Protocol

Ridding Hybridoma Cell Lines of Contaminating Microorganisms

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The long tissue culture manipulations involved in hybridoma production and maintenance sometimes can lead to contamination. If uncontaminated frozen stocks are not available, then it may be possible to rescue these cultures. Three potential methods for rescuing the lines and their success rates are provided here. If the contamination has been detected early, then the use of drugs (antibiotics) to halt the growth of the contaminating organism may succeed. For 96-well plates that have mild to moderate infections, macrophages may help clear infection. If the contamination is gross, passing the cell line through a mouse and recovering the hybridoma cells through subcloning may be the only method that may succeed.

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.

Reagents

Cell cultures of interest
Reagents for ridding contamination by antibiotics (see Steps 1–10)

- Appropriate antibiotic (see Step 4)
- Culture medium, fresh
- Phosphate-buffered saline (PBS)

Reagents for ridding contamination by passage through mice (see Steps 15–20)

- Betadine
- Culture medium supplemented with 10% fetal bovine serum
- Incomplete Freund's adjuvant
- Mice, female, from the same genetic background as the hybridoma
- Pristane

Reagents for ridding contamination with macrophages (see Steps 11–14)

- Alcohol (70%)
- Mice of the same strain as that used to generate the hybridoma
- Saline or Dulbecco's modified Eagle's medium (DMEM), sterile

Equipment

Equipment for ridding contamination by antibiotics (see Steps 1–10)

From the Antibodies collection, edited by Edward A. Greenfield.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot103275

Cell culture dishes (100 mm)
Incubator
Microscope
T-flasks

Equipment for ridding contamination by passage through mice (see Steps 15–20)

Needle (18 gauge)
Tissue culture plates

Equipment for ridding contamination with macrophages (see Steps 11–14)

Forceps, sterile
Scissors, sterile

METHOD

Ridding Cell Lines of Contaminating Microorganisms by Antibiotics

This protocol is used to stop the growth of contaminating microorganisms if the contamination has been detected early. Antibiotics may be able to kill the contaminating organisms, but the dosage required to do this is often more toxic to the hybridoma cells than the bacteria. The success rate of this method is ~20%.

1. Observe the cells under the microscope to determine whether the contaminating microorganism is bacterial or fungal. Transfer ~10^5 cells from the contaminated culture to a sterile centrifuge tube. Centrifuge at 200*g* for 5 min at room temperature.

   See Troubleshooting.

2. Remove the supernatant, being careful not to generate any aerosols. Transfer the supernatant to a suitable container for autoclaving. Resuspend the cell pellet in 10 mL of PBS and spin at 200*g* for 5 min.
3. Carefully remove the PBS as before and repeat the PBS wash.
4. Resuspend the cells in 10 mL of fresh medium supplemented with 10% FBS and an appropriate antibiotic. Normally it would be best to try several different antibiotics. Transfer the cells to a T-flask, and close the top securely. Return the cells to the incubator.

   Tissue culture medium is most commonly supplemented with penicillin and streptomycin, so the medium will now contain at least three drugs.

   For yeast infections, nystatin (Sigma-Aldrich N1638) is the drug of choice. Although it is very rough on cells in general, it is the only real treatment effective against yeast. Nystatin is a suspension and should never be autoclaved or filter-sterilized.

5. Check the cells under the microscope after 1 d. If the contamination has become worse, autoclave the flask. Try passing the cells through a mouse (see Steps 15–20) to eliminate the contamination.
6. If the contamination is the same or better, return the flask to the incubator, and check the cells over the next 2 d. After several days, if the contamination has not progressed and the cells are growing, split the cells into fresh medium with the same drug selection. Continue to grow the cells in T-flasks with tightened caps.
7. After several weeks of growth in these conditions, the cells should have outgrown the contaminating organism. At this stage, the hybridoma or myeloma cell should be single-cell-cloned in drug-containing medium (see Protocol: **Single-Cell Cloning of Hybridoma Cells by Limiting Dilution** [Greenfield 2019a] and Protocol: **Single-Cell Cloning of Hybridoma Cells by Growth in Soft Agar** [Greenfield 2019b]). Feeder cells should be included. Growing these cells on macrophage feeders has some extra advantages because these phagocytic cells may help clear the infection (see Steps 11–14).

   See Troubleshooting.

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8. Select several clones that appear to arise from single cells, and grow these to the 100-mm dish stage in medium supplemented with the same drug that has slowed the growth of the contaminating organism.
9. Remove half of each culture and centrifuge it at 400*g* for 10 min. Resuspend the cells in medium without the antibiotic. Transfer them to a T-flask and return to the incubator.
10. Grow these cells in T-flasks for several passages while checking for reappearance of the contamination. If no growth appears, handle the cells as normal.

Ridding Cell Lines of Contaminating Microorganisms with Peritoneal Macrophages

Macrophages are phagocytic cells and thus may help clear infection. The macrophages will phagocytize the bacteria, leaving the hybridoma cells untouched. After 24–48 h, the bacteria die off, leaving a contamination-free hybridoma culture. This method works well for 96-well plates that have mild to moderate infections and has a success rate of ~60%.

11. Euthanize one or two adult mice of the same strain used to generate the hybridoma.
12. Dip a mouse in 70% alcohol to keep dander down. With a pair of sterile scissors and forceps, make a midline incision, being careful not to cut through the peritoneal membrane. Peel back the skin and fur, exposing the peritoneal membrane.
13. Inject 1–2 mL of sterile saline or DMEM into the peritoneal cavity, then withdraw the injected material. It will contain many peritoneal macrophages. Caution should be exercised to avoid puncturing the intestines. Repeat this effort a few times for each mouse.
14. Wash the collected peritoneal macrophages thoroughly, then add them to the contaminated cell culture. After 24–48 h, the bacteria should die off, leaving a contamination-free hybridoma culture.

Ridding Cell Lines of Contaminating Microorganisms by Passage through Mice

This method is for gross contamination of cultures and will work with the contents of a single microwell. Therefore, it can be used to rescue a relatively small culture. It will be necessary to single-cell-clone immediately after collecting the ascites. To use this technique, you will also need to have a screening assay available to identify the hybridoma cells from other cell types that will grow out. If the hybridoma cells form a solid ascitic tumor rather than a pocket of loose cells, it is still possible to rescue the cell line using this technique. The hybridoma cells will quickly grow out of tumor fragments dispersed in tissue culture medium. When mice are injected with contaminated hybridoma cells, they should be isolated from the remainder of the mouse colony to lessen the chances of infecting the other mice. Should the mice become ill from the infection, it will be necessary to treat the mice with antibiotics in their water supply. This method has a success rate of ~80%.

15. Inject 10^7 (or less) cells into female mice that have been injected intraperitoneally ~1 wk earlier with 0.5 mL of pristane. These types of injections are also used to prime mice for ascites production, and this may serve as a convenient source of appropriate hosts. If pristane is not available, inject mice with Incomplete Freund's adjuvant and wait 4 h to 1 d before injecting the hybridoma cells. The animals must be of the same genetic background as your cell line or the immune system of the mouse will kill the cells.

    See Troubleshooting.

16. When ascites develops, shave the hair from a small region on the abdomen, apply a surface sterilant such as Betadine, then insert an 18-gauge needle and tap the fluid and transfer it into a sterile centrifuge tube.

    See Troubleshooting.

17. Centrifuge the ascites at 400*g* for 5 min at room temperature.
18. Remove the supernatant. Resuspend the cell pellet in 10 mL of medium supplemented with 10% fetal bovine serum and transfer it to a tissue culture plate. The supernatant can be checked for the presence of the antibody and used for further work if needed.

19. Handle as for normal hybridomas, except keep the cells separate from the other cultures until there is little chance of the contamination reappearing.
20. Subclone a small amount of these cells to separate out any contaminating cells from the mouse. Screen the subclone plate as if screening hybridomas from the original fusion.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 1): The contaminating organism adheres to the hybridoma.

Solution: Treat the cells at Step 3 with trypsin for 5 min. Then wash extensively. Myelomas and hybridomas can be separated from most bacteria by Ficoll density centrifugation. Try this after several washes in PBS and trypsinization (Step 3).

Problem (Step 7): The chosen antibiotic has not eradicated the microorganism.

Solution: Try a different antibiotic or cycle through a few different antibiotics with each feeding/splitting of the cells.

Problem (Step 15): The mice get sick and/or die.

Solution: The bacterial load may be too high or the animal may be susceptible to that microorganism. Treat the mice with antibiotics in their water. The staff veterinarian should be able to recommend which one and the dosage.

Problem (Step 16): Little to no ascites was produced.

Solution: There may be an MHC mismatch between the strain of mouse and the hybridoma cells. Most myeloma partners cells are BALB/c origin (H-2^d). If fused with a BALB/c splenocyte, then the hybridoma can be implanted into an BALB/c mouse or another strain that is H-2^d. If the splenocytes originate from a non-H-2^d mouse strain, then you need to use either nude, SCID, or F_1 generation mice made from a cross of a BALB/c mouse and the strain that donated the splenocytes.

If the hybridoma is not secreting antibody, ascites will not be produced. Check the cells before implantation for activity.

Problem (Step 20): None of the recovered cells are secreting antibody that recognizes the correct antigen.

Solution: The hybridoma may have been unstable and lost the ability to make the antibody. Check cells before implantation for activity.

REFERENCES

Greenfield EA. 2019a. Single-cell cloning of hybridoma cells by limiting dilution. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot103192.

Greenfield EA. 2019b. Single-cell cloning of hybridoma cells by growth in soft agar. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot103200.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot103275