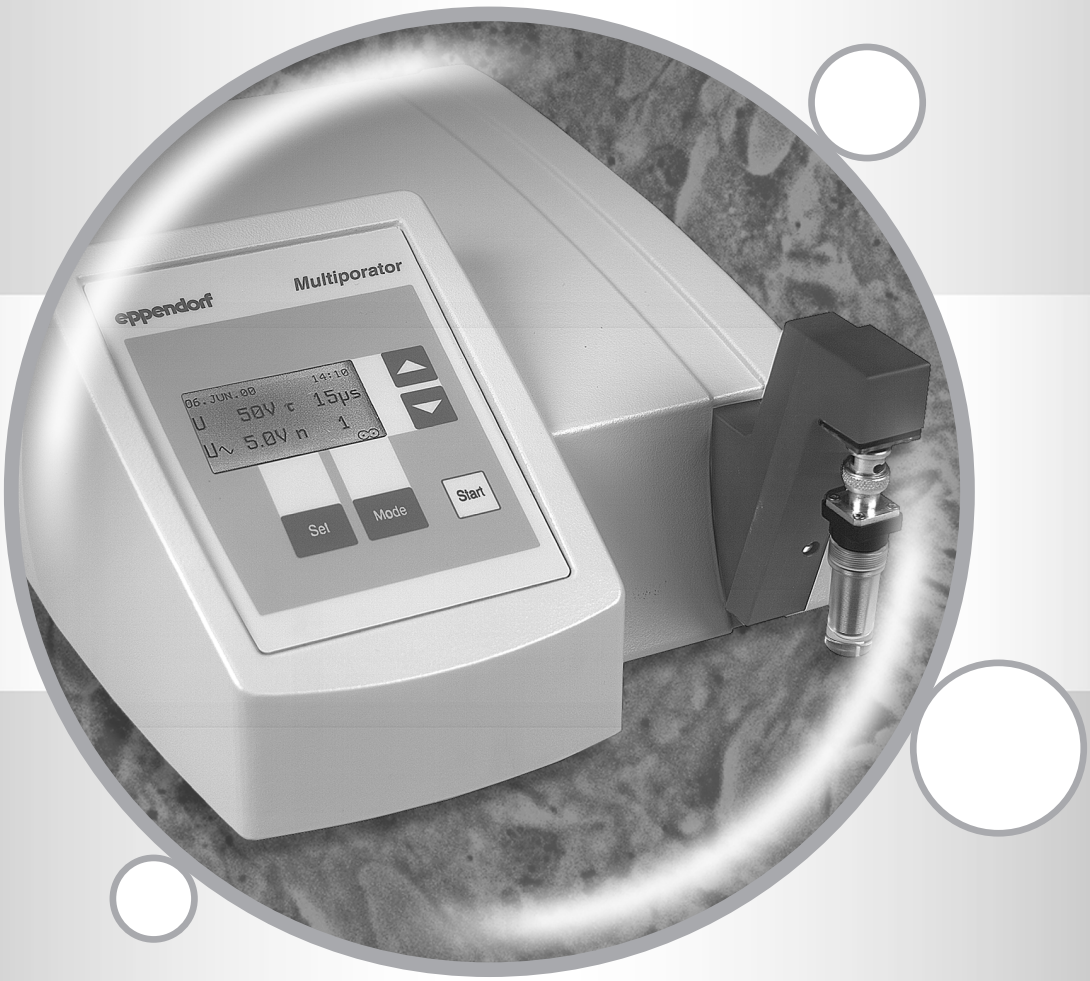

Multiporator®

Basis-Applikations-Anleitung · Basic Applications Manual
Elektroporation · Electroporation

eppendorf

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1 Introduction

The phenomenon whereby a short, intensive current surge (pulse) is used to generate reversible openings (pores) in a membrane was first used in the 1970s to introduce foreign molecules into cells. These membrane pores allow low-molecular substances (such as dyes or peptides) and high-molecular substances (such as proteins, DNA and RNA) to be introduced into cells. This procedure, which is known as electroporation, electroinjection or electropermeabilization, has developed into a standard method in many laboratories, and is used primarily for the transfection of eukaryotic cells and bacteria.

The Eppendorf Multiporator® and the special electroporation buffers form a system which allows electroporation to take place efficiently and gently under hypoosmolar conditions. The hypoosmolar buffer causes the cells to swell up, which expands the membrane and loosens up the cytoskeleton. This in turn leads to a reduction in voltage required for the formation of membrane pores. Electroporation can thus be performed in a more "cell-friendly" manner without any adverse effect on transfection efficiency.

1.1 Purpose of the applications guide

This guide is valid for the eukaryotic module of the Multiporator, by which eukaryotic cells (except yeast and some microorganisms) can be electroporated.

It contains concise descriptions of the experimental conditions that form the basis of the innovative electroporation process carried out using the Multiporator's Soft Pulse technology. Time should be taken to familiarize oneself with the effects that important parameters of the Soft Pulse technology, such as pulse voltage, time, temperature and the composition of the medium, have on the transfection yield. This will enable the best possible transfection results to be achieved with a specific cell line.

Any experiences with commonly used electroporation techniques in the past utilizing millisecond pulse times cannot be compared directly to the technology using the Multiporator® with microsecond pulse times.

Application protocols for many different cell lines can be found on the Eppendorf homepage at www.eppendorf.com. If cells are used for which no application protocol is available, Section 3, "Optimizing the parameters", and the general electroporation protocol in Section 4 should be consulted. Section 5, "Troubleshooting", contains assistance for those occasions when results have not turned out as expected.

Bacteria, yeast as well as other microorganisms can be transformed with the optional bacteria module. Application protocols are available at www.eppendorf.com too.

2 Principle of the Multiporator®

The electroporation functions of the Multiporator® are fundamentally different to those of other commercially available devices. The main differences are as follows:

- Application of extremely short pulses, in the range of 15 to 100 microseconds, versus pulses in the millisecond range.
- Electronic pulse regulation, which allows uniform, reproducible pulses regardless of the resistance properties of the media used.
- Electroporation in a hypoosmolar buffer which is non-toxic and which is adapted to the cytosolic ion composition of the cells.

The combination of these features guarantees high transfection yields without severe damage to the cells. This contrasts sharply with observations frequently made during electroporation in the millisecond range.

Soft Pulses

During electroporation, the membrane of a cell is charged up to a voltage at which the cell membrane is (reversibly) permeated. The pulse lengths (i.e. the time constant τ) used with the Multiporator® for plant and animal cells are usually between 15 µs and 100 µs. In this period, the membrane is permeated when the permeation voltage is exceeded. This leads to a drastic increase in the permeability of the membrane, which can be considered as pore-like openings in the membrane.

If the external voltage applied is up to one thousand times longer (as is the case with milliseconds), high electrical currents flowing through the inside of the cells inflict severe damage upon the cells themselves. Irreversible damage can be caused to the membrane functions and the genome, as well as irreversible changes in the ion composition inside of the pulsed cells. This is the situation with many conventional electroporation devices.

When the Soft Pulse technology of the Multiporator® is applied, however, the cell is charged up only to the point at which a breakthrough of the membrane occurs. The exponentially decaying pulse prevents significant amounts of current from flowing through the cells after the pores have formed.

In addition, the Soft Pulse is measured continuously by the Multiporator® and re-regulated every 5 µs. This unique electronic regulation enables extremely high reproducibility.

As a result, the Multiporator® ensures extremely high transfection rates.

Multiporator® buffer system

Pulse media with low electrical conductivity ensure that the current flow is markedly reduced during electroporation, thus preventing any significant damage to cells. In addition, such media guarantee that the electrically induced "pores" are much larger than those obtained from pulses in conductive solutions, such as phosphate-buffered saline solutions (1).

The Multiporator® is specially designed for the use of such low-conductivity pulse media.

Shifts in the pH value

If the current flow lasts a long time, electrolysis of water takes place on the electrodes of the cuvette. When millisecond pulses are used, the pH value directly at the electrodes changes drastically. Shifts of the pH value in the alkaline and in the acidic range are non-physiological and damage the cells. In contrast, no significant changes in the pH values in and around the electrodes are noted when the Multiporator® is used (2).

2 Principle of the Multiporator®

Release of aluminum during electroporation
Commercially available disposable cuvettes usually contain aluminum electrodes. Aluminum is released from the electrodes under both acidic and alkaline conditions. The shift in the pH value inside the cuvette, which can be observed after millisecond pulses, leads to a release of large quantities of cytotoxic aluminum ions into the pulse medium. The Multiporator's Soft Pulses prevent any cell-damaging increase in the aluminum concentration in the cuvette (2).

Hypoosmolar conditions
The hypoosmolar pulse medium contains far fewer osmotically active substances than culture media or physiological buffer solutions, such as PBS. In a hypoosmolar medium, the cell absorbs water and swells up. Both the cell and its nucleus assume a rounded form and the cell membrane becomes detached from the cytoskeleton, thereby greatly facilitating electroporation of the cell.

Na^+/ K^+ gradient of the cell
Eukaryotic cells build up a gradient in the concentration of sodium and potassium ions across the cell membrane. When electroporation has caused the membrane to become highly permeable, (particularly for small ions), the Na^+/K^+ gradient breaks down locally. The presence of sodium ions in the electroporation buffer (in PBS, for example) makes the situation even worse, since Na^+ ions enter the cell. With K^+ as its sole cation, the special Multiporator® electroporation buffers prevent sodium from entering the cell and, moreover, stops the K^+ gradient from collapsing completely.

The synergy of the Multiporator® and the buffer system
The quality of the Multiporator's performance is derived from the combination of device and pulse medium. The Multiporator® applies Soft Pulses, which lead to a gentle permeation of the cell membrane. The electronically regulated voltage curve guarantees high reproducibility. In combination with the Multiporator®, the special electroporation buffer fulfills several different functions.

1. Low electrical conductivity prevents high current flow and the resulting changes in pH values and therefore prevents an increase in the release of cytotoxic aluminum.
2. The low osmolarity of the pulse medium enables the cell to swell up and round off, thus enabling an easier and more controlled electroporation.
3. The ion composition of the buffer maintains the Na^+/K^+ gradient of the cells.

The synergy of the device and the pulse medium is a result of these fundamental electroporation factors being taken into consideration.

2 Principle of the Multiporator®

Biophysical basics of the Multiporator's technique
Crucial parameters for successful electroporation are the voltage and the length of the pulse (time constant τ) used. Both factors can be set directly on the Multiporator®. The parameters for major applications can be found in numerous existing application protocols, available on the Eppendorf homepage at www.eppendorf.com. For a new application, reference values for the optimal pulse voltage can be calculated or can be taken from the corresponding tables (see Sections 3.1 and 4.1.4).

Voltage and pulse length

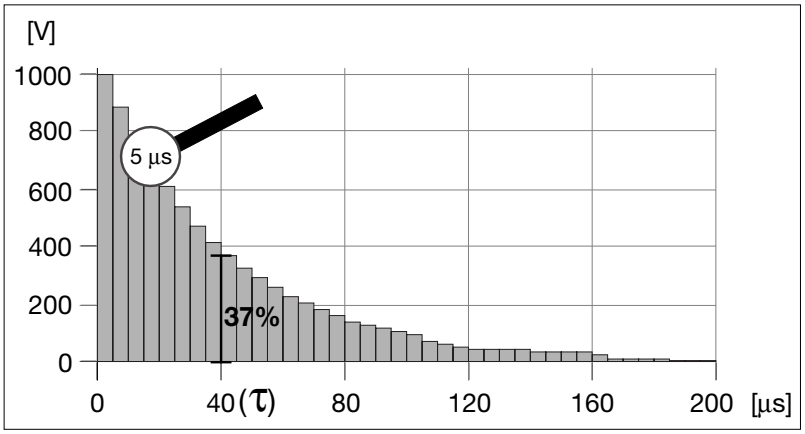

Fig. 1: Controlled exponential decay of the voltage of the microsecond pulse of the Multiporator®

The pulse voltage is readjusted every 5 µs. After the time constant τ (tau), the pulse voltage has dropped down to 37 % of its initial value. The initial pulse voltage and the time constant τ are the only parameters which define the microsecond pulse of the Multiporator®.

The voltage set on the Multiporator® corresponds to the initial voltage (V_0) of the discharge curve shown in Fig. 1. The time constant (τ) shown is the time required for the voltage to decrease to the value V_0/e (= approximately 37 % of the initial voltage). For example, if a voltage of 1,000 V and a time constant of 40 µs have been set on the Multiporator®, the initial voltage of the Soft Pulses is 1,000 V. After 40 µs, this voltage has decreased to approximately 370 V. After these 40 µs, the electronic control of the Multiporator® will have regulated the voltage eight times (i.e. at intervals of 5 µs).

Gap width and field strength
Since the strength of an electrical field depends on the distance of the electrodes, the usage of electroporation cuvettes with a gap width of 1 mm, 2 mm, or 4 mm results in a different field strength (= pulse voltage [V]/gap width [cm]) in the cuvette. As the 100 µl volume of the 1-mm cuvette is extremely small, electroporation of eukaryotic cells is normally carried out using cuvettes with a gap width of 2 mm (400 µl) or 4 mm (800 µl). If a voltage of 800 V is set on the Multiporator®, a field strength of 2,000 V/cm is produced when a 4-mm cuvette is used. However, if a cuvette with a gap width of only 1 mm is used at the same setting, the field strength is 8,000 V/cm! For this reason, particular attention must be paid to the type of cuvette used for the experiments.

2 Principle of the Multiporator®

Calculating the field strength
The critical field strength which is necessary for electropermeation of the membrane can be calculated approximately. To do so, a rough determination of the diameter (d) of the cell must be made. Based on the determined diameter, the critical field strength can be calculated using the following formula:

$$\mathbf{E_c = V_c / (0.75 \times d)}$$

$\mathbf{E_c}$ Critical field strength [V / cm]
$\mathbf{V_c}$ Permeation voltage of the membrane [1 V at 20 °C/ 2 V at 4 °C]
d Cell diameter [cm]

The following is an example for a cell with a diameter of 20 µm (2 x 10^{-3} cm) at room temperature:

$$\mathbf{E_c = 1\ V / (0.75 \times 2 \times 10^{-3}\ cm)}$$

$$\mathbf{E_c = 667\ V/cm}$$

To calculate the voltage which has to be set on the Multiporator®, it is necessary to multiply the field strength E_C by the gap width of the cuvette. In our example, a minimum voltage of 667 V/cm x 0.2 cm = 133 V must be set for a 2-mm cuvette. For a 4-mm cuvette, twice this value (667 V/cm x 0.4 cm = 267 V) is required.

When electroporation is carried out at 4 °C, the E_C value is twice as high as the value at room temperature (because V_C= 2 V at 4 °C).

At the critical field strength E_C, pores form on the poles of the cells oriented in the field direction which small molecules or ions can pass through. It is possible to test "pore formation" immediately using propidium iodide. The red fluorescence of this dye can be detected when it has been incorporated into the cell and has bound to nucleic acids. However, for large molecules, such as nucleic acids, the values used must be higher than the critical field strength E_C. In the case of suspension cells, the ideal value for introducing plasmid DNA into the cell is normally 1 to 3 times that of E_C. For adherent cells, a value 1 to 5 times that of E_C is necessary to introduce DNA into the cell.

! Eppendorf has step-by-step application protocols for the Multiporator® for many frequently used cell lines, which can be found on the Eppendorf homepage at www.eppendorf.com !

Duration of the Soft Pulse
As aforementioned, microsecond pulses are ideal for highly efficient, gentle electroporation. A general rule is that large cells require a longer time for reversible membrane permeation. With the Multiporator®, pulses with a time constant between 5 µs and a maximum of 100 µs are normally used for electroporation. These times are tailored to suit the Multiporator® buffer system.

An optimization strategy for new applications with regard to all relevant parameters (e.g. field strengths, pulse lengths) is described in detail in the following section.

Bibliography
1) Sukhorukov, V.L., Mussauer, H. and Zimmermann, U. (1998) The effect of electrical deformation forces on the electropermeabilisation of erythrocyte membranes in low- and high conductivity media. J.Membr. Biol. 163, 235-245.

2) Friedrich, U., Stachowicz, N., Simm, A., Fuhr, G., Lucas, K. and Zimmermann, U. (1998) High efficiency electrotransfection with aluminium electrodes using microsecond controlled pulses. Bioelectrochemistry and Bioenergetics 47, 103-111.

3 Optimizing the parameters

To ensure that maximum transfection rates are achieved, the electroporation parameters should be optimized for each new cell line. This section contains guidelines for determining the ideal parameters as simply and as quickly as possible.

3.1 Optimizing the field strength

The field strength (V/cm) of the electrical pulse used is an essential factor in determining the survival rate as well as the transfection rate of the cells used.

If the field strength of the pulse exceeds a characteristic value (= critical external field strength), reversible permeation occurs in the cell membrane. This so-called permeation voltage is heavily dependent on the cell diameter and the temperature at which electroporation takes place. The diagrams in Fig. 2 show the permeation voltage that has to be set in relation to the cell diameter and the temperature at which the electroporation is performed. The diameter of the cell is determined after the cells have been incubated in electroporation buffer for 10 – 15 minutes (see Sec. 3.4 + 7.1).

In addition, the gap width of the cuvettes must be taken into account when the minimum pulse voltage is determined. If the gap width is doubled, the pulse voltage must also be doubled in order to obtain the same field strength. A general rule when determining the ideal field strength is that small cells require a higher field strength in order to achieve membrane permeation. The pulse voltages in Fig. 2 and Table 2 (page 16) are the minimum values at which the membrane can be permeated. However, depending on the cell type used, optimal transfection efficiency is often only achieved at significantly higher voltages. To determine the optimal pulse voltage, it is advisable to carry out a series of experiments in which the minimum value, twice the value and then three times the value shown in Table 2 (page 16) are used for suspension cells, and up to five times the value for adherent cells. Cells which do not assume a rounded form in the electroporation buffer often require even higher pulse voltages before optimal transfection can occur.

Please note that increasing the pulse voltage can increase the transfection rate but, at the same time, can also increase the cell mortality rate.

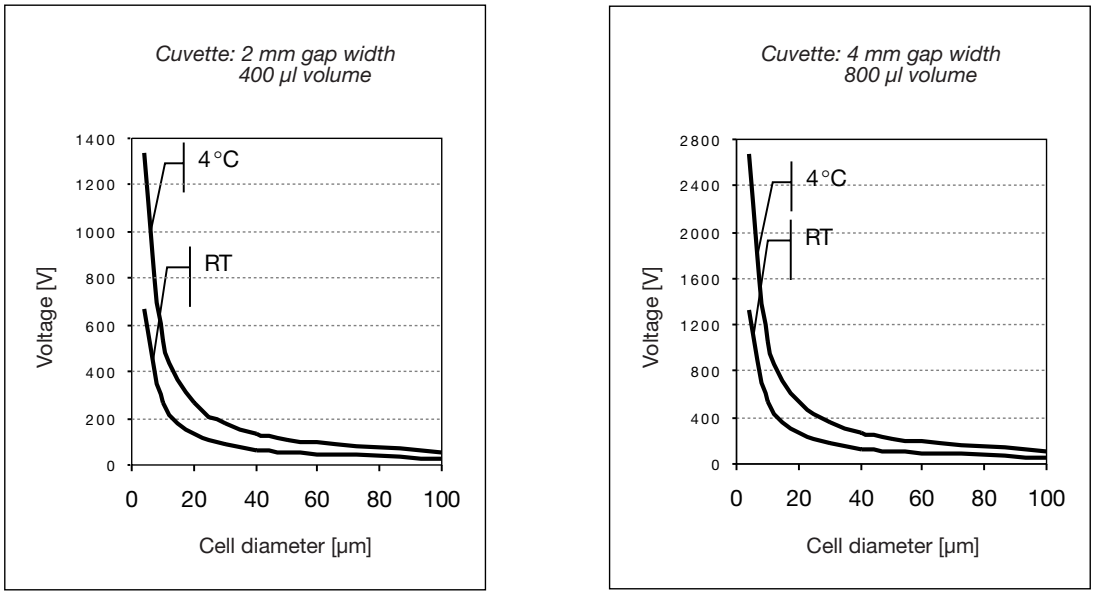

Fig. 2: Minimum pulse voltage at which the cell membrane is permeated

3 Optimizing the parameters

The minimum pulse voltage is dependent on the cell diameter following incubation in electroporation buffer as well as on the temperature and on the gap width of the cuvettes. The values shown can be used to determine the ideal pulse voltage to be set on the device, as described in Sec. 3.1.

Example

Cell type:	Suspension cells
Cell diameter in electroporation medium:	~ 20 µm
Gap width of cuvette:	2 mm
Temperature in the cuvette:	Room temperature
Minimum pulse voltage according to diagrams:	~ 130 V
Series of experiments for optimizing pulse voltage:	130 V / 260 V / 390 V

3.2 Length of the field pulse

In addition to the field strength, a crucial factor for successful transfection is the pulse length.

The length of the pulse is primarily dependent on the diameter of the cell: the larger the cell, the longer the pulse necessary for permeation of the membrane.

Empirically, the ideal pulse lengths for electroporation have proved to be 40 to 100 µs at room temperature and 15 to 40 µs at 4 °C. To optimize the duration of the pulse, three different pulse lengths should be selected within the above-mentioned ranges.

3.3 Number of field pulses

For most cell lines, electroporation is carried out with one pulse.

If one pulse proves to be insufficient, two or more pulses may be used to achieve the desired result, with a 60-second interval between pulses to allow the cell membrane to regenerate. During multiple pulsing, the Multiporator® automatically maintains this interval between each pulse. An intact and resealed cell membrane is a prerequisite for the build-up of the membrane potential, which is essential for electroporation. In addition, the cells rotate during this regeneration phase (Brownian movement), which virtually rules out the danger of further "injury" being caused to the same membrane area by a second pulse.

3.4. Adjustment of the electroporation buffer

A precondition for successful electroporation with the Multiporator® is the special buffer system with low electrical conductivity.

The best possible transfection results are obtained by using the original buffers from Eppendorf, which have been tested for sterility as well as for the absence of mycoplasma, endotoxins and pyrogen and which have also undergone a thorough cytotoxicity test.

However, users may also make up this buffer themselves (see Sec. 7.3).

Ideally, electroporation should be carried out in hypoosmolar buffer, in which the cell absorbs water shortly before the pulse and then swells up as a result. A number of effects, including a decreased optimal permeation voltage, ensure that the plasma membrane can be permeated more easily.

3 Optimizing the parameters

For most cell types, the 20- to 30-minute incubation period in hypoosmolar buffer, which is unavoidable due to the conditions of the experiment, has no effect on the viability of the cells. However, the incubation in hypoosmolar buffer may induce apoptosis, or even lysis, in very sensitive cells. Therefore, it is strongly recommended to test the tolerance of the cells to hypoosmolar conditions. The easiest way of doing so is by incubating the cells for 30 minutes in hypoosmolar buffer and then performing a viability stain using trypan blue or propidium iodide. If observation under a microscope reveals lysis in more than 10 % of the cells, the osmolarity of the buffer must be increased by adding isoosmolar buffer. To determine the optimal osmolarity, it is advisable to incubate the cells in different mixing ratios of hypo- and isoosmolar buffer for 30 minutes prior to the experiment (see Table 1, page 13). This 30-minute period is the maximum incubation time for the cells in the electroporation buffer system. A new viability test followed by observation under a microscope determines the osmolarity that can be tolerated by the cells. The mixing concentrations can then be used for all subsequent experiments with this cell type.

Irrespective of the buffer system selected, it is essential to ensure that the cells do not remain in the electroporation buffer for longer than 30 minutes.

3.5 Influence of DNA/RNA

The transfection efficiency of electroporation can be affected by the concentration, the purity and the size of the molecules used.

a) **Influence of nucleic acid concentration**
With the optimal electroporation parameters (osmolarity, voltage, pulse length), the quality of the results obtained at plasmid concentrations between 5 µg/ml and 20 µg/ml is usually satisfactory.
The efficiency of the transfection may be raised by increasing the nucleic acid concentration, but only within a limited concentration range. Tests with various different cell lines have shown that only in very few cases (e.g. when large plasmids were used) plasmid concentrations in excess of 20 µg/ml lead to an increase in the transfection rate (see Fig. 3).

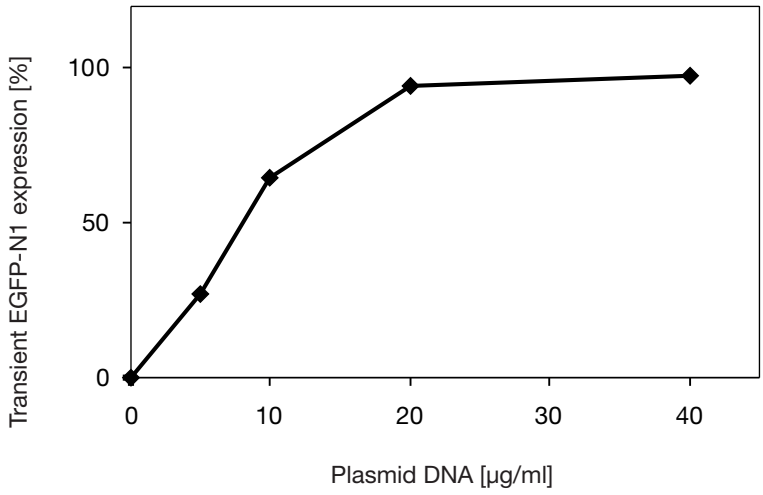

Fig. 3: Transient transfection efficiency in NIH-3T3 cells in relation to the DNA concentration (µg/ml). The cells were electroporated with different concentrations of the pEGFP-N1 plasmid. The transfection rate (max. = 100 %) was determined by FACS analysis.

3 Optimizing the parameters

b) **Influence of nucleic acid purity**
Empirical studies have shown that EDTA and buffer salts such as HEPES or TRIS can drastically reduce transfection efficiency. We therefore recommend dissolving the nucleic acid in distilled water before transfection. Any losses resulting from DNA buffer exchange are usually more than compensated for by the increased transfection efficiency. Irrespective of the preparation method used, the DNA/RNA should be ultra-pure (A_{260} nm/A_{280} nm ≥ 1.8).
It is also important to use endotoxin-free DNA. Otherwise an increase in the DNA concentration will lead to an increase in the endotoxin content in the cell suspension.

c) **Influence of the size of the plasmid**
Transfection efficiency is also affected by the size of the individual molecule that is introduced into the cell. This means that the optimal electroporation parameters that were determined for a certain plasmid, for example, have to be changed when a larger or smaller plasmid is used.

3.6 Influence of the temperature

The temperature has a direct effect on the permeation voltage of the cell membrane as well as on the regeneration of the membrane following electroporation.

a) **Influence of the temperature on the permeation voltage of the cell membrane**
Since the permeation voltage at 4 °C is twice that at room temperature, it is essential to take the temperature into account when determining the optimal field strength of the pulse. Therefore, during electroporation at 4 °C, the necessary field strength of the pulse is also nearly twice as high as those values for room temperature. However, mammalian cells are usually electroporated at room temperature.

b) **Influence of the temperature on the regeneration of the cell membrane**
Incubating cells following electroporation at low temperatures (e.g. 4 °C) slows down the healing process of the cell membrane. In the case of eukaryotic cells, the resealing of the membrane pores can take half an hour and longer under these conditions. With certain cell types, this can lead to an increase in the amount of transfection material absorbed. However, some cells are extremely sensitive to low temperatures, particularly when permeated, and can suffer from irreversible damage after short incubation times in a cold environment.
In those cases where electroporation at 4 °C leads to higher transfection rates, the cells' chances of survival can be boosted if they are resuspended in electroporation buffer at 37 °C or room temperature, cooled down to 4 °C and then transferred into precooled cuvettes. Following electroporation, the cells are incubated at 4 °C for a maximum of two minutes and then heated to 37 °C.
Electroporation at higher temperatures (e.g. >25 °C) causes the permeated membrane areas to seal up more rapidly, which accelerates membrane regeneration and thus increases the cell survival rate. However, the transfection rate may be lower than that obtained when electroporation is carried out at low temperatures.

c) **Influence of the temperature on the conductivity of the medium**
The temperature has a profound effect on the conductivity of the electroporation buffer. Increasing the temperature causes the conductivity of a solution to increase as well, which may lead to lower transfection rates. For this reason, it is advisable not to work at temperatures in excess of 33 °C.

3 Optimizing the parameters

3.7 Influence of the cell density

A cell density of 1 x 10^6 cells/ml is recommended for electroporation since the electrical field is still effective on the cells in this range. When high cell densities (>3 x 10^6) are used, homogeneous field conditions can no longer be guaranteed, i.e. the cells are no longer evenly exposed to the electrical field. This may lead to cell fusion and cause the transfection rate to decrease.

However, using a cell density of <1 x 10^6 cells/ml should have no negative effect on the transfection rate.

Bibliography

3) Zimmermann, U., Effect of high intensity electric field pulses on eucaryotic cell membranes. In: Effect of high intensity electric field pulses on eucaryotic cell membranes. U. Zimmermann and G. Neill, editors. pp. 1-106. CRC Press, Boca Raton, 1996.

4 Electroporation protocol

Cell-specific application protocols are available on the Eppendorf homepage at www.eppendorf.com. The list of applications is updated on a regular basis. (A protocol for Jurkat is included in Appendix 8.4).

If no application protocol is available for the examined cell type, the following general guidelines for the electroporation of eukaryotic cells can be used.

To obtain the best possible transfection results, we recommend determining the optimal electroporation parameters in an experiment. Information on how to optimize the parameters can be found in Sec. 3, "Optimizing electroporation parameters".

4.1 Preparing the cells

4.1.1 Mycoplasma

Mycoplasma prevent efficient and reproducible electroporation of cells. Therefore it is essential to test the cells for the presence of mycoplasma. There are several tests available.

One common method is the DNA fluorochrome staining. This test is based on DNA staining using Hoechst dye 33258 or DAPI, which makes mycoplasma-specific DNA visible under a fluorescence microscope.

The most sensitive current method for detecting mycoplasma is by PCR. PCR detection kits are commercially available but are more time-consuming and expensive than the aforementioned methods.

4.1.2 Cell culture

When electroporation occurs, the cells should already have passed several growth cycles. No freshly thawed or recently transported cells should be used since this additional stress would have a negative effect on the transfection rate.

The cells should be in the exponential growth phase when transfection takes place.

4.1.3 Setting the osmolarity of the electroporation buffer

Electroporation with the Multiporator® should ideally be carried out in hypoosmolar electroporation buffer. The tolerance of the cells to hypoosmolar conditions has to be tested in a preliminary experiment (see Sec. 3.4).

4.1.3.1 Harvesting adherent cells

Cells should be harvested as gently as possible. According to the cell type, the following methods can be used:

- Dispase (concentration: 0.01 to 0.1 % w/v). This has proved to be the most gentle method for harvesting.
- Trypsin (HPLC-grade, without EDTA, 0.1 to 0.25 % w/v). Prior to the addition of trypsin, the cells must be washed at least twice with PBS, without Ca^{2+} and Mg^{2+}.
- Scrape the cells carefully from the bottom of the culture dish.

4 Electroporation protocol

4.1.3.2 Testing the tolerance to hypoosmolar conditions

The cells are incubated in hypoosmolar electroporation buffer for 30 minutes at room temperature.
After incubation, the survival rate of the cells is determined by viability staining.

- Trypan blue: Stains dead cells under the microscope.
- Propidium iodide: Stains dead cells under the fluorescence microscope.

If the survival rate of the cells is >90 %, the hypoosmolar buffer can be used in undiluted form for electroporation.
If more than about 10 % of the cells are lysed, the optimal osmolarity of the electroporation buffer must be determined.
In a series of experiments, the cells are incubated for 30 minutes in buffers with a gradually increasing osmolarity.
These buffers are produced by mixing different volumes of the hypoosmolar and the isoosmolar electroporation buffer.
We recommend testing osmolarity according to Table 1.

Table 1
Volumes of Eppendorf Hypoosmolar and Isoosmolar Electroporation Buffers to be used to adjust the desired osmolarity (final volume: 10 ml).

Desired osmolarity	Eppendorf Hypoosmolar Buffer (ml)	Eppendorf Isoosmolar Buffer (ml)
90 mOsmol/kg	10	0
150 mOsmol/kg	6.8	3.2
200 mOsmol/kg	4.2	5.8
250 mOsmol/kg	1.6	8.4
280 mOsmol/kg	0	10

With the aid of the subsequent viability staining, the optimal osmolarity of the electroporation buffer can be determined.
The lowest osmolarity at which a survival rate of ≥ 90 % is achieved should be used for the following electroporations:

4.1.4 Determining the diameter of the cell

As the size of the cell is a crucial factor for setting the parameters on the Multiporator®, it should be estimated after an incubation of 10 to 15 minutes in the electroporation buffer. The most precise measurements can be performed with electronic instruments, such as the Coulter Counter or Schärfe CASY. As an alternative, the cell diameter can be estimated under the microscope. This can be performed with the aid of a measuring eyepiece or can be roughly approximated with the aid of Neubauer's counting chamber or a microgrid.

After the cell diameter has been estimated, the minimum pulse voltage at which the cell membrane can be permeated can be read from Table 2. The optimal pulse voltage for the electroporation experiment may be 2 to 3 times higher for suspension cells and 2 to 5 times higher for adherent cells.

4 Electroporation protocol

Table 2
Minimum pulse voltages at which the cell membranes may be permeated, in relation to the diameter of the cell after incubation for 10 to15 minutes in the electroporation buffer, the electroporation temperature and the gap width of the cuvette. Depending on the cell line, the optimal pulse voltage for the electroporation experiment may be between two and five times higher.

Diameter of cell [µm]	Voltage 2-mm cuvette room temp.	Voltage 4-mm cuvette room temp.	Voltage 2-mm cuvette 4 °C	Voltage 4-mm cuvette 4 °C
5	530	1100	1100*	*
10	270	540	540	1100
15	180	360	360	710
20	130	260	260	530
25	110	220	220	430
30	90	180	180	360
35	80	160	160	310
40	70	140	140	270
45	60	120	120	240
50	50	100	100	210
60	40	80	80	160
80	30	60	60	120

* The maximum voltage which can be applied with the eukaryotic module of the Multiporator® is 1,200 V.

4.1.5 Preparing the DNA

The DNA should be ultra-pure (A260/A280 ≥ 1.8) and endotoxin-free. Following the last purification step, it should be resuspended directly in bidistilled water and not in TE buffer.

4.1.6 Selecting the temperature

Electroporation may be carried out at room temperature or at 4 °C (see Sec. 3.6). Experiments to establish new protocols normally take place at room temperature.

However, if electroporation takes place at 4 °C in order to achieve higher transfection rates, the cells should not remain on ice for more than two minutes before being incubated at 37 °C.

4 Electroporation protocol

4.2 Electroporation procedure

Electroporation conditions must be optimized for every cell line for which no specific application protocol is available. The following protocol is a general guideline for the electroporation of eukaryotic cells. To determine the optimal electroporation conditions for highest transfection efficiency, please refer to Section 3.

1. Ensure that cells are harvested in the exponential growth phase.
2. Dilute the cells in culture medium with 0.5 to 1 % FCS and determine the number of cells and spin the cells down.
3. Resuspend the cells in Eppendorf Electroporation Buffer (at RT or 4 °C) with the determined osmolarity and set a cell concentration of between 1 x 10^6 and 3 x 10^6 cells/ml, or slightly lower.

   Caution: The overall incubation time in the Eppendorf Electroporation Buffer must not exceed 30 minutes to guarantee successful electroporation!
4. Aliquot the cell suspension (400 µl for a cuvette with 2-mm gap width and 800 µl for a cuvette with 4-mm gap width) in Eppendorf tubes. Add plasmid DNA (final concentration 5 to 20 µg/ml) or proteins (final concentration 10 to 100 µg/ml) and mix.
   When performing electroporation at 4 °C, precool the cuvettes on ice.
5. Transfer the cell suspension to electroporation cuvettes. Take care that no air bubbles are formed.
6. Electroporation: (settings on the Multiporator®)

Mode: ⦿	Eukaryotic cells	
Voltage (U):	To enable the optimal voltage to be set on the Multiporator®, it is advisable to perform a series of experiments with several pulse voltages. For adherent cells: between 1 to 5 times the minimum pulse voltage stated in Table 2. For suspension cells: between 1 to 3 times the minimum pulse voltage stated in Table 2.	
Time constant (τ):	At RT	40 to 100 µs
	At 4 °C	15 to 40 µs
Number of pulses (n):	1	
7. After pulsing, allow the cell suspension to remain in the cuvette for 5 to10 minutes.
   If electroporation was carried out at 4 °C, the cuvettes should be placed on ice for a maximum of 2 minutes after pulsing and should then be incubated in a water bath for 8 minutes at 37 °C.
8. Carefully remove the cell suspension from the cuvette using a Pasteur pipette and cultivate it in 3 to 5 ml culture medium in a 60-mm culture dish.
   When removing the cell suspension, ensure that the aluminum electrodes are not damaged so that contamination by cytotoxic aluminum ions is prevented.
   Note: After pulsing, the cells should be incubated for 2 to 3 hours at 37 °C before any centrifugation is performed, to ensure resealing of the membrane.

4.3 Follow-up treatment of the cells

After the cells have been transferred to the culture medium, they should not be subjected to stress, such as can be caused by shaking or long periods of transport.

4.4 Determination of transfection efficiency in the cells

Depending on the cell type and on the plasmid used, transient expression may be detected roughly 24 to 48 hours after transfection has taken place. In some cases (e.g. primary cells), this may require considerably longer.

5 Troubleshooting

If the transfection experiments do not turn out quite as expected, helpful information may be found in the following troubleshooting guide.

The specific problems listed are caused by a variety of factors which the researcher can easily narrow down by optimizing specific protocol areas, as described briefly below and outlined in full in Sec. 3 and 4.

Problem	Possible cause	Solution / comments
Low survival rate	Pulse is too strong.	Check determination of the cell size (Sec. 4.1.4) and minimum field strength based on this size (Table 2). Note that gap width of the cuvette and temperature during electroporation must also be taken into consideration.
	Pulse is too long.	Shorten the pulse length to decrease permeation of the cell membrane. This can increase the survival rate of the cells. Note that the optimal pulse length is affected by the temperature at which electroporation is carried out (Sec. 3.6).
	Too many pulses are applied.	Reduce the number of pulses. Multiple permeation of the same membrane areas can lead to irreversible damage to the plasma membrane.
	Conductivity of the electro-poration buffer is too high.	Check the conductivity of your electroporation buffer using a suitable measuring device. Buffers with a conductivity of >4mS/cm can lead to a reduced incorporation rate. Low-conductivity Eppendorf electroporation buffers are recommended. The addition of plasmid DNA dissolved in a buffer solution instead of distilled water can also increase conductivity.
	Cells remained too long in the electroporation buffer.	If overall incubation of the cells in the electroporation buffer exceeds 30 minutes, apoptosis maybe inducted in certain cell types. Shorten the duration of the experiment by carrying out individual steps more quickly, by shortening the washing procedure prior to pulsing, or by cutting the incubation time of the cells after the pulse. (**Attention**: An incubation time of 5 to 10 minutes at room temperature should be maintained. Then transfer the cells into culture medium and cultivate at 37 °C). Before any centrifugation is performed after pulsing, the cells should be incubated for 2 to 3 hours at 37 °C to ensure resealing of the cell-membrane.

5 Troubleshooting

Problem	Possible cause	Solution / comments
	Gene product has a toxic effect on the cell.	The gene product itself or a high expression rate of the gene product may have a toxic effect on the cells. The optimal plasmid concentration should be tested individually for each cell type and each plasmid.
	After pulsing, cells are incubated too long at a low temperature (4 °C).	An excessive incubation period on ice can lead to cell death. The incubation period on ice should not exceed two minutes.
	The cells are damaged during the harvesting procedure.	Using trypsin with an excessively high concentration or with an insufficient purity level during the harvesting of the cells may increase the mortality rate. Dispase or trypsin HPLC-grade (Sec. 4.1.3.1) is recommended.
	Osmolarity of the electroporation buffer is too low.	Low osmolarity of the electroporation buffer may cause sensitive cells to swell to such an extent that they burst during electroporation. This effect can be tested by a viability staining of the cells 2–3 hours after electroporation (Sec. 4.1.3.2). Increase the amount of isoosmolar electroporation buffer to raise the osmolarity of the buffer and repeat the viability test.
	Cells are stressed.	At the time of electroporation, the cells should have been in culture for several cycles. Freshly thawed or recently transported cells are still in a condition of stress and should not be used immediately for electroporation.
Low transfection rates	Cells are contaminated with mycoplasma.	Mycoplasma prevent successful electroporation of cells with the Multiporator®. Cell cultures must be checked for mycoplasma at regular intervals (Sec. 4.1.1).
	Pulse is too weak.	A pulse with a low field strength may be too weak to permeate the cell membrane. Check determination of the cell size (Sec. 4.1.4) and the minimum field strength based on this size (Table 2). The gap width of the cuvette and the electroporation temperature must also be considered.

5 Troubleshooting

Problem	Possible cause	Solution / comments
	Pulse is too short.	Extend the pulse length to increase permeability of the cell membrane, which can lead to a higher transfection rate. Note that the optimal pulse length is affected by the temperature at which electroporation is carried out.
	DNA concentration is too low / too high.	If the transfection rate is too low and the viability of the cells is high, the plasmid concentration may be increased. Note, however, that an increased plasmid concentration may lead to a higher transfection rate only within a limited concentration range of the plasmid.
	Large plasmids are used.	The size of the plasmid can greatly affect the transfection rate. When large plasmids are used, it may be necessary to increase the permeation of the membrane by applying pulses with a higher field strength. Be careful as this may also lead to a higher cell mortality rate.
	Cell density is too high.	If cell densities are too high during electroporation, the homogeneity of the electrical field can no longer be guaranteed. Reduce the cell concentration in the cuvette to $1 – 3 \times 10^6$ cells/ml or lower.
	Electroporation preparation contains EDTA or endotoxins.	EDTA and endotoxins have a cytotoxic effect. They are often introduced into the electroporation preparation when DNA is added (e.g. in TE buffer). Remove EDTA by carrying out a buffer exchange on bidistilled H_20. Endotoxins can be removed using "endotoxin-free" plasmid preparation kits.
	Incubation period for gene expression is too short.	Following transfection, different cell types require different incubation periods in order to reach their maximum expression rate. Expression should be checked again at a later point in time.
	Problems with the reporter assay.	Include positive controls which indicate that the reporter system is working properly.
Non-reproducible results	Washing procedure prior to electroporation was not thorough enough.	Traces of medium can have a severe effect on the conductivity of the electroporation buffer, and thus on the transfection result. Therefore, the medium in the supernatant must be thoroughly removed during the washing procedure.

5 Troubleshooting

Problem	Possible cause	Solution / comments
	Cuvette has been used several times.	Using an electroporation cuvette several times may result in a non-homogeneous electrical field during electroporation. Only new cuvettes are recommended for important experiments.
	Cells were harvested at different confluencies.	Harvesting cells at different confluencies can lead to non-reproducible transfection results. Cells should always be harvested in the exponential growth phase.
	Conductivity of the electroporation buffers varies (such as when self-prepared buffers are used).	Check the conductivity of self-prepared buffers on a regular basis using a suitable measuring device. A defined conductivity is always guaranteed with Eppendorf electroporation buffers.
No transfection	Pulse was not injected into the cell suspension due to poor contact between cuvette and Multiporator®.	Check that cuvette and the cuvette insert are correctly inserted in the Multiporator®.

6 Ordering information

Order no:	
4308 000.015	Multiporator® for eukaryotics
4308 000.023	Multiporator® for eukaryotics, bacteria and yeasts
4308 000.031	Multiporator® for eukaryotics, cell fusion, with 1 Helix fusion chamber and 1 Micro fusion chamber
4308 000.040	Multiporator® for eukaryotics, bacteria, yeast and cell fusion, with 1 Helix fusion chamber and 1 Micro fusion chamber
	Electroporation buffer
4308 070.501	Hypoosmolar buffer (PH), sterile, 100 ml
4308 070.510	Isoosmolar buffer (PI), sterile,100 ml
	Electrofusion buffer
4308 070.528	Hypoosmolar buffer (FH), sterile,100 ml
4308 070.536	Isoosmolar buffer (FI), sterile,100 ml
	Electroporation cuvettes
4307 000.569	1 mm gap width, aluminum, sterile, 50 pcs.
4307 000.593	2 mm gap width, aluminum, sterile, 50 pcs.
4307 000.623	4 mm gap width, aluminum, sterile, 50 pcs.
4308 078.006	Cuvette stand for 16 electroporation cuvettes
4308 021.004	Insert (electroporation / electrofusion) for connecting to external electrodes
4308 014.008	Helix fusion chamber for cell fusion (gap between electrodes: 0.2 mm)
4308 030.003	Micro fusion chamber (gap between electrodes: 0.2 mm)
4308 031.000	Micro fusion chamber (gap between electrodes: 0.5 mm)
4308 017.007	Stand for 10 helix fusion chambers
4308 010.002	Conversion kit for mode for bacteria (to be installed by SERVICE)
4308 011.009	Conversion kit for mode for cell fusion (to be installed by SERVICE)

7 Ordering information for North America

Order no:	
940000505	Multiporator® for eukaryotics
940000602	Multiporator® for eukaryotics, bacteria and yeasts
940000700	Multiporator® for eukaryotics, cell fusion, with 1 Helix fusion chamber and 1 Micro fusion chamber
940000807	Multiporator® for eukaryotics, bacteria, yeast and cell fusion, with 1 Helix fusion chamber and 1 Micro fusion chamber
	Electroporation buffer
940002001	Hypoosmolar buffer (PH), sterile, 100 ml
940002109	Isoosmolar buffer (PI), sterile,100 ml
	Electrofusion buffer
940002150	Hypoosmolar buffer (FH), sterile,100 ml
940001021	Isoosmolar buffer (FI), sterile,100 ml
	Electroporation cuvettes
940001005	1 mm gap width, aluminum, sterile, 50 pcs.
940001013	2 mm gap width, aluminum, sterile, 50 pcs.
940001021	4 mm gap width, aluminum, sterile, 50 pcs.
940001102	Cuvette stand for 16 electroporation cuvettes
940004209	Insert (electroporation / electrofusion) for connecting to external electrodes
940001200	Helix fusion chamber for cell fusion (gap between electrodes: 0.2 mm)
940001251	Micro fusion chamber (gap between electrodes: 0.2 mm)
940001234	Micro fusion chamber (gap between electrodes: 0.5 mm)
940001218	Stand for 10 helix fusion chambers
940004101	Conversion kit for mode for bacteria (to be installed by SERVICE)
940004128	Conversion kit for mode for cell fusion (to be installed by SERVICE)

8 Appendix

8.1 Guide to determining the minimum voltage to be set on the Multiporator®

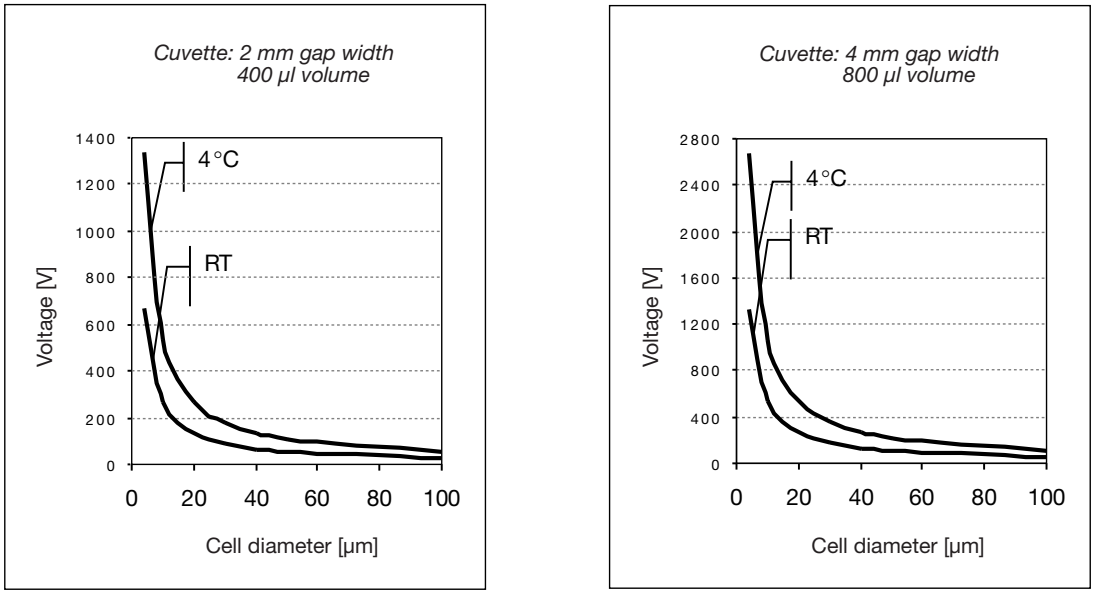

Fig. 4a:

Minimum pulse voltages at which the cell membrane is permeated
The minimum pulse voltage is dependent on the cell diameter following 10- to 15-minute incubation in electroporation buffer as well as on the temperature and the gap width of the cuvettes. These values, shown as a graph (4a) and as a table (4b), can be used to determine the optimal pulse voltage to be set on the Multiporator®. The optimal pulse voltage may be 2 to 3 times higher for suspension cells and 2 to 5 times higher for adherent cells.

Diameter of cell [µm]	Voltage 2-mm cuvette room temp.	Voltage 4-mm cuvette room temp.	Voltage 2-mm cuvette 4 °C	Voltage 4-mm cuvette 4 °C
5	530	1100	1100*	*
10	270	540	540	1100
15	180	360	360	710
20	130	260	260	530
25	110	220	220	430
30	90	180	180	360
35	80	160	160	310
40	70	140	140	270
45	60	120	120	240
50	50	100	100	210
60	40	80	80	160
80	30	60	60	120

* The maximum voltage attainable with the eukaryotic module of the Multiporator® is 1,200 V.

Fig. 4b

8 Appendix

8.2 Volumes of hypoosmolar and isoosmolar electroporation buffer for setting required osmolarity (10ml)

Desired osmolarity	ml hypoosmolar buffer (ml)	ml isoosmolar buffer
90 mOsmol/kg	10	0
150 mOsmol/kg	6.8	3.2
200 mOsmol/kg	4.2	5.8
250 mOsmol/kg	1.6	8.4
280 mOsmol/kg	0	10

Table 3

8.3 Composition of electroporation buffers

The electroporation buffers supplied by Eppendorf are tested for the following important criteria:

- Conductivity
- pH value
- Osmolarity
- Sterility
- Mycoplasma
- Endotoxins
- Pyrogens
- Cytotoxicity

	Poration (hypoosmolar)	Poration (isoosmolar)
Sterile bidistilled water	Fill up to 1000 ml	Fill up to 1000 ml
KCl	25 mM	25 mM
KH_2PO_4	0.3 mM	0.3 mM
K_2HPO_4	0.85 mM	0.85 mM
myo-Inositol *	ad 90 mOsmol/kg	ad 280 mOsmol/kg
pH value	7.2 ± 0.1	7.2 ± 0.1
Conductivity at 25 °C	3.5 mS/cm ± 10 %	3.5 mS/cm ± 10 %

* The purity of myo-Inositol may vary greatly from batch to batch. It must be pure enough to ensure that, at 280 mOsmol/kg in bidistilled water, a conductivity of 10 µS/cm is not exceeded. The conductivity of individual myo-Inositol batches **should** be measured before the buffer is prepared.

8 Appendix

8.4 Protocol for the electroporation of eukaryotic cells, based on Jurkat

Multiporator®
Transfection Protocol

Jurkat

Cell line:	Jurkat, T-lymphocyte, human leukemia (suspension cell line)
Transfection with:	plasmid pEGFP-N1 (in bidistilled H_2O)
Electroporation buffer:	Eppendorf hypoosmolar electroporation buffer (PH)
Culture medium:	RPMI 1640 / 10 % FCS
Cuvette:	Eppendorf, 2-mm gap width, 400 µl
Temperature:	RT (20 to 25 °C)

Reference:	Prof. Ulrich Zimmermann Lehrstuhl für Biotechnologie Biozentrum Universität Würzburg Am Hubland, D-97074 Würzburg, Germany	phone: fax: e-mail:	+49 931 888 4508 +49 931 888 4509 zimmerma@biozentrum.uni-wuerzburg.de

1. Harvest the cells in the exponential growth phase and centrifuge them (5 to 10 minutes, 200 x g, room temperature).
2. Resuspend the cells in RPMI 1640 / 0.5 % FCS, determine the number of cells and wash them. (5 to 10 minutes, 200 x g, room temperature).

Note: **Incubation time in the electroporation buffer must not exceed 30 minutes to guarantee a successful electroporation.**

3. Resuspend the cells in hypoosmolar electroporation buffer. When doing so, set the cell concentration to 1 x 10^6 cells/ml.
4. Add and mix plasmid DNA (5 to 20 µg/ml final concentration, in bidistilled H_2O).
5. Transfer 400 µl cell suspension into electroporation cuvettes (2-mm gap width). The cell suspension must be free of air bubbles.
6. Electroporation:
   Mode: Eukaryotes "⊙"
   Voltage (V) 240 V
   Time constant (τ) 40 µs
   No. of pulses (n) 1
7. After the pulse, allow the cell suspension to stand in the cuvette for 5 to 10 minutes at room temperature.
8. Carefully transfer the cell suspension from the cuvette to 3 to 5 ml RPMI 1640 / 10 % FCS and cultivate it in a 60-mm culture dish.
9. **Detection methods for transfection**:

   The expression of the plasmid pEGFP-N1 can be detected clearly after 24 to 48 hours with the aid of FACS analysis or under a fluorescence microscope.

   Result
   Survival rate: 70 to 85 %
   Transfection rate: 65 to 80 % based **on the number of surviving cells**.
   55 % based **on the initial number of cells used for the experiment**.

   Results were measured 24 hours after transfection.

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