. If a Countess is not available, count the cells using any standard hemocytometer.
19. Combine splenocytes and SP 2/0 in a 50-mL centrifuge tube at a 1:2 to 1:4 ratio (spleen: myeloma). Gently mix and centrifuge them at 1400 rpm for 7 min at room temperature.

Spleen–Myeloma Fusion

20. Carefully aspirate all of the supernatant off the cell pellet. Do not loosen the pellet, and aspirate as much supernatant as possible.
21. Add 1 mL of PEG 1450 (1 mL for every $\sim 0.5 \times 10^9$ cells) to the pellet slowly (drop by drop) over a period of 1 min. Mix the pellet thoroughly while adding PEG.
22. Incubate the cells for 1 min at room temperature after adding PEG.

23. Slowly add warm PBS (from the water bath) to the cells at a rate of 1 mL the first minute, 2 mL the second minute, 3 mL the third minute, and so on, up to the seventh minute, with constant stirring. In the last 2 min (eighth and ninth minutes), slowly add the remaining PBS to the cells.
24. Incubate the postfusion cell suspension for at least 10 min in the 37°C water bath. Centrifuge the cells at 1000 rpm for 7 min with break-off.
25. Pour off the supernatant, and resuspend the pellet gently in HAT medium at 10 mL per 96-well plate.
If no MRC-5 feeder layer is used, resuspend at 20 mL/96-well plate.
26. Pass the cells through a 70- μ m cell strainer to remove any debris formed during fusion. Wash the strainer with some HAT medium to avoid any cell loss.
27. Add 100 μ L of cell suspension/well to the 96-well plate (200 μ L/well if no feeder layer is used) using a multichannel pipette (preferably 12-channel).

Postfusion Cell Culture

28. Check the plates the next day for any contamination. Feed the plates 7 d after fusion. Remove at least 100 μ L from each well using a multichannel pipette or vacuum manifold, and replace it with fresh HT medium (medium with everything in HAT medium except aminopterin). Gentamicin is not required and cloning factor is optional from this stage.
See Troubleshooting.
29. Hybridoma colonies should start to grow in $\sim$ 7 d postfusion. When the medium starts turning yellow or the wells appear to be confluent, it is time to screen. This usually happens after 10–12 d (in some cases, 14 d).
See Troubleshooting.
30. Collect $\sim$ 120 μ L of supernatant from each well for screening. Add fresh HT medium as in Step 28 again. If screening has to be performed on Day 14, feed the plates one more time on Day 10. Leave the plates without changing medium for at least 3 d before screening. This facilitates accumulation of antibody in the supernatant.
31. Once positive hybridomas are identified after the fusion screen, expand them to a 24-well plate. Rescreen the hybridomas at the 24-well stage to make sure that they are still positive and are making target-specific antibody.
See Troubleshooting.
32. After the second screen (or once the 24-well plate is confluent), transfer the hybridomas to a T25 flask. Only target-specific hybridomas need to be expanded. From this stage onward, gradually reduce the serum concentration (cloning factor, NCTC medium, and gentamicin are not required for this step).
33. Once the hybridomas are expanded to a T25 flask, gradually reduce the HT concentration in the medium: that is, start adding D-10 medium 50% every time so that eventually the cells are completely in D-10 medium. HT weaning should start after four to five passages from the day fusion was performed (first passage on Day 7; second on Day 10; third on Day 12 or 14, which is the 24-well plate after fusion screening; fourth on Day 17 in T25 flask).
34. Freeze at least two vials of parental hybridomas (two vials per T25 flask). Subclone any potential positives as early as possible (i.e., immediately after identifying specific target hybridomas at the 24-well stage).

TROUBLESHOOTING

Problem (Step 28): Myeloma cells are not dying off after fusion.

Solution: Make up fresh HAT medium at 2× concentration. Remove half the medium from each well, and add the fresh 2× HAT medium.

Problem (Step 28): The fusion is contaminated with bacteria.

Solution: Collect peritoneal macrophages from one to three mice of the same strain as the spleen donor. Wash them two times and resuspend in sufficient HAT medium to add 50 µL of macrophages to each well in the fusion plate(s). Macrophages should clear the contamination.

Problem (Step 29): No hybridomas are produced.

Solution: Consider the following.

- The immunization protocol may not have produced activated B cells, an unlikely situation if an adjuvant was used. Try a different adjuvant or immunization method to increase the titer.
- Hybridoma formation and growth may be inadequate. This can occur if the cells (spleen or fusion partner) were contaminated with mycoplasma. Obtain the fusion partner from a reliable source, either a laboratory that is successfully producing hybridomas or from ATCC. Freeze aliquots of these cells as soon as possible to maintain a clean stock. Only use animals from specific pathogen-free suppliers, and cage them in uncontaminated rooms.
- The lot of fetal bovine serum (FBS) does not support hybridoma growth. Test the FBS for a lot that supports high-efficiency cloning using stable hybridoma lines or the fusion partner cells (see Protocol: Screening for Good Batches of Fetal Bovine Serum for Myeloma and Hybridoma Growth [Greenfield 2018]).
- Lack of sufficient growth factors. Add growth supplement like hybridoma growth factor or feeder cells.

Problem (Step 31): Viable hybridomas are produced at the anticipated level, but the desired monoclonal antibody is not found.

Solution: Consider the following.

- You may just have been unlucky and none of the reactive B cells present in the spleen survived fusion. Try fusing another animal.
- The timing between the final boost and when to remove the spleen may be a little different for this particular antigen. Try collecting the spleen a day later than you did before.
- It may be useful to consider alternative immunization methods, choice of animals, and screening assays.

Problem (Step 31): The hybridoma stopped making antibody.

Solution: If none of the cloned hybridomas secrete the desired monoclonal antibody, the hybridoma was not producing the monoclonal antibody initially (false positive), the hybridoma producing the monoclonal antibody died off before subcloning, or the clones have lost the ability to produce the monoclonal antibody because of chromosome loss. Check for mycoplasma contamination. If there is still some activity left in the parental culture, re-subcloning or making a transfected cell line by isolating the VH and VL genes can be tried to recover the hybridoma. Make sure that the myeloma partner cells are in log phase when collecting them for the fusion.

Problem (Step 31): There is very high titer but no stable hybridomas.

Solution: Too much of a good thing may be a bad thing. The B cells may have been overstimulated. Try fusing a mouse with a titer between 1:20,000 and 1:80,000.

RECIPES

D-10 Medium for Hybridomas

Reagent	Volume to add (mL)	Manufacturer	Catalog number	Strength
DMEM	180	Cellgro	15-017-CM	
Fetal bovine serum	75	HyClone	SH30070.03	~25%
Glutamax	3.9	GIBCO	35050	100×
Pen/strep	3.9	GIBCO	15140	100×
Gentamicin	0.3	Sigma-Aldrich	G1397	
Hybridoma Cloning Supplement ^a	9	PAA Laboratories	F05-009	3%

^aOptional.

HAT Fusion Medium

Reagent	Volume to add (mL)	Manufacturer	Catalog number	Strength
DMEM	180	Cellgro	15-017-CM	
Fetal bovine serum	75	HyClone	SH30070.03	~25%
Glutamax	3.9	GIBCO	35050	100×
Pen/strep	3.9	GIBCO	15140	100×
NCTC-109	39	GIBCO	21340	
Gentamicin	0.3	Sigma-Aldrich	G1397	
HAT supplement ^a	4	ATCC	69-X	
Hybridoma Cloning Supplement	9	PAA Laboratories	F05-009	3%

^aSubstitute HT Supplement (ATCC 71-X) after 7 d postfusion.

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Edward A. Greenfield

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