SEPTEMBER 1990

JOUAN

PS 10 - PS 15
ELECTROPULSER

APPLICATION MANUAL

CONTENTS

.c.List of accessories

1) Oscilloscope

Standard instrument (5 MHz bandwidth) Enertec 5026 (a screen hood is recommended)

2) Retort stand

3) Clamp fixer

4) Clamp

5) Tray

Items 2-5 can be obtained from any laboratory supplier

6) Experimental vessels may be: culture dishes, multi-well plates (≤ 24), disposable spectrometer cuvettes.

7) Sterile pipettes

8) Pasteur pipettes

9) Automatic pipettes with suitable tips

10) Disposable test tubes (5 and 20 ml)

11) Eppendorf tubes (1.5 ml)

12) Bench centrifuge (10-2,000 g)

12) Bench microcentrifuge (up to 13,000 g)

14) Timer and stop-watch

15) Incubator for bacteria

16) Incubator for mammalian cells (temperature and CO_2 controlled)

17) Standard laboratory equipment (Kleenex, suction bulbs, gloves, masks, etc).

.c.**Procedure**

Using the standard electrodes

1 - Mount the electrodes on the stand using the clamp. Make sure that the electrodes are horizontal with respect to the tray.
2 - Put on gloves.
3 - Connect the electrodes to the electropulser.
4 - Switch on the electropulser.
- *For treatment in culture dishes* -
5 - Lower the plate on the tray.
6 - Place a dish on the plate.
7 - Raise the plate so that the electrodes touch the bottom of the dish.
8 - Only the volume between the electrodes will be treated. The volumes on either side of the electrodes provide a useful control.
9 - Place 0.2 ml of the cell suspension between the electrodes.
10 - Apply pulses.
11 - Remove the dish.
- *For treatment in disposable spectrophotometer cuvettes* -
5 - Remove the tray.
6 - Put 0.2 ml of suspension into the cuvette.
7 - Lower the electrodes to the bottom of the cuvette.
8 - Apply pulses.
9 - Raise the electrodes.

.c.**Rinsing and sterilization of electrodes**

Certain precautions should be taken to avoid contaminating the cell preparations. A standard procedure is described below, although further details are mentioned in the sections dealing with the specific procedures.
1 - Wash the electrodes with distilled water.
2 - Wash the electrodes with 70% alcohol.
3 - Rinse the electrodes in sterile water.
4 - At the end of the experiment carefully dry the electrodes with a Kleenex, soaked in Ethanol.

.c.A) ANIMAL CELLS

.c.**Definition of operating conditions**

1) Preparation of biological material

CHO cells cultured in 35 mm dishes (Nunc)

Eagle medium (MEM 0111, Eurobio, France)

6-10% serum (Boehringer, Germany)

Streptomycin, penicillin, glutamic acid

Cells are cultured under standard conditions (37°C, 5% CO_2)

2) Pulsing buffers have the following compositions

A 10 mM phosphate pH 7.4
1 mM $MgCl_2$
250 mM sucrose

B 10 mM phosphate pH 7.4
1 mM $MgCl_2$
75 mM NaCl
125 mM sucrose

C 10 mM phosphate pH 7.4
1 mM $MgCl_2$
75 mM KCl
125 mM sucrose

3) Labelling medium

Trypan Blue (4 mg/ml) (BT) or an equivalent membrane permeability dye (such as Direct Blue) is added to one of the samples. This gives an indication of the permeability and subsequent resealing of the membrane.

Medium D = Medium A + BT

Medium E = Medium B + BT

Medium F = Medium C + BT

4) Saline medium G

10 mM phosphate pH 7.4
1 mM $MgCl_2$
150 mM NaCl

.c.Practical details

a) Volume of pulsing medium

Culture dish	· 35 mm	: 1.5 ml
	· 60 mm	: 3 ml
Multiwell	· 16 mm	: 0.4 ml

b) Preparation of culture dishes

- cell density, volume (2 ml/ ·35 mm)
- The cultured cells plated in flasks are treated with trypsin (0.1% trypsin, 0.3% EDTA) for 30 sec, then incubated at 37°C for around 5 min without trypsin and EDTA, then taken up in medium containing serum to block the action of trypsin.
- after reading the optical density in a cuvette, the samples are diluted in culture medium and the appropriate quantity of cells is placed in the culture dishes (·35 mm). The cells spread out spontaneously during the ensuing incubation at 37°C (CO_2 atmosphere). At least 2 h incubation will be required for cells in good condition. If the dishes are prepared beforehand, the calculation for dilution needs to take account of a generation time of between 16 and 22 hours.

c) Sterility

It is recommended to carry out procedures in a laminar flowhood(preferably horizontal flow, except for pathogenic material). The bench must be kept scrupulously clean, and the following procedures should be followed:

- before use, clean with alcohol and bactericidal spray (Tego Spray for example) and then leave for 10 min. It is often a good idea to carry out operations on a sheet of aluminium foil.
- switch on the air flow.
- make sure that the air flow is not disturbed above the working area.
- the electrodes and their support should be cleaned in the same way, and kept inside the sterile zone.
- rinse the electrodes with 70% ethanol and sterile water (e.g. wipe with a Kleenex soaked in ethanol, leave to dry and then soak then in sterile water).
- make sure not to contaminate the sample with one's hands, either work with an assistant or use the foot pedal.

- after the experiment, reduce the air flow, remove the sheet of aluminium foil, wash the whole area with water and then ethanol, and spray with bactericide.
- maintain the air flow.
- check the prefilters regularly.

.c.Permeabilization of mammalian cells e.g. Chinese Hamster Ovary (CHO) cells

Permeabilization of the cell membrane enables small molecules (≤ 1,000 Da) to pass into the cytoplasm. This process can be readily monitored by the use of nuclear stains such as ethidium bromide or Trypan Blue. The latter is convenient as it can be viewed with an ordinary light microscope (no requirement for fluorescence microscopy). The permeabilized cells will have blue-stained nuclei. The use of culture dishes for the permeabilization treatment has the advantage of providing an internal control since only the cells between the electrodes are subjected to the electrical field, while those on the outside serve as untreated controls.
The aim of the experiment is to show that electropermeabilization is a robust phenomenon controlled by the following parameters: electric field strength, pulse length and number of pulses applied. The interval between pulses seems to have rather little effect.
1 - Select a culture dish in which the cells are non-confluent.
2 - Remove the culture medium with a Pasteur pipette or pipetman.
3 - Add medium containing the nuclear stain (D. E or F). A volume of 1.5 ml (with pipetman) will cover the culture and provide enough liquid for electrical continuity and a uniform electrical field.
4 - Place the electrodes in contact with the bottom of the dish using the moveable tray.
5 - Set the electrical parameters (e.g. 200 V/cm, 100 µs, x 5), and apply pulses to the sample.
6 - Monitor the output pulse waveform (shape and duration of pulse) on the oscilloscope.
7 - Lower the dish, move sideways until the electrodes are above an untreated area.

8 - Raise the dish to make contact with the electrodes as in operation 4.
9 - Repeat operation 5 using different parameters.
10 - Repeat operations 7-10 if possible.
11 - Leave dish to incubate at 21°C for 5 min.
12 - Remove the staining medium, and replace it with saline medium G (1 wash).
13 - View with an inverted microscope (the phase ring is not required, although it does not have to be removed) (x20, x32 eye-piece).
14 - Observe the treated areas.
15 - Count the % of cells with blue-stained nuclei which correspond to the permeabilized cells.
16 - Repeat from operation 2 using another dish.
17 - Plot the percentage of permeabilized cells against the relevant variable (E, T, n).
Compare the effects of the three different parameters on % permeabilization P for the three different media (D, E and F).

.c.**Reversibility**

The permeabilization state is short-lived (a few minutes at ambient temperature). This can be followed by adding the indicator at different times after electropulsation.

1 - Select an appropriate culture dish.
2 - Remove the culture medium.
3 - Add pulsing medium (A, B or C).
4 - Place the electrodes in contact with the bottom of the dish using the moveable tray.
5 - Apply pulses (set parameters E, T, n).
6 - Monitor the pulse waveform on the oscilloscope.
7 - Start the timer.
8 - Lower the dish.
9 - Incubate the dish at the desired temperature (4°C, 21°C, 37°C).
10 - After a given interval (1 min or minimum 30 sec) remove the pulsing medium, and replace with the corresponding staining medium.
11 - Incubate at 21°C for 5 min.
12 - Replace the staining medium with saline medium (G) (wash once).
13 - View with an inverted microscope (eyepiece x20, x32).
14 - Determine the % of stained cells.
15 - Repeat with a new dish and another interval between electropulsation (step 5) and treatment with stain (step 10).

Leakage of cytoplasmic material

Electropermeabilization allows free exchange of small molecules between the cytoplasm and the external medium. Leakage of cellular metabolites can thus be detected in the medium after electropermeabilization.

1 - Culture CHO cells in 24 well plates or ·35 mm dishes.
2 - Remove the culture medium.
3 - Add pulsing medium A (800 µl).
4 - Place the electrodes (10 x 10 mm) in contact with the bottom of the culture dish or well.
5 - Apply pulses (set E, t, n). Repeat the operation in three different areas in 35 mm dishes, or in three different wells.
6 - Monitor the pulse waveform on the oscilloscope.
7 - After leaving for 1 min, transfer 500 µl of supernatant to an Eppendorf tube.
8 - Keep sample on ice.
9 - Repeat from operation 4.

.c.Determination of ATP content

The method based on the luciferin-luciferase reaction is convenient. The low light emission can be measured in a scintillation counter, although a luminometer can be used.

Adjustment of counter

1 - Open the window to the maximum levels (MIN-MAX).

2 - Put in "Manual, Repeat" mode.

3 - Select the shortest counting time.

4 - Switch to Mode without chemiluminescence correction.

5 - Used glass vials, washed with a mixture of sulfuric and chromic acids and then carefully rinsed in distilled water.

In each dry cooled vial, place the following:

0.7 ml of pure water (Milli Q, Millipore)

0.2 ml of 0.2 M glycylglycine buffer pH 7.4

Leave the vials to cool in the counter.

Prepare a fresh solution (solution LL) of luciferin-luciferase (Sigma) in sterile water (20 mg/ml), which should be kept cold in the dark.

For each vial

1 - Add 100 µl of supernatant.

2 - Add 100 µl of solution LL.

3 - Shake vigorously.

4 - Lower the vial into the counter.

5 - Start counting for a short period.

6 - Carry out five successive counts.

7 - Extrapolate these counts to time 0

8 - Convert this value to mmoles ATP using a previously established calibration curve.

Compare the loss of cytoplasmic ATP to the % permeabilization measured under identical conditions.

.c.Electrotransformation of CHO cells

The activity that is transferred is relatively short-lived, but can be detected 48 hours after electropulsation. This example is based on transfer of the activity of the enzyme chloramphenicol acetyltransferase (CAT). It is generally determined using a radioactive substrate, although antibody methods have also been described.

Materials

- CHO WTT cells (they grow in suspension and do not require trypsinization)
- PSV 2 CAT plasmid
- carrier DNA (salmon sperm) such as that supplied by Sigma
- pulsing medium A, B, C or a 50/50 mixture of MEM 0111 and medium A
- · 35 mm culture dish (Nunc)

The cell suspension containing 10^6 cells/ml should be concentrated 5-fold (working concentration 5.10^6 cells/ml) by centrifugation (100 g, 10 min, ambient temperature). Resuspend the concentrate in pulsing medium.

It is best to break up the carrier DNA either by passing it through a fine gauge needle or by sonication. The plasmid may be linearized, although the circular form leads to a high transfer of CAT activity.

1 - Mix the cells with the DNA at ambient temperature in the upside down lid of a culture dish from the following:
- 180 µl of cell suspension (around 10^6 cells)
- 5 µl of plasmid (1.2 mg/ml for a final concentration of 30 µg/ml)
- 10 µl of carrier DNA (1 mg/ml for a final concentration of 50 µg/ml).

Note: The ionic strength of the medium will be slightly affected by addition of the genetic material which is made up in TE medium.

2 - Place the electrodes in contact with the bottom of the dish.
3 - Place 200 µl of the mixture between the electrodes using an automatic pipette.
4 - Apply five 3 ms pulses of 1000 V/cm (1 Hz) to the suspension.
5 - Remove the electrodes from the dish.
6 - After 10 min, add 3 ml of culture medium (MEM 0111 + 6% FCS), and disperse the cell suspension.
7 - Incubate the cells (37°C, 5% CO_2).

8 - Change the culture medium after 24 h.
9 - Determine CAT activity 48 h after electropulsation.
10 - Overall survival is determined from the protein content of the cellular extract.

Note: Stages 9 and 10 can be performed using routine methods.

.c.Electrotransformation of lymphoblastoid cell lines

.c.Short-term expression

This example uses a line of lymphocytes transformed by the Epstein-Barr virus. For short-term expression, the pUC13 plasmid modified by insertion of the SV40 viral genome at site BamH1 is employed.

1 - Wash the cells in cold RPMI medium, and adjust to a concentration of 10^7 cells/ml.
2 - Add the plasmid at a concentration of 5 µg/ml.
3 - Incubate the mixture at 4°C for 10 min.
4 - Place 0.1 ml of mixtures between the electrodes.
5 - Apply ten 100 µs pulses (1 Hz) at 1.2 kV/cm.
6 - Leave the mixture to stand at 4°C for 10 min.
7 - Incubate at 37°C for 20 min.
8 - Disperse the cells in a culture medium containing serum.
9 - After 72 h post-pulse incubation, the T nuclear antigen of SV 40 can be detected by indirect immunofluorescence.

.c.Results

65% of cells are not lysed
2% of cells express the antigen

.c.Permanent expression

Cells of the Daudi line (Burkitt lymphoblastoid B) are made resistant to gentamycin by integration of the pSVTK Neo plasmid.

1 - Wash the cells in cold RPMI medium, and adjust to a concentration of 10^7 cells/ml.
2 - Add the plasmid at a concentration of 50 µg/ml.
3 - Incubate the mixture at 4°C for 10 min.
4 - Place 0.1 ml of the mixtures between the electrodes.
5 - Apply eight 100 µs pulses (1 Hz) at 1.3 kV/cm.
6 - Leave the mixture to stand at 4°C for 10 min.
7 - Incubate at 37°C for 20 min.
8 - Disperse the cells in a culture medium containing serum.
9 - 36 h after pulsation, add selection medium containing 1 mg/ml gentamycin.

10 - The transformed cells are readily detectable 4 weeks after pulsation.

.c.Electrofusion

The following procedure is designed to demonstrate some biophysical principles of electrofusion. The process of electrofusion is controlled essentially by the electrical parameters (field strength, duration and number of pulses), and so conditions can be readily optimized for each cell type. The extent of fusion is quantified by the index of polynucleation, i.e. the percentage of nuclei in polycarya in the whole population.

.c.*Cells growing on a support*

CHO WTT cells growing on a plastic support are employed. Use CHO cultures with a high cell density (1,000 cells/mm^2), which will have numerous cell contacts. Cells can be cultured in 35 mm dishes or in 16 mm wells (24 well plate).

1 - Remove the culture medium.
2 - Add pulsing medium A or B.
3 - Bring the electrodes into contact with the cell surface using the moveable tray.
4 - Apply pulses (set E, T, n). Use higher fields than for permeabilization (> 800 V/cm, 100 µs, x 5).
5 - Remove the electrodes.
6 - If need be, repeat the experiment on another area of the dish using different parameters (fields > 2000V/cm can be employed).
7 - Remove the pulsing medium.
8 - Add culture medium.
9 - Incubate at 37°C for at least 1 h.
10 - View with an inverted microscope.
11 - Determine the extent of polynucleation (percentage of nuclei in polynucleated cells with respect to the total number of nuclei).
12 - Examine the influence of E on cell fusion, compare the fusion curves with those for permeabilization (leakage and staining). Examine the effect of T and ionic strength on fusion.

.c.*Electrofusion of cells in suspension*

One must first create a film of cells

1 - Aspirate the CHO WTT cell suspension (10^6 cells/ml) through a biocompatible filter (Nucleopore polycarbonate with a diameter of 25 mm, 1 µm pore).
2 - This forms a film on the surface of the filter.
3 - Add medium A (low ionic content but isoosmotic with the culture medium).
4 - Aspirate this suspension carefully through the filter.
5 - The cells remain attached to the filter, while the surrounding medium becomes the pulsing medium.

6 - place the filter in the bottom of a culture dish with the cell side upwards. Make sure that the filter stays moist.
7 - Put the electrodes in contact with the filter.
8 - Apply pulses to the cell film (1.5 kV/cm, 100 µs, x 5).
9 - Incubate the dish containing the cells at 37°C for a few minutes.
10 - Add culture medium (MEM 0111, 6% FCS).
11 - Incubate the dish at 37°C in an air/CO_2 atmosphere.
12 - Polycarya can be then be examined under the microscope.

Post-pulsation contact
A particular consequence of electropermeabilization is the creation of a fusogenic state. Cell fusion occurs spontaneously when electropermeabilized cells are brought into contact, and agents such as polyethylene glycol are not required.
- *in culture dishes* -
1 - As described above, the cells are treated directly in cultures dishes cooled in ice.
2 - Wash CHO WTT cells in suspension twice in cold medium A.
3 - Place around 0.2 ml of the cell suspension in medium A (10^6 cells/ml between the electrodes. Lower cell concentrations can be used.
4 - Apply pulses (1.6 kV/cm, 100 µs, x 5).
5 - Removed the electrodes.
6 - Carefully transfer the cell suspension to a disposable hemolysis tube.
7 - Centrifuge the suspension (50 g, 5 min) without temperature regulation.
8 - Incubate the pellet and supernatant at 37°C for 30 min.
9 - Add 3 ml of culture medium (MEM O111, 6% FCS), and carefully disperse the pellet by repeated aspiration into a narrow pipette.
10 - Place the cell suspension in a culture dish.
11 - Incubate the suspension at 37°C for at least 2 h in an air/CO_2 atmosphere to allow the cells to spread out.
12 - Count the number of polycarya.

- *in disposable spectrophotometer cuvettes* -

1 - Wash the CHO WTT cell suspension twice in pulsing medium A.

2 - Place 0.2 ml of the cell suspension (10^6 cells/ml) in a disposable spectrophotometer cuvette.

3 - Put this cuvette inside a 20 ml centrifuge tube.

4 - Centrifuge the tube at 250 g for 5 min without temperature regulation.

5 - Insert the electrodes into the cuvette until they touch the bottom. Keep the whole set-up in the vertical position.

6 - Apply pulses (1.6 kV/cm, 100 µs, x 5).

7 - Remove the electrodes.

8 - Put the cuvette inside a 20 ml centrifuge tube.

9 - Centrifuge the tube at 50 g for 3 min without temperature regulation.

10 - Incubate the cuvette at 37°C for 30 min.

11 - Add 3 ml of culture medium (MEM 0111, 6% FCS), and carefully disperse the cell pellet.

12 - Place the resulting suspension in a culture dish.

13 - Incubate the dish at 37°C in an 5% CO_2/air atmosphere.

14 - Count the number of polycarya after several hours incubation.

.c.**Production of hybridomas**

Several procedures have been described, although they are all based on the same principle. A fusogenic state is induced by treating a system in which the relevant cells are in contact.

Procedure I

Materials

- mouse lymphocytes
- X 63 myeloma cells
- medium A
- culture dish (Nunc, · 35 mm)

1 - Mix 6.10^6 of each of the two cell samples.

2 - Wash and concentrate in medium A by centrifugation at 150 g for 10 min.

3 - Adjust to 10^7 cells/ml.

4 - Place the electrodes in contact with the bottom of the dish.

5 - Place 200 µl of the mixture between the electrodes.

6 - Apply pulses (1.5 kV/cm, 100 µs, 1 Hz, x 5).

7 - Incubate at 37°C for 45 min.

8 - Add 2 ml of culture medium (RPMI, 10% FCS).

9 - Incubate at 37°C for 2 h in an air/CO_2 atmosphere.

10 - Distribute the suspension into 96 wells.

11 - After 24 h, select hybridomas.

Procedure II

Materials

- mouse lymphocytes
- X 63 myeloma cells
- medium A
- culture dish (Nunc, · 35 mm)

1 - Wash the myelomas in 150 mM NaCl buffer.

2 - Treat with Pronase P (0.5 mg/ml) (Serva) for 30 sec at 37°C.

3 - Wash the myelomas with medium A.

4 - Mix lymphocytes and myelomas.

5 - Proceed as from step 3 in Procedure I.

Procedure III

Materials

- mouse lymphocytes
- X 63 myeloma cells
- medium A
- culture dish (Nunc, · 35 mm)

1 - Wash and concentrate a mixture of lymphocytes/myelomas in medium A (10/1 from 10^7 myelomas).

2 - Centrifuge at 2,000 g for a few seconds.

3 - Remove the supernatant.

4 - Put the electrodes in contact with the bottom of the dish.

5 - Put the cell pellet between the electrodes with an automatic pipette.

6 - Apply two pulses (2.5 kV/cm, 50 µs) separated by about 10 sec.

7 - 1 min later add 1 ml of culture medium (RPMI, 10% FCS).

8 - Incubate at ambient temperature for 1 h.

9 - Dilute to 10^5 cells/ml.

10 - Distribute to microwell plates.

.c.B) PLANT CELLS

.c.Electropermeabilization of plant protoplasts

The following description is designed to give sufficient details for the electropermeabilization and electrofusion of plant protoplasts under reproducible conditions without disrupting their biological properties.

.c.Introduction

The reversible nature of the process of electropermeabilization enables exchange of molecules between the external medium and the internal medium of living protoplasts.

The experimental conditions can be defined accurately by the use of a square wave electric field. The four main parameters: applied field strength (which stays constant throughout the duration of the pulse), the duration of the pulse (from 5 µs to several msec), interval between pulses (as low as 0.1 s if necessary) and the number of pulses, can all be adjusted to optimize the process without causing damage to the biological samples.

Protoplasts are obtained by routine methods either from leaves or cell cultures. The use of purified suspensions helps eliminate variations in quality (proportion of intact protoplasts, presence of partially digested cell or tissue debris). Under normal conditions, after enzymatic digestion of cell walls, the preparation is filtered through a Nylon or stainless steel filter (e.g. 100 µm for tobacco samples), and the collected protoplasts purified on either a Ficoll (Ficoll 400 Pharmacia) or sucrose gradient. This gives:

1) a purified preparation, which is important for samples of cultured, undigested or only partially digested cells

2) if required, a population of uniform size (more controlled response to electric pulses) and/or a sample that is physiologically adapted to spreading on a discontinuous Ficoll gradient.

After rinsing in pulsing medium, the absence of cell walls must be checked using Calcofluor (fluorescent brightener 28, Sigma) in the experiments on electrofusion.

.c.Experimental conditions

.c.Pulsing buffer

This is usually of relatively simple composition, and is designed to maintain the protoplasts during the electrical treatment. Its osmotic pressure must be matched to that used in the culture and isolation stages by addition of polyols such as mannitol and salts such as KCl, NaCl. The presence of Mg^{2+} ions (1-5 mM) along with an adequate buffer (MES pH 5.5-6, HEPES pH 7) are required to stabilize the protoplasts.

For tobacco (*N. xanthi*) for example:

Permeabilization medium

1	2
0.5 M mannitol	0.5 M mannitol
6 mM $MgCl_2.6H_2O$	6 mM $MgCl_2.6H_2O$
5 mM MES pH 5.6	5 mM HEPES pH 7.2

Fusion buffer

0.5 M mannitol
1 mM $MgCl_2.6H_2O$
50 mM NaCl
5 mM MES pH 5.6
0.01% Tween

Other media have been described, and KCl tends to be preferred to NaCl. Ca^{2+} is often employed, Mg^{2+} being used mostly for electrotransformation, somewhat contrary to what one would expect from experience with mammalian cells.

.c.Concentration of protoplasts

The method used will depend on the aim of the experiment and the conditions used (sterile or otherwise). For electrofusion, the creation of a monolayer effectively specifies the required density of organelles. For tobacco, it ranges from $0.5.10^6$ to 5.10^6/ml. As an example:

For determination of ATP leakage: $0.15.10^6$/ml

For staining: $0.5\text{-}1.10^6$/ml

With the JOUAN electropulser, the protoplasts are treated directly in the culture dishes. The concentration must be such that:
- there is sufficient material for the size of the culture dish
- the sample volume is adapted to the electrode set-up used for the dish.

Since the pulsing medium is not removed, and culture medium is added directly to the treatment dish, it is best to avoid placing large volumes between the electrodes.

.c.Experimental chamber

Under normal conditions, experiments can be carried out directly in the dishes in which the samples are cultured. They can be normal culture dishes (Nunc, Greiner, Sterilin) of an appropriate size. The following conditions can serve as a starting point:

60 mm · culture dish
Electrodes 10 x 10 mm
Sample volume 180-250 µl
Concentration 10^6/ml
Final volume 2.5-3.5 ml
90 mm · culture dish
Electrodes 10 x 10 mm
Sample volume 250 µl
Concentration $1.5.10^6$/ml
Final volume 8-11 ml

.c.Procedure

.c.Electrical parameters

On the JOUAN electropulser the field strength, pulse duration, number of pulse and interval between pulses can all be set accurately.

1 - Field strength
a - duration 100 µs
b - duration 1 s
c - number of pulses 3
The field strength should be increased in 0.1 kV/cm steps from 0 to 1.2 kV/cm. For an electrode separation of 5 mm the output voltage will thus increment in 50 V steps from 0 to 0.6 kV.
2 - Pulse duration
As an example, the following values can be employed:
a) field strength 0.6 kV/cm
b) interval between pulses 1 s
c) number of pulses 3
For an electrode separation of 5 mm the output voltage will be 0.3 kV.
Examine the effect of pulse duration in the range 5 µs to 24 ms.
3 - Number of pulses
The following values can be employed:
a) field strength 0.6 kV/cm
b) pulse duration 100 µs
c) interval between pulses 1 s
Examine the effects of applying 1, 3, 5, 7 or 10 pulses.

.c.Pulsing conditions
After setting the electrical parameters (field strength, pulse duration and frequency), the drop of protoplast suspension cooled in crushed ice is placed between the electrodes.
1 - The protoplasts should be suspended in pulsing medium which may also contain substances to be transferred into the cytoplasm.
2 - after two washes with gentle centrifugation (70 g, 10 min, ambient temperature), suspend the protoplast pellet in pulsing medium.
3 - Keep the suspension on ice.
4 - The experimental chamber (Petri dish) should be placed on ice in a stainless steel bowl placed on the moveable tray. Move the tray up until the electrodes touch the bottom of the dish, making an open chamber.

5 - Place the appropriate volume of suspension between the electrodes using an automatic pipette (yellow tip). The tip should be cut off to avoid excess pressure during manipulation of the sample. If sterile conditions are required, sterile pipette tips (autoclaved and kept in a hermetically sealed container or disposable tips provided the bore is large enough to avoid damaging the sample) should be employed.
6 - Apply pulses. For electropermeabilization, the field strength can be increased in 0.1 kV/cm steps from a minimum of 0.2 kV/cm using three 100 µs 3 pulses at a frequency of 1 Hz. The pulse waveform may be monitored on an oscilloscope.
7 - Remove the electrodes from the sample by lowering the tray.
8 - Leave the dish containing the protoplasts to stand on ice for a few minutes to allow molecules to cross the transiently permeable membrane.
9 - Return the system to ambient temperature. After a further 20 min, add culture medium and view the preparation under a microscope.
10 - Rinse the electrodes with pulsing medium, and treat the next sample (steps 4 onwards).

.c.Demonstration of permeabilization

When the membrane becomes permeable, molecules will diffuse across it in both directions. This can be visualized by measuring either the leakage of metabolites or the influx of non-permeant molecules over an interval of a few minutes post-pulse.

.c.Observation of protoplasts under the microscope

The presence of protoplasts burst by an excessive electric field can be seen by microscopic examination. In the case of tobacco protoplasts, this is observed by a release of chloroplasts at field strengths above 0.9 kV/cm (3 pulses of 50 µs in one of the media described above).

.c.Leakage of ATP

The leakage of this metabolite provides an accurate evaluation of the extent of permeabilization. The principle is identical to that used for eukaryotic cells, namely:

1 - Treat protoplasts with pulses.
2 - Transfer a 200 µl aliquot to an Eppendorf tube and leave to stand on ice for 1 min.
3 - Centrifuge this tube (80 g, 1 min) contained inside a hemolysis tube.
4 - Remove 100 µl of supernatant and keep on ice.
5 - Place this sample in a **glass** scintillation vial containing 200 µl of 0.2 M glycylglycine buffer (pH 7.8) and 700 µl of water.
6 - Cool the vial in a refrigerator, and then transfer it to the scintillation counter.
7 - The ATP content can be determined by the luciferin-luciferase reaction as described in the section dealing with mammalian cells, page 10). Ensure that the pH in the vial is compatible with luciferase (> 7.2).

.c.Penetration of non-permeant dyes

A dye is added to the pulsing medium containing the protoplast suspension. The following dyes do not normally penetrate into the cytoplasm:
- Evans blue 0.1-0.5% (W/V)
- Erythrosin 0.1-0.5% (W/V)

A 1% (W/V) solution of dye (10 g/l) is made up in pulsing medium and added at a final concentration of 1/10 (1 volume in 9 volumes) to the protoplast preparation just before inserting the electrodes. The two solutions should be mixed carefully by hand.
Direct viewing of samples on a slide is not practicable without removing excess dye, and so the protoplasts should be gently centrifuged (40 g, 5 min, ambient temperature). Take up the supernatant in pulsing medium. The percentage of stained protoplasts is determined by counting at least 200 organelles under the microscope.

.c.Penetration of non-permeant fluorescent molecules

Observation of permeation is made easier by the use of fluorescent markers, although it does require a fluorescence microscope (Leitz Ortholux or Fluovert). Calcein (Sigma) should be added to the pulsing medium at a concentration of 2.5 mM. After pulsing and

incubation, excess marker must be removed by repeated careful washing. The samples are viewed with a x20 or x40 eyepiece using the filters for fluorescein recommended by the manufacturer. The total population of protoplasts is determined by direct observation under phase contrast, while the permeabilized population is observed in fluorescence mode. The following problems may be encountered:

- photosensitization of the molecule leading to a drop in fluorescence intensity. Observations should be carried out quickly.
- presence of marker in the external medium if washing has been inadequate.
- fluorescence of vacuolar and chloroplastic substances, which may interfere with the calcein emission. A wide pass band filter (covering the fluorescein band) can be put in the observation path to eliminate the longer wavelengths (red).

An example is as follows.

Visualization of permeabilization from penetration of calcein
Calcein is readily detected by its intense fluorescence.

1 - Materials

Stock solution of calcein (Sigma) 33 mg/10 ml

Pulsing medium A

0.55 M mannitol
1 mM Mg^{2+}
10 mM MOPS buffer (pH 7.2)

Pulsing medium B

Medium A
5 mM calcein

2 - Method

1 - Suspend protoplasts in medium A (2.10^6/ml).
2 - Keep on ice.
3 - Cool medium B.
4 - Mix a volume of protoplast suspension with a volume of medium B.
5 - using 5 mm separation electrodes apply pulses to 0.2 ml of this mixture in the cold.

6 - Leave to stand for 10 min in the cold.
7 - Leave for a further 10 min at ambient temperature.
8 - Gently take up the sample drop and put it in a 5 ml polystyrene hemolysis tube (Polylabo).
9 - Centrifuge for 5 min at 50 g (600 rpm, JOUAN C500).
10 - Remove the supernatant.
11 - Take up the pellet in medium A.
12 - Repeat this wash sequence 4 times.
13 - Make an observation chamber using two slides as separators.
14 - View in fluorescence mode using the filters recommended for fluorescein (Leitz Ortholux).
17 - Calcein fluoresces in the green (around 500 nm) and is easily distinguished from the background self-fluorescence.
18 - Count the percentages of intact and broken fluorescent protoplasts.

.c.Survival

In the majority of cases, the biological samples must not be damaged by the pulse treatment. Limiting values of the electrical parameters must therefore be carefully defined. It has been mentioned above that excessive electric fields will burst cells or organelles. Viability can be evaluated by two methods.
1. Metabolism of fluorescein diacetate after 24 h culture following electropermeabilization (FDA test).
2. Ability to spread after 1 week's culture (tobacco protoplasts).
This is a biologically meaningful test, although it takes time to carry out. The two tests can be used in a complementary manner. If the survival in the FDA test is < 15% the protoplasts will no longer divide, and the spreading test need not be carried out.

.c.Electrofusion of plant protoplasts

.c.Media

The medium described above can be used:

0.5 M mannitol

10 mM NaCl

1 mM $MgCl_2$

0.01% Tween 80

3-5 mM MES/NaOH, pH 5.6

in the presence of a suitable agglutinogen. The following have been tested:

0.75 mM spermine

2.5% (W/V) PEG 6000

2.5% (W/V) PVA 1000

.c.Pulsing conditions

The experiment can be carried out in 60 mm dishes with an electrode separation of 5 mm on a sample volume of 200 µl. A suitable protoplast concentration is 5.10^5/ml

1 - Place the electrodes in contact with the bottom of the dish as for electropermeabilization. An ice bath is not required in this case.

2 - Placed the tobacco protoplast suspension between the electrodes.

3 - Leave the suspension to stand for 10 min to allow the protoplasts to sediment and form numerous contacts.

4 - Apply pulses (850 V/cm, 100 µs, x 3 for tobacco protoplasts).

5 - Remove the electrodes.

6 - Leave the protoplasts to incubate for 30 min.

7 - Add culture medium.

The preparation should be viewed at step 6 to observe fusion.

The protoplasts can be left to sediment before lowering the electrodes. In this case, only a part of the suspension will be treated (the volume between the electrodes). The yield will be less, but samples can be treated under a variety of different experimental conditions more rapidly.

.c.Electrotransformation of plant protoplasts

.c.Transfer of transient activity to tobacco protoplasts

1 - the protoplasts of tobacco (*N. xanthi*) purified on a Ficoll gradient as described above should be washed several times to eliminate nucleases derived from digestion of the cell walls.

2 - The protoplasts should be taken up in the following transformation medium:

0.56 M mannitol

1 mM $MgCl_2$.6H2O

5 mM HEPES KOH, pH 7.2

3 - Adjust the concentration to $2.5.10^6$/ml.

4 - The linearized plasmid pDW2 codes for CAT activity, and can be used at a concentration of 10 µg/ml.

5 - Carrier DNA is from calf thymus and can be used at a concentration of 50 µg/ml.

6 - Mix the two solutions in the cold, and incubate for 5 min.

7 - Keep the culture dish (90 mm diam) on ice.

8 - Put the electrodes in contact with the bottom of the dish.

9 - Place 250 µl of the protoplast/plasmid mixture between the electrodes.

10 - Apply a single 3 ms pulse at 0.5 kV/cm.

11 - Lift out the electrodes, and then lower them to touch the bottom of the dish in another place.

12 - Place 250 µl of the mixture between the electrodes.

13 - Apply a single 3 ms pulse at 0.5 kV/cm.

14 - Remove the electrodes.

15 - Rinse the electrodes with pulsing medium.

16 - Leave the samples to stand on ice for 10 min, and then incubate at 21°C for 15 min.

17 - Add 10 ml of culture medium. Seal the dish with Parafilm to prevent contamination, and incubate at 25°C.

18 - CAT activity is determined using standard methods 48 h later.

.c.Transformation of Petunia protoplasts

1 - Suspend the protoplasts in culture medium at a concentration of 10^6/ml.
2 - Add the pFR101 plasmid solution at a final concentration of 50 µg/ml.
3 - Add 5% (W/V) PEG 6000 10 min after mixing the DNA with the protoplasts.
4 - Apply a heat shock (5 min, 45°C).
5 - Place two 50 µl drops of mixture in different areas of the culture dish which is stood on ice.
6 - Place the electrodes in contact with the bottom of the dish so as to surround one of the sample drops.
7 - Apply five 100 µs pulses at 1.25 kV/cm.
8 - Remove the electrodes.
9 - Place the electrodes in contact with the bottom of the dish over the second sample drop.
10 - Apply five 100 µs pulses at 1.25 kV/cm.
11 - Remove the electrodes.
12 - Leave the sample to stand on ice for 30 min.
13 - Add 9 volumes of culture medium.
14 - 1 to 2 weeks are pulsation, add the selection medium containing kanamycin.
15 - The resistant colonies can be counted several weeks later.

.c.C) **BACTERIA**

In view of their small size (1-2 µm) and the relationship between size and applied field strength, intense electric fields are required to electropermeabilize bacteria. Special chambers with a narrow gap between the electrodes (0.15 cm in the Jouan system) should be employed. Given the relationship between the thickness of a sample and its electrical resistance, the instrument will only operate within its capacity and safety margins if the ionic strength of the sample solution is low. if these limits are exceeded, the electrical energy will be dissipated as heat and the bacterial suspension may boil. It is thus mandatory to remove ions from the culture medium as well as those used for separation of plasmids on a CsCl gradient.
The experimental chamber supplied has two separate compartments: one of 18 µl and 38 µl. The smaller compartment is used for samples with a higher ionic content, or for more drastic electrical conditions.

.c.Electropermeabilization of bacteria

Permeabilization of plasma and external membranes can be demonstrated by detection of the leakage of cytoplasmic metabolites such as ATP into the external medium.

1 - Use a suspension of 10^9 bact/ml in 10% (V/V) glycerol or other aqueous buffer. Select an appropriate medium for your particular sample.

2 - Open the lid of the experimental chamber.

3 - Place 36 µl of the bacterial suspension in the chamber using an automatic pipette (yellow tip).

4 - Apply pulses. The electrical parameters will depend on the bacterial sample, especially on the size and shape of the strain under investigation. As a general rule, short pulse durations can be used (several tens of µs, although more clear-cut effects are observed at durations above 1 ms), but the field strength should be high (several kV/cm). Repeated pulses will increase the leakage of metabolites, reflecting a higher level of permeabilization of the cell walls and membranes.

5 - Open the lid of the experimental chamber.

6 - Remove 35 µl with an automatic pipette (yellow tip) by tilting the chamber slightly.
7 - Place this sample in an Eppendorf tube.
8 - Add 200 µl of sterile water.
9 - Leave for 1 min at ambient temperature. During this time ATP continues to leak out of the cells, and will thus be more readily detected.
10 - Centrifuge for 1 min at 13,000 g.
11 - Transfer a 100 µl aliquot of the supernatant to a clean scintillation vial kept in the cold.
12 - Rinse the experimental chamber twice with 40 µl of sterile water.
13 - Determine the ATP content in the scintillation vial using the luciferin/luciferase reaction (described in the section dealing with mammalian cells, page 10).

.c.Survival of bacteria after electropulsation

The severe conditions used to electropermeabilize bacteria may lead to cell lysis. This will tend to arise at high field strengths, long pulse durations and with repeated pulsing. The effect of such conditions is highly dependent on the particular strain, its state of growth, the medium used (especially the presence of an osmotic gradient) and treatments carried out on the cell wall prior to electropulsation. It is thus important to check the viability of the cells after electropulsation.

1 - Use a suspension of 10^9 bact/ml in water or aqueous buffer. Select an appropriate medium for your particular sample.
2 - Open the lid of the experimental chamber.
3 - Place 36 µl of the bacterial suspension in the chamber using an automatic pipette (yellow tip).
4 - Apply pulses. The electrical parameters will depend on the bacterial sample especially the size and shape of the strain under investigation. As a general rule, short pulse durations can be used (several tens of µs), but the field strength should be high (several kV/cm).
5 - Open the lid of the experimental chamber.

6 - Remove 35 µl with an automatic pipette (yellow tip) by tilting the chamber slightly.
7 - Place this sample in an Eppendorf tube containing 1 ml of culture medium.
8 - Dilute suitably to obtain a readily countable number of colonies after spreading on a solid medium (around 10^5 times that of the initial sample).
9 - Spread onto the solid medium.
10 - Incubate under suitable conditions for the particular bacterial strain.
11 - Observe and count colonies after a suitable period of incubation (e.g. 24 h for *E. coli*).
12 - Compare survival to that of an untreated sample.
In *E. coli* at the start and middle of the exponential growth phase, viability will be affected by a 3 ms pulse at field strengths over 4 kV/cm, or by repeated short pulses at this value of field strength. Survival which is readily measured should be determined for each strain used, and in many cases, for each separate culture. Survival should also be checked after electrotransformation.

.c.**Electrotransformation of bacteria**

In the case of *E. coli* and *Corynebacteria*, 1% transformants can be obtained without any significant drop in viability of the population.

.c.Methods

Pulsing medium

- 1 mM Tris, pH 7.5
- 0.5 mM $MgCl_2$
- 270 mM sucrose

Positive results have also been obtained in 10% (V/V) glycerol. The medium should be sterilized (e.g. filtration).

1 - Use bacteria at the start of the exponential growth phase. Any suitable medium can be employed.

2 - Wash the bacteria three times by centrifugation and resuspension of the pellet in pulsing medium.

3 - Adjust the final volume to a concentration of around 5.10^9 bact/ml. A higher concentration may be required if many of the salts were washed out of the medium.

4 - Sterilize the chamber and lid by copious washing with 70% ethanol followed by a rinse with sterile water.

5 - Carry out the experiment in a sterile environment, e.g. in a laminar flow cupboard.

6 - Mix 30 µl of the bacterial suspension (on ice) with 5 µl of the solution of the plasmid in an Eppendorf tube just before the experiment.

7 - Final concentrations of plasmid ≥5 µg/ml are recommended.

8 - Place either 18 µl or 35 µl (small or large side of the chamber) of the bacterial/plasmid mixture (just after mixing) in the experimental chamber using an automatic pipette (yellow tip, sterile).

9 - Close the lid.

10 - Apply pulses.

11 - Open the lid.

12 - Remove the sample quickly with an automatic pipette (yellow tip, sterile) by slightly tilting the chamber. As soon as possible after

electropulsation, dilute this sample in 1 ml of culture medium at 37°C.

13 - Incubate for 1-3 h (depending on the growth time of the strain).

14 - Spread onto dishes containing the selection medium (e.g. antibiotic for transformants) or standard medium for viability studies (see section dealing with survival, page 22). The transformation yield is generally quite low, and so for the survival studies, samples must be diluted considerably before spreading, whereas little further dilution is required (10-fold dilution is commonly employed) for the transformation experiments.

15 - Rinse the space between the electrodes with sterile water.

16 - Incubate the dishes containing the treated bacteria under suitable conditions for the particular strain.

17 - Observe growth. In some cases, the transformants proliferate more slowly than the starting strain, and so they should be observed at a later time (several days) provided that the selection medium remains functional.

.c.Definition of the electrical parameters

The values of the electrical parameters will depend on the particular strain, although for transformation of intact bacteria, long duration pulses are generally required (several ms). In *E. coli*, field strengths of 5-6 kV/cm (700-900 V) and pulse durations over 4 ms can be employed. In practice, problems with heat dissipation effectively set the upper limits. A single pulse is sufficient.

In practice, if:

B = number of bacteria to be treated

S = number of surviving bacteria

T = number of transformants

P = amount of plasmids used (in µg)

V = volume of experimental chamber

B will depend on the volume of the chamber (18 µl or 36 µl) and the density of the cell suspension after washing. The effectiveness of the transformation is expressed by T, which depends on both B and P. Over a wide range of B (up to 10^{10}/ml, or 4.10^{8} treated

cells), T increases directly with B. T is also a linear function of P over a limited range. Saturation tends to occur when there is more than 1 μg of plasmid in the chamber.

The effectiveness can be expressed on a relative scale as:

$$\text{Effectiveness} = T/(B.P)$$

This produces values ranging from 10^{-5} to 10^{-1}. The number of transformants obtained will depend directly on the number of cells treated and the amount of plasmids used. In some studies, T is corrected by a survival factor (B/S) and the amount of plasmid without reference to the amount of material treated B. This can lead to figures for the production of transformants (10^{10}) that are above those of the initial population!

.c.Electrofusion of bacterial protoplasts

Electrofusion of bacterial protoplasts can lead to the formation of high yields of bacterial hybrids. The following procedure is designed for strains of *Corynebacteria*. Strain S is resistant to streptomycin (300 µg/ml), while strain R is resistant to rifampicin (100 µg/ml). Resistance is tested on solid media (containing agar).

1 - Culture both strains of *Corynebacterium glutamicum* on a rich medium (AA57, bactopeptone, yeast extract) at 37°C under gentle agitation.

2 - Incubate the bacteria in the exponential growth phase at 30°C for 2 h with penicillin (0.3 µg/ml). After washing, incubate for 16 h in the presence of lysozyme (0.3 mg/ml).

3 - After washing, take up the protoplasts in fusion medium (0.4 M sucrose, 3 mM $MgCl_2$, pH 7.2) supplemented with 0.2 mM spermine or 10% (W/V) PEG 6000. High concentrations should be used (> 10^7/ml for each strain).

4 - Place 18 µl of the mixture between the electrodes using an automatic pipette (yellow tip, sterile).

5 - Close the lid.

6 - Apply pulses (6.6 kV/cm, 100 µs, x 5).

7 - Open the lid.

8 - Take up the protoplasts in AAS 57 medium (AS 57 with 0.4 M sucrose) and incubate at 37°C overnight.

9 - Spread out on doubly selective medium (25 µg/ml rifampicin, 200 µg/ml streptomycin).

10 - The doubly resistant hybrids are detectable 5 days after electropulsation.

11 - Yields may attain 1/1000.

.c.D) FLOW ELECTRODES

The JOUAN electropulser is capable of delivering high power pulses (high voltage and current) at a high repetition rate. This enables treatment of high volumes, such as samples in continuous flow.

.c.Procedure

1 - Push the tubing into the connectors.

2 - Maintain liquid flow with a peristaltic pump. If the biological material is liable to be damaged by the pump rollers, samples can be sucked through the system by placing the pump on the output side. The reservoir at the pump input must be air-tight (e.g. Nalgene vessels ref. 2117-0250 with wash bottle tops ref. 2153-700 Polylabo refs 20413 and 20409). The threads should be smeared with high vacuum grease to make an air-tight joint. These models are sterilizable. A diagram of the set-up is illustrated in the Appendix.

3 - The flow rate will depend on the pulse frequency and volume of the chamber from relationship:

flow rate (ml/min) = volume (ml)*frequency (Hz)*60/number of pulses.

The volumes are:

0.128 ml for the 4 mm separation electrodes

0.06 ml for 1.5 mm separation electrodes (for bacteria).

The frequency is selected by the operator. The pulse repetition rate is determined by the number of pulses which must be applied to each cell.

4 - For sterile conditions, the system should be flushed out with a solution of sodium hypochlorite followed by sterile water using the system pump.

5 - The input and output tubes should be in the horizontal position to ensure a regular flow rate and prevent the formation of air bubbles.

6 - the electrode separation is marked in red on the Plexiglas containers.

7 - Grip the flow cell in a laboratory clamp fixed to a retort stand. These standard items can be obtained from any laboratory supply house.

8 - It is recommended to arrange the output tube vertically during filling to avoid formation of air bubbles in the flow cell and to turn it back to the horizontal position afterwards as described in 5.
9 - After use, flush out the system with ethanol, and dry with compressed air.

.c.Limiting conditions

As mentioned in the operation manual, the working capacity of the electropulser depends on the composition of the pulsing medium and the geometry of the electrodes. In flow systems, the pulse frequency, i.e. the recovery time between each electrical discharge, must also be taken into account.
The following limits have been defined on test for both models of flow cell.

1) 4 mm electrode separation

Medium	voltage (V)	duration (mS)	frequency (Hz)
5 mM MES, 6 mM $MgCl_2$	1000	10	1
10 mM Tris	1000	2.5	2
"	1000	1	6
"	1000	0.5	10

2) 1.5 mm electrode separation

Medium	voltage (V)	duration (mS)	frequency (Hz)
Pure water	1000	10	10
5 mM MES, 6 mM $MgCl_2$	1000	2.5	1
"	1000	1	2
"	1000	0.5	5
10 mM Tris	750	10	1
"	900	5	1
"	1000	2.5	1
"	1000	1	3
"	1000	0.5	7
2 mM Tris	1000	1.5	10
"	1000	2	8
"	1000	5	3
"	1000	10	1

These are the maximum values to be used in a flow system (8 ml/min under test conditions). They are defined by the limiting values of current, stored charge and recharge time of the internal capacitors. They also depend on the ionic content of the solution flowing between the electrodes.

.c.Continuous electropermeabilization

The incorporation of Trypan Blue (or Direct Blue) into CHO cells provides an example of the technique.

1 - Culture CHO WTT cells in suspension in MEM 0111 medium supplemented with 6% FCS.

2 - Wash the cells twice in pulsing medium A (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose).

3 - Suspend the cells in cold pulsing medium to give a final concentration of around 10^6 cells/ml.

5 - Place 5 ml of the cell suspension in the reservoir cooled in an ice bath.

5 - Place 5 ml of pulsing buffer supplemented with 3 mM $CaCl_2$ and Direct Blue (10 mg/ml) in the collector tank in an ice bath.

6 - Use the flow cell with the 4 mm electrode separation.

7 - Set the frequency to 10 Hz, the flow rate to 0.128 ml/sec. This corresponds to an average application of 10 pulses to each cell.

8 - Set the field strength to 1.2 kV/cm at a pulse duration of 100 µs.

9 - The total cell volume is thus treated in less than one minute.

10 - The percentage of cells which have taken up the dye can be determined by microscopic examination of the cells in the collector tank after electropulsation. In general over 98% of cells are permeabilized.

11 - It can be demonstrated that viability is not affected by electropermeabilization by carrying out an identical experiment except with culture medium (MEM 0111 plus 6% FCS) in the collector tank. After pulsation, place the cells in culture dishes and observe growth during 24 h. Compare to that of an untreated control.

.c.Continuous electrofusion

.c.Cells growing in suspension

CHO WTT cells are used as reference system as their fusogenic state is relatively long-lived.

1 - Culture CHO WTT cells in suspension. Wash twice in pulsing medium A.

2 - Take up the cells in cold pulsing medium to make a final concentration of 10^6 cells/ml.

3 - Place 5 ml of this suspension in the reservoir cooled in an ice bath.

4 - Place the collector tank on an ice bath.

5 - Use the flow cell with the 4 mm electrode separation.

6 - Set the pulse frequency to 5 Hz at a flow rate of 0.128 ml/sec. This corresponds to an average application of 5 pulses to each cell.

7 - Set the field strength to 1.6 kV/cm and the pulse duration to 100 µs.

8 - The whole cell volume is thus treated in less than one minute.

9 - After electropulsation, centrifuge the suspension for 5 min at 50 g.

10 - Incubate at 37°C for 30 min.

11 - Disperse the cell pellet, and take it up in an equal volume of culture medium.

12 - Place this suspension in culture dishes, and incubate at 37°C in a 5% CO_2/air mixture.

13 - The extent of polynucleation can be determined after several hours incubation.

14 - Survival is determined from the growth observed 24 h after electropulsation.

.c.Cells growing on microcarriers

Cells that normally grow in culture dishes can be cultured on microcarriers and can thus be maintained in suspension using gentle agitation.

1 - CHO WTT cells can be cultured on Biosilon (Nunc) microcarriers according to the manufacturer's recommendations (Tecne agitator, MEM 0111 medium supplemented with 6% FCS). The culture should be treated at a stage in which there are numerous cell contacts on the surface of each microcarrier.

2 - Wash the microcarrier/cell suspension in pulsing medium A.

3 - Place the suspension in the reservoir.

4 - Use the flow cell with a 4 mm electrode separation.

5 - Avoid high flow rates which tend to make cells separate from the microcarriers. Set the flow rate to 5 ml/min and a pulse frequency of 5 Hz. Under these conditions, the cells receive an average of 5 pulses.

6 - Set the field strength to 1.6 kV/cm for a pulse duration of 100 µs.

7 - Collect the suspension after electropulsation, and incubate at 37°C for 1 h. Culture medium can be added at this stage if necessary, but in this case, incubation must be carried out in a 5% CO_2/air atmosphere.

8 - The cells on the microcarriers now contain polycarya.

9 - The cells at this stage are not readily observable, and it is advisable to remove the cells from the microcarriers by digestion with trypsin under gentle agitation (according to the instructions supplied by Nunc). Culture medium should be removed if present. On stopping the agitation, the cells remain in suspension while the microcarriers sediment. Mix the supernatant with culture medium, and place aliquots in culture dishes. Incubate the dishes at 37°C in a 5% CO_2/air atmosphere. Numerous polycarya can be observed. Cells divide normally indicating that there has been no loss of viability.

xxxxxxxxxxx

BACTERIES

xxxxxxxxxxx

mise à jour Septembre 1990

1- S.P. ALLEN et H.P. BLASHEK (1990) FEMS Lett. 70, 217-220
Clostridium,
2- M.A. FARINHA et A.M. KROPINSKI (1990) FEMS Lett. 70, 221-226
Pseudomonas,
3- M. SOLIOZ et M. WASER (1990) Biochimie 72, 279-283
Enteroccus,
4- M. SOLIOZ et D. BIENZ (1990) TIBS 15, 175-177
revue,
5- Y. NATORI, Y. KANO et F. IMAMOTO (1990) Biochimie 72, 265-269
Lactobacillus casei,
6- T.D. XIE, L. SUN et T.Y. TSONG (1990) Biophys. J. 58, 13-19
E. coli,
7- M. MERSEREAU, G.P. PAZOUR et A. DAS (1990) Gene 90, 149-151
Agrobacterium tumefaciens,
8- D.M. HEERY, R. POWELL, F. GANNON et L.K. DUNICAN (1990) NAR 17, 10131
E. Coli,
9- H. MARCUS, J.M. KETLEY, J. B. KAPER et R.K. HOLMES (1990) FEMS Lett. 68, 149-154
Vibrio Cholerae,
10- D.K. SUMMERS et H.L. WITHERS (1990) NAR 18, 2192
E. coli,
11- R.F. CONCHAS et E. CARNIEL (1990) Gene 87, 133-137
Yesenia,
12- A. TAKETO (1989) J. Biochem. (Tokyo) 105, 813-817
E. coli,
13- Y. KURUSU, M. KAINUMA, M. INUI, Y. SATOH et H. YUKAWA (1990) Agric. Biol. Chem. 54, 443-447
corynées,
14- S. BONNASSIE, J-F. BURINI, J. OREGLIA, A. TRAUTWETTER, J-C. PATTE et A. M. SICARD (1990) J. Gen. Microbiol. sous presse
Brevibacterium,
15- C. BONAMY, A. GUYONVARCH, O. REYES, F. DAVID et G. LEBLON (1990) FEMS Lett. 66, 263-270
Corynées,
16- J. A. HAYNES et M.G. BRITZ (1990) J. Gen. Microbiol. 136, 255-263
corynées,
17- R. NAGEL, A. ELLIOTT, A. MASEL, R.G. BIRCH et J.M. MANNERS (1990) FEMS Lett. 67, 325-328
Agrobacterium tumeaciens, rhizogenes,
18- P.T. SCOTT et J.I. ROOD (1989) Gene 82, 327-333
Clostridium perfringens,
19- D.A. McINTYRE et S. K. HARLANDER (1989) Appl. Environ. Microbiol. 55, 2621-2626
Lactoccus lactis,
20- H. KUSAOKE, Y. HAYASHI, Y. KADOWAKI et H. KIMOTO (1989) Agric. Biol. Chem. 53, 2441- 2446
Bacillus subtilis,
21- D.A. McINTYRE et S.K. HARLANDER (1989) Appl. Environ. Microbiol. 55, 604-610
Lactococcus lactis,
22- G.A. SOMKUTI et D.H. STEIBERG (1988) Biochimie 70, 579-585
Streptococcus Thermophilus,
23- S.K. HARLANDER (1987) dans "Streptococcal Genetics" Ferretti J.J. et Curtiss R. Eds, ASM, Washington, p.229-233

24- I.B. POWELL, M.G. ACHEN, A.J. HILLIER et B. E. DAVIDSON (1988) Appl. Environ. Microbiol. 54, 655-660
Streptococcus lactis, Streptococcus cremoris,
25- N.M. CALVIN et P.C. HANAWALT (1988) J. Bact. 170, 2796-2801
E. coli, ,
26- D. Van Der LELIE, J. Van Der VOSSEN et G. VENEMA (1988) Appl. Environ. Microbiol. 54, 865-871
Streptococcus Cremoris,
27- S. BONNASSIE, A.M. GASC et A.M. SICARD (1989) dans "Genetic Transformation and expression", p.71-75 L.O. BUTLER, C. HARWOOD et B.E.B. MOSELEY eds, Intercept Ltd, Andover, UK
Streptococcus pneumoniae, Brevibacterium lactofermentum,
28- A. SUVOROV, J. KOK et G. VENEMA (1988) FEMS Lett. 56, 95-100
Streptococcus pyogenes,
29- D. LERECLUS, O. ARANTES, J. CHAUFAUX et M.M. LECADET (1989) FEMS Lett 60, 211- 218
Bacillus Thuringensis,
30- J. MAHILLON, W. CHUNGJAPORNCHAI, J. DECOCK, S. DIERICKX, F. MICHIELS, M. PEFEROEN et H. JOOS (1989) FEMS Lett. 60, 205-210
Bacillus Thuringensis,
31- W. SCHURTER, M. GEISER et D. MATHE (1989) Mol. Gen. Genet. 218, 177-181
Bacillus Thuringensis, Bacillus cereus,
32- D.M. HEERY et L.K. DUNICAN (1989) NAR 17, 8006
E. coli,
33- MATTANOVICH, F. RUKER, A. da CAMARA MACHADO, M. LAIMER, F. REGNER, H. STEIKELLNER, G. HIMMLER et H. KATINGER (1989) NAR 17, 6747
Agrobacterium,
34- J. VEHMAANPERA (1989) FEMS Lett. 61, 165-170
Bacillus amyloliquefaciens, Bacillus subtilis
35- A.M. THOMSON et H.J. FLINT (1989) FEMS Lett. 61, 101-104
Bacteroides ruminicola, Bacteroides uniformis,
36- A.V. BROEK, A. Van GOOl et J. VANDERLEYDEN (1989) FEMS Lett. 61, 177-182
Azospirillum brasilense,
37- J. JESSEE, L. UHER et T. LANDERS (1989) Focus 11, 10-11
E. coli, Pseudomonas aeruginosa,
38- R. BADII, S. JONES et P.J. WARNER (1989) Lett Appl. Microbiol. 9, 41-44
Lactobacillus plantarum,
39- P. LANGELLA et A. CHOPIN (1989) FEMS Lett. 59, 301-306
Lactococcus lactis,
40- T. AUKRUST et I.F. NES (1988) FEMS Lett. 52, 127-132
Lactobacillus plantarum,
41- B.H. BELLIVEAU et J.T. TREVORS (1989) Appl. Environ. Microbiol. 55, 1649-1652
Bacillus cereus,
42- E.J. BONE et D.J. ELLAR (1989) FEMS Lett. 58, 171-178
Bacillus Thuringiensis,
43- H. WOLF, A. PUHLER et E. NEUMANN (1989) Appl. Microbiol. Biotechnol. 30, 283-289
Corynées,
44- E.C. BOTTGER (1988) Biotechniques 6, 878-880

45- A. TAKETO (1989) Biochim. Biophys;. Acta 1007, 127-129
E. Coli,

46- J.D. OULTRAM, M. LOUGHLIN, T.J. SWINFIELD, J.K. BREHM, D.E. THOMPSON et N.P. MINTON (1988) FEMS Lett. 56, 83-88
Clostridium acetobutylicum,
47- S.P. ALLEN et H.P. BLASHEK (1988) Appl. Environ. Microbiol. 54, 2322-2324
Clostridium perfringens,
48- A. TAKETO (1988) Biochim. Biophys. Acta 946, 318-324
E coli, Salmonella typhimurium,
49- G. ZEALEY, M. DION, S. LOOSMORE, R. YACOOB et M. KLEIN (1988) FEMS Lett. 56, 123-126
Bordetella,
50- J.B. LUCHANSKY, P.M. MURIANA et T.R. KLAENHAMMER (1988) Mol. Microbiol. 2, 637-646
Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus, Propionibacterium,
51- K. ITO, T. NISHIDA et K. IZAKI (1988) Agric. Biol. Chem. 52, 293-294
Erwinia carotovora,
52- J.F. MILLER, W.J. DOWER et L.S. TOMPKINS (1988) Proc. Natl. Acad. Sci. USA 85, 856-860
Campylobacter jejuni,
53- S. DAVID, G. SIMONS et W.M. DE VOS (1989) Appl. Environ. Microbiol. 55, 1483-1489
Leuconostoc paramesenteroides,
54- B.M. CHASSY et J.L. FLICKINGER (1987) FEMS Lett. 44, 173-177
Lactobacillus casei,
55- S. FIEDLER et R. WIRTH (1988) Anal. Biochem. 170, 38-44
E. coli, Pseudomonas putida,
56- S. FIEDLER, A. FRIESENEGGER et R. WIRTH (1989) dans "Genetic Transformation and Expression", p.65-69 L.O. BUTLER, C. HARWOOD et B.E.B. MOSELEY Eds, Intercept, U.K.
nombreuses espèces,
57- E. AUBERT et J. DAVIES (1989) dans "Genetic Expression and Expression" p. 77-83 L.O. BUTLER, C. HARWOOD et B.E.B. MOSELEY Eds, Intercept, UK
Bacillus circulans,
58- J.A. HAYNES et M.L. BRITZ (1989) FEMS Lett. 61, 329-334
Brevibacterium lactofermentum, Corynées,
59- W. LIEBL, A. BAYERL, B. SCHEIN, U. STILLNER et K.H. SCHLEIFER (1989) FEMS Lett. 65, 299-304
Corynées,
60- L.K. DUNICAN et E. SHIVNAN (1989) Bio/Technology 7, 1067-1070
Corynées, Brevibacterium lactofermentum,
61- A. MERCENIER et B.M. CHASSY (1988) Biochimie 70, 503-517
revue
62- H. TAKAGI, S. KAGIYAMA, K. KADOWAKI, N. TSUKAGOSHI et S. UDAKA (1989) Agri. Biol. Chem. 53, 3099-3100
Bacillus Brevis,
63- P. BRIGIDI, E. DE ROSSI, M.L. BERTARINI, G. RICCARDI et D. MATTEUZZI (1990) FEMS Lett. 67, 135-138
Bacillus subtilis,
64- W.J. DOWER, J.F. MILLER et C.W. RAGSDALE (1988) NAR 16, 6127-6145
E. coli,
65- B.M. CHASSY, A. MERCENIER et J. FLICKINGER (1988) TIBTECH 6, 303-309
revue
66- H. POTTER (1988) Anal. Biochem. 174, 361-373
revue

67- K. SHIGEKAWA et W.J. DOWER (1988) Biotechniques 6, 742-751
revue,
68- E.S. CYMBALYUK, L.V. CHERNOMORKIK, N.E. BROUDE et Yu A. CHIZMADZHEV (1988) FEBS Lett. 234, 203-207
E. coli,
69- R. WIRTH, A. FRIESENEGGER et S. FIEDLER (1989) Mol. Gen. Genet. 216, 175-177
Biorad, gram négatif, nombreuses espèces,
70- T. THIEL et H. POO (1989) J. Bact. 171, 5743-5746
cyanobactérie filamenteuse,
71- A.W. SMITH et B.H. IGLEWSKI (1989) NAR 17, 10509
Pseudomonas aeruginosas,
72- L. MASSON, G. PREFONTAINE et R. BROUSSEAU (1989) FEMS Lett. 60, 273-278
Bacillus thuringiensis,
73- M.L. GUERINOT, B.A. MORISSEAU et T. KLAPATCH (1990) Mol. Gen. Genet. 221, 287-290
Bradyrhizobium japonicum,
74- J.E. ALEXANDER, P.W. ANDREW, D. JONES et I.S. ROBERTS (1990) Lett. Appl. Microbiol. 10, 179-182
Listeria,
75- J.T. TREVORS (1990) J. Basic Microbiol. 30, 57-61
Klebsiella aerogenes, Pseudomonas putida,
76- D.R. HATTERMANN et G. STACEY (1990) Appl. Environ. Microbiol. 56, 833-836
Bradyrhizobium japonicum,
77- A.Y. KIM et H.P. BLASHEK (1989) Appl. Environ. Microbiol. 55, 360-365
Clostridium perfringens,
78- E. PETRVALSKY et O. SOVA (1989) Stud. Biophys. 130, 177-181
E. coli,
79- R. WIRTH (1989) Forum Mikrobiologie 12, 507-515
revue,
80- D. O'CALLAGHAN et A. CHARBIT (1990) Mol. Gen. Genet. 223, 156-158
Salmonella typhimurium, typhi,
81- S.B. SNAPPER, L. LUGOSI, A. JEKKEL, R. E. MELTON, T. KIESER, B. R. BLOOM et W.R. JACOBS (1988) Porc; Natl. Acad. Sci. USA 85, 6987-6991
Mycobacterium tuberculosis, smegmatis,

xxxxxxxxxxxxxx

HYBRIDOMES

xxxxxxxxxxxxxx

mise à jour Septembre 1990

1- M.M.S. LO et T.Y. TSONG (1989) dans "Electroporation and Electrofusion in Cell Biology" chap. 16, p. 259-270, E. Neumann , A. Sowers et C. Jordan Eds, Plenum

2- M.C. GLASSY et M. PRATT (1989) dans "Electroporation and Electrofusion in Cell Biology" chap. 17, p. 271-282, E. Neumann , A. Sowers et C. Jordan Eds, Plenum

3- M.K. CONRAD, M.M.S. LO, T.Y. TSONG et S.H. SNYDER (1987) dans "Cell Fusion" chap. 21, p. 427-439, A. Sowers Ed., Plenum

4- T.Y. TSONG, M. TOMITA et M.M.S. LO (1988) dans "Molecular Mechanisms of Membrane Fusion", p. 223-236, S. Ohki, D. Doyle, T.D. Flanagan, S.W. Hui et E. Mayhew Eds, Plenum

5- S. PERKINS, U. ZIMMERMANN, P. GESSNER et S.K.H. FOUNG (1990) dans "Electromanipulation in Hybridoma Technology" chap. 3, p.47-70, C.A.K. Borrebaeck et I. Hagen Eds, Stockton Press

6- M.K. CONRAD et M.M.S. LO (1990) dans "Electromanipulation in Hybridoma Technology" chap. 5, p. 89-102, C.A.K. Borrebaeck et I. Hagen Eds, Stockton Press

7- G. Van DUJIN, J.P.M. LANGEDIJK, M. De BOER et J.M. TAGER (1989) Exp. Cell Res. 183, 463-472

8- H. ABEL, P. STOLLEY, G. DREYER, I. WALTHER, W. HANDSCHACK, K. SCHWARZ, C. PLATZER, C. GROBEL, U. KARSTEN et B. MICHEEL (1988) Bioelectrochem. Bioenerg. 19, 173-177

9- K. OHNISHI, J. CHIBA, Y. GOTO et T. TOKUNAGA (1987) J. Immunol. Meth. 100, 181-189

10- I. TSONEVA, I PANOVA, P. DOINOV, D.S. DIMITROV et D. STRAHILOV (1988) Stud. Biophys. 125, 31-35

11- S.M. BROWN, Q.F. AHKONG, A.D. SAGE et J.A. LUCY (1986) Biochem. J. 14, 297-298

12- G.A. HOFMANN et G.A. EVANS (1986) IEEE Eng. Med. Biol. 5, 6-25 revue

13- M.M.S. LO, T.Y. TSONG, M.K. CONRAD, S.M. STRITTMATTER, L.D. HESTER et S.H. SNYDER (1984) Nature 310, 792-794

14- D.M. WOJCHOWSKI et A.J. SYTKOWSKI (1986) J. Immunol. Meth. 90, 173-177

15- H. ABEL, P. STOLLEY, G. DREYER, I. WALTHER et C. JUNGE (1986) Acta Biotechnol. 3, 287-291

16- U. KARSTEN, G. PAPSDORF, G. ROLOFF, P. STOLLEY, H. ABEL, I. WALTHER et H. WEISS (1985) Eur. J. Cancer Clin. Oncol. 21, 733-740

17- J. VIENKEN et U. ZIMMERMANN (1985) FEBS Lett. 182, 278-20

18- R. BISCHOFF, R.M. EISERT, I. SCHEDEL, J. VIENKEN et U. ZIMMERMANN (1982) FEBS Lett. 147, 64-68

19- M. PRATT, A. MIKHALEV et M.C. GLASSY (1987) Hybridoma 6, 469-477

20- J.J. SCHMITT et U. ZIMMERMANN (1989) Biochim. Biophys. Acta 983, 42-50

21- J.J. SCHMITT, U. ZIMMERMANN et G.A. NEIL (1989) Hybridoma 8, 107-115

22- Y.G. MENG et J. TRAWINSKI (1988) J. Immunol. 141, 2684-2687

23- D.A. STENGER, R.T. KUBINIEC, W.J. PURUCKER, H. LIANG et S.W. HUI (1988) Hybridoma 7, 505-518

24- I. TSONEVA, T. TOMOV, I. PANOVA et D. STRAHILOV (1990) Bioelectrochem. Bioenerg. 24, 41-49

GUARANTEE TERMS

JOUAN guarantees that this unit if free from defects in materials and workmanship when it leaves the factory, and undertakes to replace or repair the unit if it proves defective in normal use or during servicing for a period of **ONE YEAR** from the delivery.

Our liability under this guarantee is limited to repairing the defective unit or any part of the unit providing it is sent, carriage paid, to an authorised service center or the SAINT-HERBLAIN office.

This guarantee is invalidated if the unit is incorrectly used, poorly serviced or neglected, mis-used or accidentally damaged.

There is no explicit guarantee other than as stated above.

FOR FURTHER INFORMATION, ASSISTANCE OR SERVICING

Please contact :

FRANCE

* Your regional office	SAINT-HERBLAIN Tel. 40.94.90.10 - Telex 711062 JOUAN NT PARIS Tel. 1.43.42.40.50 - Telex 217126 JOUAN PA LYON Tel. 78.83.04.90
USA - WINCHESTER, VIRGINIA -	Tel. (703) 665.0863 - Tlfax. (703) 665.8002
ENGLAND - TRING, HERTS -	Tel. (442) 890.202 - Tlx 826155 DANICO G.
ALGERIA	Tel. 213KL.58.16.08 - Tlx 62 357 DZ
OTHER COUNTRIES	Your local representative

or our CUSTOMER SERVICE CENTER

SOCIETE JOUAN
Service Assistance Technique
Rue Bobby Sands
CP 3203
44805 SAINT HERBLAIN
Tel. 40.94.90.10
Telex 711062 JOUAN NT
Fax. 40.94.70.16

CONTENTS

This manual deals with the technical aspects of the electropulsator, a separate manual is available which deals in more detail with the experimental procedures for a variety of cell systems.

CHAPTER 1

TECHNICAL DETAILS

1.1 - PRECAUTIONS

This instrument can deliver potentially dangerous high voltages. Check all wiring and electrode connections carefully.

The instrument is fitted with a power input filter which grounds the instrument case. Only use power leads which conform to recognized standards of safety. The power input is protected by a rated fuse. Do not replace with a fuse of a higher rating. If the fuse blows frequently, return the instrument to an authorized service center.

High capacity condensers charged to voltages in excess of 1000 V are present. Opening the case with the power on is potentially dangerous. There are no user-serviceable parts inside.

After unpacking, inspect the instrument for transport damage, and check that the following accessories are present :
- 3 lead power cable
- foot pedal and control cable
- technical manual.

Do not connect anything to the outputs which could deliver electrical energy (AC, DC or HF). The output stages or output protection circuits could be damaged.

1.2 - OPERATING PRINCIPLE

The output pulses are produced by controlled capacitative discharge. The rise time is around 1 microsecond for 1000 V output. However, with high capacitative loads, the output voltage will drop somewhat. This fall in voltage can be estimated from :

$$Q = CdV = IdT$$

For a current of 8 A and a capacity of 8 μF this gives :

$$dV/dT = 1 \text{ V per } \mu\text{s pulse duration}$$

The output is protected by an electronic circuit breaker, and in the event of an overload, the pulse duration will be reduced momentarily. The output trigger pulse (TRIGGER OUTPUT) will not be affected.

If the load is highly capacitative, the output voltage will be affected by the current limiting relationship:

$$Q = CdV = IdT$$

which gives the limiting capacitance; for $dV/dT = 10^9$ V/S and Imax = 8 A, Cmax = 8 nF. No pulses will be output for loads above this value of capacitance.

The output leads and electrodes have a nominal capacitance of 0.5 nF.

1.3 - OPERATOR SAFETY

When setting up the instrument and electrodes and after use, the output voltage should be adjusted to zero with the "V OUT" control knob.

Take particular care not to splash or spill conducting or corrosive fluids on the instrument.

1.4 - OUTPUTS

FRB output socket : high voltage pulses (negative with respect to ground) are delivered from this output. The overload protection loop is connected to ground via one of these contacts.

BNC socket "PEDAL" : TTL-compatible input for triggering a pulse in mode "EXT". The rising edge triggers the pulse. If a switch is connected (pedal), the pulse is triggered on opening the contact.

BNC socket "TRIGGER" : output synchronization pulse for observation of output signal. TTL-compatible pulse triggered on negative edge of output with a pulse duration equal to that of the output pulse.

BNC socket "V/100" : 1/100 attenuated output (10 V max) provides convenient connection to oscilloscope for observation of pulse output voltage.

1.5 - VISUAL DISPLAY

Power on light **"POWER"**

Overload light **"OVERLOAD"** lights up if the high tension power supply can no longer deliver the energy required (depends on load and pulse repetition frequency).

"OUT" light flashes with each pulse output. Triggering is also indicated by a buzzer. This light will be off if the protection loop is open. **Note:** if the output current required is over the buzzer will sound even the limit (In this case, the pulse is not delivered).

"MODE" lights

"AUTO" : the instrument delivers pulses of programmed duration and frequency as indicated on the visual display

"EXT" : an output pulse is triggered from the "PEDAL" input

"PULSE" : an output pulse is triggered by pressing the "PULSE" button

"TIME" (μs x 10) displays the pulse duration in microseconds (X1 or X100)

"FREQUENCY" (Hz) : in AUTO mode, displays the pulse repetition rate.

1.6 - OPERATION

See figure 1, chapter 4 :

- check that "V OUT" (8) is adjusted to zero.

- check that the power cable is wired correctly.

- switch on power (1), the instrument will emit a beep, the "PULSE" light (20) will come on, and the "TIME" display (17) should indicate 0.5.

- select the pulse duration by pressing key T+ (9) until the required time is displayed (17), select range X1 or X100 (19) (μs on X1 and ms on X100). To reduce duration press key T- (10). The duration steps by 5 or 10 for each key press. The output from the "TRIGGER" output (3) can be used to check the pulse duration on an oscilloscope (optional).

- choose the operating mode by pressing key "SELECT" (15). The selected mode will be indicated by the corresponding light (20-22).

- in mode AUTO (22) the display "FREQUENCY" (18) will show 0.1 (one pulse every 10 s). This can be adjusted by pressing key F+ (11) to increase the frequency or F- (12) to reduce it.

- plug the special electrode connector into the high tension socket (7). Turn the outer ring to lock the connector in place. Do not tamper with this connector assembly as it is designed to protect the operator under all operating conditions.

- specifications (including the electrode separation, l) are supplied for each electrode assembly.

- select the value of the field E by choosing the output voltage U. This voltage is the product of the electrode separation l (cm) and the field E (V/cm) :

$$V = E.l$$

The signal at the "V/100" output (4) can be used to monitor the shape of the output pulse on an oscilloscope (optional). This is a 1/100 attenuation of the signal delivered to the electrodes (Fig. 4, chapter 4).

- in PULSE mode (20), an output pulse is triggered by pressing the "PULSE" key (16). This is indicated by a beep from the buzzer and a flash from "V OUT" (6). If the "OVERLOAD" light (5) comes on, the operating conditions should be adjusted (medium is probably too conductive; reduce ionic strength or sample volume).

- in AUTO mode (22), the pulse train is triggered by pressing the "START" key (13) and stopped by pressing the "STOP" key (14). Each pulse is signalled by a beep and a flash from "V OUT" (6). If the "OVERLOAD" light (5) comes on, the operating conditions need to be adjusted (see para above).

- in EXT mode (21), pulses are triggered from the "PEDAL" input (2) (e.g. by pressing a foot pedal) indicated by a beep and a flash from "V OUT" (6). If the "OVERLOAD" light (5) comes on, the operating conditions need to be adjusted (see above).

- after use, turn the "V OUT" control (8) to zero. Wait for one or two minutes before disconnecting the electrodes.

- switch off power (1). All display lights should go out.

CHAPTER 2

MOLECULAR BASIS OF ELECTROPERMEABILIZATION

A biological membrane becomes permeable if its transmembrane potential exceeds a certain threshold (200-300 mV in general). This state which is related to the formation of transient permeation structures (TPS) is reversible, provided that :

1. the duration of the electrical disturbance is short
2. the surface area is limited
3. their rate of production is low
4. the osmotic pressure is low
5. there is no inlet of toxic substance(s).

The formation of TPS can be accounted for by one or more of the following mechanisms

a) the potential induces electrocompression of the membrane which above a certain threshold leads to rupture, as in dielectric breakdown. This mechanism regards the membrane as a homogeneous solid. This process would punch holes in the membrane.

b) there are defects in the assembly of membrane lipoproteins. The electric potential increases their size. Beyond a certain critical value, these defects give rise to reversible TPS.

c) the transmembrane potential, like temperature, can induce a phase transition in the membrane. In this new state, the membrane becomes permeable by disorganization of the interface. This unstable state disappears progressively.

Application of electric fields to cell suspensions exploits this membrane property. As mentioned above, the electric field induces a transmembrane potential when it exceeds the permeation threshold (200-300 mV), TPS are thus formed in the membrane.

CHAPTER 3

OPERATION

The electrical characteristics of the Jouan electropulsator have been designed to maximize the efficiency of treatment while preserving cell viability. Square wave pulses provide a constant field during the discharge. The duration, intensity and repetition rate can be programmed for different conditions of ionic strength of the medium. This enables operating conditions to be optimized for different types of medium and cell system.

The electric field generated between two electrodes is defined by the relationship :

$$E = U/l$$

where E is the field strength (V/cm)
U is the voltage difference between the two electrodes (V)
l is the separation between the two electrodes.

The Jouan electropulsator employs two parallel plate electrodes which produce a uniform field throughout the cell sample.

Other electrode geometries can be employed. With two parallel cylindrical electrodes, the distance between the two electrodes is not constant. The electric field will not be uniform, and not all cells will be subjected to the same influences.

Concentric electrodes have also been used, but whereas the separation between the electrodes is constant, the current density will be non-uniform. This will lead to inhomogeneities in the electric field throughout the sample.

Another important feature of the Jouan electropulsator is the use of retractable electrodes. After electropermeabilization, the cells are in a fragile state and should be manipulated as little as possible. Pipetting, for instance, can subject the cells to strong hydrodynamic pressures.

Retractable electrodes can be used for both suspension and spread cells.

Special electrodes for bacteria are available.

3.1 - SPREAD CELLS

General principle :

With spread cells, the electrodes are placed in contact with the bottom of the culture dish. The space between the electrodes will be subjected to the electric field, while the rest of the culture can serve as a control. Given the vectorial nature of the electric field, it may be necessary to lift off the electrodes and turn the dish through 90° for a second treatment. Medium is readily changed by aspirating the old medium and pipetting on new medium. These operations can be carried out with an automatic pipette with sterile tips.

Procedure :

1 - Take the dish out of the incubator

2 - Remove the culture medium

3 - Add pulsing buffer

4 - Put the electrodes in contact with the bottom of the dish

5 - Apply pulses

6 - Lift up the electrodes

7 - Turn the dish through 90°

8 - Replace the electrodes in contact with the bottom of the dish

9 - Apply pulses

10 - Remove electrodes

11 - Remove pulsing buffer at the end of incubation period

12 - Add culture medium

NB : stages 6-9 can be left out for particularly fragile specimens

3.2 - CELL SUSPENSIONS

General principles :

Suspended cells must be transferred from their culture medium into a special medium required for electropermeabilization (pulsing buffer).

The following sequence of operations should be used to change the medium :

- gentle centrifugation
- remove supernatant
- add pulsing buffer
- gently disperse the cell pellet.

The electrodes are placed in contact with the bottom of the dish, and the cell suspension is poured in between the electrodes. The seal between the electrodes and the bottom of the dish maintains the cells between the electrodes. Pulses are applied and the electrodes are lifted out. After a period of incubation, the cell suspension is dispersed in a suitable volume of culture medium.

In some situations, it may be necessary to treat cells in disposable spectrophotometer cuvettes. After washing, the cells are placed in the cuvette, the electrodes are lowered to the bottom of the cuvette. Pulses are applied and the electrodes are removed.

Procedure :

I - IN CULTURE DISHES

1 - Wash the cells in pulsing buffer

2 - Place the electrodes in contact with the bottom of the dish

3 - Pour in the cell sample between the electrodes

4 - Apply pulses

5 - Remove the electrodes

6 - Add culture medium

NB : in stage 6, the volume of culture medium is much greater than the volume of pulsing buffer, and washing is not required. If washing is required at this stage, it should be carried out in disposable tubes.

II - IN DISPOSABLE SPECTROPHOTOMETER CUVETTES

1 - Wash the cells in pulsing buffer

2 - Place the cell suspension in the cuvette

3 - Put the electrodes into the cuvette

4 - Apply pulses

5 - Remove the electrodes

NB : since the cells are in suspension there is greater latitude in the treatment conditions. For example, cells can be washed if necessary, and pulsing buffer can be eliminated completely.

3.3 - FLOW SYSTEMS

General principles :

Large volumes of cells can be treated in a flow system. The cells are subjected to the electric field for the duration of their transit between the electrodes. Flows of over 30 ml per minute can be employed.

Procedure :

- After washing, the cells are placed in the reservoir
- The suspension is pumped through the pulsing chamber using a peristaltic pump
- Cells are subjected to the electric field as they pass between the electrodes
- The treated suspension is collected in a suitable container

For fragile cells such as mammalian cells or plant protoplasts, it is advisable to place the pump after the collecting container so that the cells are drawn rather than pushed through the system.

3.4 - CHOICE OF PARAMETERS

A cell suspension placed between two metal electrodes can be regarded as an electrical resistance. This is true provided that no electrochemical reaction produces an insulating species between the electrodes. This occurs with aluminium where electrolysis leads to the formation of an insulating oxide film. The use of noble metal electrodes in the Jouan electropulsator avoids these problems.

From an electrical point of view, the system can be considered as a simple electrical resistance R across a potential difference U (Fig. 3, chapter 4).

The potential difference U is only delivered over a time T (Fig. 2, chapter 4).

By Ohm's law :

$$U = I.R$$

where I is the current (amps) flowing through the sample of resistance R (ohms).

To protect the output stage, I is limited to a maximum value of 8 A, which gives a minimum value of

$$R = U/8$$

U on the other hand can range from 0 to 1000 V provided that the current does not exceed 8 A.

The resistance R is defined by the conductivity of the cell suspension λ , the separation of the electrodes l, and the sample volume V :

$$R = l^2/(V.\lambda)$$

λ depends on the ionic strength. High salt content media such as PBS will thus have a high conductivity.

The use of square wave pulses (Fig. 4, chapter 4) enables application of a controlled and defined field throughout the duration of the pulse. In less sophisticated capacitative discharge systems, the field tends to fall off during the pulse. The Jouan electropulsator can deliver a wide range of fields (0 to 6 KV/cm) over a wide range of pulse durations (5 μs to 24 ms) at a high repetition rate (up to 10 Hz). However, some precautions need to be taken, especially with respect to the possibility of overheating the sample (Joule effect). The electric charge also needs to be limited (Fig. 5, chapter 4).

The electrical energy W (joule) transformed into heat is :

$$W = U.T$$

where U is the applied voltage (V)
I is the current flowing through the sample (A)
T is the pulse duration (S)

or

$$W = E^2.T.V.\lambda$$

where λ is the conductivity of the sample (S cm-1)
l the separation between the electrodes (cm)
V is the volume of the sample (cm3)

This energy will tend to heat the sample by $\Delta\theta$:

$$W = J.V.\Delta\theta$$

where J is the heat capacity of the sample (close to that of water 4.18 J/°C.cm3)

Thus from

$\Delta\theta = (E^2.T.\lambda)/J$

the increase in temperature depends on the pulse duration, the electrical conductivity of the sample, and the square of the electric field, but not on the sample volume.

For example: electrical conductivity of PBS = $16.3.10^{-3}$ S.cm^{-1}

A sample placed at ambient temperature (20°C) will boil under the following conditions of field and pulse duration

E V/cm	200	400	600	800	1000
T ms	600	160	70	40	25
E V/cm	1200	1400	1600	1800	2000
T ms	17	13	10	8	6
E V/cm	2500	3000	3500	4000	4500
T ms	3	2	1.6	1.3	1
E v/cm	5000	6000			
T ms	0.8	0.57			

A heating problem only arises for pulse durations above 1 ms with media of low ionic strength.

For example :

* PBS/10 = $1.9.10^{-3}$ S.cm^{-1}

E V/cm	2000	3000	4000	5000	6000
T ms	51	17	11	7	5

5 mM Tris = 2.6.10-3 S.cm-1

	37	12	8	5	3.5

1 mM Tris = 0.56.10-3 S.cm-1

	174	58	37	23	16

0.56 M mannitol, 5 mM MOPS, 6 mM $MgCl_2$ = $1.3.10^{-3}$ S.cm^{-1}

	75	25	16	10	7

In summary, the parameters E and T (pulse duration) are restricted by two physical phenomena :

- the Joule effect which can cause the sample to boil

- a capacitative effect which loads the output of the pulse generator (Fig. 5, chapter 4)

The latter effect will distort the waveform of the pulse delivered to the sample causing the field to droop (cf. Figs. 4 and 5, chapter 4).

The Joule effect will tend to predominate for high fields which, depending on the conductance of the sample, should only be applied for short periods of time. Long durations of high voltage pulses cannot be applied to media of high ionic strength such as PBS. On the other hand, at lower field strengths, the load capacitance will effectively restrict the duration of pulse that can be used.

Knowledge of the electrical conductivity of the sample enables estimation of the electrical charge absorbed during the pulse, from :

$$Q = I.T$$

where I is the current and T the duration of the pulse.

The electronic overload protection limits current to 8 A.
From Ohm's law

$$U = I.R$$
$$I = U.V.\lambda / l^2$$

hence

$$Q = E.T.V.\lambda / l$$

the absorbed charge Q will depend on the area of liquid (A) in contact with the electrodes. This means that for a given electrode separation l, the absorbed charge Q will depend directly on the sample volume (A = V/l). In situations where the pulse waveform is only slightly distorted (Fig. 5, chapter 4), this can therefore be corrected by reduction of the sample volume.

3.5 - DETERMINATION OF OUTPUT CURRENT

As described above, the discharge of the electric field corresponds to the flow of a current through the sample. The intensity of this current will depend on :

- the potential difference between the electrodes

- the nature of the sample medium

The resistance of the sample is given by :

$$R = l^2/(V.\lambda)$$

λ = the conductivity of the medium
l = the distance between the electrodes
V = the sample volume

From Ohm's law

$$U = I.R$$

the current flowing through the medium when a field E (E = U/l) is applied will be :

$$I = (U.V.\lambda)/l^2$$

For electrodes where l = 0.4 cm and for a sample volume of 0.2 ml, the current will be a function of the medium and voltage as shown in the table below

U (V)	400	500	1000
E V/cm	1000	1250	2500
PBS	8	-	-
PBS/10	1	1.25	1.25
5 mM Tris	1.2	1.5	3
1 mM Tris	0.24	0.3	0.6
0.56 M mannitol, 5 mM MOPS, 6 mM $MgCl_2$	0.64	0.8	1.6

It should be noted that the overload protection limits output current to a maximum of 8 A. Thus certain conditions cannot be achieved (U > 400 V for PBS).

For the bacteria electrodes, l = 0.15 cm and for a volume of 36 μl, the following currents will be delivered :

U (V)	400	500	1000
E V/cm	2600	3300	6600
PBS	8	-	-
PBS/10	1	1.25	2.5
5 mM Tris	1.2	1.5	0.6
1 mM Tris	0.24	0.3	0.6
0.56 M mannitol, 5 mM MOPS, 6 mM $MgCl_2$	0.54	0.8	1.6

The overload protection limits the voltage to 400 V with PBS.

3.6 - PRACTICAL IMPLICATIONS

The ionic strength of the pulsing buffer must be carefully considered. The following guidelines may be helpful :

1. Measure the conductivity of the medium

2. Check the value against values given in tables

3. Test the system without cells

4. During tests, make sure not to boil the sample. It is often advisable to monitor the pulse waveform on an oscilloscope (optional) to make sure that it is a true square wave (cf. Fig. 4, chapter 4).

5. Cells should be carefully washed with pulsing buffer in order to remove all traces of culture medium. The culture medium has a high salt content and hence a high conductivity. This is particularly important for work on intact bacteria, where high field strengths are required for cell transformation.

6. Plasmids should preferably be suspended in distilled water. Use of TE medium will increase the ionic strength and should be avoided if possible.

In summary, the operating conditions are restricted by the overload protection which limits the output current to 8 A. From Ohm's law this will define a minimum resistance R for each value of output voltage U and a maximum value of conductivity (λ max) for each electrode assembly

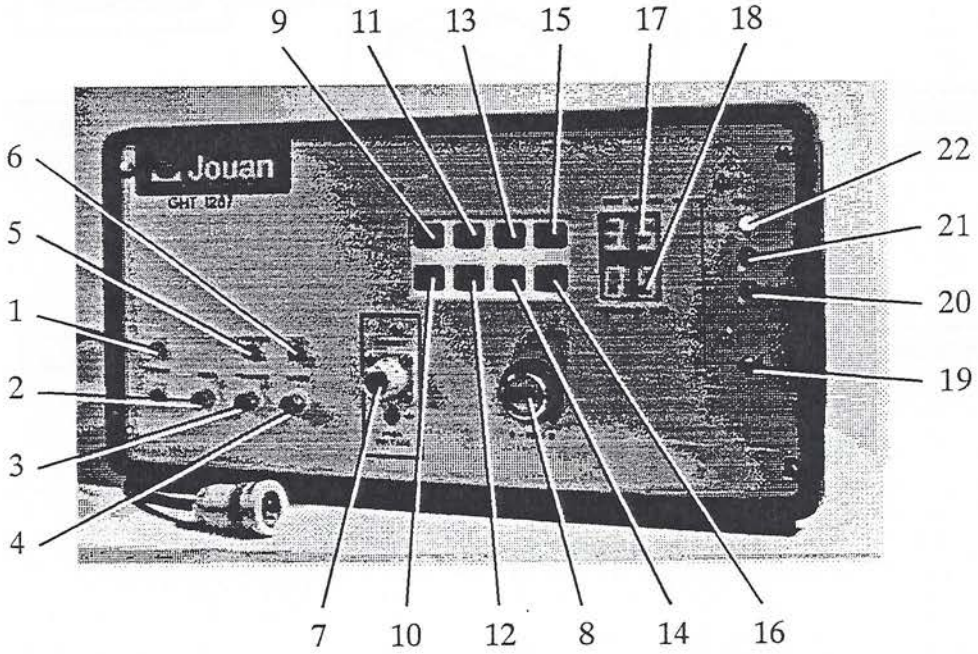
U (V)	200	400	600	800	1000
Rmin (Ω)	25	50	75	100	125
Bacteria electrodes ($\times 10^{-3}$)	22	11	7	5	4.5
Normal electrodes V = 100 μl ($\times 10^{-3}$)	64	32	22	16	12
V = 200 μl ($\times 10^{-3}$)	32	16	11	8	6
V = 500 μl ($\times 10^{-3}$)	13	6	4	3	2.5

The limiting operating conditions can thus be determined by measuring the conductance of the pulsing medium. This will avoid problems caused by overloading the output of the electropulsator.

CHAPTER 4

FIGURES

4.1 - FRONT PANEL



1 - Switch "Power"
2 - "Pedal" input
3 - "Trigger" output
4 - "V/100" output
5 - "Overload" light
6 - "V out" light
7 - Hight tension socket
8 - "V out" control
9 - Key "T+"
10 - Key "T-"
11 - Key "F+"
12 - Key "F-"
13 - Key "Start"
14 - Key "Stop"
15 - Key "Select"
16 - Key "Pulse"
17 - "Time" display
18 - "Frequency" display
19 - "x 1 - x 100" selector range
20 - "Pulse" mode
21 - "Ext" mode
22 - "Auto" mode

Figure 1

Front panel of the Jouan electropulsator

The functions marked by numbers are described in the technical reference manual

4.2 - BLOCK DIAGRAM

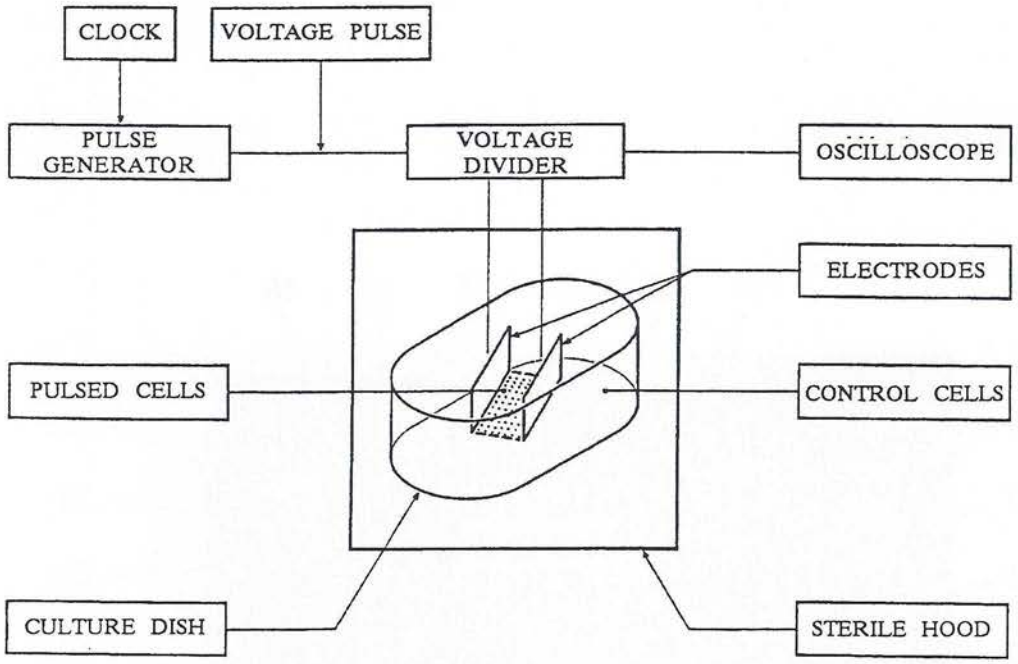


Figure 2

Block diagram of the Jouan electropulsator

4.3 - ELECTRICAL EQUIVALENT CIRCUIT

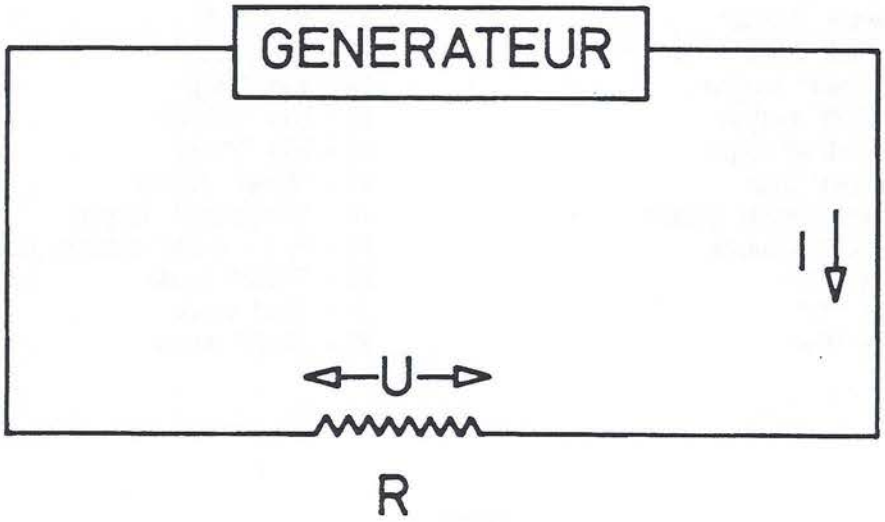


Figure 3

Electrical equivalent circuit of the Jouan electropulsator

The voltage U induces a current I in the sample of resistance R.

4.4 - SQUARE WAVE OUTPUT PULSE

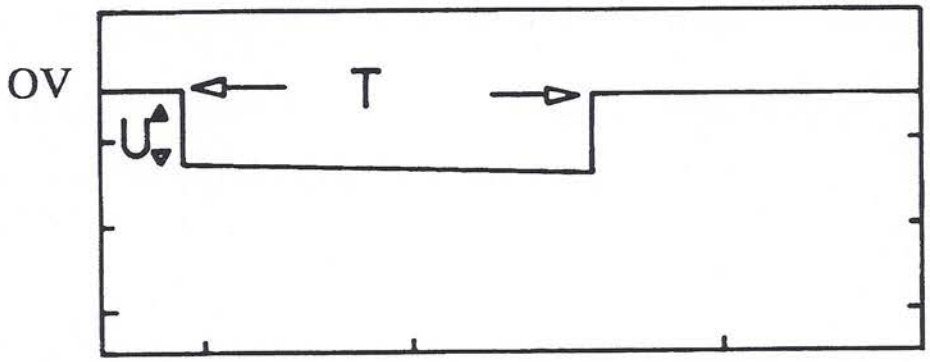


Figure 4

Square wave output pulse

The rising and falling edges of the pulse are vertical (to within 1 μs). The field remains constant over the duration of the pulse (programmable).
The operating conditions are compatible with the electrical performance of the pulse generator

4.5 - DEFORMATION OF WAVEFORM

(media with high ionic strength or large width pulse)

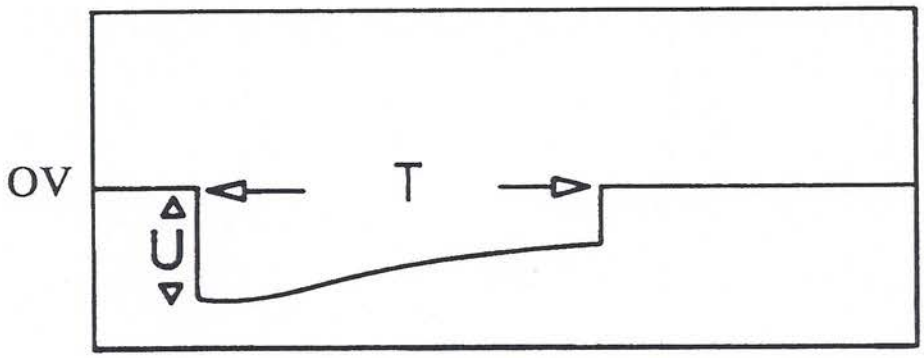


Figure 5

Deformation of waveform under extreme conditions

The intensity of the field falls during the pulse because the ionic strength of the medium is too high. The operating conditions are no longer accurately defined.

Jouan

APPAREILS DE LABORATOIRE

St-Herblain, June 3, 1994

SG/NC - 94.0603

Dr Damijan MIKLAVCIC
University of Ljubljana
Slovenia

Subject : Electropulsator

Dear Dr MIKLAVCIC,

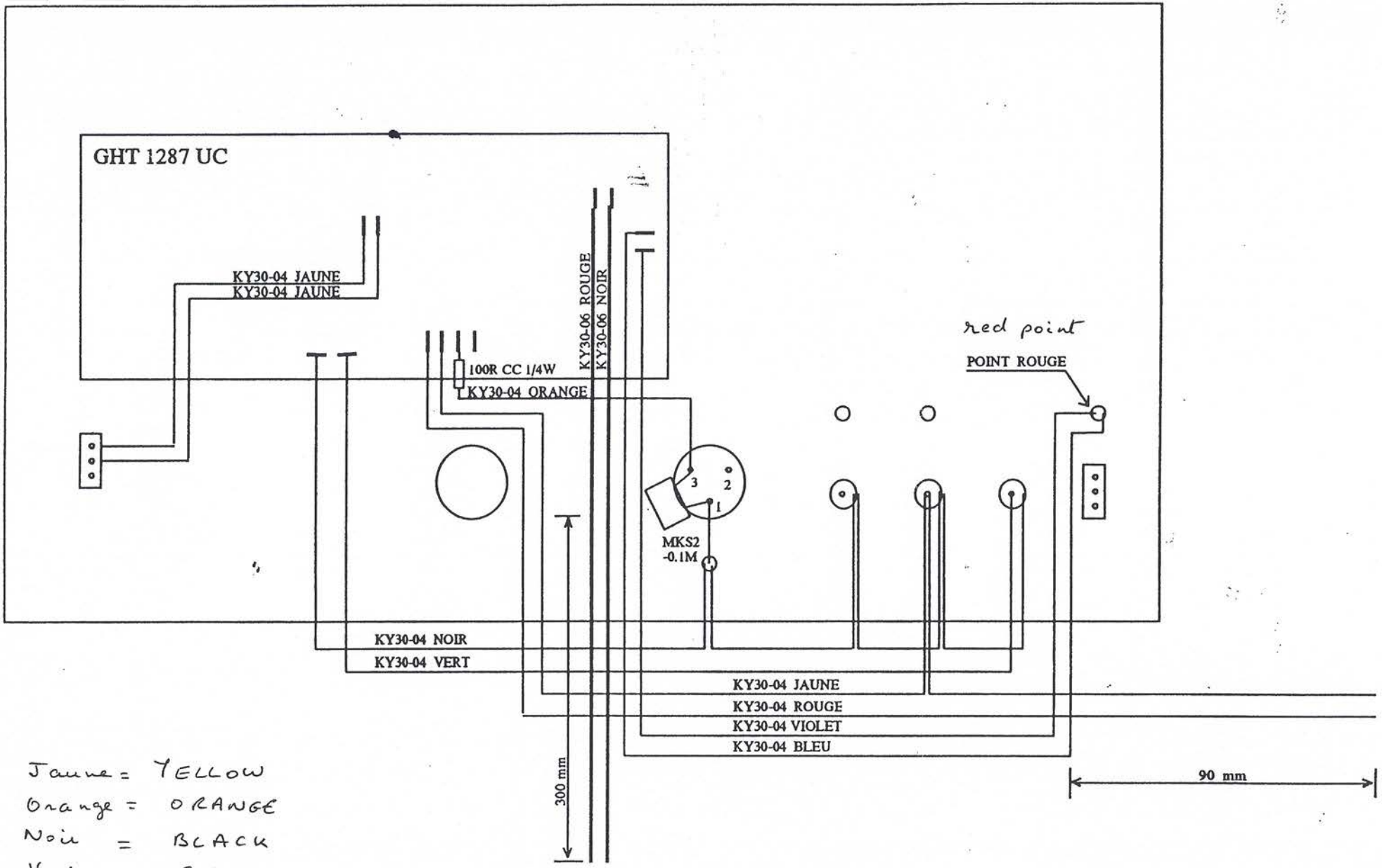
please find here enclosed the drawings concerning our PS15 electropulsator.

I hope this information will help you.

Yours sincerely,

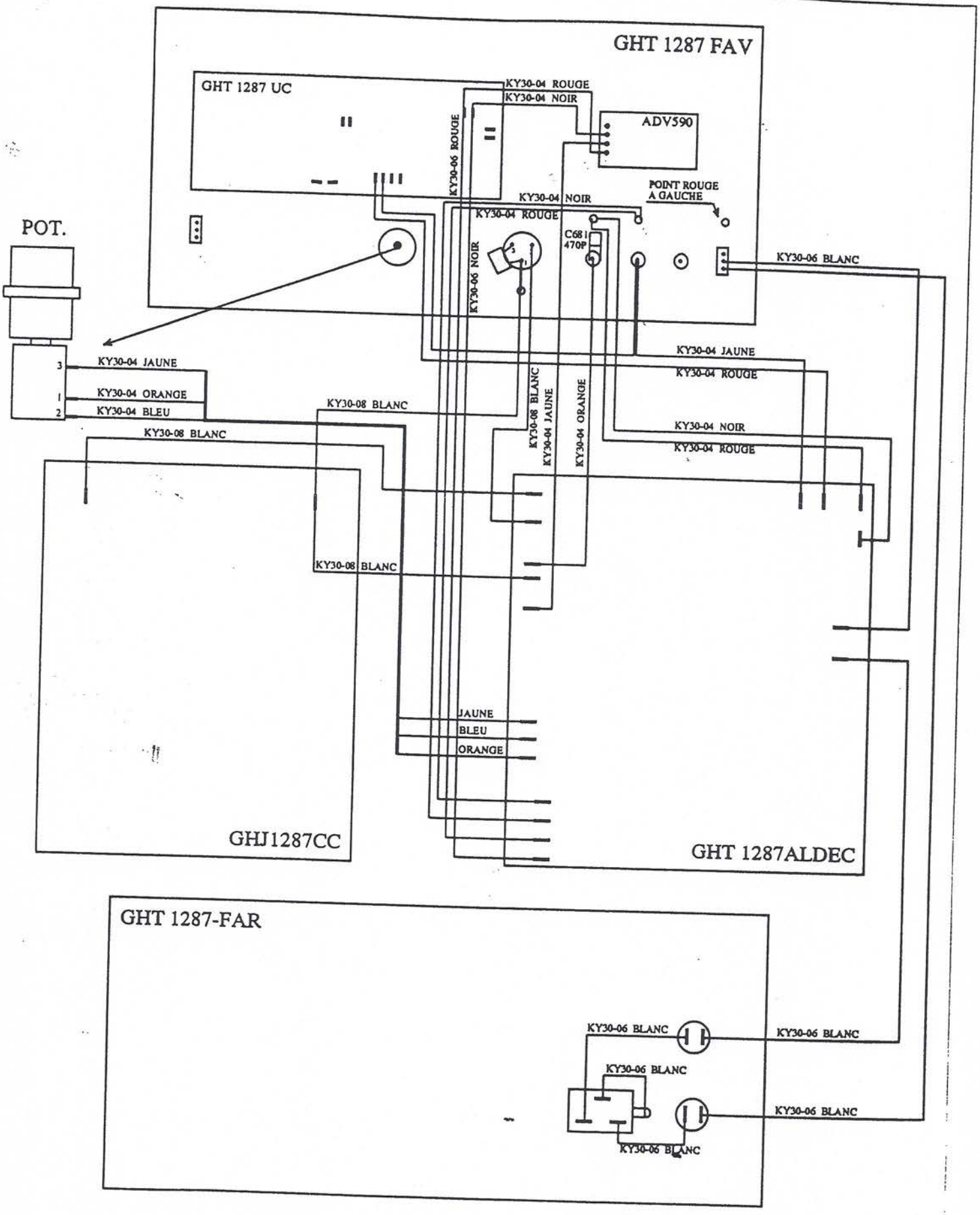
Sylvie GERARD
Product Manager

DIRECTION GÉNÉRALE RUE BOBBY-SANDS • CASE POSTALE 3203 • 44805 SAINT-HERBLAIN CEDEX • FRANCE • TÉLÉPHONE : 40 16 80 00 • TÉLEX : 711062 JOUAN NT • TÉLÉCOPIE : 40 94 70 16
USINE Z.I. DE BRAIS • B.P. 403 • 44611 SAINT-NAZAIRE • FRANCE • TÉLÉPHONE : 40 01 28 52 • TÉLEX : 700919 JOUAN ST-NAZ • TÉLÉCOPIE : 40 91 50 84
Société Anonyme au capial de 19 300 000 francs • Siège social : Z.I. de Brais - 44600 SAINT-NAZAIRE • R.C. Saint-Nazaire B 301127759 •
N° SIRET 30112775900019 - C.C.P. NANTES 3725-45 Z • APE 3404



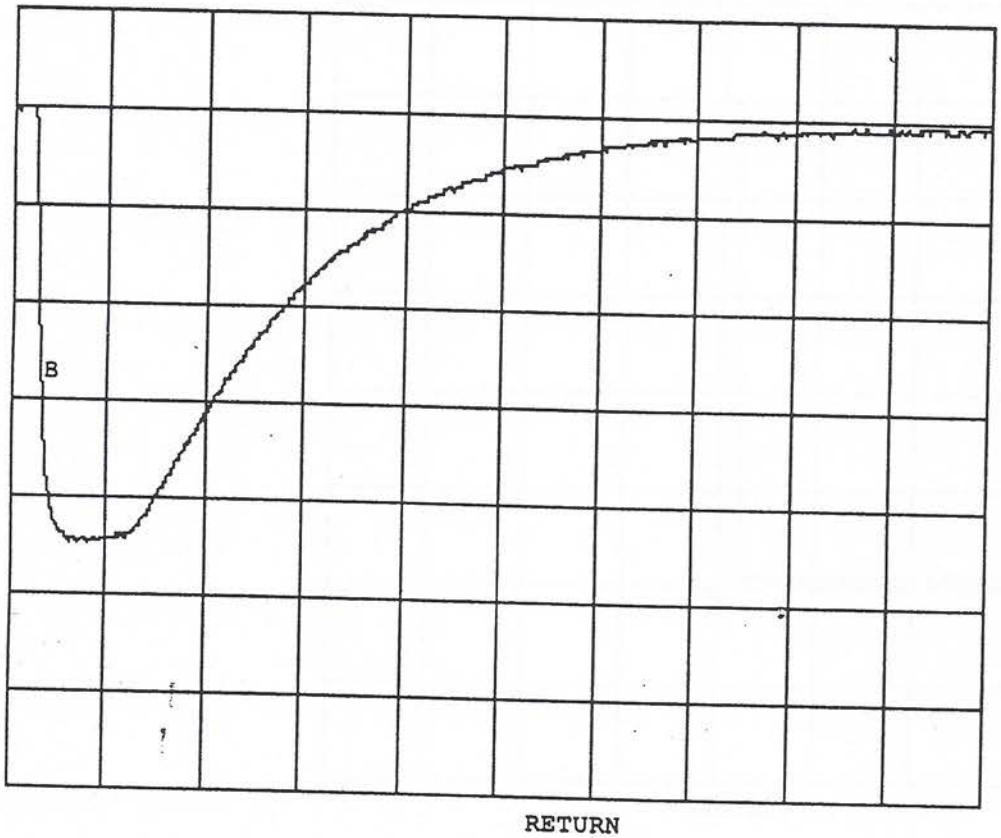
Jaune = YELLOW
Orange = ORANGE
Noir = BLACK
Vert = GREEN
Rouge = RED
Violet = PURPLE
Bleu = BLUE
Blanc = WHITE

JOUAN PS15


GHT 1287 FAV
GHT 1287 UC
KY30-04 ROUGE
KY30-04 NOIR
ADV590
KY30-06 ROUGE
POINT ROUGE
A GAUCHE
KY30-04 NOIR
KY30-04 ROUGE
POT.
C681
470P
KY30-06 NOIR
KY30-06 BLANC
KY30-04 JAUNE
KY30-04 ROUGE
3
KY30-04 JAUNE
1
KY30-04 ORANGE
2
KY30-04 BLEU
KY30-08 BLANC
KY30-08 BLANC
KY30-08 BLANC
KY30-04 NOIR
KY30-04 ROUGE
KY30-08 BLANC
KY30-04 JAUNE
KY30-04 ORANGE
JAUNE
BLEU
ORANGE
GHJ1287CC
GHT 1287ALDEC
GHT 1287-FAR
KY30-06 BLANC
KY30-06 BLANC
KY30-06 BLANC
KY30-06 BLANC
KY30-06 BLANC

JOUAW PS15

B= 1 V 10us

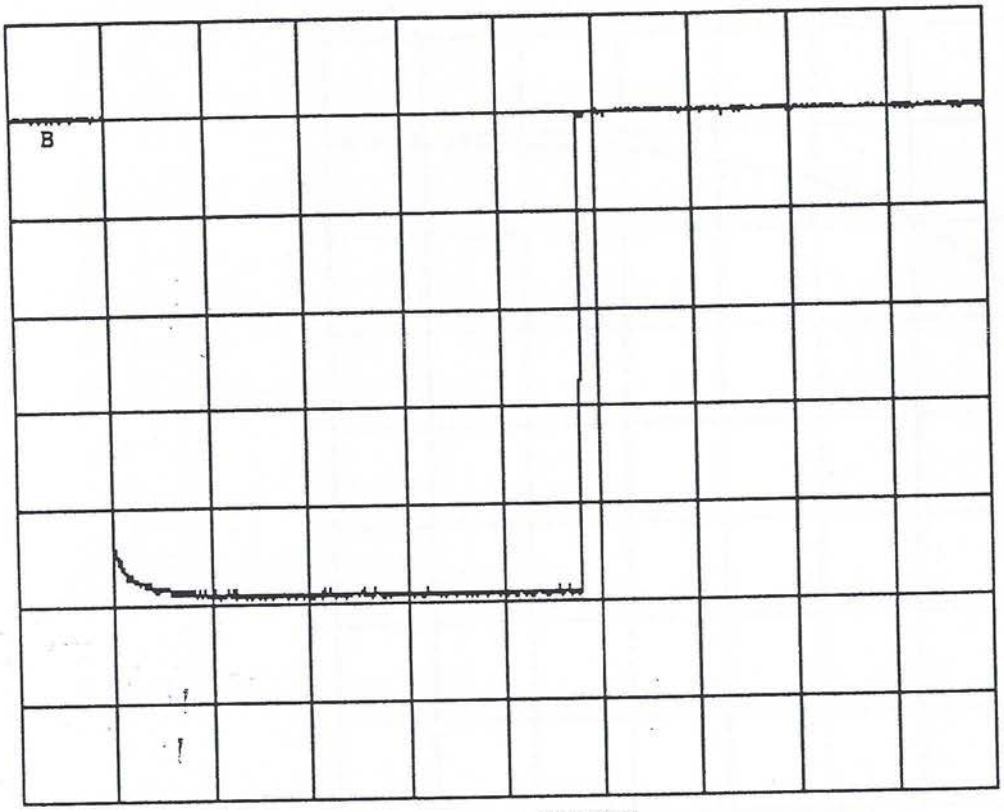


Charge infinie
"TIME" = 5 μS
"V OUT" = 500 V

Load = ∞

FIGURE 1

B= 1 V 5ms

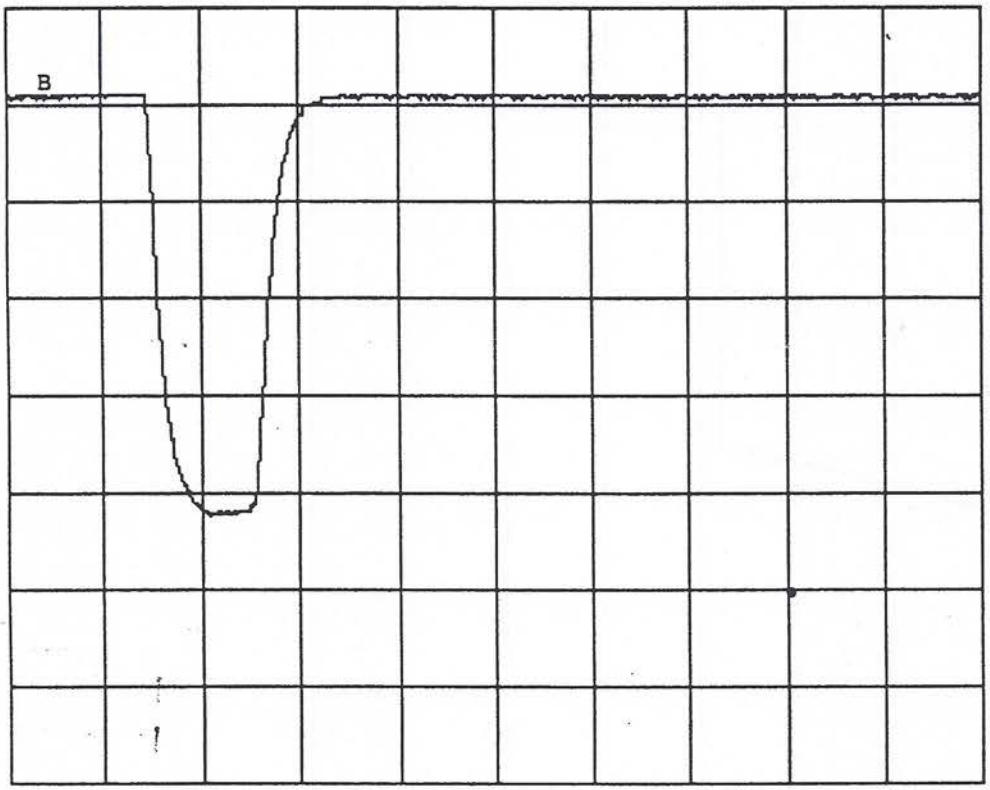


Charge infinie
"TIME" = 24 mS
"V OUT" = 500 V

Load = ∞

FIGURE 2

B= 1 V 5us

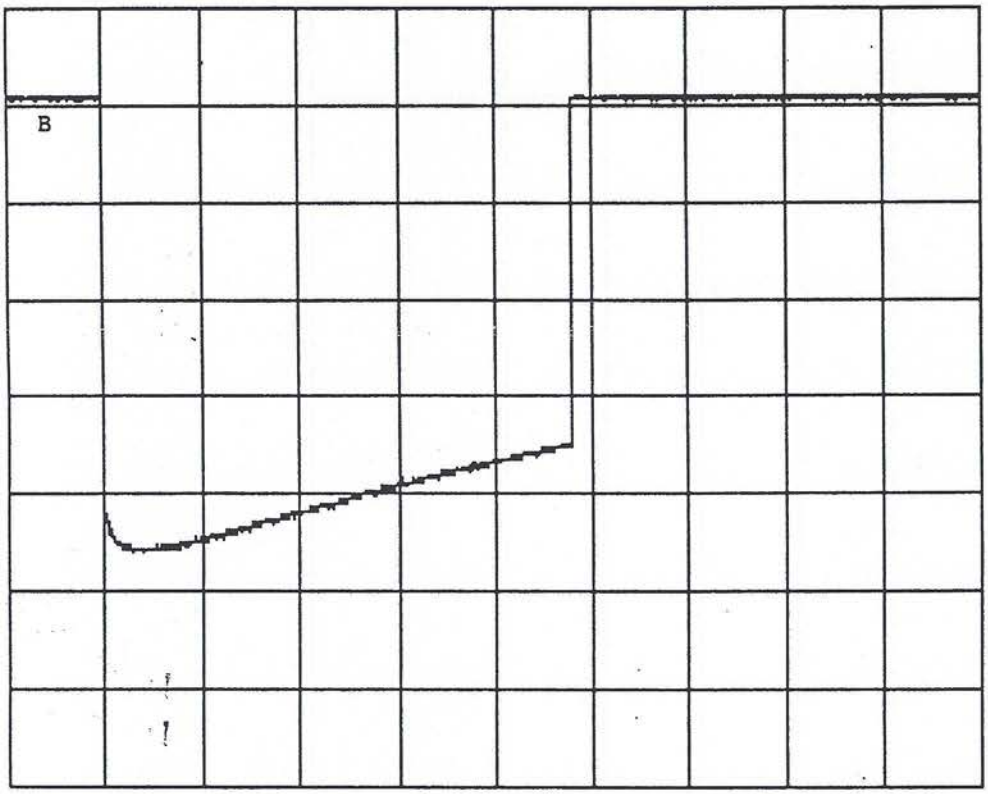


RETURN

Load
~~Charge~~ = 2 Kohms
"TIME" = 5 μS
"V OUT" = 500 V

FIGURE 3

B= 1 V 5ms

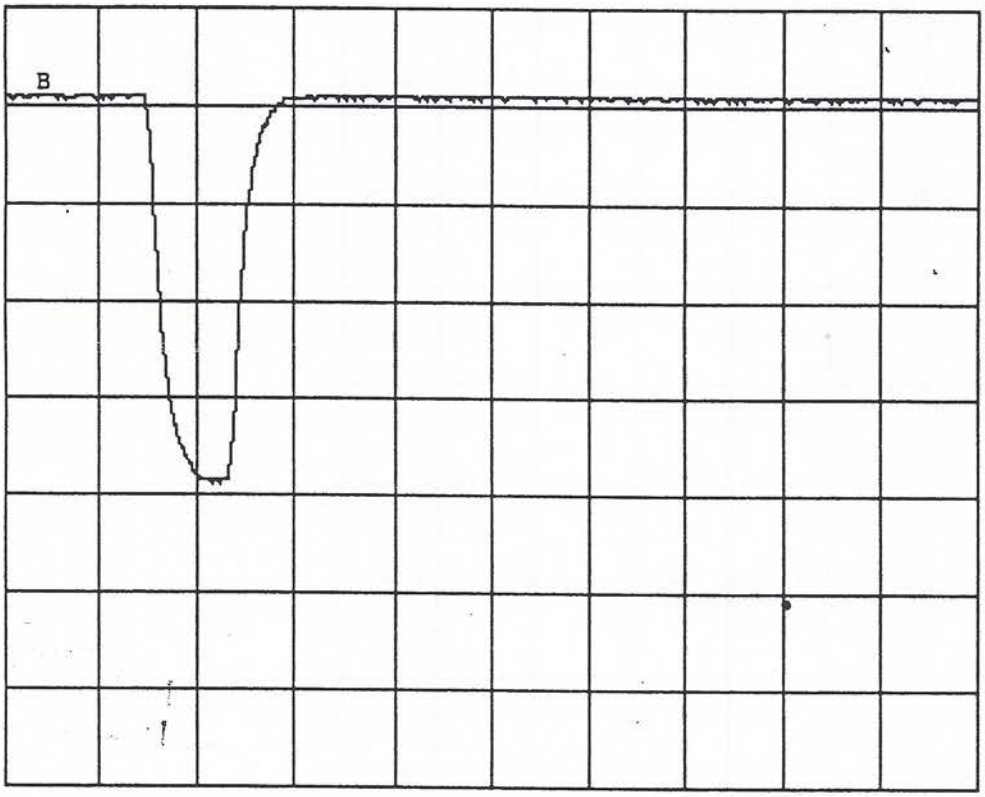


RETURN

Load
~~Charge~~ = 2 Kohms
"TIME" = 24 mS
"V OUT" = 500 V

FIGURE 4

B= 1 V 5us

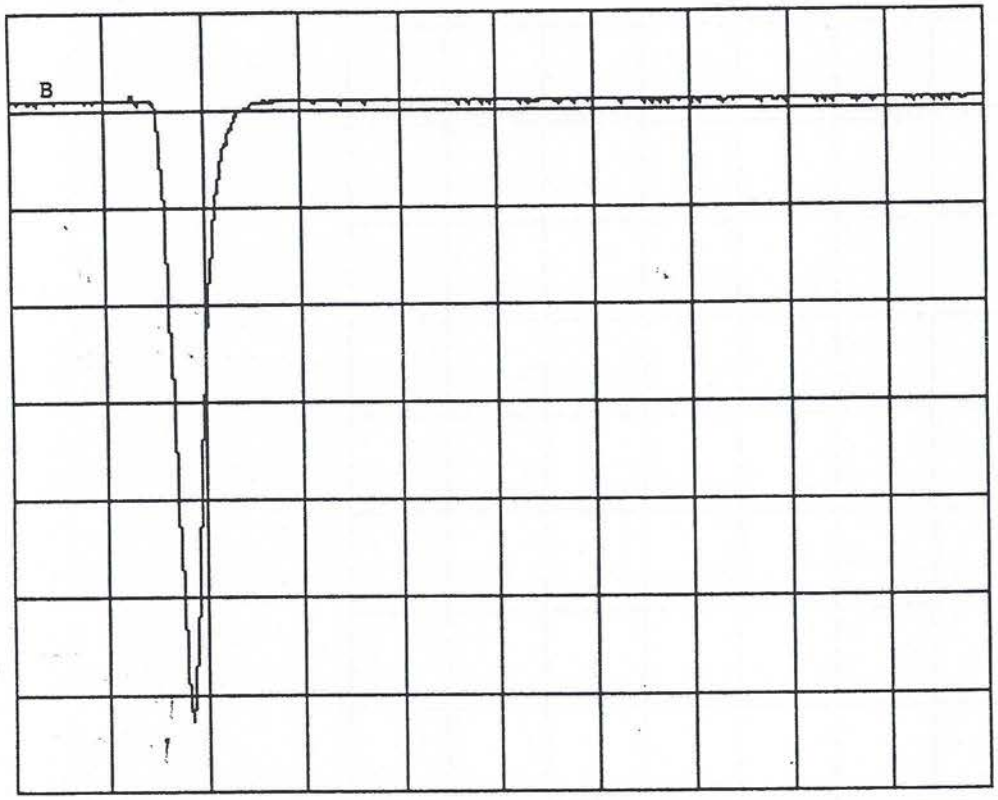


RETURN

Load
~~Charge~~ = 100 ohms
"TIME" = 5 μS
"V OUT" = 500 V

FIGURE 5

B= 1 V 5us

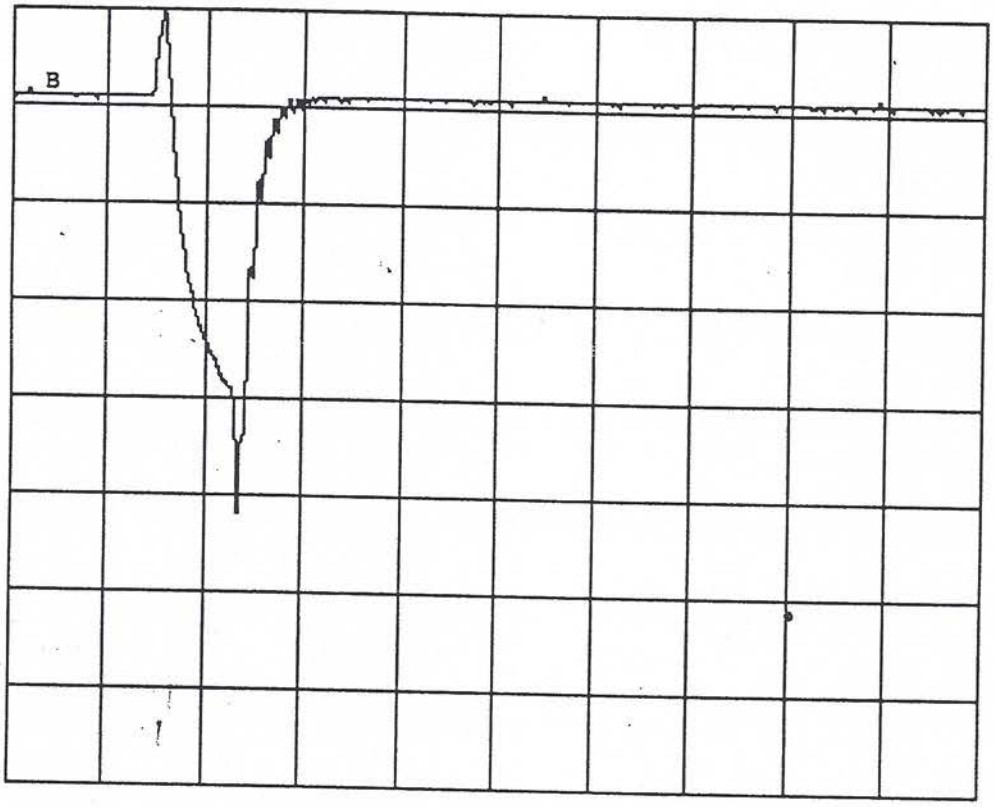


RETURN .

Load
~~Charge~~ = 100 ohms
"TIME" = 5 μS
"V OUT" = 1000 V
Limitation de courant

FIGURE 6

B=0.2 V 5us



RETURN

Load
Charge = 10 ohms
"TIME" = 5 μS
"V OUT" = 140 V

FIGURE 7