Protocol 1016

ECM™ 2001+ ELECTROFUSION PROTOCOL

Cell Line: Spleen (B cells) and P3/ SP2/ F0 Myeloma Cells

Application or Transfectant: Cell Fusion/ Hybridoma Production

Tissue Preparation

Harvest spleen to collect splenocytes (B cells)

1. Autoclave 1 pair of needle end forceps.
2. Euthanize immunized mice, harvest splenocytes using sterilized frosted glass slides in 10 ml SF RPMI or 10 ml cold DMEM.
3. Using the forceps, place the spleen into a 40 µm nylon cell strainer (BD Falcon, ref # 3523340) on top of a Falcon 50 ml conical.
4. Aspirate 15 ml D-PBS into a 25 ml pipette.
5. Gently squeeze the spleen using the forceps to gently pop the spleen's B cell contents. Gently rinse the spleen with the 15 ml of D-PBS through the cells strainer and into the 50 ml Falcon conical.
6. Once the spleen is empty of B cells, discard the spleen membrane.
7. Centrifuge cells at 1600 rpm for 8 min. Aspirate off media using an aspirating pipet attached to a 200 µl pipet tip. Tap to break the cell pellet.
    a. For RBC lysis, resuspend B cells in 5 ml of RBC lysis buffer (Sigma). Allow lysis to go for 5 min at RT. Dilute RBC lysis buffer with 35 ml SF DMEM (pellet should be white).
    b. If Lonza's ACK lysis buffer is used, resuspend cell pellet gently in 6 ml ACK lysis buffer (Lonza # 10-548E). Incubate on ice 5 min, swirling once. Dilute with SF RPMI or SF DMEM (pellet should be white).
8. Gently rinse the B cells with the 15 ml of D-PBS through the cells strainer [40 µm nylon cell strainer (BD Falcon, ref # 3523340)] and into the 50 ml Falcon conical.
9. Wash cells once with 25 ml cold serum-free DMEM by centrifuging at 1500 rpm for 7 minutes.
10. Count the cells.

Recommended Cell Ratios

After the spleen is harvested and processed, wash the P3 cells two times with serum-free (SF) media. Prepare cell mixtures as follows:

- **Ratio of spleen: P3 myeloma cells = 3:1**
- **Ratio of spleen: F0 myeloma cells = 4:1**
- **Ratio of spleen: SP2 myeloma cells = 1:1**

Chamber Preparation

Sterilize the electrofusion chamber.

1. Fill the electrode with 70% ethanol, soak for 10 min.
2. Rinse in sterile water twice.
3. Rinse with fusion medium twice before use.
4. Keep the coaxial chamber on ice prior to electrofusion (at least 30 min on ice)
5. Do NOT forget to wipe the bottom and the sides of the chamber before electrofusion to remove condensation from ice.

Electrofusion Procedure

1. Pre-warm cell fusion medium and post-fusion medium to room temperature.
2. After harvesting and counting the mouse B cells and the myeloma cells (F0 or SP2/0 or P3), calculate the amount of cells required to perform the fusion based on the number of cells or plates you wish to plate out. Wash them separately in the electrofusion medium.
3. Gently mix B cells and the myeloma cells (F0 or SP2/0 or P3) in the appropriate ratio as provided in "Recommended Cell Rations" in the Tissue Preparation section.
4. Wash B cells and myeloma cells two times (3 centrifuges) in 20 ml electrofusion medium. Spin for 7 min @ 1400 rpm.
5. Resuspend the pellet with appropriate amount of electrofusion medium in electrofusion chamber as below.
6. Resuspend in 2 ml room temperature **Cytofusion Medium C** in 2 ml coaxial chamber or 9 ml medium in 9 ml coaxial chamber at a final density of 9.9E+06 cells/ml or 10 million cells/ml or other desired cell density.
7. Place the cap on the chamber.
8. **IMPORTANT:** Immediately start electrofusion (within 30 seconds) using the recommended electrofusion parameters from the table below as a starting point.
9. View the alignment in a 2 ml coaxial optimization chamber or the 3.2 mm or 10 mm gap glass microslide under a microscope.

Note: *Once the cells have been added to the chamber, start the fusion within 30 seconds of loading the cells into the chamber or cells will settle to the bottom and result in poor alignment. If allowed to sit for more than 30 seconds, gently pipette the cells to resuspend before proceeding. You should perform the fusion within ~10 minutes after the final wash. Suspension in fusion media longer than 30 minutes will alter the electro-physical properties of the cells and reduce the fusion efficiency.*

Recommended Electrofusion Parameters

Fusion partners: B cell/SP2; B cell/P3

Step-1 Pre-AC	Step-2 Pre-AC	DC – PULSE	Post-AC
V0 = 40 V	V0 = 70 V	V = 800 V	V0 = 60 V
VF = 60 V	VF = 70 V	T = 40µs	VF = 5 V
T = 15 s	T = 20 s	N = 1	T = 30s
F = 1.4 MHz	F = 1.4 MHz	I = 0	F = 1.4 MHz

B cell and F0 cell Fusion partners

Step-1 Pre-AC	Step-2 Pre-AC	DC – PULSE	Post-AC
V0 = 40 V	V0 = 70 V	V = 800 V	V0 = 70 V
VF = 40 V	VF = 70 V	T = 40µs	VF = 5 V
T = 15 s	T = 20 s	N = 1	T = 30s
F = 1.4 MHz	F = 1.4 MHz	I = 0	F = 1.4 MHz

Key: V0 = starting AC voltage. VF = Ending AC voltage; T = Duration of AC cell focusing step in seconds. F = AC frequency

Note: *The protocols were optimized using 3.81 mm gap size 2 ml coaxial chambers. When using 3.2 mm or 10 mm gap glass microslides, please change the voltage to maintain the field strength. Electrically, the 9 ml chamber and the 2 ml chamber are identical. Protocols optimized using the 2 ml Optimization Chamber can be directly used in the 9 ml Batch Production Chamber.*

Optimization

To optimize, increase the frequency and alignment duration by 10 s (maximum 2 MHz) until pearl-chain alignment is observed. AC amplitude voltage can also be increased to a maximum of 75 V. If the cell alignment is satisfactory but the fusion efficiency is poor, increase the DC by 100 V (maximum is 3000 V).

Note: *Cell compression cannot be observed. The cells should align in an orderly manner and migrate toward the inner electrode. No turbulence or violent movements should be seen.*

The causes of turbulence, if it is observed, are listed below:

- **High current due to high ion content**
  - *Dirty electrode*
  - *Contaminated or high conductivity medium*
  - *Inadequate cell wash*
- **Excessive force applied to cells**
  - *AC amplitude too high*
  - *AC amplitude too long*

Post-Fusion Care

1. After fusion, keep cells in fusion chamber UNDISTURBED for no more than 5 min at room temperature and GENTLY remove cells from fusion chamber with a sterile transfer pipette.
2. Dilute into 40 ml 1X HAT media **w/o HAT** in a 50 ml conical tube and incubate at 37°C for 60 minutes.
3. After incubation, GENTLY add the proper amount of 4X HAT (w/HAT) to the media containing cells and dilute the cells to an appropriate volume for plating (10 cm dish, 96-well plate or 384-well plates).
4. Set up for 384-well plate: Plate cells into 384-well plates at a density of 1 x 10^3 cells/ 60 µl (pre-fusion myeloma cell count) per well.
5. Set up for 96-well plate: Cells are cultured in HAT medium at 5000 – 10,000 cells/ml. If limiting dilution method is used, GENTLY plate 200 µl/well to 10 plates in HAT media (10 ml/plate).
6. Incubate at 37°C.

Methods of Analysis & Screening

Outgrowth should be seen within a week, (usually around day 5 to 7) plates are usually ready to screen Day 10 to 12 days. Total clones are counted by screening the wells in 96 well plates by eye for hybridoma growth on day 7 to 9. The number of clones is mathematically calculated using a poison distribution analysis.

The most common method of analysis is to determine the number of wells with clones (5-10,000 cells/ well) that are secreting antibody and the number that are secreting antigen specific antibody. If low numbers of clones are obtained, it may be useful to analyze the cells immediately after the fusion to determine if there is a problem with the fusion itself.

A simple analysis is to use a cytospin to place cells on a slide and stain the cells with a Wrights or Giemsa stain. Simply count the percent of cells with two or more nuclei.

Wells are screened for presence of IgG antibody and antigen specificity using ELISA or an automated fluorescent screening system (HTRF). Data collected during the screening is normalized to 100 million cells to allow direct comparison of differed fusion.

Another method for analyzing the fusion itself by flow cytometry. For this, cells are stained prior to the fusion with intracellular fluorescent dyes of different color. A good method is published in *Analytical Biochemistry* (1994) Vol. 216:271-275.

Reagents

Complete Growth media/ 10% culture media for myeloma cell lines

- DMEM Base or RPMI 1640
- 10% FBS HyClone
- 1% PSG (Penicillin-Streptomycin-Glutamine)
- 1% NEAA (Non-Essential Amino Acid)
- 1% Na Pyruvate

HAT w/o HAT

- DMEM Base – 335 ml DMEM
- 20% FBS, HyClone – 100 ml FBS
- 20% F0 myeloma conditioned media – 50 ml myeloma conditioned media
- 1% PSG – 5 ml PSG
- 1% HFCS – 5 ml HFCS, 1 vial 50 X (Roche 11 363 735 001)
- 1% NEAA – 5 ml NEAA

Note: *Use 2% HFCS if myeloma fusion partner is P3XAg8.653.*

Note: *Use only 125 ml of HAT (w/o HAT) to make up a 4X concentrate of HAT.*

4X HAT

- 1 vial 50X HAT for 125 ml of HAT w/o HAT medium

50X HAT, Sigma, Cat # H-0262

- Final concentration = Hypoxanthine, aminopterin, thymidine

HAT Stock (50X)

- Hypoxanthine 5 mM, Aminopterin 2 mM, Thymidine 0.8 mM

BTX Reagents

- Cytofusion Medium C, 500 ml volume (47-0001)
- 9 ml Coaxial Chamber for production (47-0020)
- 2 ml Coaxial Chamber for optimization (47-0030)
- Glass Microslides with rectangular electrodes, 10 mm gap, 2.0 ml (45-0106)
- Glass Microslides with rectangular electrodes, 3.2 mm gap, 650 µl (45-0105)

Chamber Cleaning

Chamber cleaning is necessary to remove biological contaminants and ions. The electrofusion process is sensitive to the presence of ions. Clean chambers are essential to prevent excess ion contamination.

Cleaning Process

The following cleaning process is recommended:

- Immediately after use, rinse the chamber in reagent grade water.
- Fill the chamber with 4% sodium hydroxide and soak for 5 minutes.
- Empty the chamber.
- Rinse in reagent grade water for 10 seconds.
- Repeat rinse 10 times.
- Rinse once in 70% ethanol.
- Air dry.

Chemical Sterilization

- Fill chamber with 4% sodium hydroxide and incubate for 5 minutes
- Empty chamber and rinse thoroughly with running deionized (DI) water 5 times.
- Fill with 70% isopropanol or ethanol and soak for 5 minutes.
- Empty chamber and rinse thoroughly with running DI water 5 times.
- Empty chamber and fill chamber with Spor-Klenz® (Steris), soak 5 minutes.
- Rinse thoroughly in sterile reagent grade water.
- Spray the electrode with 70% ethanol, aspirate and air dry.
- Place plastic cover on chamber until ready to load with cells.

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