High School Analysis

Protocol of Cell Hybridization for Hybridoma Production

PEG Cell Fusion Protocol

Alok More
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Contents

Protocol via Polyethylene Glycol (PEG)

Introduction

This paper deals with the protocol of cell hybridization for hybridoma technology, in reference to *Polyethylene Glycol Fusion for Hybridoma Production* by Edward A. Greenfield, we will be looking at cell fusion between myeloma and mouse spleen lymph nodes. However, this experiment can be done with any mammalian cells with a high compatibility and similarity rate, and does not need to have myeloma cells, but in this case, complete randomization will occur and specific genes may not appear. After following this protocol, specific chromosomes will be kept to keep producing antibodies to combat the myeloma, however, in other experiments there will be complete random chromosomal variation during fusion unless a limiting factor like myeloma is added.

Terminology

Polyethylene Glycol: A polyether compound derived from petroleum and is used to fuse two different types of cells, most often β-cells and myelomas in order to create hybridomas.

Hybridomas: Hybrid cells produced by the fusion of an antibody-producing lymphocyte with a tumor cell, for Hybridoma Technology.

Myeloma: Also known as Multiple Myeloma, is cancer in plasma cells – a type of white blood cell which produces antibodies.

Heterokaryon: A cell that has multiple different genetic nuclei, can be formed naturally during sexual reproduction of fungi.

Hypoxanthine: a naturally occurring purine derivative. It is occasionally found as a part of nucleic acids, where it is present in the anticodon of tRNA in the form of inosine.

Aminopterin: an antineoplastic drug with immunosuppressive properties often used in chemotherapy.

Thymidine: Thymine

Fibroblast: A type of biological cell that synthesizes the extracellular matrix and collagen, produces the stroma for animal tissues, and plays a critical role in wound healing. Fibroblasts are the most common cells of connective tissue in animals. *Wikipedia*

Aspirate: To breathe or matter that has been drawn from the body by suction

Supernatant: the liquid lying above a solid residue after centrifugation, or other processes.

Pre-Protocol

When a satisfactory immune response is developed from the specified mammal, due to an exogenous antigen, the protocol for the construction of Hybridomas is ready to begin. Polyethylene glycol (PEG) is the fusing agent for this protocol, which allows rapid but manageable fusion of mammalian cells. PEG fuses the plasma membranes of adjacent myeloma cells forming a single cell with two or more nuclei. This heterokaryon keeps these nuclei until the nuclear membrane dissolves before mitosis.

Materials

Reagents

Growth Medium - a solid, liquid or semi-solid designed to support the growth of a population of microorganisms or cells via the process of cell proliferation. *Wikipedia*

Dulbecco's Modified Eagle Medium (DMEM) – A widely used basal medium for supporting the growth of many different mammalian cells. Cells successfully cultured in DMEM include primary fibroblasts, neurons, glial cells, HUVECs, and smooth muscle cells. *ThermoFisher*

HAT fusion medium - hypoxanthine-aminopterin-thymidine medium, a selection medium for mammalian cell culture. *Wikipedia*

HT medium - HAT medium without aminopterin

Mouse Spleen or Lymph Nodes Tissues – Have to be fresh, refer to *Harvesting Tissue from Immunized Mice, Rats, and Hamsters*

Myeloma Cells – Cancer cells we are experimenting on.

MRC-5 Cells - A diploid cell culture line composed of fibroblasts. *Wikipedia*

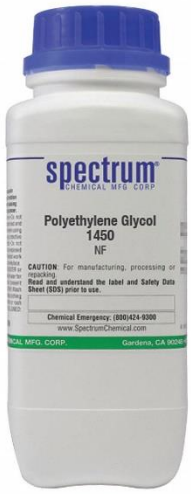
PEG 1450 – Polyethylene Glycol type 1450

PBS (50ml/fusion) - *phosphate buffered saline*, a balanced salt solution used for a variety of cell culture applications, such as washing cells before dissociation, transporting cells or tissue, diluting cells for counting, and preparing reagents. *ThermoFisher*

SP2/0 Cells – A synthesized cells from mouse spleens

Trypan Blue – Blue Dye

HAT Medium



PEG 1450

Equipment

Cell Strainer - Devices used for straining stem and primary cell samples

Centrifuge Tubes - Used in laboratory centrifuges, machines that spin samples in order to separate solids out of liquid chemical solutions

CO_2 Incubator - sealed, climate-controlled boxes to grow cultures.

Cell Counter/Hemocytometer - an instrument for visual counting of the number of cells in a blood sample or other fluid under a microscope.

Falcon Tubes – Type of Centrifuge Tubes

Multichannel Pipette – An instrument for the simultaneous multiple transfers of liquid.

Petri dishes - A shallow transparent lidded dish that biologists use to hold growth medium in which cells can be cultured.

Syringe piston

T25 Flasks – A 25cm^2 Flask for growing spheroid culture, organoid culture, and 3D culture.

Tissue Culture Dishes - Disposable or reusable shallow containers specifically designed to support the growth and propagation of cells.

Vacuum Manifold – An instrument to create a difference in air pressure.

Water Bath at 50C, 60C and 37C – An instrument for precise temperature water baths for various experiments.

Vacuum Manifold


CO2 Incubator


Water Bath

Method

Centrifuge Tubes

The Day before Fusion

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

Make sure that there are enough SP 2/0 cells. Split them at 1:1

Wash the MRC-5 cells in D-10 medium once at 1200 rpm for 5 minutes and then plate cells in tissue culture dishes, 100 µL/dish.

T25 Flask

Sample Preparation

1. Thaw the PEG 1450 by incubating it for 5–10 min at ~60°C.
2. Once thawed, add 2 mL of PBS, and leave the vial at 37°C until further use.
3. Take 50 mL of PBS in a Falcon tube, and leave it in the 37°C water bath.
4. Warm the DMEM and other medium ingredients to room temperature.
5. Collect the SP 2/0 cells and wash once in DMEM (with no additives) at 1200 rpm for 5 minutes at room temperature.
6. Resuspend the cells in 25–50 mL of DMEM. Keep the cells on ice until further use.
7. Aseptically remove the spleen and/or lymph nodes from the freshly killed mouse.
8. Place the tissues in DMEM with no additives in a 50-mL tube until further use.
9. Place the spleen in a Petri dish containing ~5 mL of DMEM.
10. Mash it using the back of the syringe piston. Make sure that the entire spleen is thoroughly mashed.
11. Collect spleen cells from the Petri dish, and pass them through a 70-µm strainer into a 50-mL conical tube.

Can skip Steps 7 to 10 if frozen splenocytes are being used

12. 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.
13. Mix 10 µL of cell suspension and 10 µL of Trypan Blue in a tube.
14. Count both SP 2/0 cells and splenocytes using a hemocytometer, while adding 10 µL of the mixture to the counting slide.
15. Combine splenocytes and SP 2/0 in a 50-mL centrifuge tube at a 1:2 to 1:4 ratio (spleen:myeloma).
16. Gently mix and centrifuge them at 1400 rpm for 7 min at room temperature.

Cell Fusion

- Carefully aspirate all of the supernatant off the cell pellet. Do not loosen the pellet, and aspirate as much supernatant as possible.
- Add 1 mL of PEG 1450 (1 mL for every 0.5 × 10^9 cells) to the pellet drop by drop over a period of 1 minute. Mix the pellet thoroughly while adding PEG.
- Incubate the cells for 1 minute at room temperature after adding PEG.
- 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 minutes (eighth and ninth minutes), slowly add the remaining PBS to the cells.
- Incubate the post fusion cell suspension for at least 10 minutes in the 37°C water bath.
- Centrifuge the cells at 1000 rpm for 7 min with break-off.
- Pour off the supernatant, and resuspend the pellet gently in HAT medium at 10 mL per dish plate.
- Pass the cells through a 70-µm cell strainer to remove any debris formed during fusion. Wash the strainer with some HAT medium to avoid any cell loss.
- Add 100 µL of cell suspension to the dish plate using a multichannel pipette (preferably 12-channel).

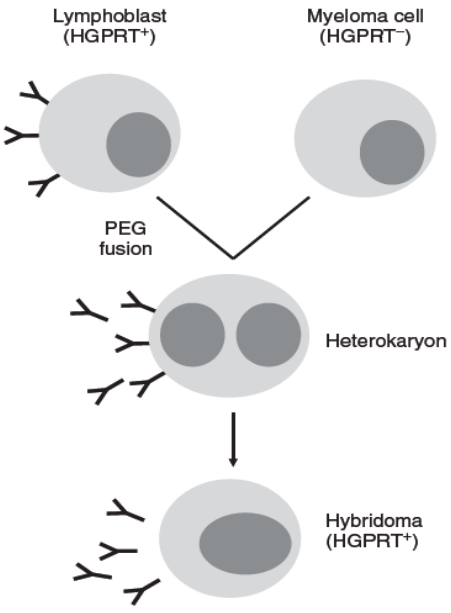


Figure 1. Simplified process of cell fusion in flow diagram.

Falcon Tubes

Post Fusion

- Check the plates the next day for any contamination.
- Feed the plates 7 days after fusion.
- Remove at least 100 µL from each well using a multichannel pipette or vacuum manifold, and replace it with fresh HT medium

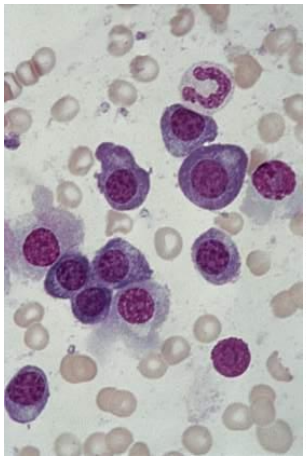
Multichannel Pipette

Hybridoma colonies should start to grow in ~7 days post fusion. When the medium starts turning yellow or the wells appear to be confluent, it is time to screen. This usually happens after 10–12 days. Collect ~120 µL of supernatant from each well for screening.

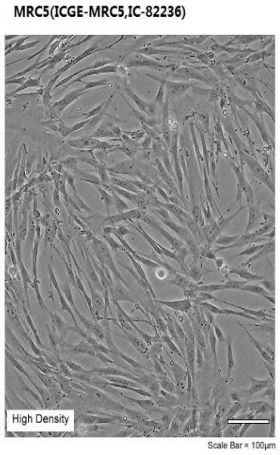
- Leave the plates without changing medium for at least 3 days before screening. This facilitates accumulation of antibody in the supernatant.

Once positive hybridomas are identified after the fusion screen, expand them to a smaller plate, and screen them again to make sure that the are still undergoing meiosis normally, and producing specific antibodies.

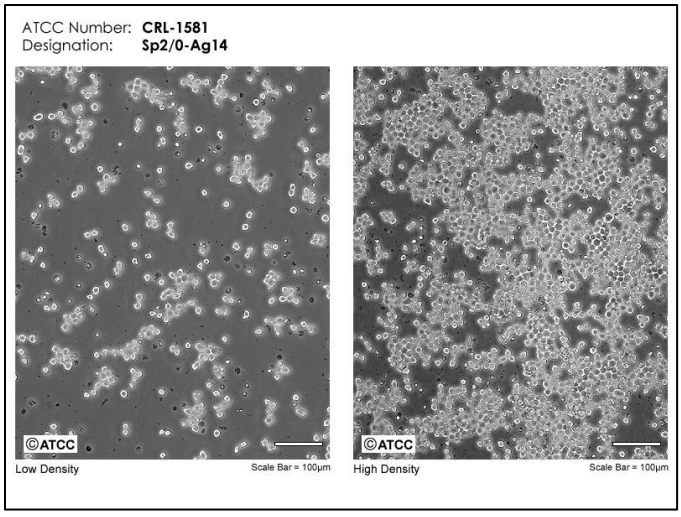
- After the second screen, transfer the hybridomas to a T25 flask.
- gradually reduce the HT concentration in the medium, that is, start adding D-10 medium 50% every time so that eventually the cells are completely in D-10 medium.
- HT weaning should start after four to five passages from the day fusion was performed



Myeloma Cells



MRC -5 Cells



SP2/0 Cells

Troubleshooting

Myeloma cells are not dying off after fusion

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

The fusion is contaminated with bacteria.

Suggested 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.

No hybridomas are produced.

This may be due to:

- The Immunization protocol may have produced activated β-cells.
- Hybridoma growth and formation may be inadequate
- Lack of sufficient growth factors

Suggested Solution: Changing immunization methods, replacing cells and checking for contamination and adding growth supplements respectively.

Hybridomas are produced, but desired antibody is not found.

You may just have been unlucky and none of the reactive B cells present in the spleen survived fusion. Try fusing another animal.

The hybridoma stopped making antibodies.

If the clones do not produce the desired antibody, the hybridoma was not producing it initially, and a false-positive will have occurred.

Suggested Solution: Redoing experiment, or attempting to re-clone any leftover cells.

References

Polyethylene Glycol Fusion for Hybridoma Production by Edward A. Greenfield, © 2018 Cold Spring Harbor Laboratory Press

Multiple myeloma

Hybridoma Technology