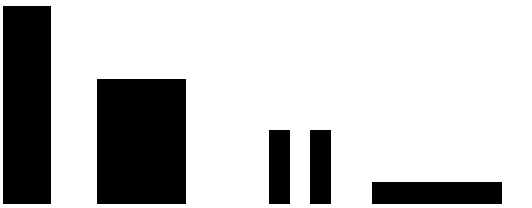


Cyto Pulse Sciences, Inc.™

Model PA-4000

Four Parameter P*ulseAgile* ®
Electroporation System

User Manual

Cyto Pulse Sciences, Inc.
P. O. Box 609
Columbia, MD 21045

1-410-787-1890
1-410-787-1891 FAX
www.cytopulse.com

Cyto Pulse Sciences, Inc. makes no warranty with respect to the product except for the warranty set forth in this Users Manual on page 7-1. **The LIMITED WARRANTY SET FORTH ON PAGE 7-1 IS EXCLUSIVE AND NO OTHER WARRANTY, WHETHER WRITTEN OR ORAL, IS EXPRESSED OR IMPLIED. Cyto Pulse Sciences, Inc. SPECIFICALLY DISCLAIMS IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE.**

License Agreement
PA-4000 Pulse Generator

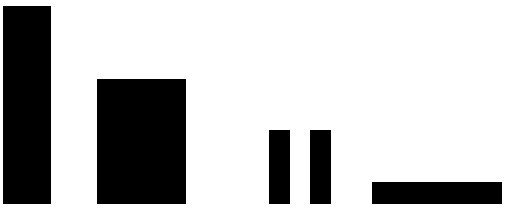
Cyto Pulse Sciences, Inc. (Licensor), conveys to the licensee, for fee paid, a nonexclusive, nontransferable license to use the ***PulseAgile***® hardware and software (equipment) for research purposes into perpetuity. Cyto Pulse Sciences, Inc., has patent allowance and patents pending covering the ***PulseAgile***® process. If the licensee wishes to use the hardware and software for production or commercial purposes, an additional license shall be required.

The equipment is not approved by the FDA for use *in vitro* or for *in vivo* diagnostics or therapy.

The information in this manual is subject to change without notice.

PA4000UMANrev.1-1/05 Price $100.00

FC CE



Cyto Pulse Sciences, Inc.™

Model PA-4000

Four Parameter P*ulseAgile* ®
Electroporation System

User Manual

Cyto Pulse Sciences, Inc.
P. O. Box 609
Columbia, MD 21045

1-410-787-1890
1-410-787-1891 FAX
www.cytopulse.com

Cyto Pulse Sciences, Inc. makes no warranty with respect to the product except for the warranty set forth in this Users Manual on page 7-1. **The LIMITED WARRANTY SET FORTH ON PAGE 7-1 IS EXCLUSIVE AND NO OTHER WARRANTY, WHETHER WRITTEN OR ORAL, IS EXPRESSED OR IMPLIED. Cyto Pulse Sciences, Inc. SPECIFICALLY DISCLAIMS IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE.**

License Agreement
PA-4000 Pulse Generator

Cyto Pulse Sciences, Inc. (Licensor), conveys to the licensee, for fee paid, a nonexclusive, nontransferable license to use the ***PulseAgile***® hardware and software (equipment) for research purposes into perpetuity. Cyto Pulse Sciences, Inc., has patent allowance and patents pending covering the ***PulseAgile***® process. If the licensee wishes to use the hardware and software for production or commercial purposes, an additional license shall be required.

The equipment is not approved by the FDA for use *in vitro* or for *in vivo* diagnostics or therapy.

The information in this manual is subject to change without notice.

PA4000UMANrev.1-1/05 Price $100.00

BlankPage

Table of Contents

List of Figures

Caution Notice

This instrument contains a high voltage power supply adjustable beyond 1,000 volts: such voltage can be lethal.

The user must read this manual carefully before the instrument is placed into operation.

Removing the cover may void the warranty.

Do not connect or disconnect the high voltage cable with the high voltage enabled. To connect or disconnect the cable, turn line power off and unplug line cord.

Do not open the cuvette holder while the high voltage is on. If a problem occurs during a run, push the **STOP/RESET** button on the front panel.

If there is any question about the operation of this instrument, call Cyto Pulse Customer service.

Blank Page

1. Introduction

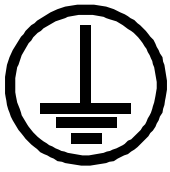
Electroporation (electropermeabilization) has many uses in the fields of cell biology, medicine and microbiology. New uses are being discovered at a rapid pace. In addition to the many *in vitro* uses for electroporation, new *in vivo* uses such as gene therapy and chemotherapy using electroporation also are being developed.

PulseAgile® technology gives research and medical scientists the tools needed for demanding new uses. ***PulseAgile***® electroporation was developed to give the operator maximum flexibility in protocol design and execution. Protocols can be optimized to give the best cell viability, the highest transfection efficiency, or the best electroporation efficiency.

This manual is designed to help the user get the most benefit from using ***PulseAgile***® electroporation. It contains information on how to operate the electroporator, safety tips, some important physical concepts, hints on how to adapt other protocols to ***PulseAgile***® and hints on developing your own protocols.

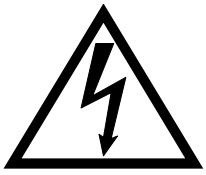
Note: The PA-4000 contains a high voltage power supply and was designed with safety features to protect both the user and the equipment. Used properly, the PA-4000 is a safe and reliable instrument. Chapter 3 explains some important concepts related to safe and accurate use of the instrument. Chapter 3 must be read and understood in order to properly set up this instrument. Our goal is the safe and productive use of the PA-4000. This product shall only be used in the manner specified by the manufacturer.

Back Panel Symbols

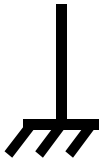


Protective Terminal Conductor

Caution: Refer to Documentation



Caution: Risk of Electric Shock



Chassis Ground

The PA-4000, including the optional PA-101, PA-201, PA-96W, or PA-301 is rated for operation with line/mains voltage of 100-240 VAC, maximum current of 2 amps, at 50-60 Hz. The AC mains power supply cord is the disconnect device for this product. The power supply cord shall be a Type SJT, rated 300 Volts AC, 18 AWG, 105° C, 3 conductor including ground.

This unit is rated for use at environmental conditions of 5-40° C, maximum relative humidity 80% for temperatures up to 31° C decreasing linearly to 50% relative humidity at 40° C, altitude to 2000 meters.

There are *no* operator replaceable parts inside the system; Cyto Pulse recommends that the user *not* remove the cabinet covers.

PA-4000 *PulseAgile*® Systems, Options, and Accessories

The PA-4000 ***PulseAgile***® electroporation system is the base of a number of advanced component systems available from Cyto Pulse Sciences.

All of the systems include:

- The ***PulseAgile***® interface software runs on a PC compatible desktop or laptop computer with the following minimum requirements: 200 Mhz Intel Pentium®, Windows® 95 , CD-ROM drive, at least one serial port.
- *Cuvette Holder and Cuvettes* - The cuvette holder is designed for maximum safety of the user and integrity of the sample. The cuvettes are available in 1mm, 2mm, 4mm gap spacing, and individually sterile-packaged.
- *Cyto Pulse System User Manual* - A User Manual is included for all systems and is written for use by life scientists. The manual details the use and safety considerations of the system

PA-4000S - Advanced *PulseAgile*® - Rectangular Wave Electroporation System (restricted license sale)

This system will run most published square wave protocols and offers the ***PulseAgile***® System parameter variations of pulse amplitude, width and interval, along with protocol file and data logging management. The system includes:

1	PA-4000	***PulseAgile***® - Advanced Rectangular Wave Electroporator
1	PA4-SW	***PulseAgile***® Application Software
1	PA-4UMAN	PA-4000 User Manual
1	CE-20	Standard Cuvette Holder
1	CUV-M	Cuvette Multi-pack (5 each 1mm, 2mm, 4mm)
1	CS-L-XX	IEC Line Cord, Serial Cable, XX = Country code

Optional Cyto Pulse Add-On Components

PA-96W - Programmable 96 Well Driver Option to PA-4000S (restricted license sale)

Provides the ability to electroporate cells in selected wells of a 96-well microplate. This add-on system includes:

1	PA-96W	Programmable 96-Well Driver
1	PA-96W-UMAN	PA-96W User Manual
1	96W-A	Electrode array for 96 Well Plate
1	96W-B	96 Well Base
1	96W-P	96 Well Polypropylene Plate, square well, flat bottom, 10 each
1	CPS-LCM-C	Low conductivity medium, 500 ml bottle
1	CS-OPT	Interface Cable Set, DB25 cable, HV cable

PA-101S - Dielectrophoresis Option to PA4000S (restricted license sale)

The sinewave AC-generator dielectrophoresis option for cell alignment and fusion. This add-on system includes:

1	PA-101	AC Generator
1	PA-101UMAN	PA-101 User Manual
1	FE-10	Fusion Plate Holder
1	FE-C25/400	Coaxial Electrodes, 2.5 mm gap, 350μL volume, set of 3
1	CPS-LCM-C	Low conductivity medium, 500 ml bottle
1	CS-OPT	Interface Cable Set, DB25 cable, HV cable

PA-201S - Programmable Pulse Switch Option to PA-4000S (restricted license sale)

A user-programmable switch that allows the PA-4000 to drive up to eight electrode elements independently. This add-on system includes:

1	PA-201	Programmable Pulse Switch, 8 Outputs
1	PA-201UMAN	PA-201User Manual
1	CS-OPT	Interface Cable Set, DB25 cable, HV cable
1	CS-201	Eight-Wire Cable with plug for electrode connections

PA-301S - Pulse Booster™ Option to PA-4000S (restricted license sale)

Capable of producing pulses up to 3000V, for higher electric-field applications. This add-on system includes:

1	PA-301	Pulse Booster™ 3:1 Step-Up Transformer
1	PA-301UMAN	PA-301User Manual
1	CS-OPT	Interface Cable Set, DB25 cable, HV cable

Optional Components Purchased Separately

DS-100	A Tektronix® digital oscilloscope with the CS-DS cable set and software
CS-DS	Oscilloscope Cable Set, Three RG-58 coaxial cables with 50-ohm terminations
PCL	Laptop computer with the ***PulseAgile***® interface software installed

Cuvettes, Electrodes, Plates and Holders

CUV-01	Standard Cuvette, 1 mm spacing, one time use
CUV-02	Standard Cuvette, 2 mm spacing, one time use
CUV-04	Standard Cuvette, 4 mm spacing, one time use
CUV-M	Multi-pack (five each of the above), one time use
CE-20	Cuvette Holder for standard cuvettes
CE-24	Cuvette Holder for reversing electric fields in a cuvette, for use with PA-201
TE-5-10	Tweezer Electrode, 5 mm x 10 mm pad
TE-5R	Tweezer Electrode, 5 mm round pad
NE-4-4	Parallel-Row Needle Array, 4 mm space, 4 needles/row
NE-4-6	Parallel-Row Needle Array, 4 mm space, 6 needles/row
NE-6-4	Parallel-Row Needle Array, 6 mm space, 4 needles/row
NE-6-6	Parallel-Row Needle Array, 6 mm space, 6 needles/row
FP-C25/400	Coaxial Fusion Electrode, 2.5 mm gap, 350μL volume
FP-C25/800	Coaxial Fusion Electrode, 2.5 mm gap, 750μL volume
FP-C20/1000	Coaxial Fusion Electrode, 2 mm gap, 1000μL volume
FE-10	Coaxial electrode holder, for use with PA-101
96W-A	Array Electrode for PA-96W
96W-P	Microplate
96W-PS	Microplate, Sterile (γ irradiated)

All of the products sold or licensed by Cyto Pulse Sciences, Inc. are for research use only and are not approved by the United Sates Food and Drug Administration for *in vitro* or *in vivo* diagnostics/therapy or any other purpose.

Blank Page

2. Tutorials

This chapter presents tutorials on electroporation and the various types of equipment used in the electroporation field.

2.1 Electroporation

Electroporation is the name for the use of a trans-membrane electric field pulse to induce an effective state of poration in a bio-membrane. The pores formed by this process are commonly called electropores. Their presence allows molecules, ions, and water to pass from one side of the membrane to the other. As Figure 2-1 shows, the electropores are located primarily on the surfaces of cells that are closest to the electrodes. If the electric field pulse has the proper parameters, then the ''electroporated'' cells can recover (the electropores reseal spontaneously) and cells will continue to grow and express their genetic material.

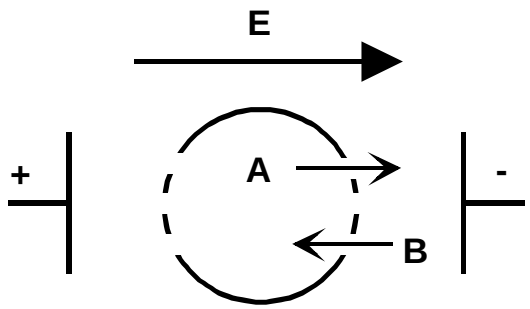


Figure 2-1: Electropores

The use of electroporation became very popular through the 1980s because it was found to be an exceptionally practical way to place drugs, genetic material (e.g., DNA), or other molecules into cells. In the late 1980s, scientists began to use electroporation protocols with multi-cellular tissue as well as cell suspensions.

Though cell-to-cell biological variability causes some cells to be more sensitive to electroporation than other cells, pore formation, number, and effective diameter is generally a function of the product of the pulse amplitude and the pulse duration (Figure 2-2). In order for pores to form, this product has to be above a *threshold*. In Figure 2-2 lines ''A'' and ''B'' identify thresholds where pore formation begins. Additionally, pore number and effective pore diameter increase with the product of pulse amplitude and pulse duration. Although other factors are involved, this threshold is now understood to be largely dependent on the reciprocal of cell size. If the upper limit threshold is reached (lines ''C'' and ''D''), pore diameter and total pore area become too large for the cell to repair by any spontaneous or biological process. Therefore the cell is irreversibly damaged. To prevent this damage, pulse protocols are empirically developed to be at some point above threshold and below lethality.

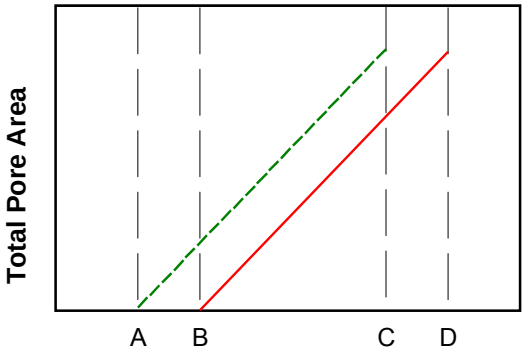


Figure 2-2: Pore Area

Since the mechanism of electroporation is not well understood, the development of protocols for a particular application to a previously uncharacterized cell or tissue have usually been achieved by empirically adjusting pulse parameters such as amplitude, duration, number, and inter-pulse interval.

Although early research on electropore-mediated transport across membranes assumed that simple thermal motion (i.e., diffusion) propelled molecules through electropores, research in the late 1980s and early 1990s began to reveal that movement of molecules through electropores depends on other experimental conditions and electrical pulse parameters in a way that indicates that other processes are involved. These reports show that certain experimental conditions and parameters of electrical pulses may be capable of causing many more molecules to move per unit time than simple diffusion. For example, referring to Figure 2-1, there is good evidence that molecular flow is in the direction of the arrow ''A'' (Dimitrov and Sowers, 1990). However, there is also good evidence that DNA movement is in the direction of the arrow ''B'' (Sukharev, et. al., 1992). This implies that electroporation has a polarity dependence. Although this apparent contradiction will have to be resolved by future basic research, it clearly shows that movement of molecules during electroporation is active rather than passive.

An additional important consideration is heat generation during electroporation. During the electroporation pulse, the electric field causes electrical current to flow through the cell suspension or tissue. Biologically relevant buffers for cells, culture medium and fluid in extra-cellular space in tissues contain ions at concentrations high enough to cause high electric currents to flow. These currents can lead to dramatic heating that is biologically unacceptable. This is explained in more detail in the tutorial on ''Equipment''. Principles of physics suggest that the early part of an exponentially decaying pulse does most of the membrane porating but the late part continues to heat the medium as well as molecular movement. One way to minimize heating is to use relatively high amplitude, short duration, rectangular wave pulse instead of an exponentially decaying pulse. If multiple pulses are used, second and subsequent pulses may be shortened to reduce the total energy input into the solution.

There are two main electroporation waveforms, exponentially decaying, and, rectangular wave. Different types of electronic equipment generate these waveforms.

2.2 Electroporation Equipment

2.2.1 Electric Fields in Aqueous Solutions and "Load"

The basic process of electroporation and electrofusion requires that cells be exposed to electric fields with special characteristics. In the most elementary form, the electric field can be viewed as a voltage applied to two rectangular plates with spacing between the plates (see D in Figure 2-3 below). The electric field is not dependent on the material between the plates.

As an example, to a first approximation, the applied electric field needed to impress a threshold voltage of one volt across a cell must be:

Paramecium	**180 μm**	**55 v/cm**
Mammalian Cell	**50 μm**	**200v/cm**
Red Blood Cell	**7 μm**	**1430 v/cm**
Bacterial Cell	**1 μm**	**10,000 v/cm**

More precise estimates of electric field requirements will involve the use of the so-called Schwann equation. For more information refer to Kinosita, etal., 1992.

In electroporation applications, a typical chamber will have an electrode spacing (D) that will range from 1 mm to 10 mm. Standard cuvettes are widely available in 1 mm, 2 mm, and 4 mm spacing. To obtain the required electric field intensities, high voltage pulse generators have adjustable pulse amplitudes from tens of volts to over 1000 volts. Figure 2.4 presents the electric field intensity for standard cuvettes and applied pulse voltages.

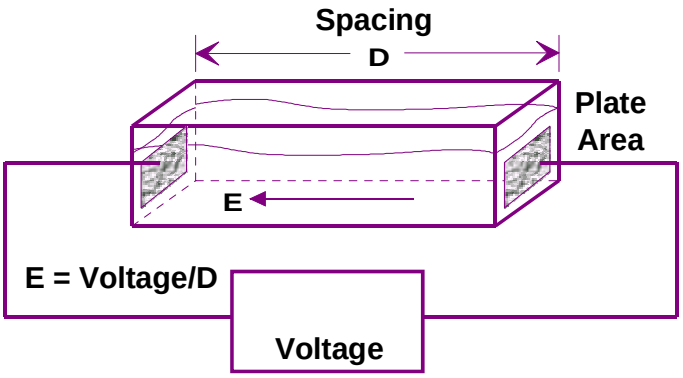


Figure 2-3: Electric Field in a Cuvette

The concept of resistance is also very important in this process. From basic physics, Ohm's Law states:

Current = voltage / resistance

Current is the quantity of electrons flowing per second. Resistance (Ω, omega), or "load," is the hindrance to that flow (measured in ohms) at the applied voltage. Current is similar to water flowing in a pipe. A smaller diameter pipe allows fewer water molecules per second to flow. In this case, water pressure is analogous to voltage.

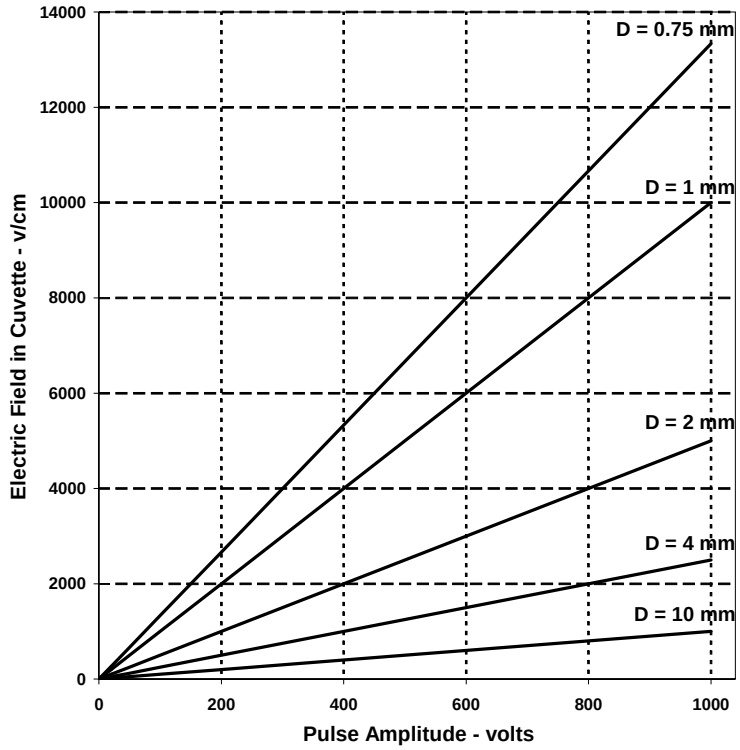


Figure 2-4: Electric Field vs. Cuvette Spacing

In biology, the solution in which the cells are contained will determine the sample's electrical resistance. Solutions such as phosphate-buffered saline (PBS) are very ionic and will conduct a large amount of current. Distilled water (DW) and solutions containing sucrose in distilled water are not ionic and will conduct a small amount of current. When discussing the conducting properties of material or solutions, a common parameter used is resistivity, represented by the Greek symbol, ρ (rho). This is given in ohm-cm and is related to resistance by the formula:

$$\text{resistance} = \rho * (D / A)$$

where:

ρ = resistivity, ohm-cm
D = plate spacing, cm
A = plate contact area, cm^2

Additionally, conductive properties are also described as conductivity. Conductivity is simply the reciprocal of resistivity:

Conductivity (σ) = $1/\rho$ in the units siemens/cm.

The reason for using resistivity or conductivity to describe the conducting properties of a material is that they are independent of electrode spacing and the electrode area in contact with the material. The resistance, however, *is* dependent on the physical dimensions. Standard cuvettes have fixed separation between plates, e.g., 1, 2, or 4 mm, and fixed electrode areas of 1 or 2 cm^2. The same-sized cuvette filled to different volumes results in samples with different resistances due to the different area of electrode contact. The table below shows resistivity and resistance data for standard cuvettes filled with phosphate-buffered saline (PBS) and distilled water (DW). Incidentally, DW is one of the most resistive (least conductive) solutions.

Table of Resistance (Load)

	ρ* Ω-cm	Resistance Ω		
Cuvette and Volume		**1 mm with 50 µl**	**2 mm with 200 µl**	**4 mm with 800 µl**
PBS[1]	**60 @ 25 °C**	**12**	**12**	**12**
Distilled Water[2]	**18x10^6**	**3.6x10^6**	**3.6x10^6**	**3.6x10^6**

1. Sigma PBS cat # D8662 2. Sigma water cat # W3500

* Resistivity is a strong function of temperature, value given at 25 °C.

If a 1000-volt pulse is applied to a cuvette with a 2 mm spacing and 200 µl PBS buffer, the current that will flow is:

1000 volts / 12 ohms = 83 amps

2.2.2 Exponential Decay (ED) Electroporators

The simplest approach to generating a high voltage pulse is to charge a capacitor (C) with a high voltage power supply, and then discharge the capacitor into the chamber containing the cells in the desired aqueous medium or buffer. The cells and the buffer represent the electrical "load" or resistance (R) for the high voltage pulse, see Figure 2.5. The charge switch is shown "closed" and the discharge switch is shown "open". When the sample is to be pulsed, these switch positions are reversed and the discharge switch remains closed until the capacitor is completely discharged. This capacitor is also called a reservoir capacitor. The number of electrons that the capacitor can store ("size") is measured in farads, and given the symbol F, which is the number of electrons per volt.

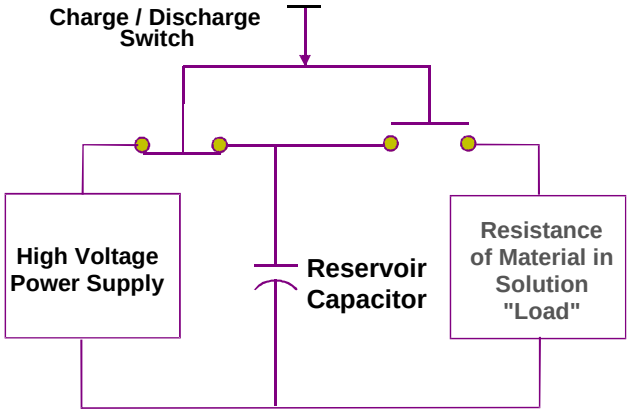


Figure 2-5: Exponential Decay Generator

The pulse width is dependent on the size of the capacitor and the resistance ("load") of the medium (solution or tissue). The pulse shape is a double exponential with a very fast rise time and a slow exponential decay fall time. The width at the 50% of amplitude point is given by:

Width (50%) = 0.7 x C (farads) x R (ohms)

For example, if an ED porator has a 500 μF reservoir capacitor and discharges into a 2 mm cuvette filled with 200 μl PBS (resistance of 12 ohms), the pulse width at the 50%-of-amplitude point is about 6 milliseconds. Below is a graph showing waveforms for a 50 μF, 500 μF, and 5000 μF reservoir capacitor and a 16 ohm Load Resistance. The waveform follows a standard exponential or "half-time" decay.

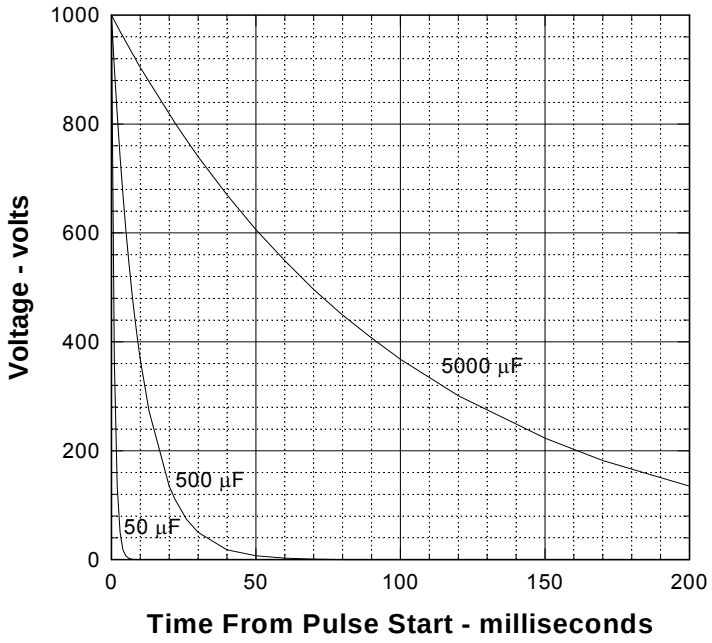


Figure 2-6: Pulse Amplitude vs. Time for an Exponential-Decay Waveform

The Exponential Decay pulser, although inexpensive, is a relatively crude device. As can be seen from the above example, the amplitude needed for electroporating is in the early portion of the pulse, but then the total area under the curve contributes to heating the sample. Additionally, the pulse width is dependent on the conductivity of the solution or tissue being porated and, without compensation; changes from one experiment to the next will cause the pulse width to change. Finally, since the capacitor is totally discharged in a single pulse, it must be totally recharged before it can be used again. This property will limit protocols where multiple pulses are required.

2.2.3 Rectangular Wave Electroporators

The next level of sophistication in generating pulses is achieved by using a high voltage solid-state switch that is turned on only for the desired pulse duration. This system still has a

reservoir capacitor, but it is only discharged by a few percent during a pulse. This approach permits the pulse width to be set to any value desired by the user. The pulse width delivered to the chamber is now independent of chamber resistance and the pulse amplitude remains relatively constant during the time the pulse is on. Together, these properties provide a more repeatable pulsed electric field. In addition, since the capacitor is only discharged a few percent, the voltage on the reservoir capacitor can rapidly be brought up to full value permitting multiple pulses with relatively short pulse intervals. The rectangular wave pulser eliminates many of the drawbacks of the ED pulser.

2.2.4 Cyto Pulse Sciences PA-4000 PulseAgile® Electroporator

The PA-4000 ***PulseAgile®*** electroporator provides the highest level of sophistication in the market today. It provides control of all pulse parameters with the ability to set pulse width, amplitude, time between pulses and the electric field direction. The PA-4000 system provides researchers with the tools to design and implement optimal electroporation protocols.

The rest of this manual is devoted to the description and use of ***PulseAgile®*** electroporation. The goal is to provide you with the ability to get the best use of this patented technology.

2.3 Using and Optimizing *PulseAgile®* Protocols

The simplest way to start using ***PulseAgile®*** protocols is to begin with published pulse parameters for the cell type with which you are working. Until ***PulseAgile®*** protocol optimization is done, standard published procedures and parameters can be used. The PA-4000 can readily deliver single pulses or pulse trains according to standard published specifications.

However, optimization may be desirable in certain circumstances when cells are difficult to replace or when high yield or viability is needed. ***PulseAgile®*** electroporation protocols give you the flexibility to achieve your goals.

Optimization of an electroporation protocol is an empirical process, but there are some principles that can be used to narrow the search for an ideal protocol. For instance, there are at least two (and probably more) mechanisms that have been proposed for movement of DNA into cells during transfection. They are:

- Electrophoresis[1,2]
- Electroosmosis[4]

Thermodiffusion and osmotic flow of medium have also been proposed as transport mechanisms, but there is little evidence that they play more than a minor role.[3]

For any of these mechanisms to work, the first pulse must permeate the cell. That means that the first pulse must be above the cell electroporation threshold. In ***PulseAgile®*** protocols, this pulse may have a shorter duration than published parameters because the first pulse does not have to do all of the work of inducing poration and transport simultaneously. Second and subsequent pulses are used to increase effective pore area and to assist in molecular transport. In general, the area of the cells that is permeable during electroporation is proportional to the strength of the applied electric field. The size of pores induced is roughly proportional to the width of the applied pulse.

According to one theory, pores are formed in cells by a rearrangement of phospholipids to a transiently stable pore shape. This rearrangement occurs normally in cells at a very low rate. The applied electric field serves to increase the probability of formation of transiently stable pores. There is an energy “hill” that pores must “climb” before rearranging from

transiently stable pores to normal bilipid layer cell membrane. Therefore pores close at a slower rate than they form. Thus for a brief time, up to seconds, a significant number of pores exist after the pulsed electric field is turned off. This is the time during which electrophoretic pulses can work to move charged molecules such as DNA into cells. For practical purposes, after about 3 seconds, the pores are closed to movement of large molecules into cells.

The optimization process should proceed iteratively, modifying one variable at a time. The following is a general outline for optimizing protocols.

2.3.1 Background

There are several components to ***PulseAgile***® protocols. It helps to breakdown the optimization process into parts to address the variables and avoid becoming overwhelmed by the number of possible combinations. An electroporation protocol can be broken down into three parts.

1. First pulse to begin initial pore formation
2. Follow-up high voltage pulses to yield further pore formation
3. Material moves into the cell

Other factors that influence the electroporation process are:

1. Cell viability factors
2. Brownian movement and vector considerations.

2.3.2 Initial Pore Formation

When an external electrical potential is applied to a cell, the cell membrane resists breakdown until a critical threshold voltage is achieved. As the voltage reaches the threshold, the cell membrane ceases to resist and a pore is formed in the cell membrane. The breakdown voltage is roughly one volt (0.2 to 2-volts) across the cell membrane. Mathematically, voltage at the cell membrane is defined as Vm = 1.5 rE cos B where r is the radius of the cell, E is the strength of the external field, B is the angle between the direction of the external field and the normal vector of the membrane at the specific site.

Since the breakdown voltage is approximately 1-volt, the critical voltage for a cell in volts/micron is E = 1/1.5 r, at the poles where *cos*B = 1. Multiplying this result by 10,000 gives the result in Volts/cm. For example, for a 40 micron diameter cell, the voltage needed to achieve critical voltage is 1/(1.5X20) = 0.033-volts/micron or 333-volts/cm. In practice, higher voltages are used since the above calculated voltage is only the *minimum* breakdown voltage.

The charge impressed upon a membrane during the application of a pulsed electric field creates a pressure across the cell membrane. This pressure is an altered energy state around the membrane and creates a condition where pores can form. Normally, the most stable state of least energy for a membrane is a continuous bilipid layer membrane. Another stable bilipid layer structure is an organized pore across the membrane. This is a slightly higher energy state than a flat bilipid layer. For a membrane to restructure from one of these stable states to another requires transitioning through a, less organized, higher energy state. According to one model of pore formation, pressure across a cell membrane created by charge redistribution reduces the transition energy and therefore makes it easier for pores to form. Once a pore forms, a path is created for electrical current to flow which relieves local pressure and maintains a favorable energy state for pores to remain open during application of the electric field.

Two practical conclusions derive from this model. One is that continued application of high electric fields can force continued enlargement of pore size. Thus, electric fields cannot be applied indefinitely. Another conclusion is that, although pore formation is fast (microseconds), pore closure is slower (milliseconds to seconds). In practice, pores are effectively closed by three seconds after application of pulsed electric fields even though some investigators have detected pores in cell membranes for more than 30 minutes.

Another factor to consider is that cells have a natural net charge across the cell membrane created by sodium pumps. It is around -70 mV in most cells. This charge is still present when an applied electric field re-distributes charges within the cell. The -70 mV them makes a negative charge that much larger and a positive charge that much smaller. This results in a different transmembrane voltage at each pole of the cell in line with the electric field. It is larger on the pole of the cell facing the negative electrode. The larger transmembrane voltage results in a larger area of the cell membrane having a voltage greater than threshold and therefore a larger area containing pores.

The movement of DNA is toward the positive electrode since DNA has a net negative charge. This means that it moves into the cell at the pole with the lower transmembrane voltage. In theory, pores could be induced in cell membranes with first pulses and the electric field reversed to move DNA into the cell on the side of greatest porosity. That process would require a PA-201 Programmable Pulse Switch option

2.3.3 Initial Pulse Width

The initial pulse width needs to be long enough to allow for pore formation and short enough to prevent excessive pore expansion or heat formation. A short period of time is needed for membranes to respond to the applied force. Minimum times are under one microsecond so this is not a practical limiting factor. Maximum pulse width is not a precise point and depends upon the cell viability desired. Over a limited range, increasing pulse width is equivalent to increasing pulse voltage. That is, effective electroporation is proportional to the area defined by voltage X pulse width. We suggest initial pulse widths in the range of 10 to 100 microseconds.

2.3.4 Follow-Up High Voltage Pulses Further Pore Formation

A follow-up pulse is defined for this manual as any pulse that 1) has a voltage above critical voltage, and 2) is applied after the first pulse. Little is known about what effect second and subsequent pulses have on the cell's pore size or number. Multiple pulses are reported to give better results than single pulses in many protocols. For practical purposes, follow-up pulses should be the same width or narrower than the first pulse.

2.3.5 Movement of Material into Cells

Two forces are known to affect transport of molecules into cells. One is electroosmosis. This force occurs as a result of charge differences between the cell membrane within the pore and water molecules adjacent to the charged membrane. The membrane is negatively charged. As a result, the layer of water immediately adjacent to the cell membrane is positively charged. This results in movement of water within the pore toward the negative electrode. Movement of water into the cell on one end and out of the cell on the other end pulls dissolved molecules in the direction of water transport.

The other known material transport force is electrophoresis. Negatively charged molecules such as DNA move toward the positive electrode (opposite to the direction of

electroosmosis). This force is linearly proportional to the voltage and time of voltage application. This means that the best transport by electrophoresis occurs in high voltage fields that are applied continuously. There are important factors such as heat production that limit the voltage and the duration of voltage application that can be applied to cell suspensions. Generally, the most practical and effective molecular movement derived from electrophoresis is obtained when lower voltages are applied in multiple, medium to long length pulses. One publication suggested that all effective movement due to electrophoresis occurs within 3 seconds of the original pulse. That time limit can serve as a guideline.

2.3.6 Cell Viability Factors - Heat

One important limit to the length of time that voltage (and the resultant current) can be applied to cells is heat production within the solution. Heat production is exponentially proportional to electrical current within the solution. After pulses are applied, there is some cooling within a solution due to a heat sink effect from the relatively large mass of metal in the electrodes. However, the cooling is not rapid enough to compensate for the rapid rise in temperature related to excessive electrical current during the application of pulses.

One method to compensate for heat production due to electrical current is to reduce the applied voltage and deliver wider pulses. While heat reduction is exponentially related to voltage reduction, the loss of movement by electrophoretic force is only linearly related. Movement due to electrophoresis is accomplished by electrical charge.

For example, a reduction of the voltage by half, coupled with a simultaneous doubling of pulse width results in the same movement of material by charge. The heat produced under the same condition is halved. In practice, multiple, wide, low voltage pulses are used to induce transport of material by electrophoresis after pores are formed by shorter, high voltage pulses. See section 3.1.5 to calculate temperature increase in an electroporation cuvette.

Another way to reduce heating is to use Cyto Pulse low conductivity medium.

2.3.7 Cell Viability Factors - Excess Voltage

Pulse voltages much beyond breakdown threshold result in formation of pores too large to spontaneously repair. As a result, cells lyse or die from loss of cytoplasmic content. In a cell suspension composed of uniform diameter cells, reducing the voltage readily solves the problem of extreme cell death due to excess voltage. In most cell suspensions, the diameter of individual cells does vary and there is a distribution of cell sizes. Because of this, some cell death is inevitable. The larger cells will be killed as the optimal voltage for average cells is applied. Conventionally, maximum poration has been observed using pulses where about half of the cells are killed. This is because traditional protocols use the same pulse conditions for material transport as those to initially form the pores. ***PulseAgile***® allows separation of desired effects with resultant increases in efficiency and less cell death. For example, in K562 cells, we have achieved 40% transfection with less than 10% cell death using ***PulseAgile***® protocols.

2.3.8 Other Cell-Associated Factors

Other cell specific factors add to variability in electroporation efficiency. Cell cytoskeletal structure is an example. Increased density of cell cytoskeleton at the site of pore formation can make the cell more resistant to detrimental effects of excessive pore expansion. Roughness of the cell due to cell processes or villi is another example.

2.3.9 Solution Temperature, Pore Closing Times

The temperature of the cell membrane (or medium) influences pore life-span. Cell membrane pores remain open for seconds to minutes at room temperature. Higher temperatures accelerate pore closure. Alternatively, at 4 ^{o}C, cell membranes are viscous and inflexible and pore closure is slower. Pore induction or formation is similarly affected by temperature variations. It is more difficult to induce pores in cold cell membranes. For maximum pore life, cells would be electroporated at 27-37 ^{o}C and brought rapidly to 4 ^{o}C. These methods of prolonging pore life are rarely practical.

2.3.10 Addition of Reagents

Electroporation efficiency is much higher if the molecules that you want to introduce into cells (DNA, proteins, and small molecules) are in the cell suspension before application of pulses rather than after. Even though electropores are theoretically open for seconds to minutes, close association of DNA with cells at the time of electroporation is essential.

2.4 Method Development

Many combinations of pulse parameters are possible using ***PulseAgile***® electroporation. Also, there are several ways to arrive at an optimal combination of electroporation parameters. The following is one suggested methodology.

1. Choose a starting point, goals, medium and reporter molecules.
2. Optimize initial pore formation.
3. Optimize follow-up pulses
4. Optimize molecular transport.
5. Repeat steps 2, 3, 4, if necessary and optimize other parameters, if desired.

2.4.1 Choosing a Starting Point

First, choose goals for the electroporation procedure. The following questions may help:

- What molecules are you trying to get into the cell?
- What are the characteristics of the molecules (size, charge in solution, etc.)?
- What type of cell are you using?
- What are the cell's characteristics?
- What is the cell size?
- Do the cells have cell walls?
- Are there any substances in the proposed medium that are toxic to the cells?
- Is cell viability important?
- Is electroporation efficiency important?
- Are single clones to be selected from the cells?
- Are cells to be part of a library?
- Are cells to be used in bulk without cloning?

- Will this protocol be used repeatedly or will this be a one-time use?
- What other factors are important?

Using this list, you should be able to choose the desired result. For instance, if the desired goal is generating a clone of cells from a group of cells transfected with the same plasmid, the percent of viable cells need not be high. If the goal is genetic engineering of rare primary cells, cell viability is very important. From this evaluation, you should be able to answer important questions regarding your electroporation goals.

2.4.2 Electroporation Medium

Choice of the electroporation medium involves compromise. Voltage drop during the pulse and heat generation are easily controlled when using high resistance, low ionic medium. The use of Cyto Pulse low conductivity medium is recommended for this purpose.

2.4.3 Reporter Molecules

Electroporation protocol development is much easier if a reporter molecule is available to readily assess the status of electroporation efficiency. Some available materials are:

DNA (with appropriate promoters)

lac-Z (B-galactosidase)
green fluorescent protein
Chloramphenicol acetyltransferase
Luciferase
antibiotic resistance

Non-DNA

FITC labeled dextrans
Calcein
propidium iodide
trypan blue

The choice of reporter molecule is based upon 1) the similarity in composition and size of the reporter molecule to the molecule of interest, and 2) the ability to assay for the reporter molecule. For example, it is a simple matter to screen for antibiotic resistance in bacteria that have been transfected with a plasmid containing an antibiotic resistance gene. Similarly, if a fluorescent microscope or a flow cytometer is available, the green fluorescent protein gene under the control of a constitutive mammalian expression promoter makes an ideal reporter gene. The fluorescent labeled dextrans are available in several molecular weights. Proteins can be directly labeled with fluorescein. Note that it is much harder to detect fluorescein labeled dextrans or proteins than it is to detect gene products because of the amplification inherent in DNA expression.

2.4.4 Cell Viability

In addition to choosing a method for measuring yield, a method for measuring cell viability is needed. Methods include

1. colony formation (colony count) before and after electroporation
2. trypan blue dye uptake (hours after the electroporation)
3. simple cell counts on tissue culture plates the day after electroporation
4. vital dye uptake of cells attached to a plate 24 hours after electroporation followed by an absorption reading of eluted dye
5. Alimar blue or other metabolic dyes
6. flow cytometric analysis, or other fluorometric analyses, of Calcein AM dye uptake
7. tritiated thymidine uptake.

There are many more methods, although the gold standard is colony formation. Note that vital dyes will penetrate permeabilized cells for some time after electroporation and cells that take up the dye may not be dead.

2.4.5 Electrical Parameters

There are at least two methods for choosing initial pulse parameters for electroporation protocols. They are:

1. Adapting to existing protocols and optimizing from this starting point.
2. Using cell diameter as a starting point

2.4.5.1 Published Protocols.

If you have a protocol that you have developed or a protocol that others have published, start with those protocol values. It is more complicated to adapt an exponential wave protocol to ***PulseAgile***® in comparison to rectangular wave protocols.

The adaptation of exponential decay protocols is as follows: The first pulse is of the same voltage as the peak exponential voltage with a pulse width of 10 to 100 microseconds. This pulse will be the pore-forming pulse. The second pulse is half the voltage and twice as wide. The third pulse is half again the voltage and twice as long as the second pulse. A fourth pulse may be optionally be added with half again the voltage and twice again the pulse width.

2.4.5.2 Cell Diameter

If published protocols are not available for your cell type, values for a similar cell type can be used or a starting voltage can be calculated using the average cell radius (in microns) of the cells in suspension. The formula described below can be used to calculate a starting point. Often, multiples of the threshold voltage are used.

Threshold in volts/cm, $\mathbf{E} \cong {}^{10{,}000}\!/_{1.5r}$, where r is the cell radius.

2.4.6 Optimize the First Pulse

There are many combinations possible using ***PulseAgile***® technology and there are several ways to arrive at the optimal combination. The following is one way.

Start with an evaluation of the effect of first-pulse electric field on cell viability. Pick a range of electric fields to work with around the chosen starting electric field. Generally, twice the threshold voltage is a reasonable starting voltage. A range of the starting voltage ± 33-50% should be sufficient. Divide the range into equal parts of 25-50 volts/cm and test the effect of each electric field on viability. Pulse widths of 10 to 100 microseconds are a good starting point.

It may be important to start with higher initial cell viability than needed to compensate for changes made to the protocol during optimization. Further optimization by changing the pulse width and number of pulses can be done at this time but it is a good idea to wait until follow-up lower voltage, pulses have been optimized.

As soon as more than one pulse is added to the protocol, either as initial pulses, follow-up pulses, or material transport pulses, a pulse interval needs to be chosen. A good initial interval is 125 milliseconds. Note that in rectangular wave or in ***PulseAgile***® protocols, pulse intervals are usually in milliseconds and pulse widths are usually in microseconds.

2.4.7 Optimize Multiple High Voltage Pulses

More than one high voltage pulse may be needed. Often 2 to 6 pulses are optimum. These pulses can be of the same voltage as the first pulse or lower than the first pulse but still above threshold voltage. It is most efficient to optimize follow-up pulses using a factorial analysis design, varying pulse voltage and pulse number simultaneously.

2.4.8 Optimize Molecular Transport

Molecular transport pulses are designed to move charged molecules into cells after pores have been induced. The electric field of the material transport pulses is lower than the first pulses. Values at or below threshold are used.

2.4.9 Further Optimization of Molecular Transport Pulses

All further optimization should focus on yield and cell viability simultaneously. It is important to monitor both yield and cell viability in order to identify positive or negative trends in electroporation efficiency. Choose a range of voltages to be tested. Values of one half, one fourth, one eighth and one sixteenth of the voltage of the first pulse are reasonable starting values. Choose a range of pulse widths to be tested for each voltage. Start with a range of 200 microseconds to 2 milliseconds. Begin the optimization process using multiple pulses since multiple pulses will often be used in the final protocol. Four pulses is a good starting point. Keeping the number of pulses and pulse widths fixed, test the effect of changing voltage within the range of voltages. Then test the range of pulse widths against the optimal voltage. Repeat this process until an optimum is found. Again, employing a factorial analysis by modifying electric field, pulse width and pulse number simultaneously may save time. The reason for the increased efficiency is that with a factorial design, interactions can be assessed and experimental variability is measured over the entire assay rather than just repetitions at individual independent variables.

Figure 2-7 shows that molecular uptake of calcein (a fluorescent tracer molecule) is enhanced with ***PulseAgile***® protocols compared to a single pulse protocol. The single pulse protocol was applied at 3.3 kV/cm for 50 μs. The ***PulseAgile***® protocols included a single pulse (3.3 kV/cm, 50 μs) followed 0.125 seconds later by 10 pulses of 1 ms and 0.4 kV/cm with interval of either (■) 0.125 s or (▒) 20 sec. DU 145 prostate cancer cells were used at 2 X 10^6 cells/ml, in a 2 mm gap cuvette, and 10 μM calcein. Molecular transport and cell viability were calculated using calibrated flow cytometry with propidium iodide as the viability stain. Figure 2.8 shows that cell viability was shown to not decrease with the ***PulseAgile***® protocols.

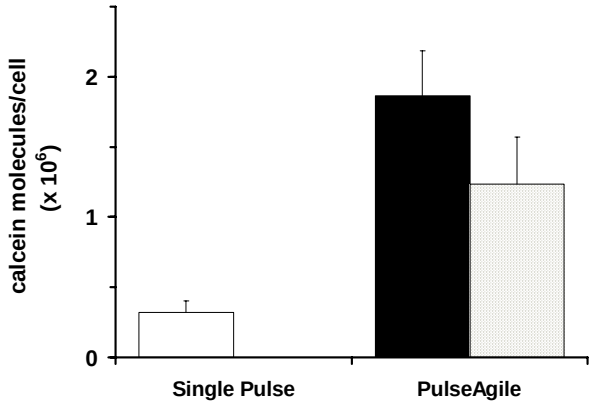


Figure 2-7: Uptake Calcein

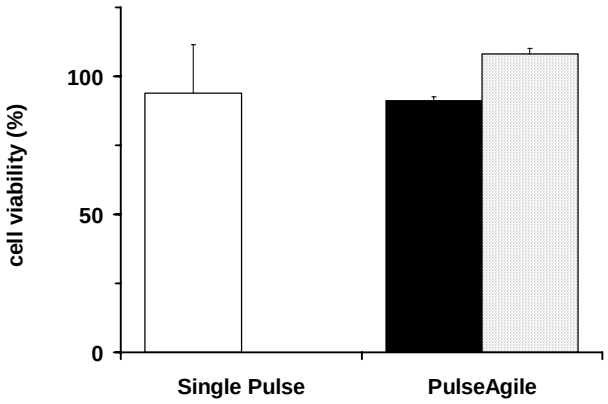


Figure 2-8: Cell Viability

Mark Prausnitz, Ph.D., Georgia Institute of Technology provided the data for these graphs.

2.5 References

1. **Sukharev S.I., Klenchin V.A., Serov S.M., Chernomordik L.V. and Chizmadzhev Y.A.**, Electroporation, and electrophoretic DNA transfer into cells: The effect of DNA interaction with electropores, 1992, Biophys J. 63; pp. 1320-1327

2. **Klenchin V.A., Sukharev S.M., Chernomordik L.V., Chizmadzhev Y.A.**, Electricaly induced DNA uptake by cells is a fast process involving DNA electrophoresis, 1991, Biophys J. 60; pp. 804-811

3. **Antonov P.A., Maximora V.A., Pancheva, R.P.** Heat shock and osmotically independent steps by DNA uptake after Escherichia coli electroporation. Biochim. Biophys Acta 1993 1216(2); pp. 286-288

4. **Sowers, A.E.** Mechanisms of electroporation and electrofusion in Guide to Electroporation and Electrofusion Editors Chang, Chassy Saunders and Sowers 1992 Academic Press; pp.119-138

5. **Nickoloff, Jac A., ed**. (1995) Electroporation Protocols for Microorganisms, Methods in Molecular Biology, Volume 47, (Humana Press, Totowa, New Jersey); p. 372.

6. **Nickoloff, Jac A., ed**. (1995) Animal Cell Electroporation and Electrofusion Protocols, Methods in Molecular Biology, Volume 48. (Humana Press, Totowa, New Jersey); p. 369.

7. **Sowers, A.E**. (1995) Permeabiliy alteration by transmembrane electric fields: electroporation, IN: Permeability and Stability of Lipid Bilayers (E. A. Disalvo and S.A. Simon, eds.), CRC Press, Boca Raton; pp. 105-121.

8. **Chang, D.C., Chassy, B.M., Saunders, J.A. and Sowers, A.E., eds**. (1992) Guide to Electroporation and Electrofusion, (Academic press, San Diego); p. 581

9. **Dimitrov, D.S., and Sowers, A.E.,** (1990) Membrane electroporation - fast molecular exchange by electroosmosis. Biochimica et Biophysica Acta 1022; pp. 381-392.

10. **Neuman, E., Sowers, A.E., and Jordan, C.A.., eds**. (1989) Electroporation and Electrofusion in Cell Biology, (Plenum Press, New York) pp. 581.

Blank Page

3. Operational Concepts

This chapter describes some important concepts with respect to the proper and safe use of the PA-4000 electroporation system.

3.1 Important Concepts

There are seven important concepts with which the user needs to be familiar in order to be able to properly use and interpret the readings provided by the PA-4000 electroporator.

- Load Resistance and Conductance
- Power Supply Voltage Monitor
- Relationship between Power Supply Voltage and Pulse Amplitude
- Changing Pulse Amplitude from pulse to pulse
- Pre-Pulse Load Estimator
- Pulse Droop
- Aqueous Solution Heating

3.1.1 Load Resistance and Conductance

The tutorials in Chapter 2 explained that applying a voltage across a cuvette produces an electric field. As a result of this electric field, current (electrons) will flow through the material contained between the plates of the cuvette. The material is said to be presenting an electrical *load* on the system and will resist the flow of current to one extent or another. From physics, Ohms Law says this resistance is related to voltage and current by:

$$resistance = \frac{voltage}{current\ (amperes)} = ohms$$

If the material is very ionic, such as Phosphate Buffered Saline (PBS), it will be very conductive, i.e., it will have a low resistance. If the material is tissue, it will be less conductive than the PBS, i.e., it will have a higher resistance and less current will flow. Inversely, conductance is given by:

$$conductanc\ e = \frac{1}{ohms} = siemens$$

The PA-4000 will estimate resistance/conductance and present both in the log report generated after each protocol run. The number printed in the log is the estimate before the protocol is run. Since the resistance of ionic solutions such as PBS is very sensitive to temperature, the resistance will change (decrease) after each pulse due to heating. The resistance estimate circuit operates between 10 ohms and 100 ohms. Below 10 ohms the system will display a message "*Output Shorted*" and the high voltage will not be enabled. Above 100 ohms the reading will be *>100 ohms*.

An example log report is given in Figure 3-1. Shown in the log is a PulseAgile protocol in which each succeeding pulse is twice the width and half the amplitude. The resistance and conductance estimates made before the protocol was run are shown at the bottom of the log.

```
Protocol File: EXP-HI.PRO
12-3-2004-6 18:21:25
Mode: Electroporation

>GRP   NUM     WIDTH     INTVL     SetV      MonV
> 1      1      0.020     1.000     1000      995

>GRP   NUM     WIDTH     INTVL     SetV      MonV
> 2      1      0.040     1.000     500       510

>GRP   NUM     WIDTH     INTVL     SetV      MonV
> 3      1      0.080     1.000     250       255

>>GRP  NUM     WIDTH     INTVL     SetV      MonV
> 4      1      0.0160    1.000     125       130

>Estimated load =  19 ohms
>Estimated conductance =   0.053 siemens
$
```

Figure3-1: Log Report Example

3.1.2 Power Supply Voltage Setting and Voltage Monitor

There are two different power supply voltage numbers that appear in the log report shown in Figure 3-1. The first is the voltage set by the user using the ***PulseAgile***® interface software, and is shown in the column labeled *SetV*. This voltage setting is converted to digital words that the internal microprocessor uses to program the output voltage of the power supply. The conversion is done in discrete levels, or counts, depending on the voltage range in use:

High Range	5 to 1100 volts	5 volts resolution
Low Range	4 to 400 volts	2 volts resolution

There are errors in these conversions and the final power supply voltage will be set to within ±5% or ± two counts, whichever is greater.

The second power supply voltage number is the value measured by an internal voltage monitor circuit at the internal reservoir capacitor. This measurement is also converted into digital counts and is presented in the log report in the column labeled *MonV*. Therefore, the user can see the intended Power Supply Voltage, *SetV*; and the measured *actual* Power Supply Voltage, *MonV*. The measurements of the actual voltage and the digitizing processes used also have errors that are on the order of ±5% or ± two counts, whichever is greater.

3.1.3 Relationship Between Power Supply Voltage and Pulse Amplitude

When the user sets a power supply voltage in the ***PulseAgile***® Interface software, that voltage will *not* be the voltage of the pulse that will appear across the material being treated (cuvette, tissue, etc.). The actual pulse amplitude can be estimated if the value of the “load” (aqueous solution or tissue) resistance is known. The circuit diagram in Figure 3-2 gives the reason for the difference.

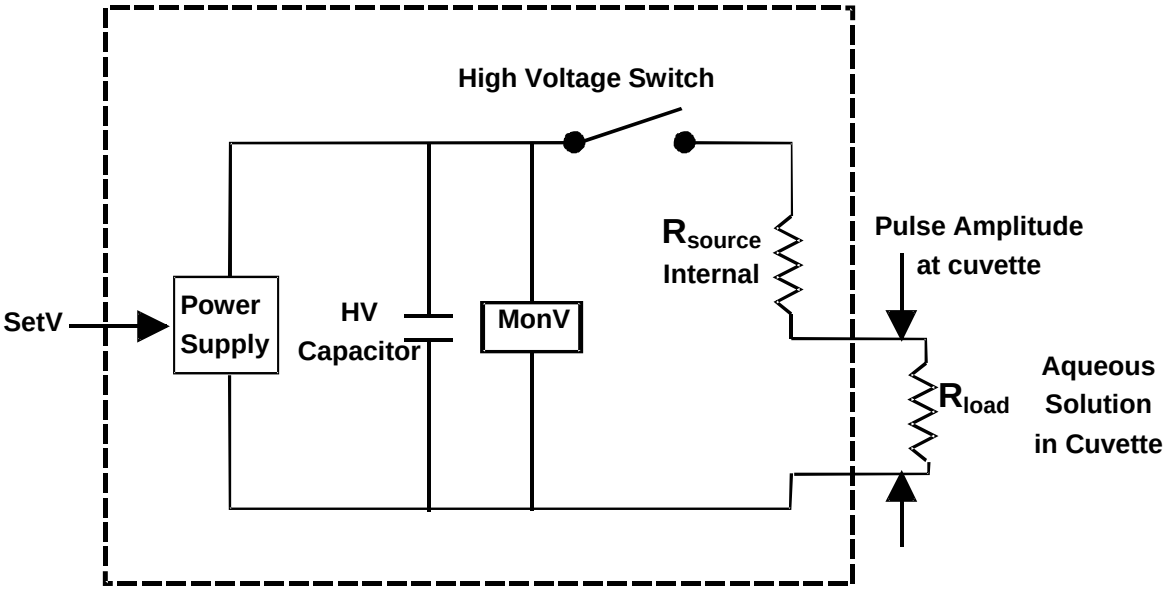


Figure 3-2: Power Supply Voltage and Pulse Amplitude Relationship

As shown, when the switch is closed, the full Power Supply voltage actually appears across two resistances, that inside the box and that outside the box (i.e., aqueous solution in cuvette or tissue). The resistance inside the box, called source resistance R_{source}, is the inherent resistance in the high voltage switch and an additional resistance included to prevent excessive current from flowing if the output is inadvertently shorted. The magnitude of the source resistance is usually a few ohms. Again, from Ohms Law:

$$Total\ Current = \frac{Power\ Supply\ voltage}{R_{source} + R_{load}} = Amps$$

From this relationship, the voltage that appears across the load is always less than the power supply voltage. If the power supply is set (SetV) to 400 volts, the high voltage capacitor is charged to 400 volts. If R_{source} is 2 ohms and R_{load} is 18 ohms, then the total current flowing from the high voltage capacitor through both resistances is 20 Amps.

The Pulse Amplitude is given by:

$$Pulse\ Amplitude\ (volts) = Total\ Current * R_{load}$$

In the example above, the pulse amplitude is 380 volts, or 95% of SetV. Another derived equation to calculate pulse amplitude is:

$$Pulse\ Amplitude = Power\ Supply\ Voltage\ (SetV) = \frac{R_{load}}{R_{load} + R_{source}}$$

Thus the voltage is divided between the source resistance and load resistance. As the load resistance goes to zero so does the pulse amplitude voltage. Figure 3-3 shows the typical power supply voltage vs. pulse amplitude relationship as a function of R_{load}. If the load resistance is larger than 100 ohms than the difference between the power supply voltage and pulse amplitude voltage is less than 2%.

Figure 3-3: Typical Relationship between Power Supply and Pulse Amplitude

3.1.4 Changing Pulse Amplitude from Pulse-to-Pulse

One of the features of the PA-4000 is the ability to change the pulse amplitude up or down from one pulse to the next. This ability is one element of ***PulseAgile***® electroporation.

It is important to note that
there are limits to how fast a voltage change
from pulse-to-pulse may be made.

3.1.4.1 Decreasing Voltage from One Pulse to the Next

To produce a pulse of one voltage followed by one of a **lower** voltage, the reservoir capacitor voltage must first be decreased, i.e., partially discharged. This voltage change takes time to occur, and that time is related to the magnitude of the desired change. The graph in Figure 3-4 shows the minimum waiting time between pulses, or Pulse Interval for this change to take place. In *High Range*, 125 ms is the minimum Pulse Interval for all changes.

3.1.4.2 Increasing Voltage from One Pulse to the Next

To produce a pulse of one voltage followed by one of a **higher** voltage, the reservoir capacitor voltage must first be increased, i.e., additionally charged. This voltage change takes time to occur, and that time is related to the magnitude of the desired change. The graph in Figure 3-5 gives the required time between pulses necessary in order for the pulse amplitude to be increased from one pulse to the next.

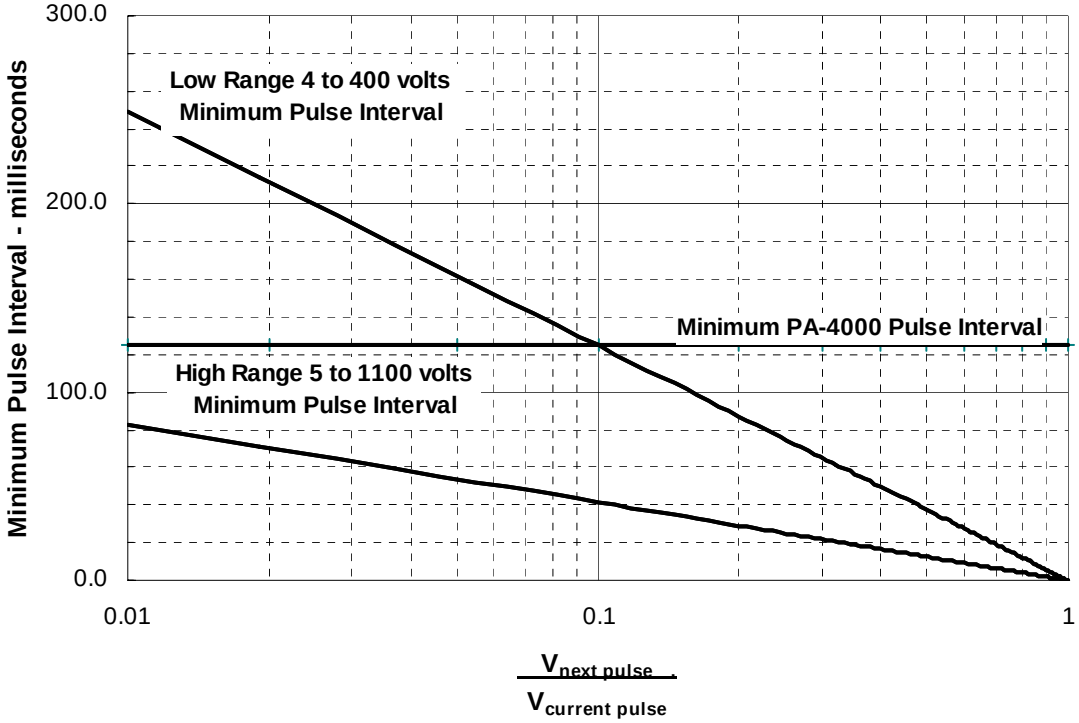


Figure 3-4: Minimum Pulse Interval for Decreasing Voltage between Pulses

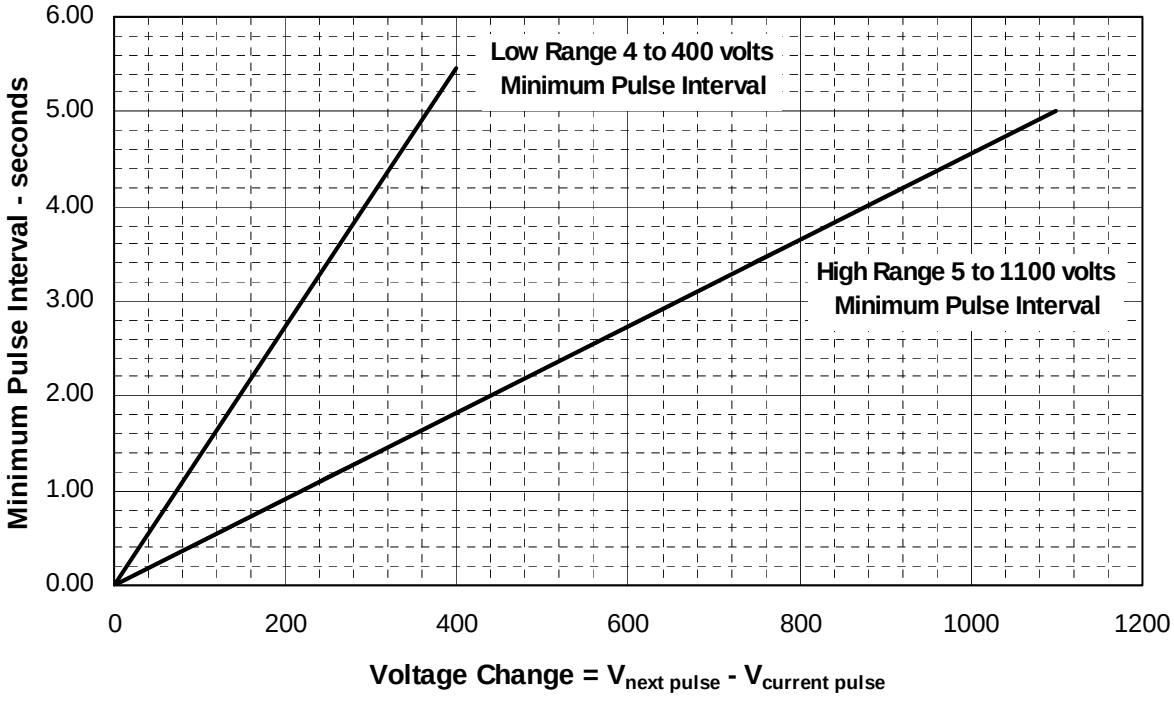


Figure 3-5: Minimum Pulse Interval for Increasing Voltage between Pulses

3.1.5 Pre-Pulse Load Estimator

The load is what the pulse voltage "sees" and it is a function of the material being electroporated. In a cuvette, this is a function of the cuvette plate spacing, the cuvette plate area and the conductivity of the aqueous solution. In tissue, it is a function of the electrode spacing and the conductivity of the tissue. The user may not know these. In order to estimate the load so an estimate of pulse amplitude can be provided, the PA-4000 uses a pre-pulse generated by the computer before the high voltage is turned on and the protocol is started. This pre pulse is 2.2 μs in duration and 2 volts in amplitude. The pulse is placed across the electrodes and the resulting current measured. Since the voltage is known, the resistance is calculated. This is done by the microprocessor and presented at the bottom of log report, as a resistance in ohms and a conductance in siemens, see Figure 3-1.

The pre-pulse data is also used by the microprocessor to detect an output short or excessive conductivity of the ionic solution. If the load estimate is less than 10 ohms the high voltage will not be enabled and an "Output Shorted" message will appear.

3.1.6 Pulse Droop

As explained in Section 2.2.3, rectangular wave electroporators also use storage or reservoir capacitors. When the high voltage is turned on this capacitor is charged to an initial voltage by the internal power supply. A pulse is generated when the capacitor is momentarily connected to the load and electrons in the capacitor are drained off like water running out of a reservoir. After a set time, the pulse width, the capacitor is disconnected from the load. Long pulses allow more electrons to run out and the voltage decreases (reservoir level drops) just as in an exponential discharge pulser. In rectangular wave electroporators the maximum pulse width is usually defined at the point that the pulse voltage at the end of the pulse is 95% of the initial voltage level. This voltage drop is called droop and is determined by the size of the internal reservoir capacitor and the load resistance. Droop is calculated by:

$$Droop\ (\%) = \frac{pulse\ width\ (\text{seconds})}{C * R_{load}}$$

When using highly ionic loads, the electrons are depleted faster. Caution is required is setting pulse widths in these situations.

Typical pulse widths yielding 5% droop or less in the PA-4000 are:

Load ohms	Low Range 4 to 400 volts	High Range 5 to 1100 volts
10	420 μs	150 μs
20	840 μs	300 μs
40	1.68 ms	600 μs
100	4.20 ms	1.50 ms
400	20.0 ms	6.00 ms

3.1.7 Aqueous Solution Heating

Heating in the material being treated is a very important consideration. The material is heated by energy from the pulses. Energy is a function of many variables. Energy in a single pulse is given by:

$$Energy\ in\ one\ pulse = \frac{[\,Pulse\ Amplitude\,]^2}{Resistance\ of\ material} * Pulse\ Width$$ watt-seconds or joules

The total energy in the pulse sequence used (protocol) is the sum of the energy in all of the pulses. For rectangular wave pulses, the temperature increase in the material being treated is (Neumann, Sowers, Jordan, p66):

$$Temperature\ Increase = \frac{Energy\ in\ all\ Pulses}{c_p * s * \nu}$$ °K

where:

c_p = specific heat , joule/gm K, approximately 4.186

s = specific mass, gm/cm^3 , approximately 1

ν = volume, cm^3

For example, the resistance of 100 μl of PBS in a 2 mm cuvette is:

$$Resistance = \rho * \frac{[\,spacing\,]^2}{volume} = 60\,\Omega - cm * \frac{[0.2\,cm]^2}{100\,\mu l * 0.001\,cm^3/\mu l} = 24\,\Omega$$

For two pulses, with amplitude 1000 volts, and widths 100 μs,

$$Total\ Energy = 2 * \frac{1000^2}{32} * 10^{-4}\ sec = 8.3\ joules$$

and,

$$Temperature\ Increase = \frac{6.3\ joules}{4.186\ j/gmK * 1\ gm/cm^3 * 100\,\mu l * 0.001 cm^3/\mu l} = 20\ ^0K$$

NOTE:

The most effective method of monitoring temperature increases during the pulse is to use an oscilloscope to monitor the pulse current. The pulse current should be constant over a pulse. If the pulse current is increasing during the pulse, then the material being treated is increasing in temperature during the pulse.

3.2 Safety Features

There are many safety features designed into the equipment that will protect both the user and the PA-4000. This section will describe them.

3.2.1 Cuvette Holder

The *Cuvette Holder* was designed to prevent accidental contact with the high voltage pulse electrodes. The electrodes can only apply voltage to the cuvettes when the handle is pushed all the way in.

3.2.2 Cuvette Holder Interlock

If the Cuvette Holder handle is withdrawn from the plastic shield, an interlock is tripped and the high voltage cannot be enabled. A red Light Emitting Diode (LED) will illuminate on the front panel and *Open* will appear in the *Electrode Holder* status window. The interlock is provided by a second cable that must be connected for the system to fully operate.

3.2.3 Short-Circuit Detection

As described in Section 3.1.5, a load estimator circuit is used to determine the approximate resistance of the test sample before the high voltage is turned on. If the value of resistance is too low (below 10 ohms), the PA-4000 software will display a message “Output Shorted” and will not turn on the high voltage or run the protocol. If this happens, there is a problem with the conductivity of the cuvette solution. This problem will need to be fixed before the system will operate fully.

3.2.4 Over Peak-Current Limit Sensor

The peak current sensor is used to detect excessive load current during a pulse. If such a condition is detected, the unit will shut down within a few microseconds and a red LED on the front panel called *External Fault* will be illuminated. There are generally two causes of excessive load current:

- **Arc in the Cuvette during a pulse:** If this fault occurs, the cuvette or chamber must be examined to see what caused the fault and the situation corrected.
- **Over-heating the aqueous solution in the cuvette:** This type of fault will occur if an ionic buffer such as PBS is used and/or the user sets the pulse repetition rate too high. Each pulse heats up the solution. Heat decreases the resistance of the solution. Eventually, the resistance becomes so low that excessive current will flow. In some cases, the temperature increase can be so large that the aqueous solution in the cuvette boils. Creating excessive heat must be avoided. Cell lysis will occur long before the temperature reaches the boiling point. In a 1mm or 2mm cuvette, this can happen in a few pulses at high voltage.

3.2.5 Over Average-Current Limit Sensor

The average current sensor is used to detect excessive charging and discharging of the reservoir capacitor during complex protocol runs. ***PulseAgile***® capability permits an infinite number of waveform combinations, so it is not possible to describe each condition that will trigger this fault circuit. In general, protocols with a large number of repetitive pulses of very wide pulse widths and short pulse intervals can trigger this fault. In such cases, it is likely that substantial heating of the test sample would take place, further enhancing the current draw and probably doing harm to the cells under treatment.

3.2.6 Microprocessor Protection

A microprocessor is used in the PA-4000 to control system operation and safety. As a result there are a number of protection and sensing circuits used to monitor the microprocessor operation to insure it is operating correctly. The sensing circuits monitor proper power supply voltage, proper cycle execution, and adverse effects of sever line transients, which could effect logic operation. If any of these conditions are detected an UNIDENTIFIED FAULT window will appear in the operator interface. To clear the fault, the system must be reset by clicking *OK* in the operator window. In some cases, pushing the red *Stop/Reset* button on the front panel is required.

3.3 Pulse Voltage and Current Monitors

All Cyto Pulse waveform generators have built-in monitors to safely view replicas of the pulse amplitude and pulse current waveforms. This permits the monitoring of these values for critical applications. This method is the only precise way of determining the delivered pulse characteristics, including load resistance and aqueous solution heating. A digital oscilloscope and cable kit may be ordered from Cyto Pulse for this purpose. It consists of three 50-ohm coaxial cables with three 50-ohm terminations.

The scale factors of the pulse monitors are:

Pulse Amplitude = (Pulse Voltage Monitor voltage) X 200, into 50 ohms

Pulse Current = (Pulse Current Monitor voltage) X 20, into 50 ohms

For example, a 1000-volt pulse will produce a 5-volt replica at the connector. A 100-amp pulse current will produce a 5-volt replica of the current pulse. An oscilloscope must be connected to the monitor ports on the back of the unit for viewing. A complete description of the monitor circuits is given in Appendix B.

Blank Page

4. Set Up

! NOTE: THERE ARE SEVERAL IMPORTANT SAFETY AND OPERATIONAL CONCEPTS DESCRIBED IN CHAPTER 3. FOR YOUR SAFETY AND PROPER OPERATION OF THE PA-4000, CHAPTER 3 MUST BE READ FIRST !

4.1 Introduction

It is recommended to first setup only the basic system consisting of the PA-4000, a computer, and the cuvette holder. Test this configuration *before* connecting any optional equipment that may have been purchased. The connections and set-up for using the optional equipment (PA-96W, PA-101, PA-201, and PA-301) are described in their respective User Manuals. If you purchased the Laptop Option, the ***PulseAgile***® software was installed at the factory.

! DO NOT PLUG IN THE LINE/MAINS CORD UNTIL ALL OF THE SET-UP PROCEDURES DESCRIBED BELOW HAVE BEEN COMPLETED !

4.2 PA-4000 Pulse Generator

4.2.1 Front Panel Features

Place the PA-4000 Pulse Generator on a tabletop. There are no connections to be made to the pulse generator front panel shown in Figure 4-1. The three functions on the front panel are the *Line/Mains Power* switch, the system *Stop/Reset* button and the indicator light-emitting-diodes (LEDs), that display the equipment status.

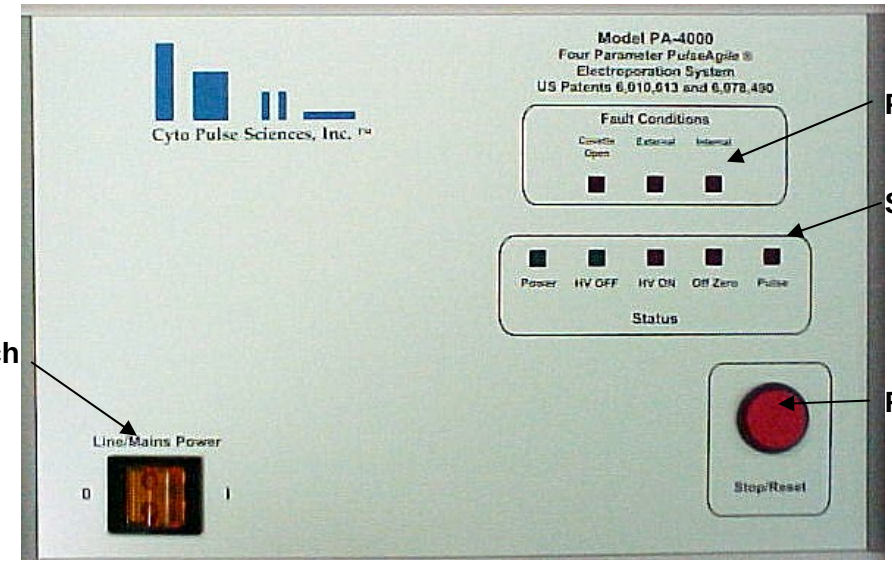


Figure 4-1: PA-4000 Front Panel Features

4.2.2 Back Panel Connections

Line/Mains Power Cord - The line/mains cord supplied with a grounded IEC connector must be plugged into the back of the unit. Figure 4-2, Detail 1 shows the location.

! DO NOT CONNECT THE OTHER END OF THE LINE/MAINS CORD INTO THE WALL UNTIL ALL INSTALLATION STEPS ARE COMPLETE !

Serial Cable - the supplied serial cable must be connected between the D-Subminiature 9-position (DB9) connector on the back of the unit and a computer. This is the communication link between the microprocessor in the PA-4000 and the computer. This is a standard RS-232 serial communication interface. See Figure 4-2, Detail 2.

Cuvette Interlock Cable - The interlock cable with the RCA-type phono jack from the Cuvette Holder must be connected to the jack labeled *Cuvette Interlock* at the top of the back panel. See Figure 4-2, Detail 3.

High Voltage Cable - the high voltage coaxial cable from the cuvette holder must be plugged into the *Pulse Out* MHV jack. There is only one such jack on the back panel. See Figure 4-2, Detail 4. The MHV connector is *similar* to the low voltage BNC connector, however:

! DO NOT ATTEMPT TO FORCE A BNC PLUG ONTO THE MHV JACK. BOTH CONNECTORS CAN BE DAMAGED, VOIDING THE PA-4000 WARRANTY !

Ground Stud - It is good practice to ground electronic equipment. A wire from the ground stud on the back panel connected to any good earth ground, such as a metal water pipe, is satisfactory. See Figure 4-2, Detail 4.

Pulse Voltage Monitor (Optional) - this BNC jack is available for the user who wishes to observe/measure the pulse amplitude using an oscilloscope. (Figure 4-2, Detail 4) Connection to this jack is not required. A 50-ohm termination at the oscilloscope is required. A 50-ohm cable with terminations may be ordered from Cyto Pulse.

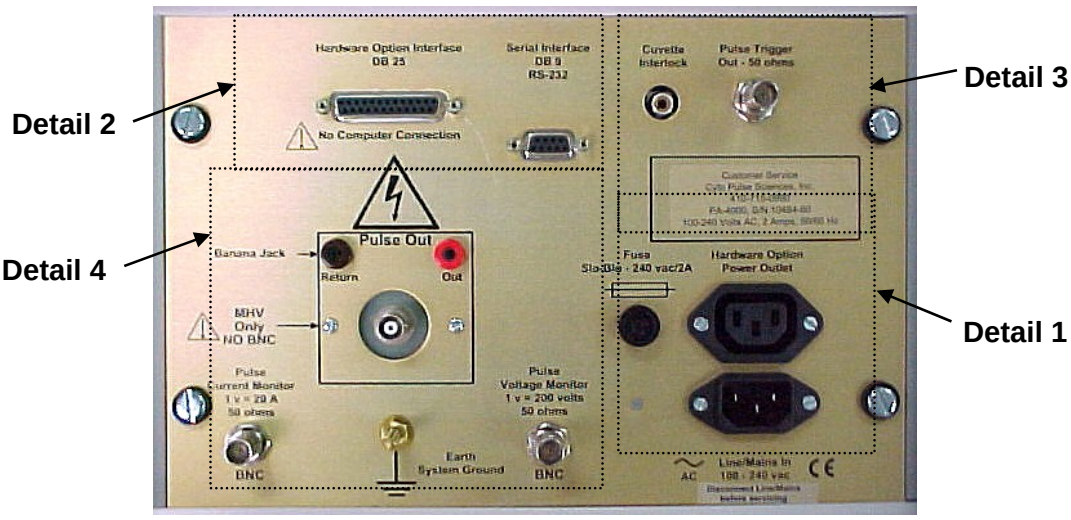
Pulse Current Monitor (Optional) - this BNC jack is available for the user who wishes to observe/measure the pulse current using an oscilloscope. (Figure 4-2, Detail 4) Connection to this jack is not required. A 50-ohm termination at the oscilloscope is required. A 50-ohm cable with terminations may be ordered from Cyto Pulse.

Pulse Trigger Out (Optional) - this BNC jack is available for the researcher who wishes to observe/measure the pulse signals using an oscilloscope. (Figure 4-2, Detail 3) It is connected to an oscilloscope trigger input. The trigger signal precedes the pulse amplitude or current signals by a few hundred nanoseconds. Connection to the trigger connector is not required. A 50-ohm cable and termination at the oscilloscope should be used.

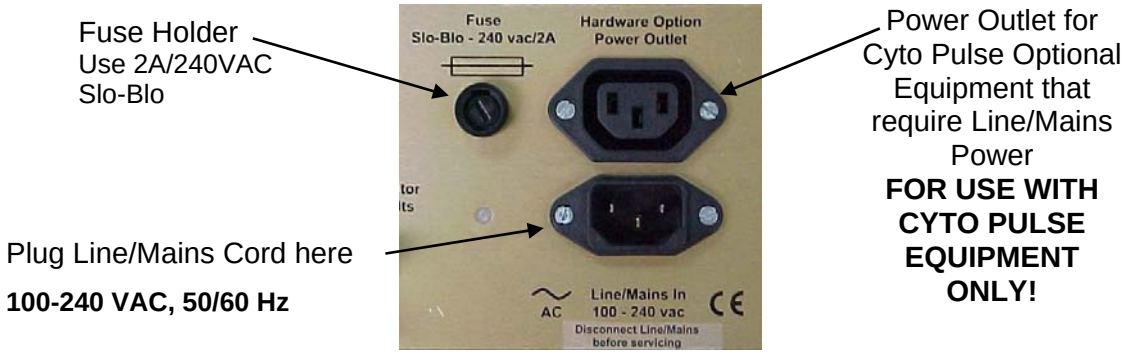
Hardware Option Interface - this is the D-Subminiature 25-position (DB25) connector located at the top left of the back panel. (Figure 4-2, Detail 2) It is the control line over which the PA-4000's internal microprocessor commands the optional equipment. A shielded DB25 cable is supplied with the optional equipment. This connector is the same type as that used for computer parallel interfaces. However,

! DO NOT USE THIS CONNECTOR TO CONNECT TO A COMPUTER PARALLEL PORT OR TO A PRINTER. DAMAGE TO BOTH PIECES OF EQUIPMENT MAY OCCUR, VOIDING THE PA-4000 WARRANTY !

PA-4000 Back Panel



Detail 1. Line Power



Detail 2. Interface

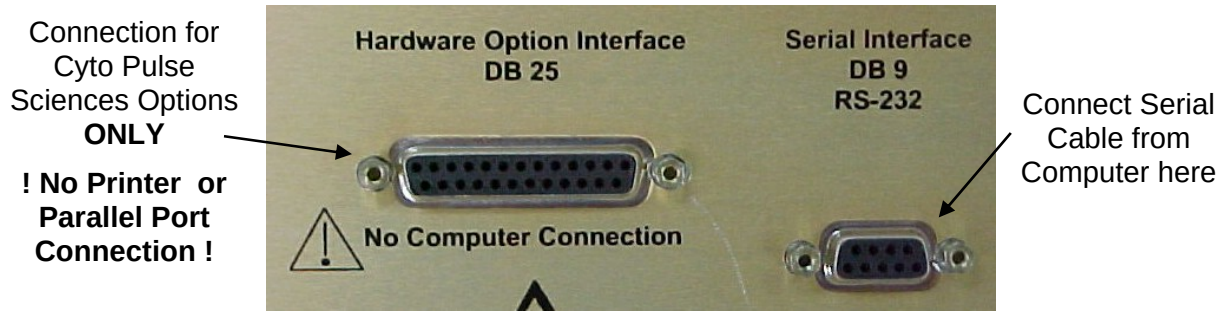
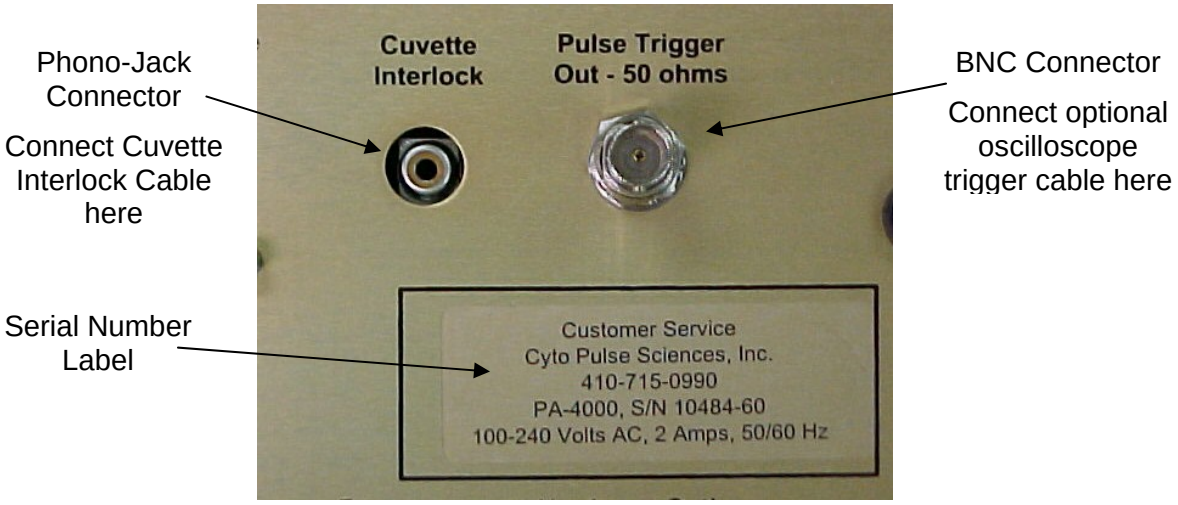


Figure 4-2: PA-4000 Back Panel Features

Detail 3. Cuvette Interlock, Pulse Trigger Out



Detail 4. HV Out, Monitors, and System Ground

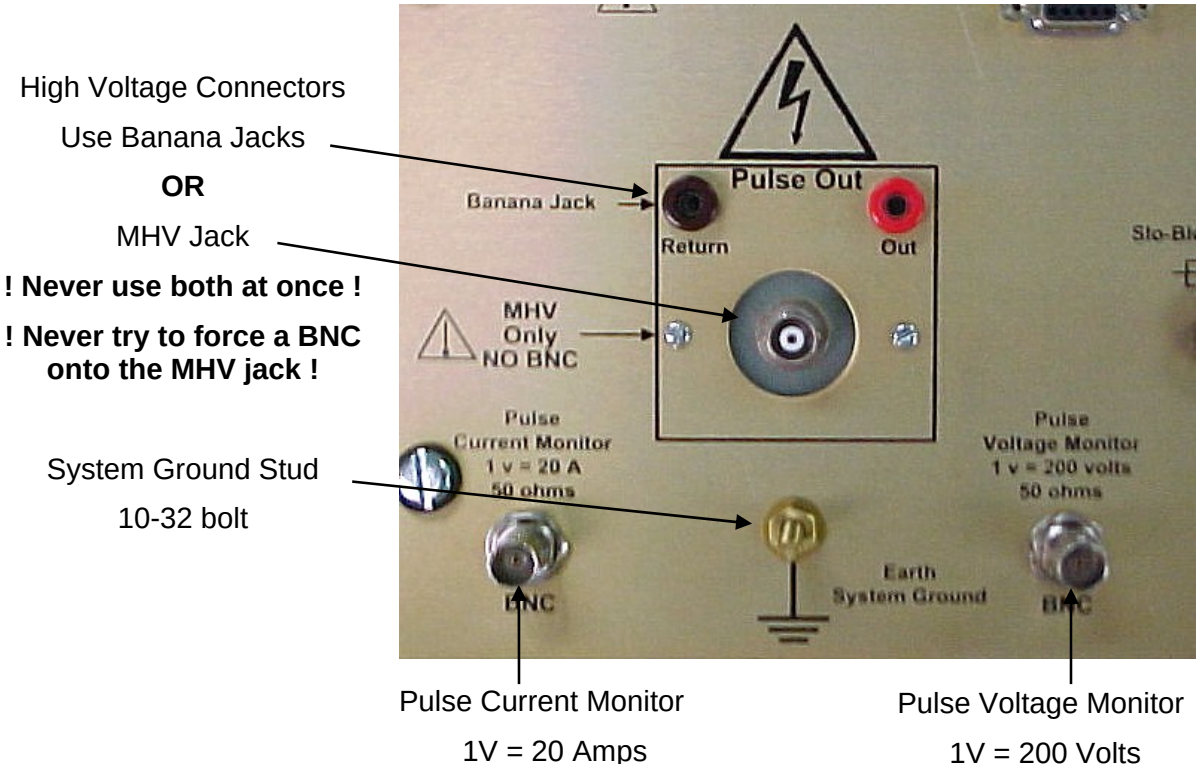


Figure 4-2 (cont'd): PA-4000 Back Panel Features

4.3 The CE-20 Cuvette Holder

The CE-20 cuvette holder will accept industry-standard cuvettes. The holder has two cable assemblies; one for the pulsed high voltage connection and one for a safety interlock. The larger diameter cable must be plugged into the *Pulse Out* MHV jack on the back panel of the PA-4000. This connector is the only one that will accept the high voltage cable. The safety interlock cable must be plugged into the RCA-type phono jack labeled *Cuvette Interlock* on the back panel of the PA-4000. The interlock must be satisfied, for the system to properly operate.

PLACE AN EMPTY 4 MM CUVETTE IN THE CUVETTE HOLDER AND SLIDE THE HANDLE FORWARD UNTIL IT STOPS

DO NOT CLOSE THE HANDLE WITHOUT A CUVETTE INSTALLED

4.4 Computers

Place a computer next to the PA-4000. Ideally, the computer will be equipped with an RS-232 serial port with a DB9 connector. Connect the serial cable provided with the system between the serial ports on the PA-4000 and the computer.

If only USB ports are available on the computer, then a USB-to-Serial converter will be necessary. These converters are widely available; however they are *not* available from Cyto Pulse. (Cyto Pulse Sciences makes no claim that the use of any USB-to-Serial converter is 100% applicable to the functionality of a PA-4000 Electroporation system. The use of a RS-232 serial interface is recommended). Use the manufacturer's instructions to install the converter first, then connect the serial cable between the PA-4000 and the converter and proceed.

4.5 *PulseAgile*® Interface Software Installation

The ***PulseAgile***® PA-4000 interface software must be run under Windows® 95 or higher (32 bit). If the software needs to be installed, use the following procedure:

1. Close all other programs and insert the CD-ROM.
2. Click **Start** from the Windows® lower menu bar.
3. Select "Run...", which will display a dialog box.
4. Type "{*CDRomDriveDesignation*}:\setup", then click "OK".
5. The setup program will begin; follow the instructions from this point.

4.6 System Test

The following is a test of the basic system (PA-4000 system with no optional equipment attached) using a preprogrammed protocol located in the default */protocol* folder. A more detailed explanation of the use of the ***PulseAgile***® software is covered in Chapter 5.

First:

- Plug in the Line/Mains cord for the PA-4000.
- Turn on the Line/Mains power for the PA-4000.

What should happen…

The green *Power* LED on the front panel will illuminate followed by the green *HV Off* LED. This delay is due to system checks performed by the PA-4000's internal microprocessor. If the CE-20 handle has not been fully pushed-in, the red *Cuvette Open* LED will be illuminated. If so, slide it forward to engage the interlock, and then the red *Cuvette Open* LED will turn off.

Then:

- Start the ***PulseAgile***® PA-4000 Interface software.

What should happen…

The screen should appear like that as shown in Figure 4-3. The *CommLink* window should show **OK**. This display indicates that the computer is communicating with the internal microprocessor of the PA-4000. Additionally, the *Electrode Holder* window should display **OK**, the *High Voltage* window should display **OFF**, and the *Status* window should say **Ready**. The *Power Supply Voltage* window will display **5** volts.

Troubleshooting tip:

If **ERROR** appears in the *CommLink* window, then click *Settings>Communications…* on the upper toolbar. Click *Test* in the *Comms Test* area. If **TEST OK** does not appear in the box, then try choosing another COM port in the *Port Selection* area and repeating the test. If communication can't be established, then turn off the PA-4000 and check the serial cable connection to both the PA-4000 and the computer (or USB-to-Serial adapter, if applicable). Once communication is established, the test may proceed.

Then:

- Click on the file folder icon on the left side of the screen
- Open the protocol file called *PA-4000Test.pro*.
- Click *Turn HV ON*

What should happen…

The protocol settings are downloaded to the PA-4000's internal microprocessor. The red *Pulse* LED will flash once, indicating that the low voltage load estimator pulse has been sent to the cuvette holder. Soon thereafter the red *HV On* LED will turn on, and the *High Voltage* window should display **On**. Then the red *Off Zero* LED will illuminate. The high voltage power supply is now enabled and the reservoir capacitor is being charged. After approximately seven seconds the *START* button is enabled. The *Power Supply Voltage* window will display **50** volts and the *Load Ohmmeter* window will display **100** Ohms.

Then:

- Click *START*

What should happen…

The PA-4000 runs the protocol. A protocol log as shown in Figure 4-4 should appear in the *Last Protocol Log* window. Again, a more detailed description of the software features follows in Chapter 5.

This completes the set-up and testing of the basic PA-4000 system.
If there is a problem, contact the factory for assistance.

Phone: 410.787.1890

Internet: www.cytopulse.com

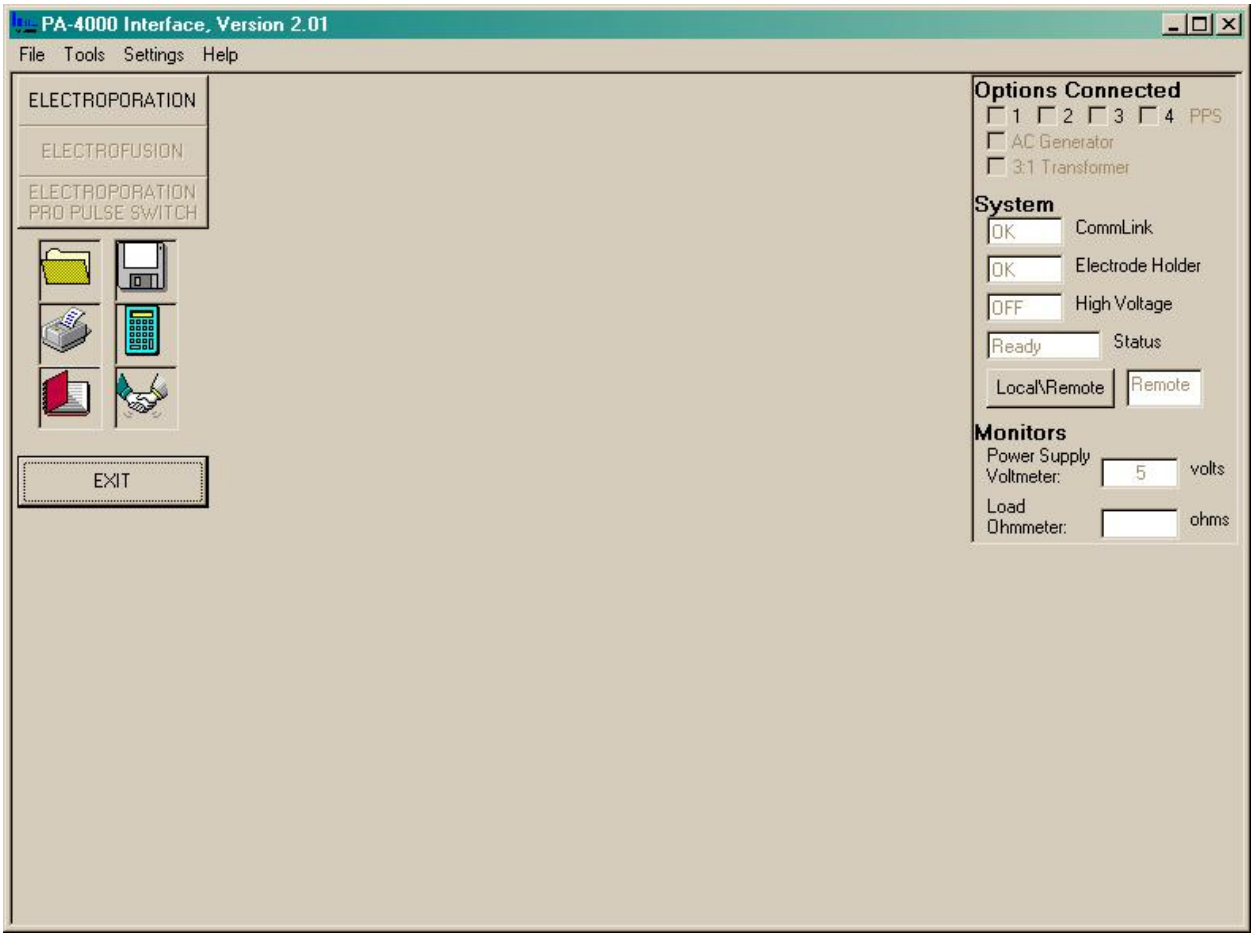


Figure 4-3: *PulseAgile*® Opening Screen showing current system status

```
Protocol File: PA-4000TEST.PRO
12-14-2004-3 15:51:24
Mode: Electroporation

>GRP   NUM      WIDTH      INTVL      SetV       MonV
> 1       1        0.020      0.50       50         50

>Estimated load > 999 ohms
>Estimated conductance <   0.001 siemens
$
```

Figure 4-4: *PA-4000Test.pro* Protocol Log

4.7 Oscilloscope Installation (Optional)

This installation procedure is for the digital oscilloscope option ordered from Cyto Pulse. The scope-package ordered from Cyto Pulse is composed of a 2-channel Tektronix® digital storage oscilloscope and a set of three coaxial cables with 50-ohm terminations.

1. Place the scope on top of or beside the PA-4000.
2. Connect one coaxial cable from Ch1 of the scope to the *Pulse Voltage Monitor* BNC jack; located at the bottom-center of the back panel of the PA-4000.
3. Connect one coaxial cable from Ch2 of the scope to the *Pulse Current Monitor* BNC jack; bottom-left of the back panel of the PA-4000.
4. Connect one coaxial cable from EXT Trigger of the scope to the *Pulse Trigger Out* BNC connector located at the top-right of the back panel of the PA-4000.
5. Connect the scope line/mains cord and turn power on.
6. Set-up the scope as follows (refer to the scope manual for assistance):

Push Ch1 Menu, set:

- Coupling DC
- BW Limit Off
- Volt/Div Coarse
- Probe 1X
- Volts/DIV knob 2.00V
- Vertical Position 0.00 div (using the position knob)

Push Ch2 Menu, set:

- Coupling DC
- BW Limit Off
- Volt/Div Coarse
- Probe 1X
- Volts/Div 2.00V
- Vertical Position 4.00 div (using the position knob)
- Sec/Div 25 μs

Push Trigger menu, set:

- Edge
- Slope Rising
- Source Ext
- Mode Normal
- Coupling DC
- Level 1.00V

5. Software Operation

5.1 Introduction

This chapter will describe the various ***PulseAgile***® interface software features and functions. The software conforms to standard Windows® conventions and this manual assumes that the user is familiar with Microsoft Windows® 95 or later.

The software performs the following functions:

- Select operating mode
  - Electroporation
  - Electroporation with dielectrophoresis (electrofusion)
  - Electroporation with programmable pulse switch
- Setting up Protocols
- Running Protocols
- File Management to save and recall protocols
- Data Log Display, printing and saving
- Status Display

The following sections describe operation based on the above bullets.

Some things to remember:

- There are three operating modes: *Electroporation*, *Electrofusion* and *Programmable Pulse Switch*.
- *Electrofusion* mode may only be selected when a PA-101 is connected to the interface on the back panel.
- *Programmable Pulse Switch* mode may only be selected when a PA-201 or a PA-96W is connected to the interface on the back panel.
- If an item is grayed out, it cannot be accessed. Either it is an option that is not installed or not available in the current mode.
- Some commonly used functions have redundant control features, i.e. they can be accessed from several places on the screen.
- The default installation directory is C:\Program Files\Pagile. It will be different if you have selected another directory during installation.
- The screen is divided into four areas within the main window. The Title Bar and pulldown menus (top), the *Tool* buttons (left), the *Control Panel* for each operating mode (center), and the *Status* area (right), see Figure 5-1.
- When the cuvette holder is open, The *Electrode Holder* window will display **OPEN**. As a safety feature, the high voltage power supply cannot be enabled and protocols cannot be run while the cuvette holder is open. However, protocols can be programmed and saved in this state.

5.2 The *PulseAgile*® PA-4000 Interface Software

Start the ***PulseAgile***® Interface software. A screen will appear that shows standard Windows® -type pull-down menus at the top, a *Tools* area on the left, and a *Status* area on the right (shown in Figure 4-3). Now click the button labeled *Electroporation*, which will add the *Control Panel* area and the *Last Protocol Log* window to the center of the display. The screen should now appear as shown in Figure 5-1.

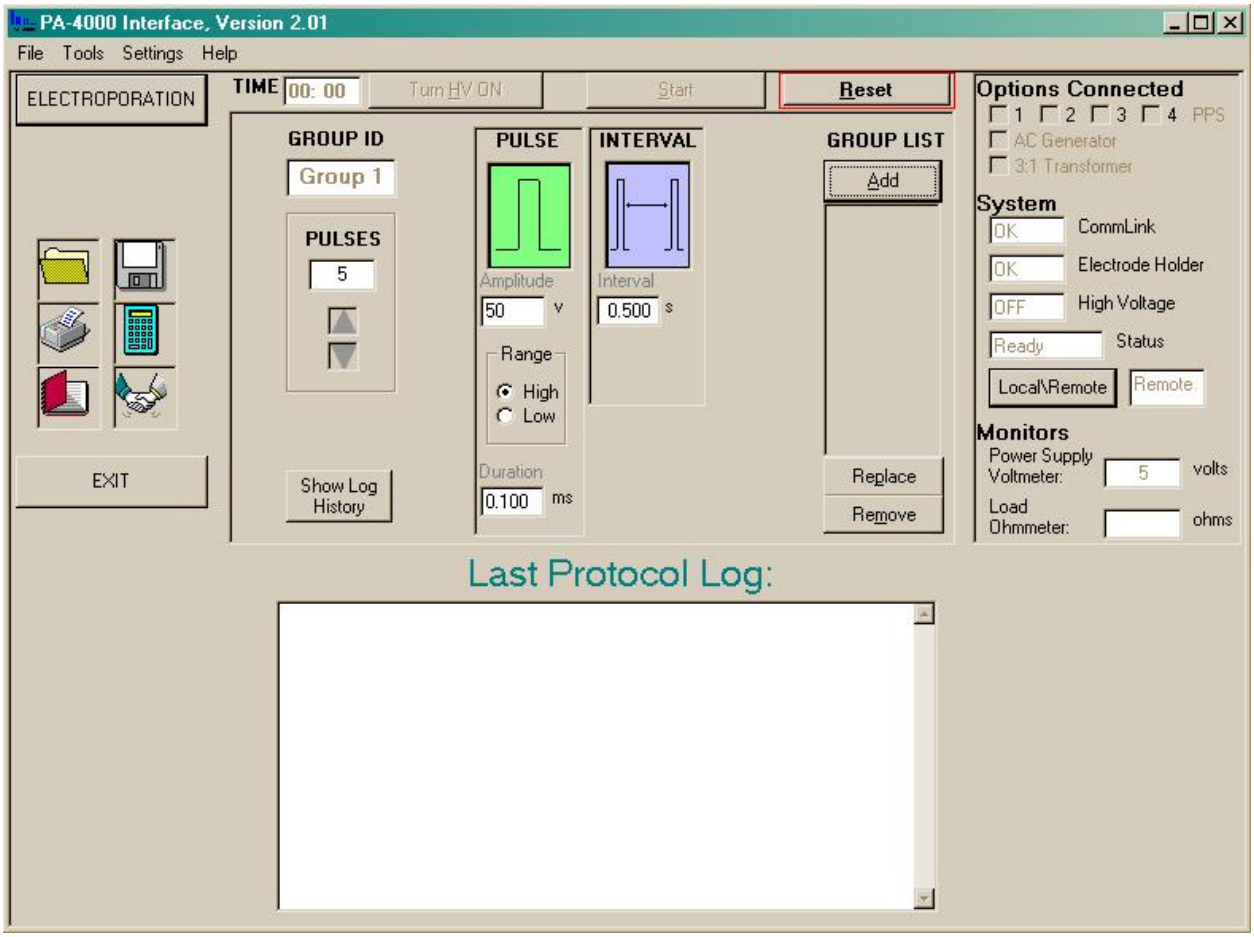


Figure 5-1: *PulseAgile*® Interface Electroporation Mode Screen

5.2.1 *The Toolbar*

Across the top-left of the main window is a list of standard Windows® pull-down menus.

5.2.1.1 File Pull-down Menu

- *Open*: Opens a previously saved protocol.
- *Save*: Saves the current protocol, overwriting the file if it had been previously saved.
- *Save Protocol As*: Saves the current protocol. The user is prompted for a filename.
- *Log Save As*: Saves the protocol *Log History*. The user is prompted for a filename.
- *Print Log*: Prints the *Last Protocol Log* or the *Log History* (user selectable).
- *Exit*: Exits the program.

5.2.1.2 Tools Pull-down Menu

- *Calculator*: The Microsoft Windows® calculator
- *Session History*: Opens a panel that allows the user to scroll through the Protocol Logs that have been run during the current session.
- *Electroporation*: Selects the electroporation mode.
- *Electrofusion*: Selects the Electrofusion mode *only* if a PA-101 is connected.
- *Programmable Pulse Switch*: *S*elects the Programmable Pulse Switch mode *only* if a PA-201 or a PA-96W is connected.
- *Quick Pulse*: Runs the open protocol without additional user input. Pressing F12 achieves the same result. The software activates *Turn HV ON* and *START*. May be used to run the same protocol repeatedly. A protocol must be open for this to work.

5.2.1.3 Settings Pull-down Menu

- *Communications*: This selection opens the communications dialog box. Within the box are two functions: 1) Port selection and 2) Communication link testing.
- *Sound*: This selection turns the event beeper on and off.
- *Reset Device*: Resets the system (same as *Reset* button at top right).

5.2.1.4 Help Pull-down Menu:

- *About*: Shows the Cyto Pulse logo, software version, and phone numbers.

5.2.2 *Tools Area*

The *Tools* area is found on the left side of the screen and contains a set of frequently used function buttons. These functions can also be accessed from the *Toolbar* as described above.

5.2.2.1 Mode Select Buttons

- *Electroporation* Button: Selects the Electroporation mode.
- *Electrofusion* Button: Selects the Electrofusion mode *only* if a PA-101 is connected.
- *Electroporation Pro Pulse Switch* Button: *S*elects the Programmable Pulse Switch mode *only* if a PA-201 or a PA-96W is connected.

5.2.2.2 Tool Buttons

- *Folder* Button: Opens a protocol
- *Disk* Button: Protocol "Save As…" Saves a protocol under a user specified filename.
- *Printer* Button: Prints the *Last Protocol Log* or the *Log History* (user selectable) to a file or printer
- *Calculator* Button: The Microsoft Windows® Calculator
- *Notepad* Button: Saves the protocol *Log History* under a user specified filename.
- *Handshake* Button: Communications: Opens the Communications dialog box.
- *Exit* Button: Exits the program.

5.2.3 *Status Area*

This area at the upper right corner of the screen displays up-to-date information about the system conditions.

5.2.3.1 Options Connected

This section shows which optional device is connected to the PA-4000. A check is shown in the appropriate box if a device is properly connected. Only one optional device can be connected at a time.

5.2.3.2 System

This section shows the current PA-4000 system status. These conditions frequently change during operation.

- *CommLink*: This box displays the status of the RS-232 serial communications link.
  OK: Communications is established and functional.
  ERROR: There is a problem with the communications link.
- *Electrode Holder*: This box displays whether or not the electrode holder in use is ready to receive pulses, and the current status of the safety interlock.
  OK: The electrode holder is closed; the safety interlock is satisfied.
  OPEN: The electrode holder is open; the safety interlock is not satisfied.
- *High Voltage*: Displays the state of the internal high voltage power supply (HVPS).
  ON: The power supply is on and the reservoir capacitor is charging.
  OFF: The power supply is off.
- *Status*: This box shows the current functional status of the PA-4000. It displays:
  Ready: The PA-4000 is ready to accept instructions to begin a protocol.
  Download…: The computer is loading protocol to the internal microprocessor.
  Charging: The system is charging the reservoir capacitor to the set voltage.
  Pulsing: The protocol is being delivered to the electrode holder.

5.2.3.3 Monitors

- *Power Supply Voltage*: Displays the voltage of the reservoir capacitor, *not* the pulse amplitude. This display is operational at all times except when pulses are being delivered.
  During charging: Displays the voltage, updated every second or so.
  After charging: Displays the final voltage on the reservoir capacitor.
  PA-4000 at rest: Displays an approximate measure of the pre-pulse voltage used by the Load Estimator function.
  in *Low Range*: **2** volts
  in *High Range*: **5** volts
- *Load Ohmmeter*: Displays an estimate of the external load as determined by the Load Estimator function. The estimate is displayed during the period from when the high voltage power supply is turned on to the finish of the protocol deilvery. This value is then listed at the end of the *Protocol Log*.

5.2.4 *Last Protocol Log Window*

The window at the bottom center of the screen is the Last Protocol Log window. The window displays the last protocol executed. However, all protocols run during a session are stored in memory. At any time during a session, the entire log history can be saved as a text file using the *Log Save As* tool described above.

5.2.5 *Electroporation Mode Control Panel Area*

The *Electroporation Mode Control Panel* Area in the center of the screen is where the pulse-protocol parameters are set, edited, and reviewed. The following is a description of each of the sections, and input/display boxes found in the *Control Panel*.

- *Group ID*: Displays the group number whose characteristics are shown in the rest of the *Control Panel* Area. The *Group ID* is also shown highlighted in the *Group List*. The Cyto Pulse Sciences concept of Pulse Groups is explained in Section 5.3.
- *Pulses*: Displays the number of pulses in the active group. The number can be changed by directly typing in the number or by using the up and down arrows shown on the screen.
- *Pulse*
  Amplitude: Displays the reservoir capacitor charge voltage level setting of the active group. The user sets the value, within the allowed limits, by typing in the number desired. The unit is volts.

  Range: Two buttons to select High or Low, which changes the range and resolution of the reservoir capacitor charge voltage.
  Low Range allows from 4 to 400 volts in 2 volt increments.
  High Range allows from 5 to 1100 volts in 5 volt increments.

  Duration: Displays the pulse duration (width) setting of the active group. The user sets the value, within the allowed limits, by typing in the number desired. The unit is milliseconds. Since most pulse durations will likely be set in the microsecond range, the duration will be frequently displayed in decimal values. For example, a display of 0.020milliseconds is 20 microseconds.
- *Interval*: Displays the pulse interval setting of the active group. It is the amount of time from the beginning of one pulse to the beginning of the next pulse. The unit is seconds. The minimum value is 0.125 seconds.
- *Group List*: Displays the list of groups in the current protocol. The groups will be executed in the order displayed. There are three buttons to control the list:
  Add (or Alt-A): Used to add a group to the list.

  Replace (or Alt-P): Used to change data within a group. Changes made to values within a group will not take effect until *Replace* has been selected. If a pulse parameter is changed, but not *Replaced*, an error dialog box will appear if the user tries to run the protocol. The user will be prompted to replace the values first.

  Remove (or Alt-M): Deletes a group. If a group is deleted, the group numbers below the deleted group (if any) are renumbered accordingly.

5.2.6 *Running a protocol*

The following function buttons are used to run the current protocol:

- *Turn HV ON (or Alt-H)*: After all of the electroporation parameters are set, and the user is ready start the protocol (cuvette in place, safety interlock engaged), then click *Turn HV ON*. The internal high voltage power supply (HVPS) turns on and charges the reservoir capacitor to the level set in Group 1. After seven seconds, the *Start* button is highlighted and the system is ready to deliver pulses.
- *Start (or Alt-S)*: Clicking *Start* begins delivery of the protocol to the test sample. The pulse groups are executed in sequential order. A double beep signals the end of protocol execution, if sounds were enabled. If the *Start* button is not clicked within 80 seconds after turning on the HVPS, the PA-4000 system will automatically reset itself.
- *Reset (or Alt-R)*: Clicking *Reset* stops the capacitor charging and/or the delivery of pulses, and can be used at any time.
- *F12*: Pushing F12 on the keyboard is the same as clicking *Turn HV ON* followed by *Start*. Please note that the reservoir capacitor takes time to charge, so there will be delay of approximately seven seconds before pulsing starts. A double beep signals the end of protocol, if sounds were enabled.

5.3 Using the *PulseAgile*® Interface Software

This section will provide instruction regarding the conventions used to create Cyto Pulse ***PulseAgile***® protocols, the setting up of a basic electroporation protocol, and the data logging and file management features.

5.3.1 *P*ulse*A*gile® *Protocol Conventions, the Pulse Group*

PulseAgile® electroporation allows pulse-to-pulse changes of parameters such as amplitude, duration and interval. In order to achieve this capability, Cyto Pulse introduces the concept of the *Pulse Group*. A *Pulse Group* is a set of pulses (1 to 99 in number) of the *same* amplitude, duration (a.k.a. pulse width), and interval. Figure 5-2 is a graphical representation of the concept. Additionally, the *Pulse Group* can contain other defined parameters specifically for PPS and electrofusion modes *if* those optional devices are in use.

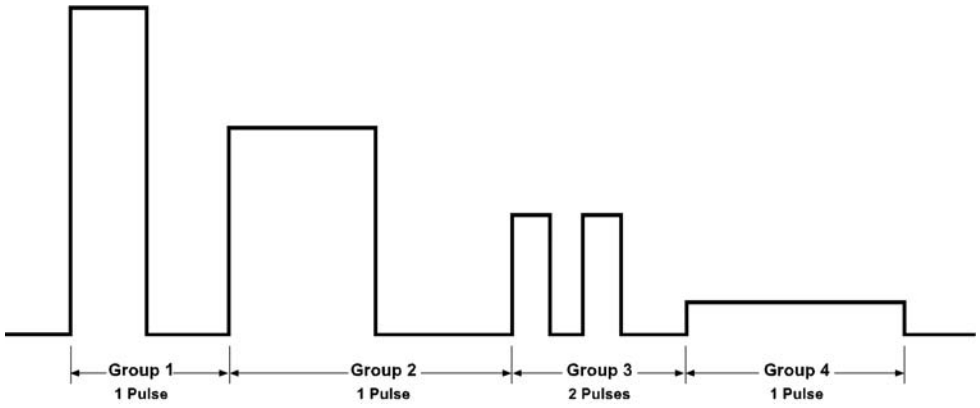


Figure 5-2: A Five-Pulse Protocol Divided into Four Groups of Pulses of Varying Parameters

5.3.2 *Setup and Run a Basic Protocol*

The following section is a guide to begin programming ***PulseAgile***® electroporation protocols. It is meant as an exercise to familiarize the user with the functions and features of the PA-4000 system.

5.3.2.1 A Basic *PulseAgile*® Electroporation Protocol

As an example, the following pulse train will be programmed below and then run using the ***PulseAgile***® interface software.

Pulse 1	500 volts, 20 μs, wait 0.2 seconds
Pulse 2	500 volts, 20 μs, wait 0.2 seconds
Pulse 3	200 volts, 100 μs, wait 0.3 seconds
Pulse 4	200 volts, 100 μs, wait 0.3 seconds
Pulse 5	200 volts, 100 μs, end

5.3.2.2 Program the Basic Electroporation Protocol

- Start the ***PulseAgile***® interface software.
- Click the *Electroporation* mode-select button. When the mode is first selected, there is no protocol loaded, but default values are contained in the various input boxes.
- Click the *Add* button above the *Group List* to begin creating the protocol. This action adds Group1 to the list.
- Change the following parameters:
  In *Pulses*, type **2**
  In *Pulse Amplitude*, type **500**
  In *Pulse Duration*, type **0.020**
  In *Interval*, type **0.200**
- Click the *Replace* button for the changes to take effect. Group1 is now programmed.
- Click the *Add* button. This action adds Group2 is now added to the list.
- Change the following parameters:
  In *Pulses*, type **3**
  In *Pulse Amplitude*, type **200**
  In *Pulse Duration*, type **0.100**
  In *Interval*, type **0.300**
- Click the *Replace* button. Group2 is now programmed.

5.3.2.3 Save the Basic Protocol

At this point the protocol can be saved to disk, if desired. Follow these steps to save:

- Click the *Floppy-Disk* tool button, or click *File>Protocol Save As.*
- Type a name for the file in the dialog box.
- Click *Save.*

5.3.2.4 Prepare a Test Sample and Run the Basic Protocol

Once the protocol has been setup, it can be executed.

- First place 400 µL of PBS in a 4mm cuvette. An empty cuvette can be used as well, but the Load Estimator result will be different than that discussed below
- Insert the cuvette in the CE-20 Cuvette Holder slide the handle forward until it is closed. Check the *System Status* box to see that *Ready* is displayed.
- Click the *Turn HV-ON* button. A low voltage (approximately 2.5-volts) "pre-pulse" is delivered to the cuvette. This pulse is used by the internal microprocessor to estimate the resistance of the load (the aqueous solution in the cuvette).

Troubleshooting hint:

If the pre-pulse current detected is *too high*, the protocol run will cease. An *Output Shorted* window will appear. Click OK to acknowledge. The solution in the cuvette *must* be checked for excessive conductivity before proceeding. If the test cuvette was prepared *exactly* as described above, the conductivity should not pose a problem

- The internal high voltage power supply will turn on if the load-current test is passed. The system allows about seven seconds for the reservoir capacitor to charge. On the front panel, the *HV ON* LED illuminates followed shortly by the *Off Zero* LED. The *Off Zero* LED turns on at the following conditions:
  In Low Range: for all voltage settings 4 – 400 Volts
  In High Range: for voltage settings above 10 Volts (15 – 1100 Volts)
- The *Start* button will be enabled when the reservoir capacitor is fully charged.
- Click the *Start* button. The programmed pulses are delivered to the cuvette, beginning with Group1. The *Pulse* LED illuminates as each pulse is delivered. The System Status box will display *Pulsing*. A double beep signals the end of protocol execution, if sounds are enabled.

Try *Quick Pulse*

Pressing F12 on the keyboard will perform both functions of clicking *Turn HV-ON* and *Start*. If this option is used, remember that there is a built-in seven second charging delay before pulses are delivered.

- When the protocol run is complete, the high voltage power supply will be turned off and the reservoir capacitor will be discharged. The system will return to the following status:
  The *HV OFF* LED will be **on**.
  The *HV ON* LED will be **off**.
  The *Off Zero* LED will be **off**.
  The *System Status* box will display **Ready**.
  The *Turn HV On* button will be **highlighted**.
- At the end of protocol execution, it is a good safety practice to confirm that the *HV Off* LED is illuminated to be sure that the protocol run has indeed completed before removing the cuvette from the holder.

5.3.3 *Reviewing the Last Protocol Log and Log History*

The *Last Protocol Log* is now ready for viewing and saving. It provides a record of the protocol that was last delivered to the test sample. A description of *Last Protocol Log* for this basic example is given in Figure 5-3. A representation of the actual pulse-train is shown below in Figure 5-4. The parameters for every pulse delivered are recorded. Note that the *SetV* and *MonV* parameters are referring to the voltage of the HVPS and reservoir capacitor, *not* the pulse amplitude. Each time the protocol is run, the *Last Protocol Log* is refreshed, and the previous results are added to the *Log History*. The *Log History* is the record of the entire electroporation

Protocol Information:
Filename (if applicable)
Date: MM-DD-YY-DoW
Time: 24-Hour Clock
System Mode

Delivered Pulse Parameters subdivided by Group number where:
GRP = Pulse Group #
NUM = Pulse # in Group
WIDTH = Pulse Duration
INTVL = Time between pulses
SetV = Set HVPS Voltage
MonV = Measured HVPS Voltage

Load Estimator Result

Calculated Conductance

System Message

Protocol File: BASICTEST.PRO
12-17-2004-6 18:51:35
Mode: Electroporation

>GRP	NUM	WIDTH	INTVL	SetV	MonV
> 1	1	0.020	0.20	500	500
> 1	2	0.020	0.20	500	500

>GRP	NUM	WIDTH	INTVL	SetV	MonV
> 2	1	0.100	0.30	200	205
> 2	2	0.100	0.30	200	205
> 2	3	0.100	0.30	200	205

>Estimated load > 19 ohms

>Estimated conductance < 0.053 siemens

>Normal Completion
$

Figure 5-3: The *Last Protocol Log* for the Basic Test Run

session that has occurred since the software was started. It can be viewed by clicking the *Show Log History* button. The *Log History* may then be saved as a *.txt* file by clicking *File>Log Save As* or the *Notebook* button. The *Log* may be printed to file or by a printer by selecting the *Printer* button.

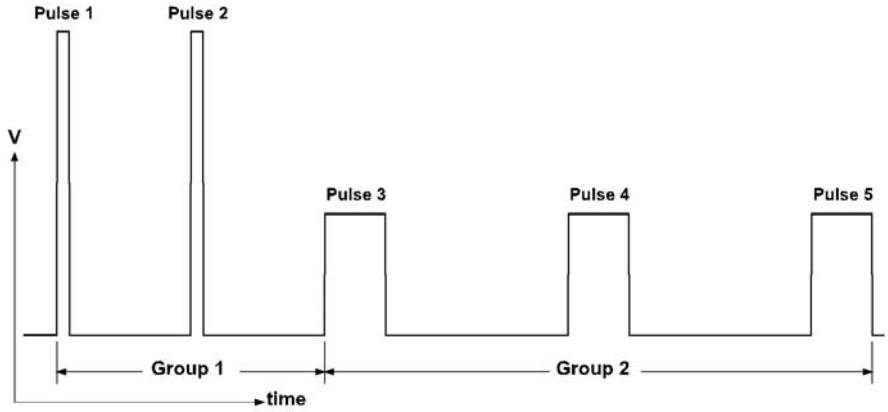


Figure 5-4: The Pulse-Train Delivered by the Basic Protocol

5.3.4 *File Management*

File management conforms to standard Windows® conventions. The protocols are saved to and retrieved from a folder created at the time of software installation or to any user created folder. The file extension used for a protocol file is *.pro*. The software automatically applies this extension when a protocol is saved. *Log History* files are saved as plain text and are given the *.txt* extension.

6. Getting Started with *PulseAgile*® Electroporation

6.1 Introduction

This chapter provides general guidelines to help with the building and optimizing of electroporation protocols. Referring to published work is one way to start the electroporation protocol optimization process. The following checklist may help:

Starting point for Electroporation Optimization	
1. What is the cell type?	
2. What is the cell diameter? (Can be estimated - See examples below.)	cm
3. Published pulsed-electric field. (If known. See examples below.)	V/cm
4. Published pulse width. (If known. See examples below.)	
5. Published number of pulses. (If known. See examples below.)	
6. Estimate the minimum electric field required to electroporate the cell. [1]	V
7. Desired transfection efficiency. (Helps define the endpoint for optimization)	
8. Desired cell viability. (Helps define the endpoint for optimization)	
9. Molecule for transfection (DNA, dye, etc.) [2]	
10. What is the conductivity, σ, of the medium? [3]	siemens/cm
11. Cuvette electrode gap. (0.1, 0.2 or 0.4 cm)	cm
12. Estimate the load resistance. [4]	Ω
13. Percent of voltage delivered to cuvette. (Use Figure 3-3, on page 3-4.)	$\%_{applied}$
14. Calculate the necessary PA-4000 pulse voltage setting. [5]	V

[1] Use the formula $V_t = 1.5rE(cos\theta)$, solving for E where:
- V_t = the minimum required (threshold) voltage across the cell. Assume $V_t = 1$.
- r = the radius of the cell in cm.
- E = the strength of the applied electric field in V/cm.
- θ = the angle between the applied field direction and the normal vector of the membrane. Assume $cos\theta = 1$ for an electric field normal to the membrane.

[2] Optimization for DNA transfection may be different than that for other small, soluble molecules. It is known, for instance, that additional low voltage pulses enhance DNA transfection after the initial high voltage pulse. See Chapter 2 for a further explanation.

[3] This value is important for highly conductive medium such as PBS or tissue culture medium. *If* the medium is highly conductive but the actual conductivity is unknown, an approximation can be made by using the conductivity of PBS whose ρ = 0.0333 siemens/cm.

[4] Use the formula R = ρ ***x*** (cuvette gap, cm)^2 ÷ (sample volume, cm^3), with ρ = Ω-cm = 1/σ

[5] Use the formula V = 2 ***x*** E ***x*** (cuvette gap) ÷ ($\%_{applied}$), with E = V/cm, and cuvette gap = cm.

Examples of several published protocols:

Cell Line	Cell diameter*	Voltage	Pulse Width	Reference
CHO	30 microns	1.5 kV/cm	50 μs	Zerbib, 1985
CHO	30 microns	600-1500 V/cm	100-4000 μs	Wolf, 1994
Human RBC	7 microns	2-4 kV/cm	10 μs	Serpersu, 1985
3T3 fibroblasts	40 microns	1.2-1.5 kV/cm	100 μs	Mir, 1988
Murine fibroblasts	40 microns	1-4.2 kV/cm	40-500 μs	Liang, 1988
B lymphoblasts	30 microns	1.2-1.4 kV/cm	100 μs	Press, 1988
Polymorphonuclear leukocytes	50 microns	5-10 kV/cm	1-5 μs	Hashimoto, 1989
Yeast	5 microns	7.5-8.5 kV/cm	50 μs	Bartoletti, 1989
Fish eggs	200 microns	750 V/cm	50 μs	Inoue, 1990

* Estimated cell diameter. Actual diameter not mentioned in articles.

6.2 An Example of Protocol Optimization

Chapter 2 presented details regarding the factors that affect electroporation protocol optimization. We will work through an example using Chinese Hamster Ovary, CHO cells.

6.2.1 Choose a Starting Pulse Voltage and Pulse Width

The most difficult initial decisions are the choices of the starting pulse amplitude and the duration (pulse width). A method for doing this is to first calculate the minimum required applied electric field for the cell and compare it to published work on the cell in question (or similar cell), and confirm whether or not the published value is reasonable starting point.

6.2.1.1 Calculate the Minimum Required Electric Field

Our example cell is the CHO cell with an average diameter of 30 microns. Our goal is to transfect the cells with a plasmid containing a gene that we have inserted.

Note 1 from the checklist gives $V_t = 1.5rE(cos\theta)$.

Solving for electric field, $E = V_t/1.5r(cos\theta)$

Assume V_t=1 volt and $cos\theta$=1, $E = 1 \text{ volt}/1.5(15x10^{-4} \text{ cm})1$

Therefore, $E = 444.4$ V/cm

This is the applied electric field that would produce the threshold voltage required to electroporate the average diameter cell at the poles nearest the electrodes. Since all of the cells are not the same size and are not located near the electrodes, higher electric field strength would be required to electroporate the maximum number of cells.

6.2.1.2 Compare to Published Electric Field Data

From the table above, we see that Zerbib, et al, used a rectangular wave pulse that produced an electric field of 1500 V/cm for a period of 50 microseconds in his electrode. That field strength is over three times the threshold value. Note that for any given cell size, there is a

wide range of fields used in the published work. This variability is due, to some extent, to the differing balance of transfection efficiency and cell viability goals of the experimenters. So it would likely be reasonable to start with a pulsed electric field of 1500 V/cm.

6.2.1.3 Calculate the Starting Pulse Amplitude and PA-4000 Set-Voltage

The starting pulse amplitude will be influenced by two cuvette-related factors: the electrode gap and the resistance of the electroporation medium.

For this example, assume that a standard cuvette with a gap of 2-mm and PBS (or similar ionic medium) will be used. The following calculations determine the load resistance, the pulse amplitude, and the PA-4000 set-voltage if the cuvette is filled to a volume of 180 μL:

From Chapter 2, the resistance of the solution is given by:

$R = \rho(D/A)$ where

ρ = resistivity, Ω-cm (for PBS ρ = 60Ω-cm)
D = electrode gap, cm
A = contact area, cm^2

Since $A = Volume/D$,

then $R = \rho(D^2/Volume)$

Substituting $R = (60\ \Omega\text{-cm})(0.2cm)^2/0.180cm^3)$

gives $R = 13.3\ \Omega$ **Load Resistance**

The electric field desired in the cuvette is 1500 V/cm, and is given by:

$E = V/D$ where

V = pulse amplitude, volts

So $V = E(D)$

$V. = (1500\ V/cm)(.2cm)$

$V = 300$ volts **Pulse Amplitude**

Recall that due to the PA-4000's internal resistance, the actual pulse voltage delivered to a 13 Ω load is 84% of the set voltage (see Figure 3-3). So the actual set voltage needs to be:

$V_{Set} = (V_{Pulse})\ /\%_{applied}$

$V_{Set} = 300\ V/0.84$

$V_{Set} = 357$ volts **PA-4000 Set Voltage**

If a 4-mm cuvette were used with the same medium and filled to a volume of 400 μL (half full), about 90% of the set voltage would be applied to the solution. So,

R = 24 Ω	4-mm Load Resistance
V = 600 volts	Pulse Amplitude
V_{Set} = 667 volts	PA-4000 Set Voltage

6.2.1.4 Quick Test of Starting Voltage

Next we need a method to see if our starting voltage is reasonable. Our starting values are:

- Cuvette chosen = 4 mm gap cuvette
- Medium or buffer = PBS
- Minimum Electric Field = 444 V/cm. (PA-4000 setting of 197 volts)
- Published Electric Field = 1500 V/cm. (PA-4000 setting of 667 Volts)
- Pulse width = 50 μs

One quick method to test the range is to do a Trypan blue dye exclusion test. This test evaluates total cells porated, including dead cells. Choose a range of voltages to test that includes the minimum voltage of 197 volts something above the highest published voltage (667 volts). For instance, perform single-pulse protocols using the PA-4000 set from 200-volts to 700-volts in 50-volt increments.

The assay is performed by applying a 50 μs duration pulse at a voltage within the chosen range to the CHO cell mixture in medium. Immediately after the pulse is applied, add an equal volume of commercial 0.4% Trypan blue dye. Incubate for 5 minutes and count clear vs. blue cells. Draw a graph of total cells vs. blue cells. Since this test does not differentiate between dead porated cells and living porated cells, it serves as a first approximation. The mid-range voltage for the rest of our optimization studies will be a voltage that induces less than 50% blue cells. The exact value to choose will depend upon your electroporation goals. For this example we will chose the voltage that yields 25% blue cells. At this value, we know that at least 25% cell viability is maintained and that effective poration can be measured. For this example, we will **assume** that 500-volts (*not* an actual measurement) yields 25% blue cells.

6.2.2 Amplitude of Low Voltage Pulses

Since our goal for this electroporation protocol is to transfect CHO cells, we will use a two-voltage protocol since we know that it will be more efficient than a one-voltage protocol. Low voltage pulses move DNA into cells by electrophoresis and, as far as we know, do not porate cells. For this example, let's choose arbitrary parameters for the low voltage pulses, such as 200 V/cm or about 90-volts, and long durations (wide pulses) of 2 ms. Again, as an arbitrary starting number, we will apply 6 low voltage pulses.

6.2.3 Optimization of First Pulse

Two measurements need to be made for each pulse protocol tested. One measurement needs to be a measurement of transfection efficiency. The other measurement needs to be a measurement of cell viability.

Since our hypothetical DNA molecule does not have an easily identified product, we will choose a reporter molecule to determine transfection efficiency. For this we will use the Green Fluorescent Protein reporter gene. (See the list of reporter molecules in Chapter 2). A positive transfection is recorded if cells are fluorescent under a fluorescent microscope 24 hours after returning to tissue culture. Alternatively, a flow cytometer can be used to measure fluorescence. The percentage of positive cells is recorded. The concentration of DNA may have to be optimized but we will use 10 μg/ml as a starting point.

For cell viability, we will use colony-forming units. This is done by plating a dilution of the cells onto a tissue culture plate, allowing cells to adhere for four hours and overlaying the cells with agarose made with cell culture medium. Any of the viability tests listed in Chapter 2 will do.

Since 500 Volts is our starting point (from trypan blue experiments), we will test the first pulses in 50-Volt increments ranging from 350 to 650-volts using a starting pulse width of 50 μs. Since two pulses are usually better than one pulse for the high voltage pulses, we will use two initial pulses. The first two pulses will be followed by six 90-volt pulses of 2 ms duration. Plotting percent viability vs. transfection efficiency gives us our optimal first pulse voltage. At this point, our optimization gives us the desired efficiency and viability.

Also at this point, a factorial analysis would be useful if the first optimization did not yield the desired results. A 2^4 factorial analysis would allow simultaneous examination of voltage and pulse width of the two groups.

There are many paths to protocol optimization and this was one example.

6.3 References

1. **Zerbib, D., Amalrick, F., Teissie, J.** (1985) Electric-field mediated transformation: Isolation and characterization of a TK+ subclone. Biochem. Biophys. Res. Commun. 129;611

2. **Bartoletti, D.C., Harrison, G.I., and Weaver, J.C**. (1989) The number of molecules taken up by electroporated cells: quantitative determination. FEBS Letters 256: 4-10

3. **Wolf, H., Rols, M.P., Boldt, E., Neumann, E., Tiessie, J.** (1994) Control by pulse parameters of electric field-mediated gene transfer in mammalian cells. Biophys. J. 66: 524-531

4. **Mir, L.M., Banoun, H., Paoletti, C**. (1988) Introduction of definite amounts of nonpermeant molecules into living cells after electropermeabilization: direct access to the cytosol. Exp. Cell Res. 175:15-25

5. **Serpersu, E.H., Kinosita, K., Tsong, T.Y**. (1985) Reversible and irreversible modification of erythrocyte membrane permeability by electric field. Biochem. Biophys. Acta 812;779

6. **Liang, H., Purcker, W.J., Stenger, D.A., Kubiniec, R.T., Hiu, S.W**. (1988) Uptake of fluorescent-labeled dextrans by10T ½ fibroblasts following permeation by rectangular and exponential-decay electric field pulses. BioTechniques, 6; 550

7. **Press, F., Quilet, A., Mir, L., Marchio-Fournigalt, C., Fuenteun, J., Fradelizi, D.** (1988) An improved electro-transfection method using square shaped electric impulsions. Biochem. Biophys. Res. Commun. 151; 982

8. **Hashimoto, K., Tatsumi, N., Okuda, K**. (1989) Introduction of phalloidin labeled with fluorescein isothyocyanate into living p[olymorphonuclear leukocytes by electroporation. J. Biochem Biophys. Methods. 19;143-154

9. **Inoue, K., Yamishita, S., Hata, J., Kabeno, S., Asada, S., Nagahisa, E., Fujita, T**. (1990) Electroporation as a new technique for producing transgenic fish. Cell Differ. Dev. 29; 123-128

Blank Page

7. Customer Service

7.1 Limited Warranty

Cyto Pulse products are warranted against defect in materials and workmanship. If the customer provides notice of such a defect during warranty period, Cyto Pulse, at its option, will either repair or replace the products, which were found to be defective. The limited warranty set forth above is exclusive and no other warranty whether written or oral, is expressed or implied. Cyto Pulse specifically disclaims implied warranties of merchantability and fitness for a particular purpose.

EXCEPT AS SET FORTH ABOVE, CYTO PULSE MAKES NO WARRANTY WITH RESPECT TO THE PRODUCT, AND IN NO EVENT, REGARDLESS OF CAUSE, SHALL CYTO PULSE BE LIABLE FOR INDIRECT, SPECIAL, OR CONSEQUENTIAL DAMAGES OR OTHER LOSSES OF ANY KIND ARISING FROM BREACH OF WARRANTY OR OTHER USES OF THIS PRODUCT. CYTO PULSE'S OBLIGATION TO REPAIR OR TO REPLACE, TO THE EXTENT SET FORTH ABOVE, CONSTITUTES THE EXCLUSIVE REMEDIES OF THE CUSTOMER FOR ANY BREACH OF WARRANTY.

This warranty shall not apply to products that after inspection by Cyto Pulse were found to be improperly used or to have been modified in any manner. Cyto Pulse recommends that the user not open the product cabinet. This limited warranty is valid for one year from the date of shipment.

7.2 Customer Service

If the user believes that there is a defect in the CYTO PULSE product, the customer should contact CYTO PULSE Customer Service through our website at **www.cytopulse.com** or phone 410-787-1890, or contact the local CYTO PULSE representative. A determination if the product is still in warranty will be made. If the warranty period is still in effect, the user will be given an authorization number (RMA) to return the product. If after receipt and inspection the product is found to be defective, it will be replaced or repaired and returned to the customer. If the product is found to have been modified or misused, the user will be given a quote for repair. If the warranty period has expired and the user requests repair, CYTO PULSE will inspect the product and provide a written quote for repair. The user must provide a purchase order number before the product will be repaired. If the unit is damaged in shipment, the user must recover the insured value to replace or repair from the carrier

Blank page

Appendix A
Pulse Specifications

Power Supply	
High Range:	
Voltage	5 to 1100 volts
Step Size	5 volts
Set Accuracy	±5% ±5 volts
Low Range:	
Voltage	4 to 400 volts
Step Size	2 volt
Set Accuracy	±5% ± 2 volt
Pulse Amplitude	
at 10 ohm load	5 to 970 volts
at 20 ohm load	5 to 1030 volts
at 100 ohm load	5 to 1070 volts
at 1000 ohm load	5 to 1100 volts
Droop	< 5% at 20 ohms, 150 μs
Pulse Over-Current Shut Down	>125 amps
Maximum Average Power	> 50 watts
Pulse Width	1 μs to 20 ms
Pulse Width Step Size	1 μs
Pulse Interval	0.125 to 400 sec
Pulse Interval Step Size	0.001 second
Number of Groups	20
Number of Pulses per Group	99
Line/Mains Power	100-240 VAC, 50/60 Hz IEC 320

Blank Page

Appendix B
Pulse Voltage and Current Measurements

This appendix describes how to make pulse measurements using the internal monitors or by using external customer supplied equipment, which will improve accuracy by a few percent. Both of these techniques require the use of an oscilloscope, which usually has a measurement accuracy of 3% to 4%. Also described are the internal circuits of the monitors and how the scale factors are derived. A Tektronix digital oscilloscope may be purchased from Cyto Pulse.

How to Use the Internal Monitors

The PA-2000 and PA-4000 have internal pulse voltage and current monitors. The monitors are available via BNC connectors on the back panel of the unit. These monitors provide a signal that is a scaled down replica of the actual pulse voltage and pulse current. The monitor signal must be viewed with an oscilloscope. The monitors must operate into a 50-ohm load to provide a properly calibrated signal. This may be accomplished by selecting the 50 ohm input impedance option on oscilloscopes that have that option or by using an external 50 ohm coaxial termination. A kit containing three 1 meter coaxial cables and three 50 ohm attenuators may be purchased from Cyto Pulse.

Three connections must be made to use the monitors:

Connection 1 - External Trigger, this BNC connector is at the top-right corner on the back panel. A coaxial cable is connected between this connector and the oscilloscope external trigger input. The signal is identical to the low voltage pulse that drives the high voltage switch. This signal has the same width and interval as the high voltage pulse but is always the same voltage. The level of this trigger pulse is about 1.5 volts into 50 ohms. When used in this manner the scope will be triggered independent of the pulse voltage or pulse current amplitude. A trigger level, on the oscilloscope, of 1.0 volt is recommended.

Connection 2 - Pulse Voltage Monitor, this BNC connector is located at the bottom center of the back panel. A coaxial cable is connected from this connector to oscilloscope Channel 1. As stated, this replica is calibrated into 50 ohms. The amplitude of the signal is 1/200 of the actual high voltage pulse. That is, a 1000-volt pulse will appear as a 5.0 volt pulse into 50 ohms at the oscilloscope. To calculate an estimate of the actual high voltage pulse:

Pulse Amplitude Estimate = Pulse Voltage Monitor in volts x 200 volts/volt

The pulse width and interval are the same as the high voltage pulse. The pulse rise time out of this monitor is slower than the actual pulse rise time. If rise time measurements are critical than an external high voltage probe must be used (see below, external measurements).

Connection 3 - Pulse Current Monitor, this BNC connector is located at the bottom left of the back panel. A coaxial cable is connected from this connector to oscilloscope channel 2. As stated, this replica is calibrated into 50 ohms. The amplitude of the signal is 1/20 of the actual pulse current resulting from the high voltage pulse. That is, pulse current of 100 Amps will appear as a 5.0 volt pulse into 50 ohms. To calculate an estimate of the pulse current:

Pulse Current Estimate = Pulse Current Monitor in volts x 20 Amps/volt

The pulse width and interval are the same as the high voltage current pulse. The pulse rise time out of this monitor is slower than the actual pulse current rise time. If pulse current rise time measurements are critical than an external torroidal type current transformer should be used (see below, external measurements).

In addition to pulse voltage and current, two other parameters of interest may be calculated, resistance of the external load (buffer in cuvette or tissue) and charge.

The resistance in vitro or in vivo is calculated by:

External Resistance = Pulse Voltage / Pulse Current ohms

The total charge transferred by a rectangular-wave pulse is calculated by:

$$\text{Total Charge} = \int_0^{PW} i(t)\,dt$$

$$= I \bullet PW \quad \text{in coulombs}$$

where:

I = flattop pulse current in Amps
PW = pulse width in seconds

Combining with the current monitor equation above:

Total Charge = Current Monitor x 20 x Pulse Width coulombs

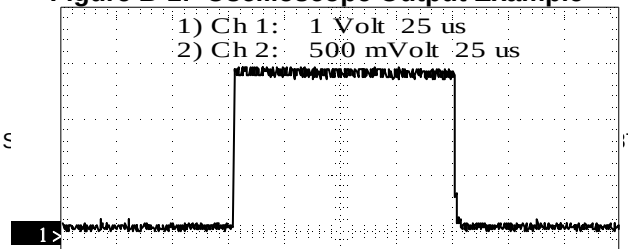
An example of an oscilloscope output is presented in Figure B-1. The top trace, Channel 1 is the Voltage Monitor and the bottom trace, Channel 2 is the Current Monitor. The following are the calculated values. In this example, a 400 μl volume of PBS in a 4 mm cuvette at 19 ^{o} C and power supply voltage of 1,000 volts was used.

Estimated Pulse Amplitude	= 2.24 div x 2 v/div x 200 v/v = 896 volts
Estimated Pulse Current	= 1.80 div x 1 v/div x 20 A/v = 36 Amps
Estimated PBS Resistance	= 896 v/ 36 A = 24.9 ohms
Estimated Total Charge	= 24.9 A x 100 μs = 2.5 millicoulombs

Internal Pulse Voltage and Current Monitors

The Pulse Voltage Monitor and Pulse Current Monitor signals are derived from the high voltage pulse. The circuit diagram is shown in Figure B-2 below.

Figure B-1: Oscilloscope Output Example



Voltage Monitor Error Due to CVR

The voltage monitor circuit is a resistive divider. It is across the External Load plus the Current Viewing Resistor. This is important because the voltage applied to the External Load is slightly less than that measured by the Pulse Voltage Monitor. This error is 1.78%, at the lowest

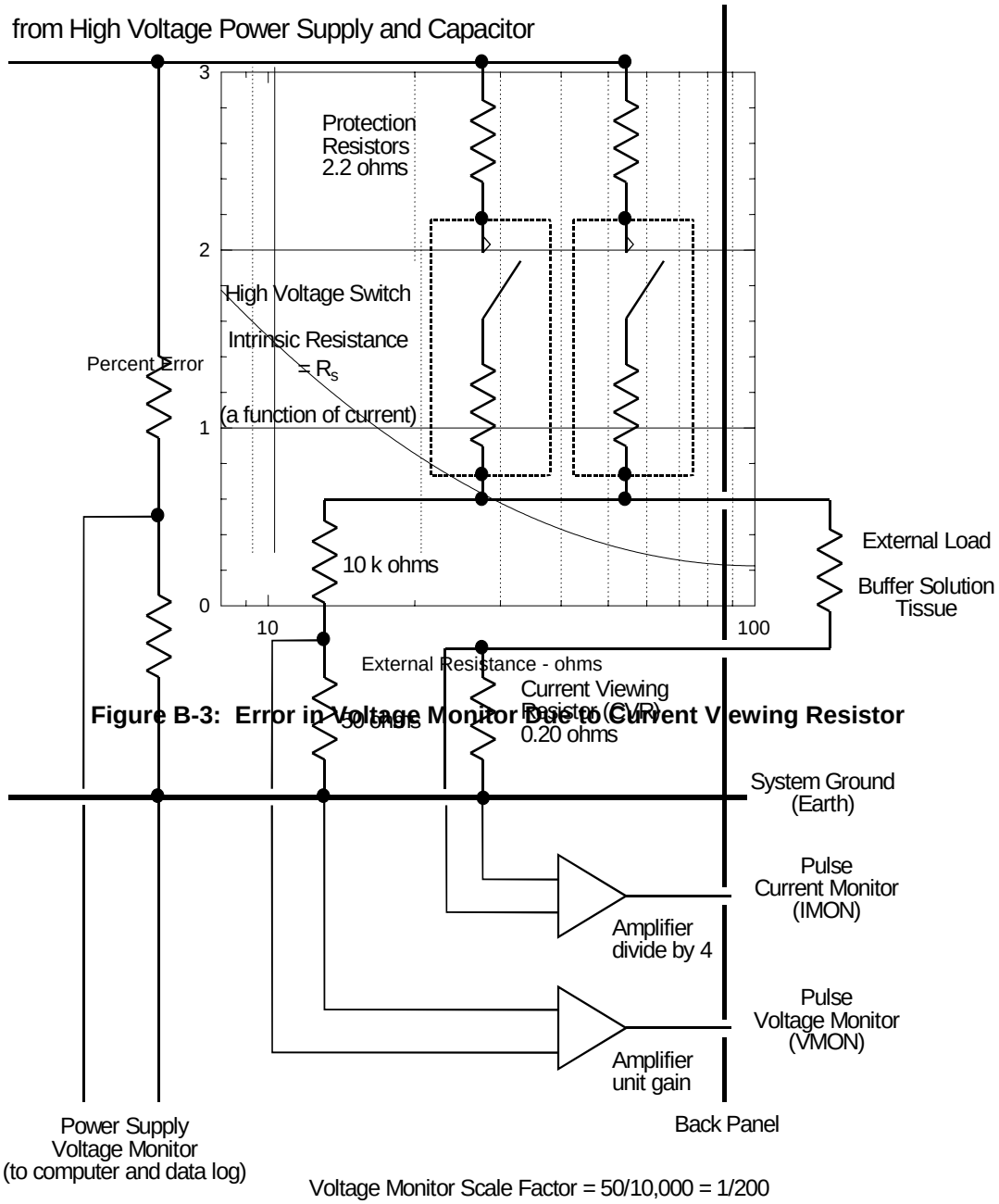


Figure B-3: Error in Voltage Monitor Due to Current Viewing Resistor

Voltage Monitor Scale Factor = 50/10,000 = 1/200

Current Monitor Scale Factor = 0.20/4 = 1/20

HV Switch $R_s = a + b\,Rext + c\,Rext^2 + d\,Rext^3$

Figure B-2: Simplified Circuit Diagram of Monitors

Figure B-4: Typical Relationship between Power Supply and Pulse Amplitude

value of permitted External Resistance of 8 ohms. Shown in Figure B-3, as the External Load Resistance gets larger, the error gets smaller.

Voltage Across External Load

If the Pulse Voltage Monitor/Oscilloscope combination is not used, the voltage across the External Load Resistance can be estimated. The voltage across the load is:

$$\text{Pulse Voltage across External Load} = R_{ext}/R_{total} \times \text{Power Supply Voltage}$$

where:

$R_{total} = R_{internal} + R_{ext}$

$R_{internal}$ = 2.5 ohms (nominally)

This is a standard voltage divider relationship. An estimate of R_{ext} is given in the Ohmmeter window *Status* area of the ***PulseAgile***® software interface and at the bottom of the *Protocol Log* report. The above computation is performed by the ***PulseAgile***® software and also presented in the log report. The number presented is the ratio of pulse voltage to power supply voltage. As the External Load Resistance gets lower, more voltage appears across the internal resistance and less voltage appears across the External Load. This is shown in the graph in Figure B-4. This ratio estimate is accurate to about 10%. The R_{ext} estimate should not be used for precise analysis.

Using External Equipment to Measure Pulse Voltage and Current

The most accurate method to measure pulse voltage and pulse current is with an external high voltage oscilloscope probe and an external current transformer.

High Voltage Probe

Most oscilloscope manufacturers offer a high voltage probe. One example is the Tektronix TEK5100. To use the probe, connect the probe tip to the high voltage side of the external load such as the cuvette contact. The ground side *must* be connected to the System Ground Screw on the back panel, not the other side of the external load. If connected to the other side of the external load, the internal current monitor circuit will distort the current measurement.

Caution

When connecting to the high voltage side, care must be taken so it is not possible to come in contact with any high voltage while the system is operating.

Current Transformer

The most accurate and safest current measurement is with a torroidal current transformer. This is a coil through which the low potential side of the External Load current is passed. The current through the return lead induces a voltage in the transformer, which is in turn measured. A Pearson Model 411 is recommended for this type of measurement. Contact Pearson Electronics, Palo Alto, CA.

Load Resistance and Electric Field Predicting Model

A Microsoft EXCEL model that will predict the external load is available from the Cyto Pulse. This prediction is useful when setting up a protocol to determine the cuvette type to use, the buffer type, the volume to be treated, and the electric field intensity in the cuvette.

Appendix C
Declarations of Conformity

CE Declaration of Conformity

CB Test Certificate

FCC Compliance: CFR 47, Part 18, Subpart C, Class A Equipment

Industry Canada Compliance: ICES-003 Category II, Class A Equipment

Blank Page

Replace with CE

Blank Page

Replace with CB

Blank Page

Replace with FCC

Blank Page

Replace with Industry Canada

Blank Page