

Guide for generating hybridomas by electrofusion

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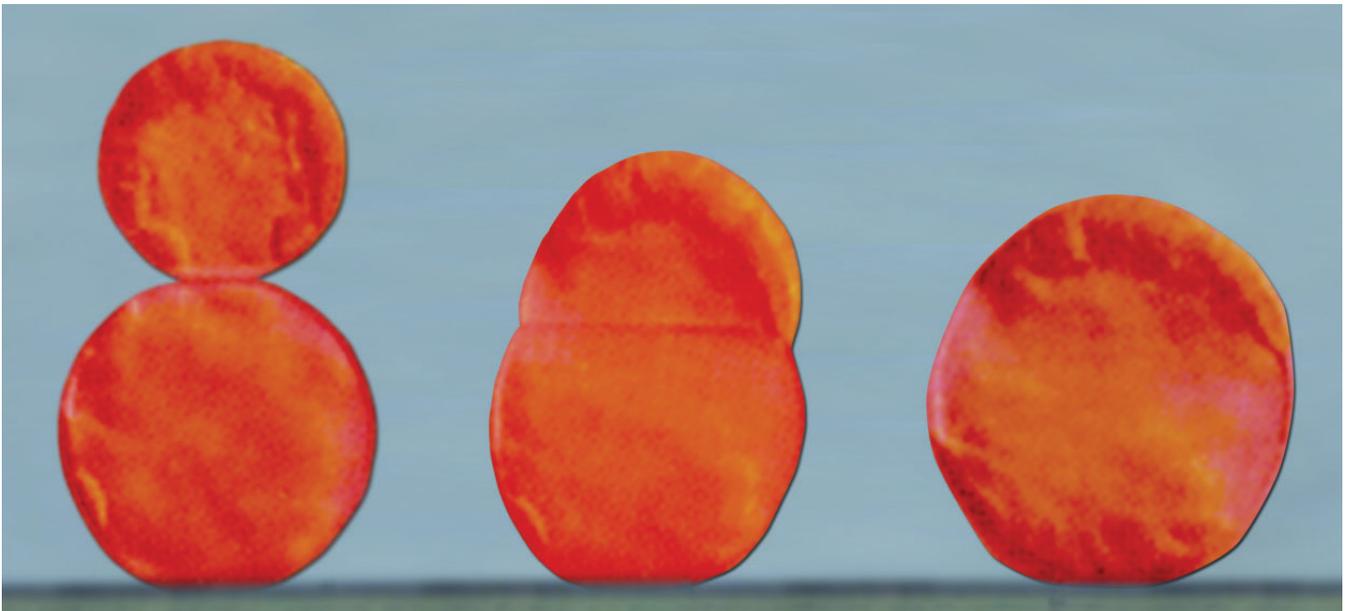


Table of contents

Section	Page
I. Introduction	03
1. Hybridoma technology	03
2. Basics of electrofusion	04
II. Materials for cell culture and electrofusion	04
1. Cell culture devices	04
2. Cell culture disposal	04
3. Cell culture media	04
4. Reagents for cell isolation	05
5. Reagents for B-cell stimulation	05
6. Electrofusion system	05
III. Preparation of cells	06
1. General information concerning cell culture	06
2. Isolation and cultivation of fusion partners	06
Cultivation of myeloma cell lines	06
Isolation of lymphocytes	07
Activation/stimulation of lymphocytes	
IV. Electrofusion	07
1. Points to consider before starting	07
2. Preparing the electrofusion experiment	08
Testing of the fusion buffer	08
Washing of cells	08
Adjusting the cell density	08
3. Optimizing parameters for one cell line	09
4. Optimizing parameters for electrofusion of two different cell lines	10
5. Cleaning the Micro fusion chamber	10
6. Transfer parameters to Helix fusion chamber	10
7. Cleaning the Helix fusion chamber	11
8. Post-fusion treatment	11
Feeder layers	11
Selection	11
Screening	11
9. Short protocol: Electrofusion in the Helix fusion chamber	12
V. Optimization guide	12
VI. Appendix	13
1. Relation between cell radius and fusion voltage	13
2. Example for an optimization table sheet	13
3. References	14
4. Ordering information	15

I. Introduction

1. Hybridoma technology

In 1975, Köhler and Milstein developed a procedure to fuse myeloma cells with B lymphocyte cells from the spleen of an immunized animal. The aim was to generate a cell that is characterized by both the lymphocyte's property of specific antibody production and the immortal character of the myeloma cells. The screening of the resulting hybrid cells together with limiting dilutions, enabled the generation of clones from a single cell fusion [1]. Hybridoma clones can be maintained in culture, and will continue to secrete antibodies.

Today, monoclonal antibodies (Mab) set the stage for many different applications, e.g. tests for the presence of specific antigens, studies of cross-reactivity among antigens, and antigen purification. The development of techniques to produce murine monoclonal antibodies in combination with a variety of applications has led to advances in many fields of biomedical research and diagnostic procedures.

The hybridoma technique is now a routine application performed world wide and modified in many details since its first publication. Up to now PEG (polyethylene glycol) is the most frequently used agent for inducing cell fusion. Nevertheless, PEG-induced fusion is only partly controllable since the success of a fusion depends on minute details such as the size and shape of cells as well as the intensity of shaking, which are difficult to standardize [2].

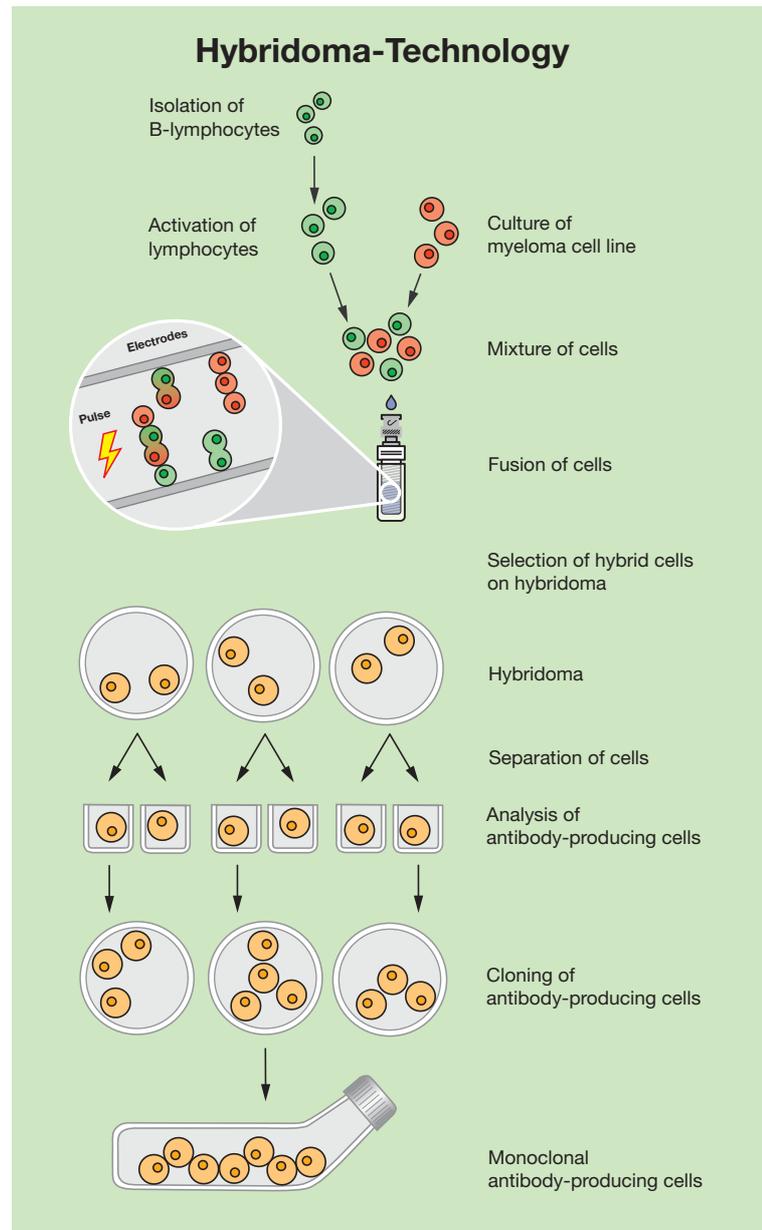


Fig. 1: Generation of antibody-producing hybridoma cells.

An efficient alternative is electric field-mediated cell fusion, which offers several advantages over conventional biological (virus-induced), and chemical (PEG-induced) fusion methods:

- Considerably higher efficiencies for many cell types
- Better reproducibility

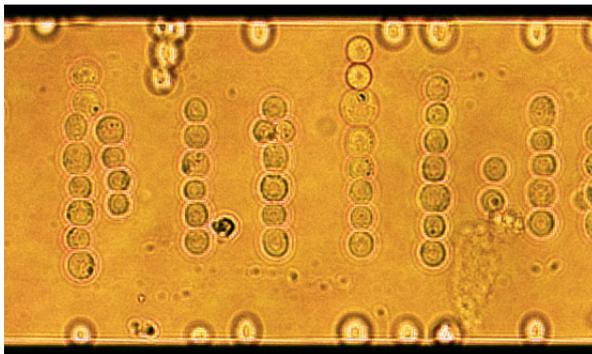
- Significantly lower amount of B cells required
- Fast and easy-to-use protocol
- Better growth properties in the early stage following fusion
- Possibility of tailor-made fusion protocols for specific cells
- Direct control of fusion-results

2. Basics of electrofusion

Applying an electrical field to cells in the form of short, intense pulses increases the permeability of the membrane as well as the membrane flow. The resulting local perforation of the cell membrane allows cell fusion to form a new cell with the properties of the fusion partners. The prerequisite for successful electrofusion is the direct membrane contact between the individual cells before the electrical pulse is applied.

The process of electrofusion comprises three steps:

- **Convergence and contact of the cells (pre-alignment)**
The close membrane contact required for cell fusion is induced by positive dielectrophoresis. An inhomogeneous electrical field causes a dipole to build up within the cells, in turn causing them to move toward the point of maximum strength (i.e. the electrodes) and attract each other, forming long strings ("pearl chains").
- **Membrane fusion**
Very short field pulses with high intensity lead to a reversible electrical breakthrough of the cell membranes. This results in the formation of temporary pores in the contact zone of two cells, which may then become fused.
- **Rounding off of the fusion product (post-alignment)**
Rounding off the fusion products is carried out by expanding the primary fused membrane sections, resulting in a so-called plasmogamy.



II. Materials for electrofusion

1. Cell culture devices

- Sterile bench
- Incubator at 37 °C with CO₂ atmosphere
- Microscope with a slider holder
- Centrifuge for cell centrifugation (e.g. Eppendorf Centrifuges 5702 R, 5804 R, 5810 R)
- Neubauer chamber or electronic cell analyzer
- Automated pipetting device (e.g. Eppendorf Easypet)

2. Cell culture disposal

- Cell culture flasks
- Conical centrifuge tubes
- Serologic pipettes (since many cells will adhere to glass, plastic pipettes or tips should be preferred)
- 24-well plates or 96-well microplates for cultivating the fusion products

3. Cell culture media

- Complete cell culture growth medium (CGM): e.g. RPMI 1640, DMEM or IMDM as a basic medium supplemented with FCS
Adding serum to media has a crucial effect on the growth of hybrids. When fetal calf serum (FCS) is used in the cell culture medium, the endotoxin content must be kept to a minimum. Different manufacturers offer FCS which has been specially tested for hybrid growth after fusion.

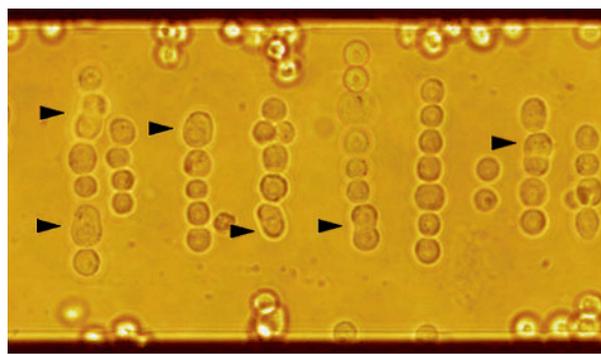


Fig. 2: Microscopic image of cells in the Micro fusion chamber. Alignment (left), fusion products (right, labelled)

- Post-fusion medium: e.g. complete cell culture growth medium without phenol red

In a cell culture medium, phenol red acts as a pH-indicator. However, it has a toxic effect within the cell. Treating the cells only with phenol red-free medium following fusion, i.e. for as long as the cell membranes are partially permeable, is therefore recommended. Phenol red may be added to the medium 24 hours after fusion.

- Selection medium: e.g. HAT (hypoxanthine-aminopterin-thymidine) medium

100 x HT

Dissolve 136 mg hypoxanthine and 38.75 mg thymidine in 100 ml of 0.02 M NaOH pre-warmed to 60 °C. Cool, filter sterilize, and store in aliquots at -20 °C.

100 x A

Dissolve 1.9 mg aminopterin in 100 ml of 0.01 M NaOH, filter sterilize and store in aliquots at -20 °C.

HAT medium

Add 1 % (v/v) 100 x HT and 100 x A to the cell culture medium.[3]

- Trypan blue (0.5 %) or propidium iodide (2 µg/ml) for viability tests.

4. Reagents for cell isolation

- FicolTM separating solution (density: 1.077 g/ml)
- PBS (without Ca²⁺ and Mg²⁺)
- Collagenase II/DNase solution, final concentration 10 U/ml DNase, 1.5 mg/ml collagenase II (e.g. dissolve 2,000 units of DNase in 10 ml cell culture growth medium (CGM). Add 30 mg collagenase II to 1 ml of this solution and fill with CGM until the total volume is 20 ml. Carry out sterile filtration.)

5. Reagents for B cell stimulation

Since small, unstimulated lymphocytes are usually very difficult to fuse, fusion is always performed with activated B cells [4]. The stimulation can be performed *in vivo* or *in vitro*.

One of the following reagents/methods can be used for the *in vitro* stimulation, depending on the antigen system and the source of B cells.

- LPS (lipopolysaccharide) activation [5,6]
- PHA (phytohemagglutinin) activation [7,8,9]
- EBV (Epstein-Barr-Virus) activation [10]
- PWM (pokeweed mitogen) activation [11]
- PWM plus IL-2 [7,12]
- IL-6 plus IL-2 plus muramyl dipeptide [13]
- Anti-CD40 antibodies in combination with IL-4 [7,14,15]

6. Electrofusion system

Multiporator with fusion module

The Eppendorf Multiporator is an innovative, compact and easy-to-use tool for the electrofusion of cells. In combination with a special hypoosmolar, low conductive buffer system, factors which damage the cells (e.g. long pulse times and excess voltage) can be reduced to levels that have no adverse effect on the cell physiology [16]. The Multiporator regulates the voltage impulse used for electrofusion as well as the alternating voltage required for cell alignment.



Fig. 3: Multiporator with insert for cell fusion and Helix fusion chamber.

Micro fusion chamber

Microscopic monitoring of the cells on the Micro fusion chamber allows the optimization of the parameters for alignment and for fusion. The ideal values can be transferred into the Helix fusion chamber for further fusion purposes. The Micro fusion chamber consists of a housing containing a transparent reservoir with two electrodes located 200 μm apart. It is connected to the Multiporator by a coaxial cable.



Fig. 4: Micro fusion chamber for observing electrofusion under the microscope.

Helix fusion chamber with insert

The Helix fusion chamber is specially designed for obtaining large amounts of fusion products (hybrids). It consists of a conically tapered core which bears the electrodes (space between electrodes: 200 μm) and a beaker in which the cells are placed (250 μl). Both parts are screwed together and linked to the Multiporator via the insert.



Fig. 5: Helix fusion chamber.

Fusion medium (Eppendorf Hypoosmolar and Isoosmolar Electrofusion Buffer)

The electrofusion media from Eppendorf are tailor-made for the Multiporator system. An optimal result can be expected under hypoosmolar conditions,

i.e. the cells should swell slightly. The membrane and cytoskeletons are temporarily loosened and fusion in the electric field takes place much more efficiently.



Fig. 6: Eppendorf Electrofusion Buffer system.

III. Preparation of cells

1. General information concerning cell culture

- It is important to develop a consistent system of cell selection, care, and feeding.
 - Fusion parameters can vary with different states of cell activation, different feeding schedules, different cell culture media and the individual cell populations. Even a cloned cell population is not 100 % homogeneous. Feeding the cells the day before fusion ensures that they are all in the same cell cycle [17]. The cell population should be in the exponential growth phase.
- Cell cultures must be tested for mycoplasma.
 - If the cell membranes are attacked by mycoplasma, the necessary cell contact during the alignment is not possible, and fusion cannot take place.
- The centrifugal force necessary to pellet your cells optimally with your equipment must be known in order to reduce cell loss in the wash steps [10].

2. Isolation and cultivation of fusion partners

If not otherwise stated, all centrifugation steps are performed at room temperature.

Cultivation of myeloma cell lines

An appropriate myeloma cell line with a selectable characteristic (e.g. sensitivity to HAT medium) has to be chosen as a fusion partner. The cells should be cultivated at least six days before the fusion experiment. If they do not grow rapidly and healthy a longer time may be necessary [18]. Split the cells one day before fusion. The cultivation and harvesting procedure depends on the cell line.

Isolation of lymphocytes

Isolating lymphocytes from peripheral blood

- Add 20 ml Ficoll™ separating solution to a 50 ml centrifuge tube.
- Mix 10 ml blood sample with 10 ml PBS.
- Coat the Ficoll™ separating solution carefully with the same volume of diluted blood sample.
- Centrifuge for 20 min at 1,200 x g and room temperature (start with the slowest acceleration ramp and switch off the brake!).
- Transfer the lymphocyte band with a pipette to a new centrifuge tube.
- Dilute the lymphocytes in 10 ml PBS and centrifuge for 10 min at 300 x g and 8 °C.
- Dilute the lymphocyte pellet in complete cell culture growth medium and wash once again for 8 min at 200 x g and room temperature.
- Dilute the lymphocytes in complete cell culture growth medium. Set the cell density to 1-2 x 10⁶ cells/ml.

Isolating lymphocytes from tumor material or from lymph nodes

- Cut the tumor/lymph nodes using a pair of scissors.
- Incubate for 60 min in collagenase/DNase at 37 °C in a CO₂ atmosphere. Shake occasionally.
- Strain the tumor material/lymph nodes through a stainless steel sieve (Sigma).
- Dilute the cell material in 10 ml complete cell culture growth medium and centrifuge for 10 min at 200 x g.
- Resuspend the pellet in complete cell culture growth medium and set to a cell density of 1-2 x 10⁶ cells/ml.

Activation/stimulation of lymphocytes

In vivo activated B cells

In traditional murine MAb technology, subjecting the mouse to extensive hyper-immunization programs with highly immunogenic compounds usually causes B cell activation. Human subjects may only be immunized with a very limited number of immunogens (mostly after vaccinations or naturally occurring infections) [4]. In this case an *in vitro* activation is generally not necessary.

In vitro activated B cells

In vitro activated B cells, which are highly suitable for electric field-mediated hybridization, can be obtained by stimulation with polyclonal activators (see sec. II.) [4].

Note: If feeder cells are required (see sec. IV., 8.), prepare these 1 to 3 days before electrofusion.

IV. Electrofusion

For defining the electrofusion protocol the following steps have to be performed:

- Determination of parameters for each cell population separately in the Micro fusion chamber.
Before carrying out electrofusion in the Helix fusion chamber, it is important to gain experience with the myeloma cell line and a variety of stimulated B cells in the Micro fusion chamber [10].
Determine the range and limits for the myeloma cell line in advance. This cell line represents the only constant of the fusion system [17].
- Optimization of parameters with the two cells together in the Micro fusion chamber.
Use a range between the parameters determined in previous experiments to begin the optimization [10].
- Electrofusion experiment in the Helix fusion chamber.
Parameters determined in the Micro fusion chamber can be transferred directly to the Helix fusion chamber.

1. Points to consider before starting

- Make a table for the different conditions you will test in one round ahead of time so that everything important can be recorded quickly (see supplement for an example) [17].
- Have all equipment and reagents ready and available for use.
It is important to minimize the length of time the cells spend in sub-optimal growing conditions (e.g. incubation at room temperature, without CO₂, in solutions without nutrients) [10].
- The fusion temperature should be between 20 °C and 30 °C.
Outside of this temperature range, the fusion rate, and thus the yield of hybrid cells, decreases drastically.

2. Preparing the electrofusion experiment

The following steps have to be carried out for each fusion partner separately.

Testing of the fusion buffer

Prior to the fusion experiment an empirical test has to be performed to determine the optimal conditions. A minimum of 90 % should survive in the Eppendorf Hypoosmolar Electrofusion Buffer after an incubation period of 30 minutes at room temperature.

For sensitive cells which undergo lysis, the osmolarity should be increased by gradually mixing the Eppendorf Hypoosmolar Buffer with the Eppendorf Isoosmolar Buffer until the survival rate of the cells exceeds 90 %. In some cases it might be necessary to use pure Eppendorf Isoosmolar Buffer.

Determine the diameter of the cells after an incubation period of 15 minutes in the optimized buffer mixture under a microscope. The diameter will be the starting point for the calculation of the pulse voltage (see equation in supplement).

Washing of cells

Cells must be washed free of serum protein prior to fusion. They should therefore be collected and washed twice with the previously tested electrofusion buffer. Care must be taken not to lose cells by centrifuging too gently, using too great a volume of washing medium or centrifuging too harshly.

Some populations, such as EBV activated B cells, have increased fusibility in hypoosmolar media for a very short period of time only. In this case washing is performed in isoosmolar medium and cells are then fused after 10 minutes in the hypoosmolar media.

After washing, the cell number is set as required [10].

Note: The cells should not remain in the Eppendorf Electrofusion Buffer longer than 30 minutes!

Adjusting the cell density

Count the cells before you start. A cell density of $1-3 \times 10^6$ cells/ml will be roughly equivalent to a fusion of $3 \times 10^5 - 8 \times 10^5$ cells in the Helix fusion chamber. A higher cell density, especially with large cells, will make it very difficult to observe cell fusion in the Micro fusion chamber [17].

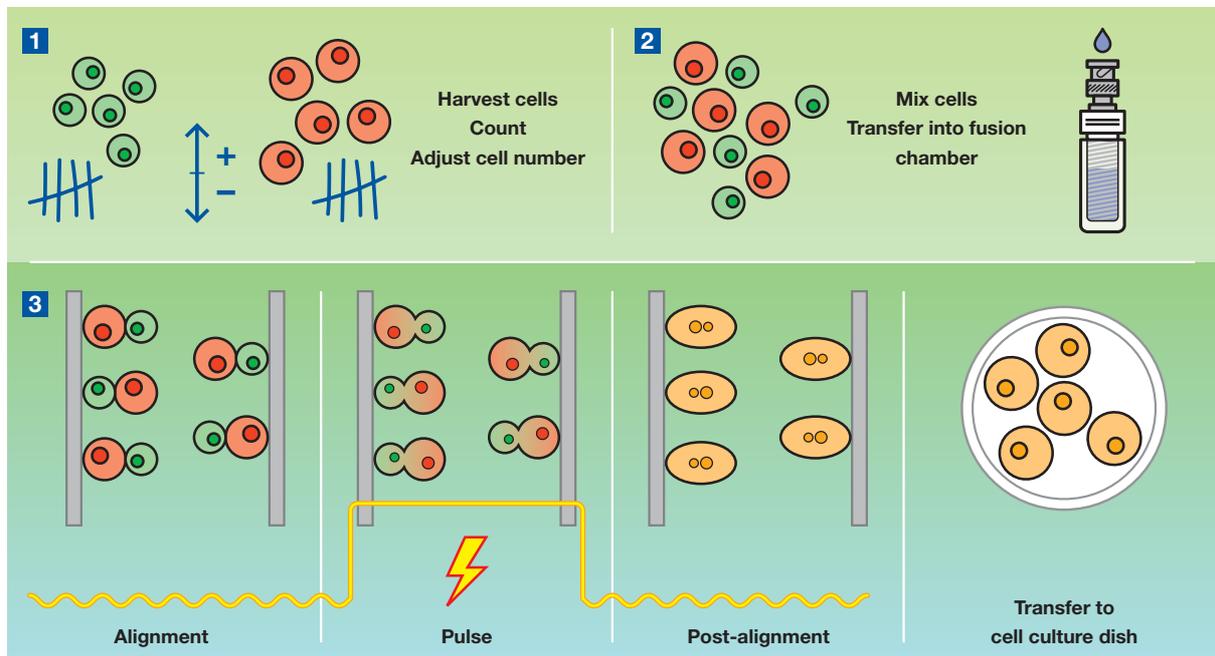


Fig. 7: Schematic sequence of cell fusion with the Multipiporator.

3. Optimizing parameters for one cell line

- Make Micro fusion chambers available to optimize fusion parameters and as many cell preparations as possible beforehand [17].
Since timing is critical it is important that more than one condition can be tested after washing [17].
- Pipette an aliquot of the cell suspension (20 to 50 μ l) onto both electrodes of the Micro fusion chamber so as to completely cover them.
- Place the chamber under a microscope.
Either a normal or an inverted microscope is suitable. A slide holder is advantageous to prevent the chamber from moving. Connect the Micro fusion chamber to the insert of the Multiporator. Focus the microscope on the electrodes under high magnification [10].
- In general standard values for electrofusion protocols will be found in the following range:
Alignment: 4-6 V, 20-30 sec
Pulse: 20-60 V, 15-20 μ sec, no. of pulses 1-3
Post-alignment: 4-6 V, 20-30 sec
These fusion parameters can be used as basic values for optimization.
- The first step is to determine the alignment parameters.
Before a square wave pulse can fuse the cells, they must be aligned in chains between the electrodes. Fusion will occur at the flattened areas of the cell membranes, where the cells are in close contact with one another. Using a higher field strength than necessary during alignment will lead to fewer cells remaining viable through the fusion process. An insufficient field strength will cause the cells to rotate and fail to produce the required membrane contact between cells [10].
Observe the voltage at which the cells start to align themselves. The length of time it takes cells to align depends on several parameters, e.g. how well the cells are washed, their number, viability and size.
For fusion with about $3 \times 10^5 - 8 \times 10^5$ cells, 30 sec are adequate for alignment. For greater numbers of cells, the alignment time shows greater dependence on the specific cells being fused [10,17].
Cells not washed sufficiently or with very poor viability will not align well [10].
- The second step is to determine the square wave pulse needed for fusion.
The pulse voltage required for fusion depends on the cell (e.g. size, shape and state of activation) and the fusion medium. Higher voltages are required with smaller and less activated cells, and lower voltages with larger and more activated cells [10].
The field strength necessary for permeating the membrane of a spherical cell (e.g. lymphocytes, myeloma/hybridoma cells) can be calculated using an equation (see sec. IV., 2. and VI., 1.).
To analyze the fusion process, observe the behavior of the cells under the microscope. Fusion does not take place immediately. Observe intermittently for 10-30 min without removing the chamber from the microscope during this period. If a removal of the chamber is necessary be careful not to disturb the cells [10].
Ideally, in a very homogeneous population chains of fused cells form within 10-15 minutes in Hypoosmolar Electrofusion Buffer and within 20-30 minutes in Isoosmolar Electrofusion Buffer. If the voltage is too high, many cells will lyse or start to lose membrane integrity immediately. In this case try a lower voltage. If no cells fuse, try a higher voltage [10]. If too many giant cells are seen after the pulse a lower cell density should be used [17].
- The last step is the optimization of the post-alignment parameters.
Often, the parameters established for the first alignment step can also be used for the post-alignment. Sometimes, however a new optimization may be required, especially for sensitive cells. In this case try a lower voltage and a shorter time [10].

4. Optimizing parameters for two different cell lines

After determining the optimum fusion parameters for each cell population, the two cell populations can be fused at once. Following is an outline of the process [10].

- Wash each cell population separately as described above [10].
- Pool cells in different ratios to work with in the Micro fusion chamber. Start with a 1:1-2 ratio to determine the voltage and time range. The optimal pulse voltage depends on the particular cell ratio [10].
The goal of the process is to establish optimized parameters for generating hybridomas consisting of one B cell fused to one heteromyeloma. Because the cell populations differ, they will tend to migrate to the electrodes at different rates rather than randomly distributed. This often leads to two cells of one population fusing with a third cell of the other population. With uneven cell ratios this tendency is increased, leading to the formation of unstable hybrids [10].
- Start with the voltage and time range determined in the previous experiments (see sec. IV. 3.).
Observe which population is lost when the voltage becomes too high. In general, a relatively high voltage is used to insure fusion of the activated B cells. However, this intensity will tend to lyse significant numbers of heteromyelomas. The loss can be tolerated because the objective is the fusion of activated B cells and their number is limited [10]. Therefore parameters for the valuable B cells should be favored.

5. Cleaning the Micro fusion chamber

The content of the Micro fusion chamber is rinsed out using bi-distilled water from a spray bottle. Particularly stubborn cell residue can be removed by carefully cleaning the electrodes with a soft toothbrush using vertical strokes (the space between the electrodes must not be changed during this procedure!). Rinsing the chamber with 70 % non-denaturated ethanol accelerates the drying process.

6. Transfer parameters to Helix fusion chamber

- Based on the parameters optimized with the Micro fusion chamber determine the cell ratio and the cell density necessary for fusion [10].
- Pool an appropriate cell number from both populations of cells in one 15 ml conical tube and centrifuge as above. Aspirate the medium [10].
- Wash the cells twice with 5-10 ml of fusion buffer (by using lower cell numbers e.g. 3×10^5 cells, the number of washing steps and volume must be reduced) [10].
Note: In order to guarantee a successful electrofusion the overall incubation time in the Eppendorf Electrofusion Buffer must not exceed 30 minutes.
- After the second wash aspirate to a dry pellet [10].
- Resuspend the cell pellet in Eppendorf Electrofusion Buffer [10].
The exact volume to add depends on the cell number. Hypoosmolar Electrofusion buffer fusions in the Helix fusion chamber are performed with more than 2×10^5 and less than 10^6 cells total. In Isoosmolar Electrofusion Buffer more than 10^6 cells are required [10,17].
- Carefully pipette approx. 250 μ l cell suspension onto the bottom of the beaker of the Helix fusion chamber. Avoid wetting the edges or the inner wall, since this impairs the filling of the Helix fusion chamber. Air bubbles may form, which reduce the effectiveness of the experiment. Insert the electrodes carefully into the beaker and screw both parts of the chamber together slowly but continuously. This action causes the gap to decrease in width, which forces the cell suspension evenly upwards. Leave the closed Helix fusion chamber(s) upside-down on the coaxial connection until starting the fusion process.

- After attaching the Helix fusion chamber to the coaxial connection of the insert by screwing it a quarter-turn, place the insert into the Multiporator.
- Program the device and start the electrofusion. Usually, in Hypoosmolar Electrofusion Buffer the optimized parameters from the Micro fusion chamber can be transferred directly to the Helix fusion chamber. Using Isoosmolar Electrofusion Buffer parameters can be approximated only because cell density in the Helix fusion chamber is generally much greater than what can be observed in the Micro fusion chamber [17].
- Following fusion, the cells remain in the Helix fusion chamber for 10 minutes at room temperature. Using Eppendorf fusion buffer with higher osmolarity than the Eppendorf Hypoosmolar Buffer may require a longer time [17].
- After the chamber is opened, the core is rinsed into the beaker with 1 ml post-fusion medium. Transfer the contents of the beaker to a conical tube. The desired cell concentration can be set by adding post-fusion medium as required.
- The cells are plated out on special cloning plates or microplates and incubated for 24 hours at 37 °C in a CO₂ incubator. If the cell density of the fused cells is low, feeder cells can be plated as well in order to support the initial growth of the cloned cells.

7. Cleaning the Helix fusion chamber

The beaker and core of the Helix fusion chamber should be rinsed with distilled water directly after the experiment in order to prevent drying of the cell and buffer residue. If heavily contaminated, the Helix fusion chamber should be cleaned for 3 minutes in an ultrasonic bath (possibly with a supplementary cleaning agent, such as Edinosite Super) or with a very soft (tooth) brush. When cleaning using brushes, make sure that to brush in the same direction as the windings, since the electrodes may otherwise move out of their correct position and thus render the Helix fusion chamber unusable. Disinfect the parts with ethanol (70 %, non-denatured) by filling the beaker with 250 µl of ethanol and screwing beaker into the core. Unscrew the core after 10 seconds and remove the alcohol. Then set the beaker and the core in the stand to dry under sterile conditions. After drying, the Helix fusion chamber can be used again.

8. Post-fusion treatment

Feeder layers

Whether a feeder layer is required depends on the cell line, the fusion parameters and the plating density. These are found to be most useful under harsh conditions, such as high voltage, and low plating density [17]. Feeder layers are commonly prepared from embryonic fibroblasts, peripheral blood lymphocytes or peritoneal exudate cells. Peritoneal or peripheral blood cells can be used directly since the cells do not divide in culture. Fibroblast feeder cells must be pre-treated by exposing the cells to gamma radiation or incubating them with mitomycin C [19].

Selection

A selective medium (HAT medium) which contains aminopterin (A) to inhibit the growth of the myeloma fusion partner is used following the fusion. Since lymphocytes will die after a few days in culture, only the hybrid cells which result from fusion of a myeloma cell and a lymphocyte will survive [13, 20].

To eliminate all undesired cells, the selective pressure must be maintained for at least 15 days. Following this, the cells are transferred to medium containing a supplement of HT (hypoxanthine, thymidine) for the same length of time, allowing the adaption to the main pathway. In the last step H and T can be removed and the cultures can be kept in normal cell culture growth medium [20].

Hybrids are fed 2-3 times/week after their removal from the selection medium. When the medium becomes yellow as a result of cell growth the supernatant is ready to test for antibody secretion. Mouse/human heteromyeloma fusion partner fusions are ready to assay for activity approximately 2-6 weeks after fusion [17].

Screening

Many different procedures can be used to screen hybridoma culture supernatants. Bound monoclonal antibodies can be detected by using tagged secondary antibodies (e.g. fluorescent, radio-labeled, enzyme-linked) or functional assays, e.g. inhibiting the binding between the ligand and its receptor. Here it is important that the assay is fast, reliable, and specific. Most hybridomas grow rapidly with a generation time of 10-12 hours (rat) to 15-24 hours (mouse), precluding long-term assays [3].

9. Short protocol

Electrofusion in the Helix fusion chamber

- Harvest both cell lines by centrifugation.
- Count the number of cells and set the cell density.
- Pool an appropriate cell number of both populations of cells, centrifuge and aspirate the medium.
- Wash cells twice with Eppendorf Electrofusion Buffer.
Note: In order to guarantee a successful electrofusion the overall incubation time in the Eppendorf Electrofusion Buffer must not exceed 30 minutes.
- After the second wash aspirate to a dry pellet.
- Resuspend the cell pellet in Eppendorf Electrofusion Buffer.
- Carefully pipette approx. 250 µl cell suspension onto the bottom of the beaker of the Helix fusion chamber and fuse as soon as possible (during the waiting period leading up to fusion, turn the chambers upside-down).
- Attach the Helix fusion chamber to the insert and place the insert into the Multiporator.
- Program the device and start the electrofusion.
- Following fusion, the cells remain in the Helix fusion chamber for 10 minutes at room temperature.
- Unscrew the chamber(s) and rinse the electrode core with 1 ml post-fusion medium in the beaker.
- Transfer the contents of the beaker to a conical tube.
- Plate the cells on special cloning plates or microplates and incubate at 37 °C.

V. Optimization guide

During the optimization process using the Micro fusion chamber the following phenomena can occur. Below are some suggestions to help you obtain better results. These parameters must not only be modified separately, but also in combination with each other to achieve efficient fusion [17].

1. What to do if not enough cells fuse?

- Higher pulse voltage
- More pulses
- Longer pulses
- Buffer with lower osmolarity
- Longer alignment times

2. What to do if too many cells die?

- Lower pulse voltage
- Fewer pulses
- Shorter pulses
- Buffer with higher osmolarity (more isoosmolar)

3. What to do if too many giant cells form?

Optimization of two cell fusions rather than multiple cells

- Reduce the total cell number in the chamber
- Decrease the alignment time
- Change the fusion ratio
- Try less hypoosmolar buffer

VI. Appendix

1. Relation between cell radius and fusion voltage

Calculation of the critical field strength

The cell diameter is determined by incubating the cells in (previously tested) Eppendorf Electrofusion Buffer (15 minutes at room temperature) either microscopically or by using a cell counter. With the aid of the diameter, the critical field strength can be calculated:

$$E_c = V_c / 0.75 \times d$$

V_c : Permeation voltage

(for eukaryotic cells: 1 V at 22 °C) (V)

E_c : Critical field strength at which the membrane is permeated at the points on the cell surface oriented vertically to the applied field (V/cm)

d: Cell diameter (cm)

$2 \times E_c$ = Critical field strength for two-cell and three-cell fusions.

Calculation of the fusion voltage

$$U = 2 \times E_c \times d$$

U: Voltage to be set on the device (V)

$2 \times E_c$: Critical field strength for two-cell and three-cell fusions (V/cm)

d: Distance between electrodes (2×10^{-2} cm for Micro fusion chamber and Helix fusion chamber) (cm)

Table of pulse voltages for different cell sizes

Cell radius	Pulse voltage
5 μm	53 V
10 μm	27 V
15 μm	18 V
20 μm	13 V
25 μm	11 V
30 μm	9 V

2. Example of an optimization table sheet [17]

Cell line

Chamber #	Cell density (and cell ratio)	Fusion buffer	Fusion parameter (voltage, time, no.)	Timing of steps	Results	Observations*
			Alignment Pulse Post-alignment	Time to wash medium Time to fusion buffer Time to chamber Time fused	Fusion rate Survival rate	
			Alignment Pulse Post-alignment	Time to wash medium Time to fusion buffer Time to chamber Time fused	Fusion rate Survival rate	
			Alignment Pulse Post-alignment	Time to wash medium Time to fusion buffer Time to chamber Time fused	Fusion rate Survival rate	

*Observations: Examine the cells fusing under the microscope with 10 x phase

- Are the cells still aligned?
- How quickly does fusion take place?

3. References

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General literature (available on the Eppendorf homepage at www.eppendorf.com)

- Multiporator Basic Applications Manual Electrofusion (B 4308 900.105)
- Multiporator Operating Manual (B 4308 900.016)
- Cell specific electrofusion protocols

4. Ordering information

Article		Order no. Intenational	Order no. North America
Multiporator®	for eukaryotics, cell fusion with 1 Helix fusion chamber and 1 Micro fusion chamber	4308 000.031	940-00-070-0
Multiporator®	for eukaryotics, bacteria, yeast and cell fusion with 1 Helix fusion chamber and 1 Micro fusion chamber	4308 000.040	940-00-080-7
Helix fusion chamber	for cell fusion	4308 014.008	940-00-120-0
Micro fusion chamber	gap width 0.2 mm	4308 030.003	940-00-125-1
Stand	for 10 Helix fusion chambers	4308 017.007	940-00-121-8
Electrofusion buffer	hypoosmolar (FH); sterile; 100 ml	4308 070.528	940-00-215-0
Electrofusion buffer	isoosmolar (FI); sterile; 100 ml	4308 070.536	940-00-220-6
Conversion kit for fusion		4308 011.009	940-00-412-8
Insert	for connecting to external electrode (electroporation / electrofusion)	4308 021.004	940-00-420-9

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