Hybrimune™

Hybridoma Production System

USER'S MANUAL

BTX®

a division of **Harvard Bioscience, Inc.**

Publication 015-100191 REV 4.0

A Message from BTX

Thank you for investing in a Hybrimune system.

Since its founding in 1983, the main focus of BTX has been in the area of applying controlled electric fields for genetic engineering applications. Because of this, we quickly established a reputation as the technological leader in the fields of electroporation and electrofusion. Our systems have been installed in many prestigious institutes around the globe where they are used successfully for high efficiency transfection, transformation and cell fusion applications. We offer a variety of waveforms, electrodes and chamber options to provide you with the best tools to achieve your goals.

We are vested in your success. To that end, the BTX technical support team constantly tracks published literature for any reference to electroporation and electrofusion. We extract the pertinent experimental conditions and yields from these papers to help us in our efforts to help you. In addition to tracking publications, we are available to you for support at any time for advice in experimental design, product recommendations, troubleshooting, and any other relevant technical advice.

We thank you again for your investment and we look forward to assisting you in any way we can.

Finally, please read this manual carefully before attempting to operate the electroporation system. If you have any questions about the unit or about particular applications, please contact us:

BTX
84 October Hill Road
Holliston, MA 01746 USA

Toll Free: 1-800-272-2775
International Callers: 508-893-8999
Fax: 508-429-5732
Web: www.btxonline.com
Email: support@hbiosci.com

For any customers outside the US or Canada, please call your local BTX dealer or call us directly.

BTX®
a division of **Harvard Bioscience, Inc.**

Table of Contents

Table of Contents

Table of Contents

Warranty Information

RESEARCH ONLY

BTX
84 October Hill Rd
Holliston, MA 01746, USA

Phone: 1-508-893-8999
Fax: 1-800-429-5732
Web: www.btxonline.com

Warranty

BTX warranties Hybrimune systems for a period of two years from the date of purchase. At its option, BTX will repair or replace the product if it is found to be defective as to workmanship or materials. This warranty does not extend to any device which has been (a) subjected to misuse, neglect, accident or abuse, (b) repaired or altered by anyone other than BTX without BTX express and prior approval, (c) used in violation of instructions furnished by BTX. This warranty extends only to the original customer purchaser. **IN NO EVENT SHALL BTX BE LIABLE FOR INCIDENTAL OR CONSEQUENTIAL DAMAGES**. Some states do not allow exclusion or limitation of incidental or consequential damages so the above limitation or exclusion may not apply to you. **THERE ARE NO IMPLIED WARRANTIES OF MERCHANTABILITY, OR FITNESS FOR A PARTICULAR USE, OR OF ANY OTHER NATURE.** Some states do not allow this limitation on an implied warranty, so the above limitation may not apply to you. Without limiting the generality of the foregoing, BTX shall not be liable for any claims of any kind whatsoever, as to the equipment delivered or for non-delivery of equipment, and whether or not based on negligence. Warranty is void if the BTX Hybrimune system is changed in any way from its original factory design or if repairs are attempted without written authorization by BTX. Warranty is void if parts, connections or electrodes not manufactured by BTX are used with the BTX Hybrimune instrument. If a defect arises within the warranty period, promptly contact BTX, 84 October Hill Road, Building 7, Holliston, Massachusetts, USA 01746-1388 using our toll free number 1-800-272-2775 (US Only) or 508-893-8999 (E-mail: support@hbiosci.com). Goods will not be accepted for return unless an RMA (Returned Materials Authorization) number has been issued by our customer service department. The customer is responsible for shipping charges. Please allow a reasonable period of time for completion of repairs, replacement and return. If the unit is replaced, the replacement unit is covered only for the remainder of the original warranty period dating from the purchase of the original device. This warranty gives you specific rights, and you may also have other rights, which vary from state to state.

Out of Warranty Service

Proceed exactly as for Warranty Service above. If our service department can assist you by phone or other correspondence, we will be glad to help at no charge.

Repair service will be billed on the basis of labor and materials. A complete statement of time spent and materials used will be supplied. Shipment to BTX should be prepaid. Your bill will include return shipment freight charges.

Disassembly by the user is prohibited. Service should only be carried out by experienced BTX technicians.

Repair Facilities and Parts

BTX stocks replacement and repair parts. When ordering, please describe parts as completely as possible, preferably using our part numbers. If practical, enclose a sample photo or drawing.

Caution Notice

The BTX Hybrimune systems are intended for laboratory use only and can be used in research and development applications. These systems have been designed to meet the standards for electromagnetic compatibility (EMC) and safety intended for laboratory equipment applications.

This product should not be used in the presence of a flammable atmosphere such as an anesthetic mixture with air, oxygen, or nitrous oxide.

Safety Information

Please read the following safety precautions to ensure proper use of your generator. If the equipment is used in a manner not specified, the protection provided by the equipment may be impaired.

To Prevent Hazard or Injury

Emergency Stop

If a problem occurs during a run, push the STOP/RESET button on the front panel.

Use Proper Line Cord

Use only the specified line cord for this product and make sure line cord is certified for country of use. The operating voltage range for the Hybrimune is 100 to 240 VAC, 50/60 Hz.

Ground the Product

This product is grounded through the grounding conductor of the power cord. To avoid electric shock, the grounding conductor must be connected to earth ground. Before making any connections to the input or output terminals of the product, ensure that the product is properly grounded.

Make Proper Connections

Make sure all connections are made properly and securely. Any signal wire connections to the unit must be no longer than three meters.

Observe All Terminal Ratings

Review the operating manual to learn the ratings on all connections.

Use Proper Fuse

Use only specified fuses with product.

Avoid Exposed Circuitry

Do not touch any electronic circuitry inside of the product.

Do Not Operate with Suspected Failures

If damage is suspected on or to the product do not operate the product. Contact qualified service personnel to perform inspection.

Orient the Equipment Properly

Do not orient the equipment so that it is difficult to operate the disconnection device.

Place Product in Proper Environment

Review the operating manual for guidelines for proper operating environments.

Observe All Warning Labels on Product

Read all labels on product to ensure proper usage.

High Voltage Risk

These instruments contain a high voltage power supply adjustable to 1,000 V. High voltage power supplies present a serious risk of personal injury if not used in accordance with design and/or use specifications, if used in applications on products for which they are not intended or designed, or if they are used by untrained or unqualified personnel.

- The user must read this manual carefully before the instruments are placed into operation.
- Removing the cover will 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 (mains) cord.
- Do not touch the electrode tip while the waveforms are being applied.
- Do not under any circumstances turn the power switch off while the system is pulsing; if an emergency occurs push the emergency button on the front panel

If there are any questions about the operation of this instrument, call BTX Customer service at 1-800-272-2775, or 1-508-893-8999.

Caution Notice

The BTX Hybrimune Systems are intended for laboratory use only and can be used in research and development applications. These systems have been designed to meet the standards for electromagnetic compatibility (EMC) intended for laboratory equipment applications as well as the applicable safety requirements for electrical equipment for measurement, control, and laboratory use. The unit itself does not generate waste, but may be used to treat samples that are hazardous. Please use appropriate PPE and ensure disposal in accordance with local regulations and practices.

This product should not be used in the presence of a flammable atmosphere such as an anesthetic mixture with air, oxygen, or nitrous oxide.

Caution

Protective Ground Terminal

Caution Risk of Electric Shock

Hybrimune™ System Components & Set-Up

Figure 1: Hybrimune™ System

Introduction

The Hybrimune™ Hybridoma Production System was developed for large volume commercial applications using cell electrofusion. This includes hybridoma production and tumor/dendritic cell immunotherapy. The system has not been approved for clinical or in vitro diagnostic use. The system can fuse up to 180 million cells in one process run. The Hybrimune is designed to be simple to set-up and to operate. The system is shown in Figure 1.

The Hybrimune System is comprised of four components: the Hybrimune Waveform Generator, the user-interface software, two large volume coaxial chambers (2 ml optimization chamber and 9 ml production chamber), and the low conductivity BTXpress Cytofusion® Medium C in which the electrofusion takes place. These components were all designed to operate as a system. Using other components may damage the system and may result in degraded performance. A computer is required for the application software but is not included in the system.

The Hybrimune Hybridoma Production System is the most sophisticated system available for fusing dissimilar cells. The system is available only under license directly from Cellectis. Please note the following important information about the system:

1. The System must remain in the possession of the licensee.
3. The System shall only be used as described in this User Manual.
4. Certain concepts and processes are covered by BTX patents and patents pending.
5. BTX Cytofusion Medium C is a required component of the Hybrimune system. Other ionic medium should not be used; it will generate significant heating and kill cells.
6. The chamber must be cleaned after each use to prevent ionic build up which will cause heating, kill cells, and reduce efficiency. Please refer to chamber cleaning procedure, page 27.

The Hybrimune™ Voltage Waveform Generator

The waveform generator produces the voltage waveforms programmed by the user. The desired waveform parameters and sequences are set by the user in the user-interface application software. When the Start button is clicked, the software is downloaded from the computer to the microprocessor in the waveform generator. The waveform is generated and applied to the Electrofusion Chamber producing an electric field. Depending on the user-input instruction, this microprocessor-controlled waveform generator will generate:

- **Pre-Fusion Pulse AC Wave:** a low voltage, megahertz frequency sinusoidal alternating voltage that results in an alternating electric field that aligns and compresses the cells in a process called dielectrophoresis
- **Fusion Pulse:** a high voltage fusion pulse that forms pathways in the cell membranes that results in fusion
- **Post-Fusion Pulse AC Wave:** a second sinusoidal alternating electric field that holds the fused cells together while they mature

A detailed description of the waveforms is presented in page 37. The parameter values available are listed on page 42. The User-Interface software provides the user with the ability to fine-tune each of the three parts of the waveform. The instructions on how to program the waveforms may be found on page 18.

Hybrimune™ System Components & Set-Up

Figure 2: Large-Volume Fusion Chamber

Figure 3: Cytofusion Medium C

The Large-Volume Fusion Chamber

The Hybrimune Waveform Generator connects to a coaxial large-volume fusion chamber. The chamber produces the various electric fields required in the hybridoma production process. These chambers were very carefully designed to provide optimum alignment forces and intense electric fields necessary for efficient electrofusion processes. When the alternating sinusoidal voltage is applied to the inner and outer electrode rings of the chamber, a highly non-uniform electric field is established that causes the cells to align into ”pearl chain” formations.

Two types of chambers are provided. The first is the 2 ml chamber (left image in Figure 2) that holds a maximum volume of 2 ml. This chamber permits light to enter in from the bottom so that the cell fusion process can be observed with a microscope. The 2 ml chamber is used for process optimization. The second chamber is the 9 ml chamber (right image in Figure 2) that holds a maximum volume of 9 ml. This chamber is used for large batch production. The electrical characteristics of the two chambers are equivalent. The electrodes are coated with MEDCOAT 2000™, a chromium coating by Electrolyzing Corporation of Ohio.

Cytofusion® Low Conductivity Medium

BTX has developed a proprietary formula cell fusion medium over several years that has under gone extensive testing. The conductivity of this medium is low in order to minimize electrical current flow during the application of the intense alternating electric fields generated by the Waveform Generator. By limiting current flow, aqueous heating is minimized which improves fusion efficiency and viability. The medium is shown in Figure 3.

The Hybrimune Waveform Generator and the large-volume fusion chamber were designed to work with low conductivity cell suspensions. Do not use highly ionic buffers in the chambers. If ionic buffers are used, two catastrophic problems will occur. First, the cell suspension will rapidly heat causing cell death. Second, significant convection currents will be generated in the suspension that will inhibit cell alignment.

WARNING: ONLY CYTOFUSION® MEDIUM MAY BE USED IN THE LARGE-VOLUME FUSION CHAMBER CHAMBERS!

Setting Up the Hybrimune™ System

All of the cables necessary to install the equipment are included in the Hybrimune System. They are:

1. **Serial Interface Cable, contained within the cable set**
2. **Line/Mains Power Cord, contained within the cable set**
3. **Chamber Connection Cable, Item Number 47-0302**

BEFORE PROCEEDING: MAKE SURE THAT THE LINE/MAINS SWITCH AT THE LOWER LEFT OF THE FRONT PANEL IS IN THE OFF (O) POSITION AND THAT THE POWER CORD IS NOT PLUGGED INTO THE LINE/MAINS INPUT.

Serial Cable: Connect the serial cable between the D-Subminiature 9-position (DB9) connector labeled "Computer Control" on the back of the unit and a serial port on the computer. This is the communication link between the microprocessor within the waveform generator and the computer. It is a standard RS-232 serial communication. 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 BTX. See next page for more information.

BTX HARVARD APPARATUS DOES NOT GUARANTEE THAT A USB-TO-SERIAL CONVERTER IS 100% APPLICABLE. THE USE OF AN RS-232 SERIAL INTERFACE IS RECOMMENDED.

Line/Mains Power Cord: Plug this cord into the power input receptacle labeled "Line/Mains In". Plug the other end into an outlet supplying suitable electric power.

Chamber Connection Cable: There are two connections made with this cable assembly. Using the figures shown in the "Chamber Cable Connections" area of the back panel, connect the SHV-type coaxial plug to the jack labeled "Waveform Out". Then connect the RCA-type phono-plug to the jack labeled "Chamber Enable". Generally, this cable only needs to be connected at the time that a chamber is to be used.

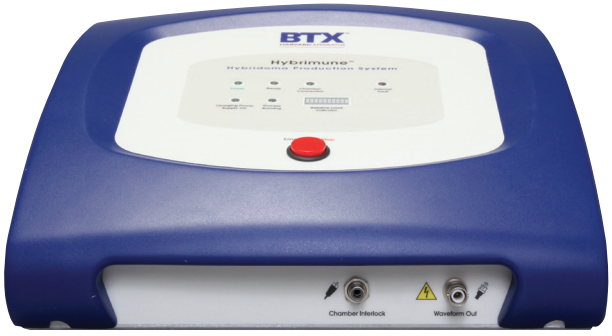


Figure 4: Cable Connections

Waveform Generator Front Panel Display

There are eight status indicators on the front panel, divided into two groups. Before powering-up the system, the user should become familiar with them.

System Status Group

The function of this group is to inform the user of the overall state of the waveform generator when it is powered-up. This group consists of four LED (light emitting diode) indicators.

- "Power", green LED: Indicates that the internal power supplies and the system microprocessor are receiving adequate power. It should be illuminated at all times when the waveform generator is turned on.
- "Ready", green LED: Indicates that the waveform generator is ready to deliver a electrofusion waveform to an attached Chamber. It will only be illuminated when the internal microprocessor detects no problems and when the Chamber Cable is properly connected.
- "Internal Fault", red LED: Indicates that a problem occurred inside of the unit during a protocol run. If illuminated, the system requires a Reset. If the problem persists, BTX Customer Service should be contacted before proceeding. Under normal operating conditions, this LED will not be illuminated.

Process Status Group

The function of this group is to inform the user of state of an electrofusion protocol run. This group consists of three LED indicators, and a multi-color bar graph.

- **"Chamber Connected", Green LED:** Indicates that the Chamber Cable is properly connected. If a Chamber is plugged into the cable, then it is ready to receive a waveform from the waveform generator.
- **"Charging Power Supply On", Green LED:** Indicates that the internal reservoir capacitor is charging (or is fully charged). When not illuminated, the reservoir capacitor is at zero volts. It is only illuminated after the user clicks Turn HV On and remains illuminated during the entire process run.
- **"Process Running", Green LED:** Indicates that the waveform generator is delivering an electrofusion waveform to an attached Chamber. As the process runs, the LED will alternate between a steady illumination and a brief flashing state. This behavior indicates that the pre-pulse AC, the HV pulse(s), and the post-pulse AC parts of the waveform are being delivered sequentially.
- **"Relative Load Indicator", Multi-Color Bar Graph:** This indicator is a series of 10 LED segments that provides an indication of the amount of electrical current flow being delivered to the coaxial chamber during waveform delivery. The number of segments that illuminate is dependent on a number of factors: buffer conductivity, cell concentration, cell solution temperature, the volume of cell solution in the chamber, the sine wave voltage and frequency being applied, and the length of time the wave form is applied. If many segments illuminate, then significant heat and turbulence is being generated in the coaxial chamber, which will have a negative effect on the fusion process. During the factory calibration procedure, the bar graph is set to display seven bars (three green, four yellow) when a 70 V_{pk}, 1 MHz sine wave is delivered to a 47-0020 large-volume fusion chamber Chamber filled with 9 ml of a 100 µS/cm Conductivity Standard at 25°C.

Waveform Generator Interface Software Installation

The Hybrimune waveform generator interface software must be installed on a local computer with these minimum specifications: Windows® 98 or higher, 2Mb free disk space, and at least one RS232 Serial Port. If only USB ports exist, then a USB-to-Serial adapter will be required, and should be installed first. 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. The default file locations are **\Program Files** for the application and **\My Documents\BTX** for protocol and log files.

Set-up using a USB-to-Serial Converter

This system requires an RS-232 interface. However, if only USB ports are available, a converter will be required. Please note that all converters types may not be compatible with the system. The following procedure was developed using the Dynex USB PDA/ Serial Adapter Cable (Model: DX-UBDB9) not currently provided by BTX.

Use the manufacturer's instructions to install the converter's software on the computer. After installation, disconnect the converter from the computer's USB port. Connect the serial cable between the D-Subminiature 9-position (DB9) connector labeled "Computer Control" on the back of the waveform generator and the DB9 connecter on the converter. Turn on the waveform generator. Plug the USB connector from the converter into an available USB port on the computer. The computer should recognize the connection.

System Test

The following is a basic test of the waveform generator system using the preprogrammed protocol **HYBRIMUNE Test.pro** located in the default \ protocol folder. For this initial test, the Chamber Cable and Chamber should be fully connected, but it is not necessary to fill the chamber.

First:

- Plug in the Line/Mains power cord for the waveform generator.
- Turn the Line/Mains Power rocker switch at the lower left front of the front panel to the ON (I) position

What should happen…

The rocker switch, “Power”, “Ready”, and “Chamber Connected” LED’s should illuminate.

Troubleshooting tips:

If the rocker switch fails to illuminate, then return it to the OFF position (O), and:

- Verify that the Line/Mains cord is properly plugged into the receptacle on the back panel and into the source of electric power.
- It may be necessary to check the fuse. See Maintenance section for instructions, proper replacement of the fuse, and the correct fuse type.
- If only the rocker switch and “Power” LED are illuminated, then connect the Chamber Cable and verify that the “Ready” and “Chamber Connected” LEDs turn on.

Then:

Start the Waveform Generator Interface software.

What should happen…

The screen should appear like that as shown in Figure 5. The CommLink window should show **OK**. This display indicates that the computer is communicating with the internal microprocessor of the waveform generator. Additionally, the Electrofusion Chamber window should display **OK**, the High Voltage window should display **OFF**, and the Status window should say **Ready**. The Power Supply Voltmeter window will display 0 volts.

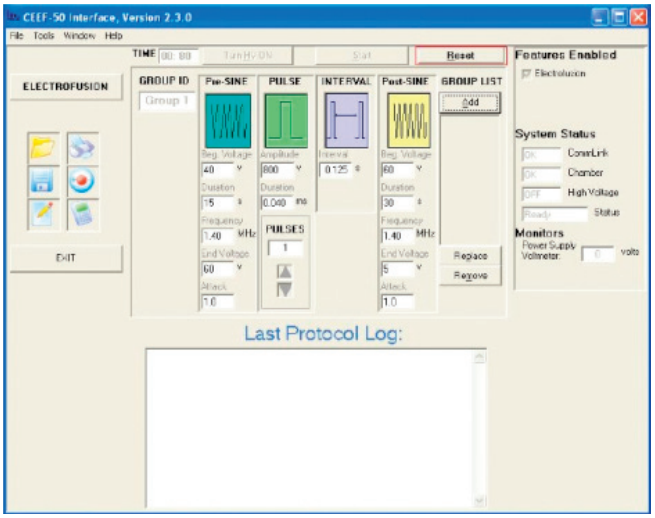


Figure 5: User Interface Application Opening Screen

Troubleshooting tip:

If **ERROR** appears in the CommLink window, then turn off the Waveform Generator and check the serial cable connection to the waveform generator and 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 **HYBRIMUNE Test.pro.**
- Click Turn HV **ON**

What should happen…

The protocol settings are downloaded to the waveform generator microprocessor. The green "Charging Power Supply On" LED will turn on, and the High Voltage window should display On. The internal 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 Voltmeter window will display 500 ± 5 volts.

Then:

- Click Start

What should happen…

The waveform generator runs the protocol. The green "Process Running" LED will illuminate, cycling through its display phases. The "Relative Load Indicator" display will not illuminate since there is no conductive medium in the chamber. A protocol log as shown in Figure 6 on the next page should appear in the Last Protocol Log window.

Delivered Waveform Parameters Subdivided by Group Number Where:

GRP	=	**Pulse Group #**
Dur	=	**AC duration, sec**
Freq	=	**AC Frequency, MHz**
BegV	=	**Start AC Amplitude**
EndV	=	**Stop AC Amplitude, volts**
NUM	=	**Pulse # in Group**
WIDTH	=	**Pulse Duration, msec**
INTVL	=	**Time between pulses, sec**
SetV	=	**Programmed PS Voltage**
MonV	=	**Measured PS Voltage**

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

System Message

Auto-Saved Log Filepath

System Message

Protocol File: HYBRIMUNE TEST.PRO
Date: 1-03-2019-4 7:52:24
System Serial Number: 08-9937HY
Firmware Version: 5.81
Mode: Electrofusion
Delivered Waveform Parameters

>Pre-Sine

>GRP	Dur	Freq	BegV	EndV
>1	5.0	1.4	50	50

>Pulsing

>GRP	NUM	WIDTH	INTVL	SetV	MonV
>1	1	0.100	0.50	500	500
>1	2	0.100	0.50	500	500
>1	3	0.100	0.50	500	500
>1	4	0.100	0.50	500	500
>1	5	0.100	0.50	500	500

>Post-Sine

>GRP	Dur	Freq	BegV	EndV
>1	5.0	1.4	50	50

>Normal Completion
$
Log: C: \My Documents\Harvard Apparatus\Hybrimune\ log\ PulseAgile 2006-06-27 15_52_44.txt

>CPS System v5.81

Figure 6: The Last Protocol Log for the TEST.pro Run

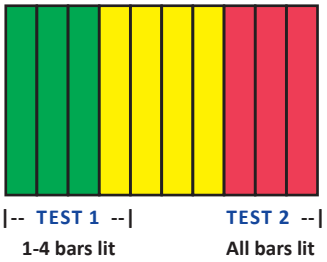
Elementary Verification Tests

There are two elementary tests that can be run to determine if the AC waveforms are operating properly:

Test 1

Fill the 47-0020 large-volume fusion chamber with 9 ml of medium. Allow the medium to reach room temperature (25°C). Set the AC start and stop to 70 volts, the duration to 10 seconds and the frequency to 1 MHz. The LED bar segment display should show between one and four bars. This test shows:

- The cable between waveform generator and chamber is OK.
- The conductivity in the chamber is OK.
- The AC waveform is working properly.



Test 2

Set the AC waveform to start and stop 70 volts, the duration 10 seconds and the frequency to 2 MHz. All bars should be illuminated. This test demonstrates the effects of frequency. Although the conductivity of the medium in the chamber is still the same as Test 1, since the chamber is also a capacitor, at 2 MHz it begins to look like a short circuit to the Wave Form Generator. Therefore a significant amount of current will begin to flow. In general, frequencies above 1 MHz should not be used.

The Waveform Generator Interface Software

The Waveform Generator Interface conforms to standard Microsoft Windows® conventions and this manual assumes that the user is familiar with Windows®. The software performs the following:

- **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 list. Some things to remember:

- 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\BTX\Hybrimune**. 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 pull down menus (top), the Tool buttons (left), the Control Panel for each operating mode (center), and the System Status area (right), see Figure 5.
- When the electrofusion chamber cable is not attached to the system, the Chamber window will display OPEN. As a safety feature, the high voltage power supply cannot be enabled and protocols cannot be run while the system is in this state. However, protocols can be programmed and saved in this state.

Hybrimune™ System Components & Set-Up

The Toolbar

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

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. The user is prompted for a filename.
- **Print Log:** Prints the Last Protocol Log.
- **Exit:** Exits the program.

Tools Pull-down Menu

- **Electric Field Converter:** Application to help convert between absolute voltages and electric field.
- **Standard Calculator:** The Microsoft Windows® calculator.
- **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.
- **Options:** General options to set sound mode, auto save, provide communications check, etc.

Help Pull-down Menu

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

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.

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.
- **Electric Field Converter Button:** Opens the Electric Field Converter application.
- **Calculator Button:** The Microsoft Windows® Calculator.
- **Notepad Button:** Saves the protocol Log under a user specified filename.
- **Exit Button:** Exits the program.

Status Area

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

Features Enabled

This feature shows that there is proper internal connection of the system when the box labeled Electrofusion is checked.

System Status

This section shows the current 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.

Chamber: This box displays whether or not the chamber cable is connected and ready to receive pulses.

OK: The chamber cable is connected; the safety interlock is satisfied.

Open: The chamber cable is not connected; 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. It displays:

Ready: The system 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 chamber.

Monitors

Power Supply Voltmeter: 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.

W. Generator at reset: Displays the system rest voltage.

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.

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.
- **Pre-SINE**

  Beg. Voltage: Beginning voltage

  Duration: Duration in seconds

  Frequency: Frequency in Megahertz

  End Voltage: End voltage

  Ramp k: Non-linear ramp constant
- **Pulse**
- **Amplitude:** Voltage of the electroporation fusion pulse
- **Duration:** Length of the pulse
- **Pulses:** Number of pulses to deliver
- **Interval:** Delay between the start of each pulse
- **Post-Sine**

  Beg. Voltage: Beginning voltage

  Duration: Duration in seconds

  Frequency: Frequency in Megahertz

  End Voltage: End voltage

  Ramp k: Non-linear ramp constant

- **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.

Non-Linear Step Protocols

Non-linear step protocols containing multiple constant-amplitude sine waves are programmed by the concatenation of multiple groups. Typically, the first group would only execute a single pre-SINE parameter set followed by a second group that executes a second pre-SINE parameter set with both fusion pulse and post-SINE healing waveform.

A sine parameter set can be skipped by setting the duration to zero seconds. Likewise, a pulse parameter set can be skipped by setting the number of pulses to zero.

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 (chamber in place), 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 the pulse protocol.
- **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 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.

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 files are saved as plain text and are given the .txt extension.

This completes the set-up and testing of the Hybrimune System. If there is a problem, please contact Technical Support for assistance.

phone: 508.893.8999
website: www.btxonline.com

Programming Waveform Protocols

This section describes various methods to program waveform protocols. As stated previously there generally are three waveform components:

- **Pre-fusion Pulse AC**
- **Fusion Pulse**
- **Post-fusion Pulse AC**

The waveform generator user interface uses the concept of Groups. A group is defined as a set of AC + Pulse +AC waveforms in which the parameters of the first AC, the pulse and the last AC do not change. The one exception is the change in AC amplitude. The AC amplitude can be varies by changing the Ramp k parameter. A group does not have to consist of all thee components, only one of the three is required for a valid group.

- **To inhibit or cause an AC waveform not to appear select zero for the duration**
- **To inhibit or cause the pulse not to appear select zero for the number of pulses**

The software automatically concatenates all of the Groups in the proper order to be executed in series.

The programming of the recommended protocol will be demonstrated.

Programming the Protocol

This protocol requires two groups because the pre fusion pulse AC has two parts:

Note that setting pulse number equal to zero inhibits pulses and setting AC duration to zero inhibits AC.

GROUP 1		
Pre-sine	Start	40 volts peak
	Stop	40 volts peak
	Function	Linear (Ramp k =1)
	Duration	15 seconds
	Frequency	0.8 Mhz
Pulse	Amplitude	set at Group 2 value
	Width	set at Group 2 value
	Interval	set at Group 2 value
	Number	0
Post-sine	Start	set at Group 2 value
	Stop	set at Group 2 value
	Function	set at Group 2 value
	Duration	0
	Frequency	set at Group 2 value

GROUP 2		
Pre-sine	Start	75 volts peak
	Stop	75 volts peak
	Function	Linear (Ramp k =1)
	Duration	20 seconds
	Frequency	0.8 Mhz
Pulse	Amplitude	800 volts
	Width	40 µs (entered as 0.040 milliseconds)
	Interval	0.125 seconds
	Number	1
Post-sine	Start	60 volts peak
	Stop	5 volts peak
	Function	Linear (Ramp k=1)
	Duration	30 seconds
	Frequency	0.8 MHz

To program:

- Enter the values for Group 1 in the user interface
- Click Add
- Enter the values for Group 2 in the user interface
- Click Add
- Save as BTX

This can also be programmed as four groups:

- AC1
- AC2
- Pulse
- AC

Producing Fusion Products Using Hybrimune™

This section describes a protocol to produce cell fusion using the Hybrimune System. This is a starting protocol; every lab has its own procedures which need to be considered. The cleaning procedure and process optimization techniques are also described. The protocol is presented in the following chapters:

The description in this section refers to Hybridoma production. For immunotherapy refer to Trevor (2004).

Cell Preparation Before Electrofusion

Myeloma Cells

Myeloma cells are B cell leukemia cells that have been selected for or engineered to have certain properties. One is that the myeloma should not secrete antibody of its own. Some myeloma cells produce a single heavy or light chain of their own but it is preferable to use one that does not produce either.

Another trait is that the myeloma cells should be susceptible to killing using selected reagents. Ideally, a hybridoma is immortal because the myeloma contributes immortality to the B cell and the B cell contributes resistance to the selection agent. This leaves B cells to die on their own and myeloma cells to die because of the selection agent.

A common selection medium is hypoxanthine-aminopterin-thymidine (HAT) containing medium. Aminopterin blocks the de novo biosynthesis of purines, resulting in inhibition of cell growth. Specifically, the folic acid antagonist aminopterin interferes with the donation of methyl and formyl groups by tetrahydrofolic acid in the early stages of de novo synthesis of glycine, purine nucleoside monophosphates, and thymidine monophosphate. Thus de novo synthesis of purines is blocked. Normal cells, but not myeloma cells, have enzymes that allow production of the necessary nucleotides using purine bases and thymidine (a salvage pathway). Thus the chemicals thymidine and hypoxanthine are provided as source material for the salvage pathway. Myeloma cells do not survive in HAT medium because they lack the salvage pathway enzymes. Fused B cells have limited growth potential and thus die in vitro within two weeks. Myeloma/B cell hybridoma cells survive because they have received salvage pathway enzymes from the B cells and immortality from the myeloma cells.

Early preparation of myeloma cells is relatively simple. The cells should be grown using good sterile technique and the recommended medium for that myeloma. The cells should be in log phase of growth on the day of the fusion. Passage level of the myeloma cells should be monitored. Use low passage level cells when possible. If cultured for extended passage levels, the cells can lose the susceptibility to HAT medium selection. Culture of the myeloma cells in either 8-azaquanine or 6-thioquanine will eliminate cells that are not sensitive to HAT.

Producing Fusion Products Using Hybrimune™

Available Myelomas or Heteromyelomas

Note: This list is not exhaustive. It was made from literature searches and a search of the ATCC and Coriell Institute's web sites. Carefully research intellectual property issues relating to these cells before using them since some of these cell lines may be patented. It does not indicate which cell lines are better suited as fusion partners for the species indicated. It is meant to provide a starting point for finding a good fusion partner.

Human

- U-226AR
- GM1500GTGAL2 (Coriell Institute, GMO4672)
- HuNS1 (ATCC CRL8644)
- SHM-D33 (ATCC CRL-1668)
- KR-12 (ATCC CRL8658): Derived from GM1500

Mouse

IgG secreting mouse myeloma cells:

- MOPC 21
- P3X63AG8 (ATCC #TIB9)

Non-antibody mouse secreting cells (some produce light chains):

- SP2/0-Ag14 (ATCC #CRL 1581)
- NS-1 (ATCC #TIB18)
- P3.X63AG8.653 (ATCC #CRL 1580)
- F0 (ATCC CRL1646)
- S194/5.XXO.BU.1 (ATCC #CRL 1580, TIB20)
- FOX-NY (ATCC #CRL 1732).

Rat

- 210. RCY3.Agl.2.3

Rabbit

- 240E1-1 (Spieker-Polet 1995)

B Cells

Preparation of B cells is more complicated. The general steps are in vivo or in vitro immunization, collection and purification of B cells, and optional activation of B cells. There are many ways to do this series of procedures and all of them will not be covered.

Immunization

One immunization procedure follows. Immunize mice every 3 to 4 weeks. The first immunization can use complete Freund's adjuvant. Subsequent immunizations should employ other adjuvants such as incomplete Freund's adjuvant. Other more modern adjuvants can be used.

Bleed the mice periodically to determine if they are producing polyclonal antibody to the antigen of interest. Three days before harvesting spleen cells, a final boost of antigen without adjuvant can be given intravenously to activate B cells and localize B cells to the spleen.

An alternate immunization procedure is to use DNA vaccines delivered using intradermal inoculation of plasmid plus electroporation. Mice are immunized every 3 to 4 weeks. The immunization can be either a series of DNA vaccines or a DNA prime with protein boost. A final boost is given 3 to 7 days prior to harvest. For details, consult BTX regarding the AgilePulse™ DNA vaccine delivery system.

Screening

Test Bleeds

- Identify mice with ear tags or ear clips.
- Apply grease to tail (an area of 3/4 inch, from base toward tip).
- Use extremely sharp blades to make slice (EM blades). Be careful not to cut through the tail but make cut large enough to draw about 2.5 heparinized capillary tubes.
- Place blood into mouse clot tubes.
- Apply pressure to tail wound to stop the bleeding.
- Spin down the blood at max speed for 5 minutes in the microfuge.
- Draw off and store sera at -20°C until you can test on a blot or ELISA at 1:100 or at 1:50.

Evaluating sera: Use an ELISA or Western Blot to evaluate antibody production in mice.

Harvesting Spleen Cells

- Euthanize immunized mice.
- Immerse mice in alcohol.
- Sterilely collect spleen and place into 10 ml cold DMEM.
- Pour spleens onto Cellector screen.
- With a 25-27 gauge needle, inject 0.5 ml sterile PBS into spleen.
- Tease spleen apart using two syringes with 21 gauge needles. Use the needle tips to cut the spleen into small parts.
- Express spleen cells through 100 mesh sterile screen using Cellector pestle or rubber end of a syringe plunger.
- Drop-wise, flush screen with 7 ml PBS.
- Pipette cells into 15 ml conical tube.
- Let settle for 5 minutes to remove large pieces.
- Collect cells in supernatan.
- Centrifuge 300 Xg for 7 minutes.
- Re-suspend pellet in 5 ml red blood cell lysis buffer (pre-warmed to 37°C).
- Mix well and incubate 3 minutes.
- Add 5 ml PBS.
- Centrifuge cells and wash cells once in complete medium.

In Vitro Activation

Activated B cells fuse to produce more functional antibody producing myelomas than unactivated B cells. The purpose of the intravenous immunization 3 days before harvest is to increase the percent of activated B cells in the spleen. A number of in vitro B cell activation methods are available as alternatives or as supplements to in vivo B cell activation. See references 8-14 for additional methods.

The method for PHA-L stimulation follows:

- Wash spleen cells in complete culture medium.
- Re-suspend cells at 1 million cells/ml in complete growth medium plus 2.4 µg/ml PHA-L.
- Place cells in T-75 flask and incubate at 37°C , 5% CO_2 for 3 days.

Cell Preparation on Electrofusion Day

Medium

BTX Cytofusion Medium must be used for the fusion. Establishing the conductivity and purity of this medium was part of the system design process. The medium was developed over a two year period and has been used in commercial applications since 2003. The medium is produced in the BTX facility under strict quality system including mixing, bottling and labeling. Each batch is full tested for pH, endotoxin, conductivity, sterility and other quality control parameters.

One example of a poor medium would be one containing excessive ions due to the addition of inorganic acids or bases in order to adjust the pH. The presence of the ions will increase conductivity enough to result in excessive heat that will kill the cells.

Cells need to be in a high resistance (low conductivity) medium for several reasons. One is that the cell alignment step, dielectrophoresis, is dependent upon there being a difference between the conductivity of the cell cytoplasm and the conductivity of the medium outside a cell. (See Figure 13, page 34) Second, the high current generated in a highly ionic media will cause excessive heating. The heating will, at first, cause convection currents and disrupt the cell alignment process. If the heating persists, the temperature of the medium will rise to critical levels and kill the cells.

Producing Fusion Products Using Hybrimune™

Chamber Cleaning

The chamber must be clean for two reasons; to minimize the chance for contamination and to eliminate ions that increase conductivity of medium placed in the chamber. (See the chamber cleaning instructions on page 26) There have been many cases where cell fusion has failed due to improperly cleaned chambers.

The chamber comes with two plastic caps. One size fits the top snugly the other loosely. It is a matter of laboratory preference which one to use. However, the caps should be kept on the chamber when they are not in use and must be kept on the chambers when the waveforms are being applied. The chamber electrodes will have as much as 1000 volts between them.

Washes and Other Preparatory Needs

It is important that all tissue culture medium be washed from the cells prior to electrofusion. This requires a minimum of one centrifugation plus two washes in Cytofusion medium. Residual ions from an incomplete wash will dramatically reduce hybridoma production.

For the cell washes, it may be useful to wash the two cell populations separately. This has two advantages. One is that if there is differential cell loss during the washes, compensation can be made during the mixing of the cells after the last wash. For instance, myeloma cells are larger than B cells and may centrifuge differently. Another advantage of combining the cells after the last wash is that the two cell populations may be stained with fluorescent dyes to differentiate the two cells after fusion and to identify fused cells. Stained cells must be washed in separate washes to prevent bleeding of the dye from one population to the other during the washes.

While most labs mix the cells in the last step, others obtain better yields by mixing the cells before the first wash.

If the cells used in the electrofusion are immortalized tissue culture cells (the myeloma cells), the process is most efficient when cells in log growth phase are used. Primary cells may not divide in tissue culture and therefore may not be obtained in log phase.

It is also advisable to do the washes immediately prior to electrofusion. Electrofusion medium is non-toxic but fusion efficiencies have been known to decrease if the total time in the Cytofusion medium (pre and post fusion) exceeds 30 minutes. However, 30 minutes is a sufficient amount of time to complete the process.

Generally cells should be mixed in a 1:1 ratio. Any decrease in the myeloma cells-to-B cell ratio may result in decreased efficiency. If one cell type is particularly rare, such as antigen specific B cells, then more myeloma cells can be added to increase the chance that a B cell will fuse to a myeloma cell rather than another B cell. Keep in mind though that this will reduce the total yield.

Cell Preparation Process

- Harvest cells.
- Optionally mix the cell populations at a ratio of 1:1
- Connect the chamber to the Hybrimune Waveform Generator with the provided cable
- Wash cells 2 times (3 centrifugations) in 20 ml Cytofusion Medium, if the cell pellet is large, a third wash will be needed. It is recommended that the cells be centrifuged at 400 x g for 6 to 8 minutes to assure minimal cell loss during was steps
- Re-suspend the cells in Cytofusion medium at a cell density of 10 million cells/ml or other desired cell density
- Mix the cell populations at a ratio of 1:1 if not already mixed
- Place cells in the electrofusion chamber
- Important: the fusion steps must be performed within 30 seconds (e.g., as soon as possible) of loading the cells into the chamber or cells will settle to the bottom and give poor alignment. If allowed to sit more than 30 seconds, gently pipette the cells to resuspend before proceeding
- Place the cap on the chamber
- Start the previously loaded fusion protocol

Electrofusion

The electrofusion process is done by applying a series of electrical waveforms. These are described in this section. The physics and theory of these processes are described in the Cell Electrofusion Tutorial, pages 29–43. Follow the set-up procedures described on pages 19–22.

Pre-Fusion Pulse AC

The first step is to align the cells in a chain and then compress by increasing the force. This is done by applying a high frequency sine wave with varying amplitude to the cells in Cytofusion Medium immediately prior to applying the fusion pulse. Initially the cells are aligned using a lower amplitude waveform. Once aligned, additional force is applied to compress the cells. This provides intimate contact of the cell membranes for optimum fusion. There are two published approaches for this first step which are presented in the table on page 43.

The optimum process will be dependent on cell type. The user is encouraged to develop protocols for the specific cell line in use. BTX recommends starting with the two level sine wave. Use a low amplitude to align and a high amplitude to compress, Figure 19 Non-linear Step. Reasonable values are:

40 volts peak for 15 seconds at 0.8 Mhz

75 volts peak for 20 seconds at 0.8 Mhz

The 2 ml Optimization Chamber supplied with the Hybrimune System has a clear bottom, see Figure 15. This is so a microscope can be used to observe the cell alignment. Cell compression cannot be observed. The cells should align in an orderly manner and migrate toward the inner electrode. No turbulence or violent movements should be seen. Electrically, the 9 ml chamber and the 2 ml chamber are identical. Protocols optimized using the 2 ml Optimization Chamber can be directly used in the 9 ml Batch Production Chamber.

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

- **High current due to high ion content**

  Dirty electrode

  Contaminated or high conductivity medium

  Inadequate cell wash

- **Excessive force applied to cells**

  AC amplitude too high

  AC amplitude too long

On the front of the Hybrimune voltage waveform generator there is a bar graph labeled “Relative Load Indicator”. It will illuminate bars during waveform delivery depending on a number of factors. See Pages 11 and 14. Generally, the load Indicator should have green and possibly yellow lights illuminated during sine wave delivery. If the indicator shows red bars illuminated then the current is too high and aqueous heating and turbulence can occur.

Fusion Pulse

The next step after aligning and compressing the cells is to apply one or more high voltage pulses to induce pores in adjacent cell membranes followed by fusion of the same adjacent cell membranes. The principles of pore formation for electrofusion are the same as those for pore formation for electroporation. The difference is that in electrofusion, the pores are forming in intimately touching cell membranes. This is a short but essential step in the electrofusion process. An example of an electrofusion pulse used for the Hybrimune System is the application of one 1000 Volt pulse for 40 microseconds.

Post-Fusion Pulse AC

After the fusion pulse has been applied, the cell fusion process starts. The connections between cells are delicate until the fusion process begins to mature. A post pulse AC waveform is applied for 30 seconds to 1 minute after the pulse to hold delicately connected cells together immediately after the pulse.

BTX recommends starting with a decreasing linear ramp, see Figure 19. The start amplitude is lower than the ending amplitude of the pre fusion pulse sine wave amplitude. If 70 was used then start at 50 or 60 volts. End at 5 volts over 30 to 60 seconds. Set frequency to 0.8 Mhz.

Cell Handling After Electrofusion

Immediate Post-Fusion Care of Cells

After the electrofusion process, cells begin to completely fuse. Even though the pores that are responsible for the fusion were formed in a few microseconds, the full fusion of cell membranes takes many minutes. In a practical sense, this fusion maturation occurs over the next 30 minutes. During this time, the cells should be handled gently. The initial pores are very small (nanometers). While the pores are small, they are fragile. As the cell membranes finish their fusion, the fused pores expand to the size of the cell. The following is a recommended procedure:

- Allow the cells to rest 5 minutes undisturbed.
- Harvest cells from the chamber using a sterile disposable transfer pipette.
- Flush chamber with culture medium to remove cells that have settled to bottom.
- Add cells to 50 ml conical tube and fill tube to 40 ml mark with complete culture medium.
- Allow cells to rest an additional 25 minutes.
- Dilute the cells to an appropriate volume for plating.
- The cells can be immediately plated and cultured without washing.

Materials Needed for Post Electrofusion Culture

Cloning Medium 1

- DMEM
- 20% Fetal Bovine Serum
- 10% Origen Supplement
  (Note: this supplement is no longer commercially available. A filtered supernatant from J774A.1 cells can substitute)
- 1% OPI Supplement (Sigma O5003)
- Penicillin-Streptomycin 1 X
- Sodium pyruvate, 110 mg/L
- Glutamax
- 50 µM 2-mercaptoethanol
- HAT diluted to 1X

Cloning Medium 2

- RPMI 1640
- HFCS (50X) diluted to 1X (Roche 11 363 735 001)
- HAT diluted to 1X
- 24 µM 2-mercaptoethanol
- Penicillin-Streptomycin 1 X
- Sodium pyruvate, 110 mg/L
- Glutamax

Complete Growth Medium

- DMEM or RPMI 1640
- 10% Fetal Bovine Serum
- Penicillin-Streptomycin 1 X
- 50 µM 2-mercaptoethanol
- Glutamax
- Sodium pyruvate, 110 mg/L

HAT Stock (50X)

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

HT Stock (50X)

- Hypoxanthine 5 mM
- Thymidine 0.8 mM

Producing Fusion Products Using Hybrimune™

Method, Culture of Cells

Culture of Cells

- Centrifuge cells.
- Select one of the above cloning media.
- Dilute cells to 30,000 C/ml in cell cloning medium plus HAT.
- Mix well and add 100 ml/well to individual wells of a U bottom 96 well plate.
- Incubate 37°C, 5% CO_2.

Feeding Cells

- Optionally, replace half of the medium daily for 4 days (not done by everyone).
- At one week, note wells that are showing signs of metabolism by the medium turning yellow.
- Take 50 ml aliquots of supernatant for antibody screening.
- Re-feed with 150 ml complete medium plus HAT.

Cloning Cells

- Choose selected positive wells.
- Mix cells in the wells.
- Count cells.
- Dilute cells to 3 cells/900 ml (3.3 cells/ml) in cloning medium with HAT.
- Add 100 ml/well to individual wells of a 96-well plate.
- At one week, note wells that are showing signs of metabolism by the medium turning yellow.
- Take 50 ml aliquots of supernatant for antibody screening.
- Re-feed with 150 ml complete medium with HAT medium.

Weaning Clones

- Clones are selected based upon immunoassays.
- Expand clone to T-25 flask.
- After growth in T-25 flask, separate out aliquot for freezing.
- Pass cells in HT medium for one passage.
- Gradually reduce the HT concentration to wean cells off HT.

Methods of Analysis

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

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

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

Demonstration Experiment to Illustrate Turbulence

A simple demonstration can be done in the lab to show what proper cell alignment looks like and what it looks like when it is not working correctly.

- Wash 10 million myeloma cells twice in Cytofusion Medium C.
- Set-up the Hybrimune System.
- Program a 70 V pre-pulse sine wave for a 10 second duration (other parameters can be set at minimum).
- Place the cells in the two ml chamber, place the chamber on an inverted microscope and connect the chamber to the Hybrimune Waveform Generator.
- Click turn on HV Pulse then Start when the start bar becomes active.
- Observe orderly alignment of cells.
- Next, add 50 ml PBS to the cells in the chamber.
- Click turn on HV Pulse then Start when the start bar becomes active.
- Observe turbulence and that the current indicator shows red bars lit.

Optimization

The protocol presented on page 43 is a good starting point for producing hybridomas by electrofusion using the Hybrimune System. The cells from each lab probably have a different optimum.

The factors that most dramatically affect fusion efficiency are:

- Pre-pulse sine wave amplitude and duration
- Pulse voltage and pulse width

The pre-pulse sine wave second step amplitude is 75 volts peak. If there is excessive turbulence, it may be reduced slightly. Decrease the duration until the turbulence cannot be visually detected. Another function such as Ramp k may also improve performance.

Next try decreasing pulse voltages and then increase pulse width in steps of 10 µs.

Unless the post-pulse sine wave is causing excessive movement of the cells, changing that parameter will have minimal effect on the results.

Chamber Cleaning

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

Cleaning Process

The following cleaning process is recommended:

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

Chemical Sterilization

- Fill chamber with 4% sodium hydroxide and soak for 10 minutes.
- Empty chamber and fill with 70% isopropanol or ethanol and soak for 10 minutes.
- Empty chamber and fill chamber with Spor-Klenz® (Steris), soak 10 minutes.
- Rinse thoroughly in sterile reagent grade water.

Spor-Klenz® is a registered trade mark of Steris, www.steris.com.

Other Sterilization

Alternatively, the chambers can be gas sterilized. Do not autoclave the chambers.

Hybrimune™ Electrofusion Protocol Summary

Electrode Cleaning and Sterilization

1. Load 9 ml of 1N NaOH into the chamber, incubate 5 minutes.
2. Dump out the NaOH into the sink and rinse thoroughly with running DI water.
3. Fill and dump the chamber ~10x.
4. While under a sterile hood, aspirate water from the chamber with a plastic pipette.
5. Spray the electrode inside and out with Sporklenz™, rinse with DI water and aspirate.
6. Spray the electrode with 70% ethanol and aspirate.
7. Place plastic cover on chamber until ready to load with cells.

Cell Preparation

1. Prepare and count spleen cells.
2. Count myeloma cells.
3. Centrifuge splenocytes and SP2/0 cells together at a 1:1 ratio or separately at 400 x g for 8 minutes.
4. Resuspend cells 5 to 10 mls of BTX Cytofusion® media.
5. Spin at 400 x g for 8 minutes.
6. Repeat wash.
7. After the second wash, resuspend in less than the expected final volume (resuspend fully by pipetting up and down several times to achieve a single cell suspension).
8. Count the cells, mix the appropriate number of SP2/0 cells with the splenocytes if not done before and dilute cells at a final concentration of 1 x 10^7 cells per ml.

Note: You should perform the fusion within ~10 minutes after the final wash. Suspension in fusion media any longer than 30 minutes will alter the electro-physical properties of the cells and will reduce the fusion efficiency.

Computer Preparation

1. Turn on the computer.
2. Turn on the BTX Hybrimune system.
3. Load the “Hybrimune PulseAgile” program from the “Start” button in Windows (or shortcut from the desktop).
4. Click the “electrofusion” button.
5. Load the optimized protocol for this set of conditions (Found under the “file” menu).

Overall Fusion Summary

1. Sterilize the hood.
2. Clean and sterilize the electrode.
3. Turn on computer and load fusion protocol.
4. Wash and count the cells.
5. Plug the machine into the electrode.
6. Carefully load the resuspended cells into the chamber and place the plastic cover on the chamber.

Note: The following step should be performed within 30 seconds (e.g., as soon as possible) following loading the cells into the chamber or cells may settle to the bottom and give poor alignment. If allowed to sit more than 30 seconds, resuspend the cells before proceeding

7. Click the “Start” button. The fusion process will start, and the computer will run the pre-sine, pulse and post-sine steps.
8. Allow the cells to sit undisturbed for 5 minutes.
9. Gently re-suspend the cells. It is important to get settled cells back into suspension.
10. Transfer the cells to a 50 ml conical tube with a disposable transfer pipette.
11. Gently rinse the chamber with electrofusion media to remove all the cells and transfer these cells to the 50 ml conical tube.
12. Add selection media to fill the tube.
13. Incubate 10 to 20 minutes more minutes to allow fused cells to stabilize.
14. Do not wash or centrifuge the cells prior to plating.
15. Dilute the fused cells to the appropriate volume for plating.
16. Plate the fused cells and incubate at 37°C.

Hybrimune™ Electrofusion Protocol Summary

Staining (Optional QC Step)

1. Dilute a small aliquot of the fused cells 1:2 in complete media.
2. Allow the cells to rest 30 minutes.
3. Spin fused cells onto a slide at 350 RPM for 2 minutes using the cytospin machine method.
4. Allow slide to air dry a couple of minutes.
5. Dunk it into fresh 95% ethanol (5 seconds).
6. Allow to air dry.
7. Place the slide in the Wright's Giemsa for 1 minute.
8. Dunk the slide up and down in the first tube of DI water to remove most of the stain.
9. Repeat the dunking in the second tube of water; keep dunking until most of those little precipitates of stain are no longer on the slide.
10. Knock the side of the slide on paper towels to get most of the water off.
11. Set the slide up so any water and ppt'ed dye will flow off the slide and allow it to dry.

Applications

There are three common uses of electrofusion.

- Hybridoma production as one step in monoclonal antibody production
- Producing therapeutic hybrids for immunotherapy
- Nuclear transfer from one cell cytoplasm to another cell cytoplasm

Hybridoma Production

One step in monoclonal antibody production is producing hybridomas. The following are the basic concepts for making antibody-producing hybridomas:

1. Standard myeloma cells are B-cell cancer cells that are immortal. They have several important modifications. One is that they have been made susceptible to being killed by specific chemicals that do not kill B cells. Another is that they generally do not produce antibody on their own.
2. In humans and animals there are millions of different types of B cells (lymphocytes) with different antibody specificities. They are not immortal. B cells expand in number in response to external events, stimulation by antigen and T cells.
3. A hybridoma is produced by fusing an immortal B-cell line (myeloma) that does not secrete antibody with B-cells that have been extracted from blood. The fused cells take on characteristics of both cells (the myeloma's immortality and the B cell's specific antibody).
4. The resulting hybrid is cultured in a special medium that kills unfused myeloma cells while unfused B cells die naturally over time.
5. The hybrids are then placed in a 96-well plate with a dilution where approximately one antibody secreting hybridoma exists per well. After some growth, antibody is detected in the culture supernatant. Cells from positive wells are cloned.

A figure of merit is the number of productive wells with antibodies and the number of cells required to give, on average, a positive well. The number of functional, antibody-secreting, hybridomas is less than the number of fusions.

An example of E-fusion versus PEG was produced by M. Coccia, Ph.D., Platform Development Group at Medarex, Inc., Milpitas, CA. Transgenic human antibody-producing mice were used in experiments comparing efficiencies of E-fusion to PEG fusion. Each experiment used a portion of the same splenocyte preparation from mice immunized with tetanus toxoid (TT) for comparison of the E-fusion and PEG fusion methods. Results shown are the number of TT antigen-specific clones generated by each method normalized to the same number of cells.

These data are representative of additional experiments (not shown) utilizing four different antigens. Taken together, all experiments showed E-Fusion generated approximately ten-fold more antigen-specific antibody clones relative to PEG fusion.

Experiment Number	Antigen Specific	
	E-Fusion	PEG
1	20	0
2	10	0
3	400	23
4	151	21
Mean	145	11

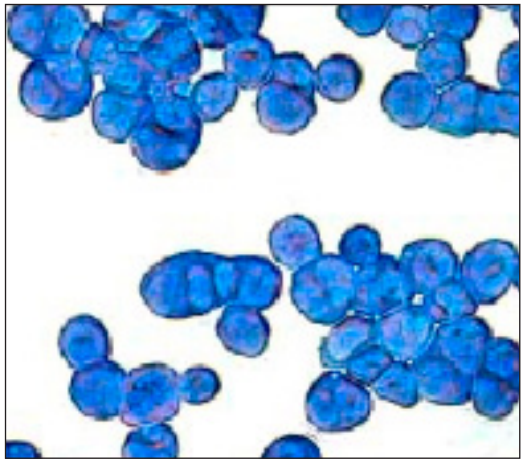


Figure 7: Medarex Fusion Example

Figure 7 shows Cytospin™ prepared and Wrights-Giemsa stained SP2/0 cells 30 minutes following large scale E-fusion. In these E-fusion experiments more than 75% of cells in the final population were fused with 2 to 3 nuclei per cell. Additional Medarex data is presented in Appendix B.

Another process using human B cells is described in Li, et al. *PNAS*. 2006;103(10):3557-3562.

Immunotherapy

Another application is the ex vivo fusion of human dendritic cells and human tumor cells removed from a patient to produce a personal immunotherapeutic vaccine against the tumor. A significant number of experiments were conducted at the Arizona Cancer Center. The results of this research were published in Trevor et al. *Cancer Immunol Immunother.* 2004;53(8):705-714..

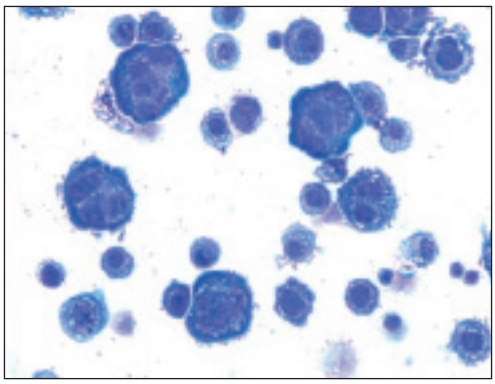


Figure 8: Arizona Cancer Center Fusion Example

Figure 8 shows an electrofusion of dendritic cells with A549 human lung carcinoma cells. Fusion efficiencies of 10% were obtained. Cells were fused at 8 million cells/ml. With that concentration, in a full 9 ml electrofusion chamber, up to 7.2 million therapeutic hybrid cells can be produced. Data provided courtesy of Dr. Katrina Trevor, Arizona Cancer Center.

Figure 9 was produced in the laboratory by fusing an immune system cell and a tumor cell. The image was taken 45 minutes after electrofusion.

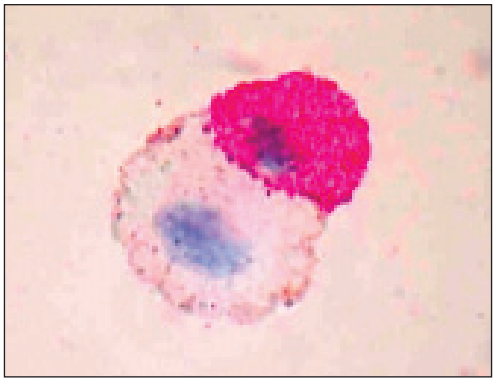


Figure 9: Electrofusion Example

Nuclear Transfer

Nuclear transfer is the transfer of a nucleus from one cell to the cytoplasm of another cell. The donor cell can be either from an embryo or from somatic cells. The recipient cell is usually an enucleated oocyte. The process uses electrofusion in the final steps of nuclear transfer. First a recipient oocyte is enucleated. Second, a nucleus from another cell is inserted in the recipient egg under the zona pellucida and outside of the cell membrane. Finally, electrofusion is used to fuse the nucleus with the recipient cell. The electrofusion step also serves to activate the cell. An image furnished by an unidentified Chinese researcher is presented in Figure 10.

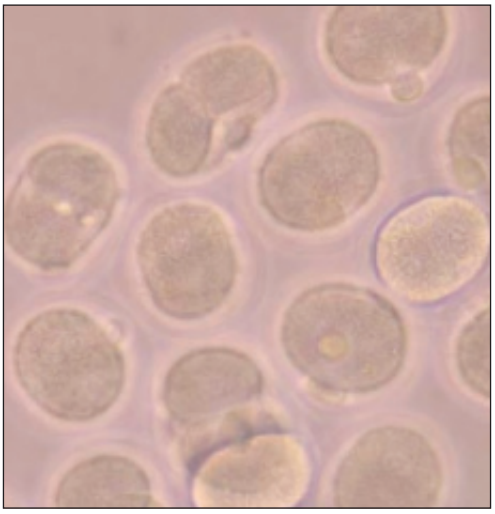


Figure 10: Nuclear Transfer

Cell Electrofusion Tutorial

Cell Fusion Methods

Cell fusion is the formation of a single hybrid cell containing the nuclei and cytoplasm from more than one cell. It is accomplished by:

- Bringing cells together so that they touch
- Compressing the cells to increase contact area
- Disrupting the cell membrane
- Allowing cell membranes to fuse and providing a good environment for growth of cells.

There are several methods to accomplish these steps. The following table summarizes some of these:

Contact	**Fusion**
Centrifuge	PEG
Tissue Culture	Virus
Electrical	**Electrical**
Centrifuge	Electrical
Vacuum	Electrical

Any approach must overcome the natural negative surface charge on a cell. The net charge on a cell as a whole is neutral.

Of these, electrical methods to bring cells into contact and electrical methods to fuse cells are the methods used by the Hybrimune System and also tend to be the most efficient methods.

There are three distinct steps in cell fusion. Each will be discussed in the pages that follow:

1. Pre-fusion process cell processing
2. Electrofusion
3. Post fusion process cell processing

Electrofusion

The process of electrofusion has three steps:

1. **Cell alignment and compression**
2. **Fusion**
3. **Stabilization**

The first step is to align the cells and then compress using special electric fields. The Hybrimune System uses an alignment and compression technique called dielectrophoresis. The process of manipulating matter using electric fields has been discussed for hundreds of years. The process of using non-uniform electric fields has been used for a number of applications in chemistry, physics, biology and engineering for several decades (Pohl, Dielectrophoresis, Cambridge University Press, 1978). Cell electrofusion was described by Zimmerman in a 1982 paper (Zimmerman and Vienken, J. Membrane Biology 67, 165-182,1982).

This section covers the basic physics of dielectrophoresis which includes the chamber in which the cells are fused. Page 37 presents more detailed information on the special waveforms used.

Cell alignment (Dielectrophoresis)

The first step is in electrofusion is to bring the cells together and compress using special electric fields. This is a critical and complex step. Cells have a net zero charge but do have a local negative charge on the surface which acts as a repelling force. The Hybrimune System uses an alignment technique called dielectrophoresis that applies a force to overcome the surface charge. From basic physics we know that the force on a charged particle in an electric field is:

FORCE = ELECTRIC FIELD X CHARGE

Therefore if a cell, which has a net charge of zero, is placed between two parallel flat electrodes which produce a uniform field, the force applied to the cell is zero. However, there are charges inside the cell such as salt ions. A force is applied to these charges and they will move until stopped, in this case by the cell membrane (an insulator) creating a dipole inside the cell. In a uniform field the force applied is equal and opposite and the cell will not move. However, if a non-uniform field is used the forces are not equal and opposite and the cell will move in the direction of the highest field intensity (see Figure 11).

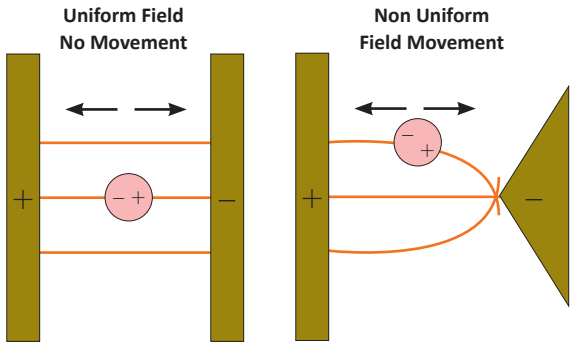


Figure 11: Uniform and Non-Uniform Fields

If there are a number of cells in the non-uniform field, then the cells will line up due to the attraction of unlike charges as shown in Figure 12.

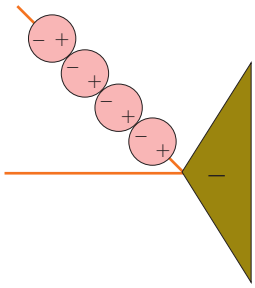


Figure 12: Cell Alignment

The actual force applied is a complex formula, see Pohl, Chapter 4:

$$F_{dep}=r_c^3\ [2\pi\ \varepsilon_{medium}\ K\ (\varepsilon,\sigma,\omega,r)]\nabla E^2$$

The larger the force the faster the cells will move. Although the equation is complex much can be learned by looking at the terms in the equation.

r_c is the radius of the cell

The equation shows that the force is proportional to the radius of the cell cubed. As a practical matter when the radius of the cell gets below 3 or 4 micrometers the force falls below that of other forces and the process becomes ineffective. Thus attempting to electrofuse bacteria is not a productive endeavor. This parameter is also of practical interest when attempting to fuse a large cell with a smaller cell. The forces applied to the large cells are larger causing them to move faster.

THE CELL RADIUS IS NOT UNDER USER CONTROL

ε_{medium} is the permittivity of the medium

Permittivity, also called electric permittivity (ε), is a constant of proportionality that exists between electric displacement and electric field intensity. This constant is equal to approximately 8.85 x 10^{-12} farads per meter (F/m) in free space (a vacuum). In other materials it can be much different, often substantially greater than the free-space value, which is symbolized ε_o. The medium used in electrofusion is close to high purity water which has a permittivity of 80 x ε_o. The permittivity of BTX Cytofusion medium used in the electrofusion process is about 78.5 x ε_o. This term is constant over all conditions specified in this User Manual.

THE PERMITTIVITY IS NOT UNDER USER CONTROL

$$K\ (\varepsilon,\ \sigma,\ \omega,\ r)$$

This term is called the Clausius-Mossotti function. This function represents the time constants of the various ions, medium in the cytoplasm, medium external to the cell, characteristics of the cell membrane and the cell radius. The term w = 2π f where f is the frequency of the applied alignment waveform.

A computer model of this formula is presented in Figure 13.

K IS NOT UNDER USER CONTROL

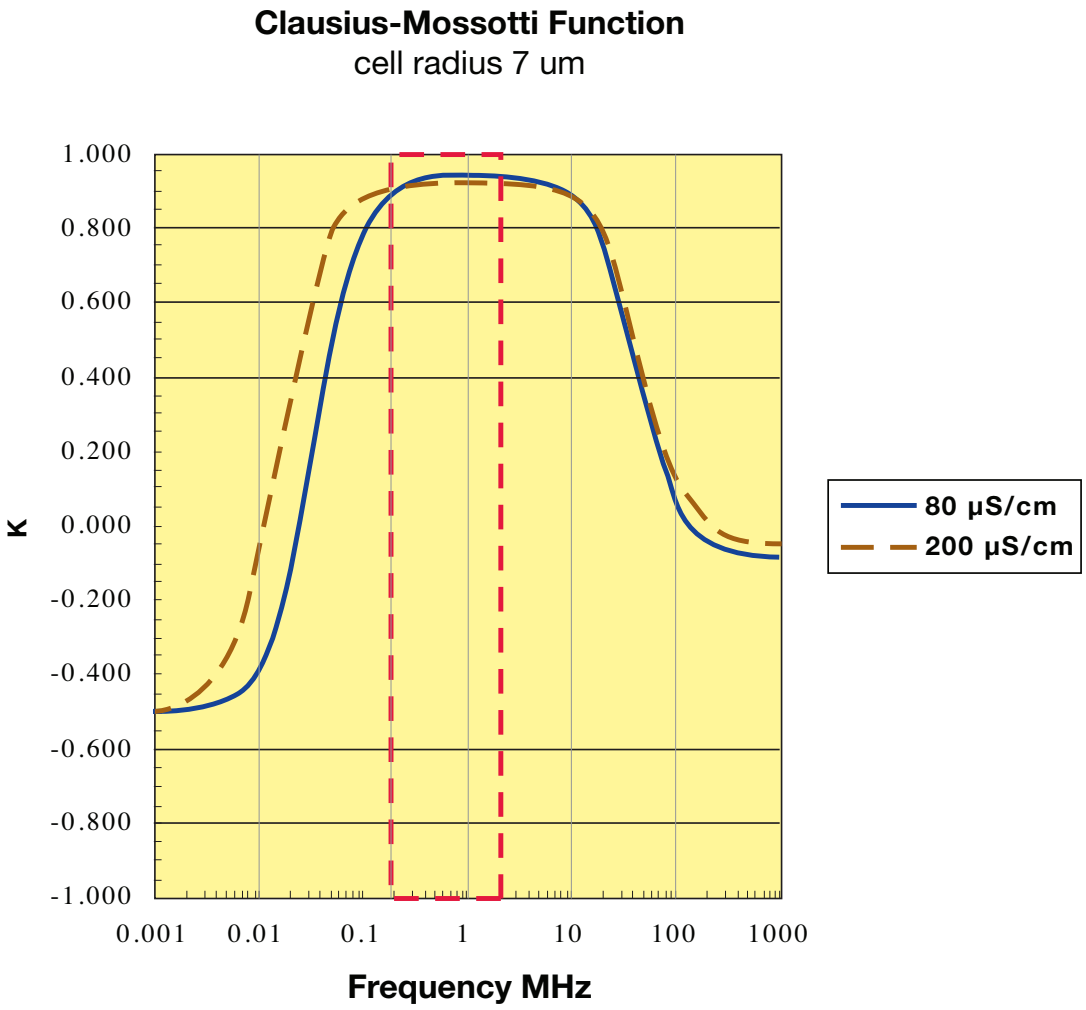


Figure 13: Clausius-Mossotti Function

A cell radius of 7 µm is used in the model which is typical. The model shows, that within typical conductivities of 80 to 200 µS/cm, the Clausius-Mossotti function is generally in the +0.90 to +0.95 range as long as waveform frequencies are kept within the range of 0.2 to 2 MHz (red rectangle). The Hybrimune System including waveform and medium was designed to operate in this range. It should be noted that the nominal conductivity of Cytofusion™ medium is 80 µS/cm. After adding properly washed cells the suspension will generally have a conductivity of 100 to 200 µS/cm.

∇E^2 is the characteristic of the chamber used

The character ∇ is the mathematical operator to differentiate with respect to spatial volume. It is important to note that the E is the electric field vector and the electric field intensity is squared. Thus the force on the cell is always in one direction; the force does not reverse direction when a sine wave voltage waveform goes from plus to minus. If the electric field is uniform then:

$$\nabla E^2 = 0$$

Thus with uniform electric fields, such as those produced in a cuvette (parallel plate), the force is zero. There have been publications claiming to have fused cells in a cuvette.

The geometry of the chamber and the cell radius are the two major elements that determine the force on the cells. Non-uniform fields can be produced in a number of ways: by two parallel wires, by a wire and a plate, etc. The optimum chamber is two coaxial cylinders called a coaxial chamber. The mathematical model of a coaxial chamber is given by the following (see Pohl). Chamber dimension definitions are given in Figure 14:

$$V = V_o \frac{\ln [r_g / b]}{\ln [a / b]}$$

V varies with r_g the radial position from a to b in the chamber gap

$$E_r = \frac{V_o}{r_g \ln [a / b]}$$

E field varies with r_g and is non-linear

$$\nabla E^2 = \frac{-2\, V_o^2}{r_g^{\,3} \ln [a / b]^2}$$

∇E^2 varies with r_g, and force is from the outer to inner electrode

- V_o = **The voltage applied from the inner electrode to the outer electrode**
- a = **The radius of the outside of inner electrode**
- b = **The radius of the inside of the outer electrode**
- a / b = **Always less than 1**
- r_g = **The variable radial distance in between a and b.**

As a / b gets small, the gap gets large and ∇E^2 gets small and V_o must increase to compensate. As a / b approaches 1 the gap gets smaller and ∇E^2 gets large and less voltage is required. However, as the gap decreases so does the volume. Increasing the chamber height to hold volume constant has practical limits. It becomes more difficult to maintain a homogeneous cell suspension.
A simulation of ∇E^2 and the complete force equation and has been used to define the optimum radius of the inner electrode and the outer electrode in the BTX Fusion chamber to provide the optimum combination of parameters to:

1. **Have a quasi-uniform force on the cells in the gap**
2. **Maximize volume**
3. **Minimize voltage required**

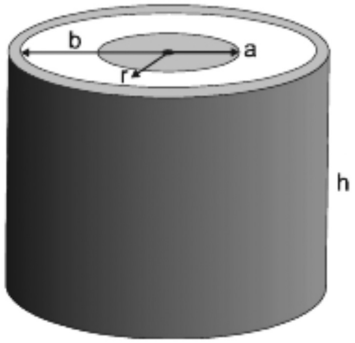


Figure 14: Chamber Definitions

The following is an equivalent presentation:

$$\nabla E^2 = V_o^2 * \frac{-2}{r_g^3 \ln [a/b]^2}$$

This shows the ∇E^2 term has two distinct terms, the first is the amplitude of the AC waveform the second is the geometric configuration of the chamber.

Chamber Geometry

The chamber geometry is fixed by the BTX design. A significant amount of effort was expended to determine an "optimum" dimension. The dimensions of the Fusion chambers are:

Parameter	Optimization Chamber	Batch Production Chamber
Volume	2 ml	9 ml
Outer Inside Radius (b)	22.86 mm	22.86 mm
Inner Outside Radius (a)	19.05 mm	19.05 mm
Gap	3.81 mm	3.81 mm
Well Height	5 mm	18 mm
a/b	0.8333	0.8333

The two chambers are electrically equivalent. As shown in the table above height is not a factor, only the radius of the inner and outer electrodes. An image of cell alignment in the 2 ml Optimization Chamber is presented in Figure 15.

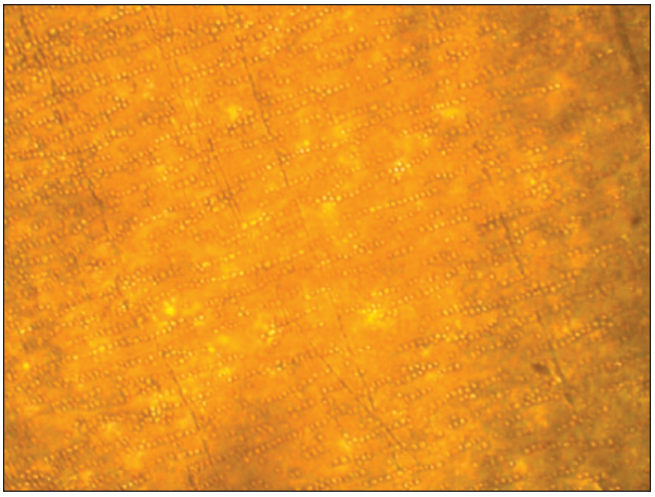


Figure 15: Cell Alignment in Optimization Chamber

The direction of the force on the cells in the fusion chamber is from the outer to inner electrode. The field is non-uniform from outer to inner electrode. The force does not change as a function of chamber height which is a very significant advantage over other electrode configurations.

AC Waveform Amplitude

The AC waveform amplitude and duration are the only variables under user control. The user can control force by changing amplitude. In addition the duration of the AC waveform applies the force for a given period of time which is also under user control. Frequency is available but Figure 13 shows it has little effect. Waveforms for Electrofusion are described in the next section. A graph is presented in Figure 16 which presents the approximate force applied to a cell as a function of cell radius of 5, 10 and 20 µm as a function of AC peak voltage. The AC peak voltage range is that provided by the Hybrimune Waveform Generator. The effect of the cell radius cubed is quite dramatic. This force is on a cell in isolation.

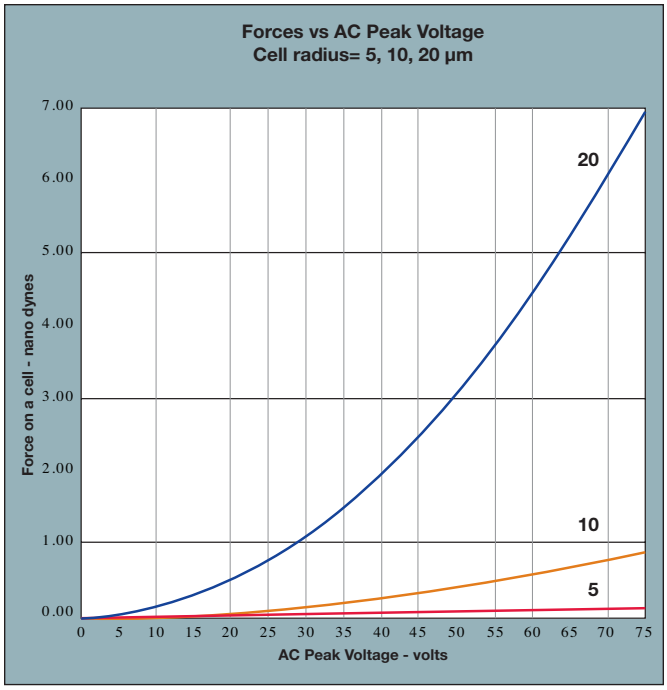


Figure 16: Force on a Cell

In summary, the design of the Hybrimune System has been optimized for electrofusion and many of the variables are fixed by system design such as the chamber configuration and the conductivity of the medium. The one powerful tool available to the user is the selection of the waveform protocol. The waveform protocol sets the force on the cells for alignment and compression, the pulse to fuse the cells, and the waveform to stabilize the fused cells after the pulse. The next section covers the available waveforms.

Waveforms for Electrofusion

The two most important metrics in an electrofusion system for a given cell radius are the chamber configuration and the voltage applied to the chamber to produce the electric field. In the last section the chamber parameters were discussed. In this section the voltage waveform parameters will be discussed.

There are two types of waveforms used, alternating current (AC) in the form of a sine wave and pulsed direct current (DC). The functions of these waveforms in the electrofusion process are:

Function	Waveform Type	Typical[1]
Cell alignment and compression	AC sine wave[2]	Non-linear increasing amplitude
Fusion	DC Pulse	One short pulse
Fusion partner stabilization	AC sine wave[2]	Linear decreasing amplitude

Notes:

1. There have been optimized protocols that use waveforms other than those shown as typical, for example see the Trevor et. al, 2004.
2. There are many types of AC waveforms, rectangular, square, trapezoid, triangle, and sine wave. Hybrimune technology uses sine wave AC waveforms due their smooth amplitude transition.

Cell Alignment and Compression

Pages 32–37 described the method of aligning cells using dielectrophoresis. There were two important parameters, the chamber configuration and the voltage amplitude. The chamber configuration has been optimized and is fixed. However, the voltage parameters can be set by the user. The objective of this step in electrofusion is to align the cells in an optimum manner and then apply the fusion pulse. There are a number of important considerations:

1. A perfect alignment process would alternate the two cell types to be fused. In general that does not occur in any known process. The way the cells align is a statistical event. If the cells all have the same radius and equal numbers of each are mixed, then (see Figure 17):

   XY = 25%
   YX = 25%
   XX = 25%
   YY = 25%

   XY + YX = 50%

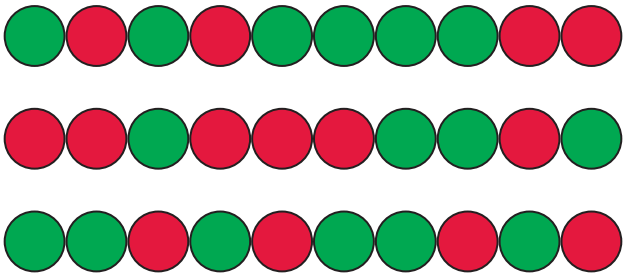


Figure 17: Cell Alignment Sequence

   In addition not all cells fuse in pairs; some products have 3, 4 or more nuclei. This can be controlled but not eliminated by optimization of waveform amplitude.

   A practical result of the stochastic nature of cell alignment is that the maximum yield is obtained when the percent of each cell population is 50% (shown above). However, when 3 cells of X are present for every one cell of Y (75%:25%), then the following yield is obtained:

   YX = 19%
   XY = 19%
   XX = 56%
   YY = 6%

   XY + YX = 38%

   Unequal ratios of cells are sometimes used to conserve a rare cell population. However, when this is done, the total yield decreases.

2. As shown in the equations on pages 32–33, the force on the cell during alignment is a function of the cell radius cubed. The radius of the cell varies within the same type and the mean radius of the two cell types to be fused varies. This will affect the number of fusion products produced.

3. Some cell types are much more difficult to fuse than others. This is inherent in the cell characteristics.

4. In the initial alignment process the cells will accelerate when the force is applied. If the acceleration is significant then, the cells will move toward the center electrode and bounce off like a billiard ball. It is possible to create such acceleration that the cells begin circulating in a “race track” pattern. High acceleration is very detrimental to good cell alignment and must be avoided. The principal use of the BTX 2 ml “Optimization” Chamber is waveform protocol optimization. The alignment process must be optimized while observing the cell alignment.

5. The initial alignment must be done slowly and deliberately so good alignments can be produced. However if a small force is used to align as required then compression will not occur. This still leaves the function of compression to increase the surface contact between adjacent cells to increase the probability of a successful fusion pair. After alignment the cells will be in contact tangentially but not compressed. The solution to this problem is to use a non-linear AC amplitude. The first part of the AC sine wave will be low amplitude and then immediately increase the amplitude to compress while the cells are in contact. The alignment chains are much less likely to break up at this point since they have cells touching on each side. The Hybrimune voltage waveform generator will produce a number of these types of waveforms. These are described below.
6. Care has to be taken with the amount of time the AC sine wave is applied. If left on too long the current will heat up the medium like the heating element in a coffee maker. Heat causes convection currents which in turn causes the chains to break apart.

AC Sine Wave Waveforms

The definition of AC sine wave amplitude and frequency is shown in Figure 18. Electrical engineers use several definitions for amplitude. As shown half of the sine wave is positive and half is negative. The concept of average value is meaningless since that is zero. There are three common definitions in use:

- **Peak Voltage** is the amplitude from zero to the peak positive or negative value.
- **Peak-to-Peak Voltage** is the value from the negative peak to the positive peak.
- **rms Voltage** is square root of the mean value of the sine wave squared, this is the power in the waveform (root mean squared).

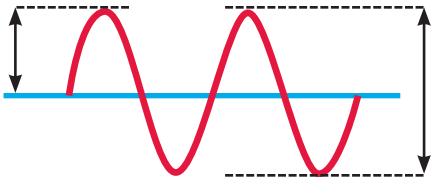


Figure 18: Sine Wave Definition

The Hybrimune voltage waveform generator uses the definition of Peak Voltage or Vpk. The table below shows the relationship between the various definitions. Also included is the standard USA mains voltage. Note: the 120 V (240 in many other countries) rating on appliances is actually rms. The bottom of the table shows that peak-to-peak at the wall socket (power mains) is actually 340 volts.

Vpk-to-pk	Vpk	Vrms
10	5	3.5
20	10	7.1
30	15	10.6
40	20	14.1
50	25	17.7
60	30	21.2
70	35	24.7
80	40	28.3
90	45	31.8
100	50	35.4
110	55	38.9
120	60	42.4
130	65	46.0
140	70	49.5
150	75	53.0
340 (US mains)	170	120
680 (EU mains)	340	240

The voltage range of the Hybrimune voltage waveform generator can be set from 5 to 75 volts peak in 5 volts-peak steps using several algorithms. These algorithms and their names are presented in Figure 19. The method of programming these functions is described on page 18.

Constant Amplitude

This function is the classic electrofusion waveform and has appeared on a number of vendors systems. Generally the amplitude is set and the duration is set. This function is available on the BTX laboratory instrument system.

LINEAR RAMP AMPLITUDE (SET BY RAMP K = 1)

This function appeared on the original Zimmerman system a number of years ago. That system has not been sold for many years. The linear ramp can be set to increase or decrease at a constant rate volts per second by setting the start voltage, the stop voltage and the duration. This function is also available on the BTX Hybrimune Interface Software.

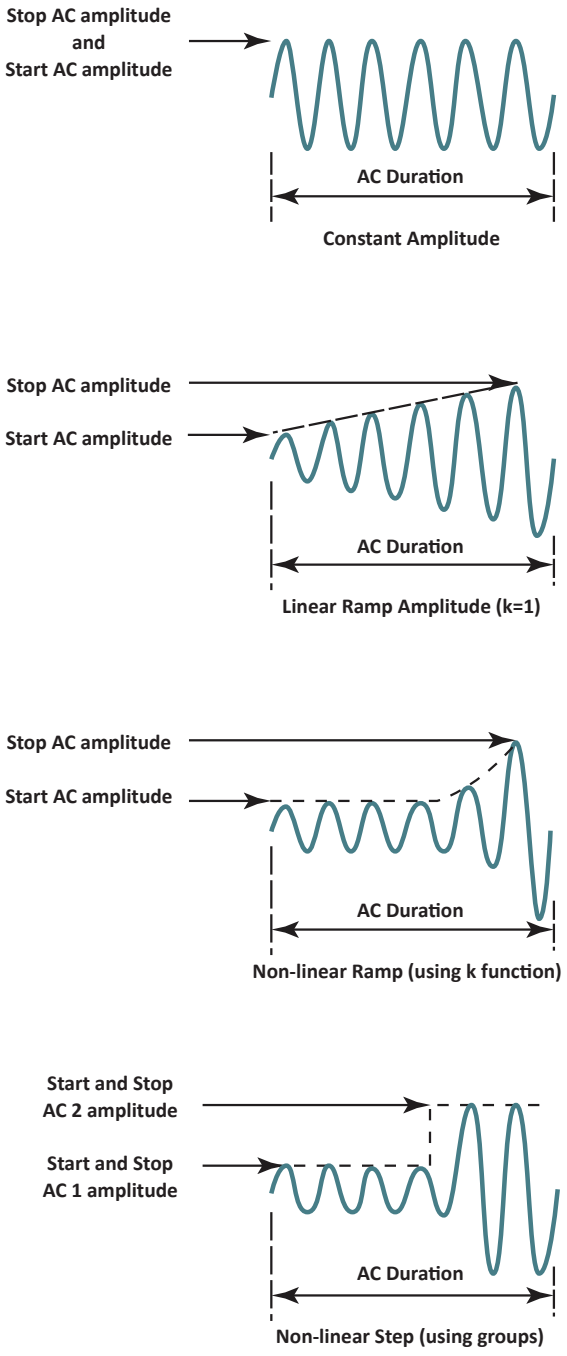


Figure 19: AC Sine Wave Amplitude Functions

Non-Linear Ramp (k) Amplitude

This function is set by programming the start amplitude, the stop amplitude, the duration and the “Ramp k” factor. The “Ramp k” factor determines how fast the amplitude becomes non-linear. These functions are presented in Figure 20 for a Start Amplitude of 20 volts, a Stop Amplitude of 50 volts and duration of 10 seconds.

AC Amplitude = [(t / AC Duration)k x (Stop Amplitude - Start Amplitude)] – Start Amplitude

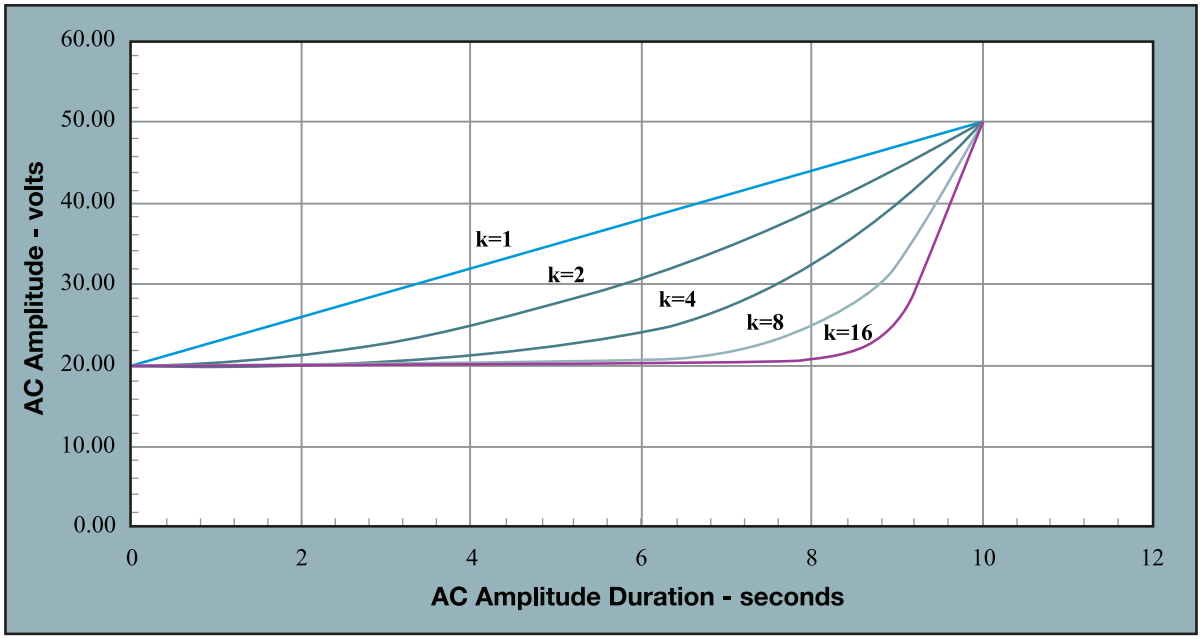


Figure 20: Ramp k Factor in Non-Linear Sine Wave Amplitude

“Ramp k” may be set from 0.1 to 20 in steps of 0.1. This function is used for slow alignment and then compress. If k=1 then the ramp is a constant.

Non-linear Step

This function steps the AC Amplitude instead of continuously changing it. Up to 10 groups or steps can be programmed.

Cell Electrofusion Tutorial

Electrofusion Pulse

The function of this waveform is to form pathways in the cell membranes to allow the cytoplasm to mix and the cells to fuse. One or more high electric field pulses are needed. This process is electroporation and is illustrated in Figure 21. An electric field is applied that is high enough to induce a voltage across the cell membrane that induces pore formation. The minimum transmembrane voltage is approximately one volt. This requires applying electric fields of hundreds of volts/cm to achieve the at least one volt across the cell membrane. Small diameter cells require higher electric fields than do large diameter cells.

Theoretically, the formation of a pore in one cell increases the likelihood that a pore will form in an adjacent cell membrane in the same location as the original pore. This is because pores in cell membranes conduct electricity through the pore.

Whatever the actual mechanism of formation, pores in cells whose membranes are touching can re-seal in one of two ways. One is that the pore will simply close in the cell membrane. Another is that adjacent pores in the membranes of two touching cells will fuse together, combining the cell membranes of two cells.

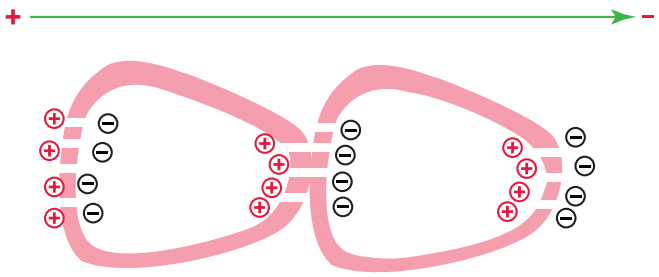


Figure 21: Electric Field Pulse Forms Pathways

Post Fusion Pulse AC Sine Wave

The objective of this function is to hold in place, the cells that have been fused together, to allow them to mature. Immediately stopping the AC sine wave causes the cells to break apart by thermal motion. The same AC Amplitude functions described in Pages 39–41 can be used. Experience has shown that a simple decreasing Linear Ramp Amplitude (k=1) has proven to be the most effective.

Parameter Range of Hybrimune™ Voltage Waveform Generator

The following parameters may be programmed in the waveform generator.

AC Before Fusion Pulse and AC After Fusion Pulse	Amplitude	5 to 75 v peak, 5 volts steps
	Amplitude function	Constant Linear increase or decrease (k=1) Step Ramp k = 0.1 to 20
	Frequency	0.2 to 2.0 MHz in 0.2 MHz steps
	Duration	0 to 60 seconds in 1 second steps Set zero inhibits AC
Fusion Pulse	Amplitude	100 to 1000 volts in 5 volt steps
	Width	20 to 1000 µs in 10 µs steps
	Interval	0.125 seconds in 0.001 second steps
	Number	0 to 10 in steps of 1 0 inhibits pulse

Published Waveform Protocols

Waveforms which have been published are as follows:

Parameter	Medarex Appendix B	Li, J (2006) PNAS	Trevor, et al. (2004) Cancer Imm Immoth
AC Pre-Pulse	40 to 60 volts peak 15 seconds 1.4 MHz	40 volts peak 15 seconds Freq unknown	40 volts peak 30 seconds 0.8 MHz
		75 volts peak 30 seconds	75 volts peak 10 seconds
	Linear	Step	Step
Pulse Amplitude	800 volts	800 volts	800 volts
Pulse Width	40 µs	40 µs	40 µs
Pulse Number	1	1	4
Pulse Interval	NA	NA	0.125 seconds
AC Post-Fusion	60 to 5 volts peak 30 seconds 1.4 MHz	60 volt peak 30 seconds Freq unknown	45 volts peak 55 seconds 0.8 MHz
AC Post-Fusion	Linear	Constant	Constant

BTX recommends the following parameters as a starting protocol in the optimization process.

Waveform	Parameter	Value
AC Before Fusion Pulse	Amplitude-Duration Step function	40 volts peak for 15 seconds 70 volts peak for 20 seconds
	Frequency	0.8 MHz
Fusion Pulse	Amplitude	1000 volts
	Width	40 µs
	Interval	0.125
	Number	1
AC After Fusion Pulse	Amplitude-Duration Linear decreasing	Start 60 volts peak Stop 5 volts peak Duration 30 seconds
	Frequency	0.8 MHz

The method of programming this protocol is shown on Page 18. These waveforms are covered by BTX patents.

Specifications

Waveform Generator	
Pulse In a Group	
Pulse Amplitude	100 to 1000 volts, in 5 volt steps
Pulse Width	20 to 1000 µs, in 10 µs steps
Number of Pulses	0 to 10, in steps of 1
Pulse Interval	0.125 to 10 sec, in 0.001 sec steps
Pre-Pulse AC and Post-Pulse AC In a Group	
Start AC Amplitude	5 to 75 vpk, in 5 volt steps
Stop AC Amplitude	5 to 75 vpk, in 5 volt steps
Frequency	0.2 to 2.0 MHz, in 0.2 MHz steps
Duration	0 to 60 sec in 1 sec steps
Amplitude function (k)	0.1 to 20, in steps of 0.1
Inhibit	
Pre-pulse AC	Set AC Duration = 0
Fusion Pulse	Set Pulse Number = 0
Post-pulse AC	Set AC Duration = 0
Number of Groups (Concatenation)	1 to 10 groups
Maximum Separation Pre-Pulse AC End to Pre-Pulse AC start	1 ms
Maximum Separation Post-Pulse AC End to Post-Pulse AC Start	1 ms
Maximum Separation Pre-Pulse AC End to Pulse Start	10 ms
Maximum Separation Pulse End to Post-Pulse AC Start	20 ms
Electrical and Mechanical	
Current Ratings	2A
Input Voltage Frequency Ratings	100 to 240 VAC, 50/60 Hz
Dimensions (HxWxD)	6.5 x 12 x 16 in, 16.5 x30.5 x 40.6 cm
Weight	12.5 lb, 5.7 Kg
Warranty	2 years
Operating Temperature	4°C to 40°C (40°F to 104°F)
Storage Temperature	-10°C to 70°C (14°F to 158°F)
Operating Humidity	20% to 80% RH, non-condensing
Storage Humidity	20% to 80% RH, non-condensing
Mode of Operation	Continuous
Classification	Class I
Pollution	IP2X
Installation	Category II
Other Specifications	
Supplier Name	BTX
Supplier Address	84 October Hill Rd., Holliston, MA 01746
Supplier Phone Number	508-893-8999
Regulatory Certifications	CE, ETL (UL, CSA), FCC, WEEE, EU& RoHS

Specifications

Chamber		
	Optimization Chamber	**Production Chamber**
Volume	>2 ml	>9 ml
Outer ID	45.72 mm	45.72 mm
Inner OD	38.10 mm	38.10 mm
Gap	3.81 mm	3.81 mm
Well Height	5 mm	18 mm
r1/r2	0.8333	0.8333
Overall Dimensions	13.5 cm x 7.7 cm x 2.2 cm	11 cm x 10 cm x 5 cm
Weight	210 g (7 oz)	270 g (9 oz)
Operating temperature	20 to 30°C	
Storage temperature	0 to 50°C	

Cytofusion Medium Formula C		
Parameter		**Tolerance**
Sterility	Sterile filtered (no growth on random samples using four bacterial test media)	
pH	7.2	±0.2
Conductivity	80 µS/cm	±5 µS/cm
Endotoxin	<0.2 IU/ml	
Osmolarity	270 to 290 milliOsmoles	
Storage	4 to 8°C	
Packaging	500 ml bottle	

Maintenance

The Hybrimune Instrument requires no special maintenance other than keeping it clean.

If the surface of the touch screen display or enclosure needs to be cleaned, use a standard (non-ammonia) glass cleaner or mild detergent with warm water and a soft, lint free paper or cloth towel. Do not apply the cleaning solution directly to the screen or enclosure, to avoid liquid running into other parts of the cabinet. Put a small amount of cleaner on the towel and gently rub the screen or enclosure. Avoid hard rubbing, abrasives, or harsh solvents like alcohol or ammonia.

Fuse Replacement

1. Make sure the power cord is disconnected from the main supply before servicing the fuse.
2. Replace fuse with (250V/2A slo-blo) 5 x 20 mm.

Turn Off power and remove power cord from power module. Use a straight blade screwdriver and follow instructions in figure below.

1. To remove fuse use screwdriver, push in and turn to the left.
2. Remove fuse from cap and replace with new fuse.
3. Return fuse and cap insert into holder, push in and turn to the right

Figure 22: Fuse Replacement

Ordering Information

Item No.	Description	Qty.
Electrofusion Systems		
47-0300N	Hybrimune Electrofusion System	1 Ea
47-0305N	Hybrimune Electrofusion Generator Only	1 Ea
Chambers		
47-0020	9 ml Coaxial Chamber for Production	1 Ea
47-0030	2 ml Coaxial Chamber for Optimization	1 Ea

Item No.	Description	Qty.
Accessories		
47-0001	BTXpress Cytofusion Medium C, 500 ml Volume	1 Ea
47-0302	Hybrimune Chamber Cable	1 Ea
47-0301	Hybrimune User Interface Application Software	1 Ea
Service Offerings		
45-9996	Hybrimune 1 Year Extended Warranty	1 Ea

References

Darveau, A, et al. Efficient preparation of human monoclonal antibody-secreting heterohybridomas using peripheral B lymphocytes cultured in the CD40 system. *J Immunol Meth.* 1993;159:139-143.

Hui, SW, Stenger, DA. Electrofusion of cells: hybridoma production by electrofusion and polyethylene glycol. *Methods Enzymol.* 1993;220:212-227.

James K, Bell GT. Human monoclonal antibody production. Current status and future prospects. *J Immunol Methods*. 1987;100:5-40.

Jaroszeski MJ, Gilbert R, Heller R. Detection and Quantitation of Cell-Cell Electrofusion Products by Flow Cytometry. *AnalBiochem*. 1994;216:271-275.

Köhler G and Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *J Immunol.* 2005 Mar 1;174(5):2453-2455.

Larrick, JW, et al. Characterization of human hybridomas secreting antibody to tetanus toxoid. *Proc Natl Acad Sci USA.* 1993;80:6376-6380.

Lee R. et al. A high-throughput hybridoma selection method using Fluorometric Microvolume Assay Technology. *J Biomol Screen.* 2008;13(3):210-217.

Li J, et al. Human antibodies for immunotherapy development generated via a human B cell hybridoma technology. *Proc Natl Acad Sci U S A.* 2006 Mar;103(10):3557-35662.

Lobito AA, et al. Murine insulin growth factor-like (IGFL) and human IGFL1 proteins are induced in inflammatory skin conditions and bind to a novel tumor necrosis factor receptor family member, IGFLR1. *J Biol Chem*. 2011 May 27;286(21):18969-18981

Lobito AA, et al. An optimized electrofusion-based protocol for generating virus-specific human monoclonal antibodies. *J Immunol Methods.* 2008 Jul;336(2):142-151.

Niedbala WG, Scott DI. A comparison of three methods for production of human hybridomas secreting autoantibodies. *Hybridoma.* 1998;17:299-304.

Neil GA, Zimmerman U. Electrofusion. *Methods Enzymol.* 1993;220:174-196.

O'Shannessy, DJ, et al. Novel antibody probes for the characterization of endosialin/TEM-1. *Oncotarget.* 2016 Oct;7(43):69420-69435.

Schmidt, et al. CD19+ B lymphocytes are the major source of human antibody-secreting hybridomas generated by electrofusion. *J Immunol Methods.* 2001;255:93-102.

Shirahata S, et al. Cell hybridization, hybridomas, and human hybridomas. *Meth Cell Biol.* 1998;57:111-145.

Steenbakkers PG, van Meel FC, Olijve W. A new approach to the generation of human or murine antibody producing hybridomas. *J Immunol Methods.* 1992;152:69-77.

Sugasawara RJ, Cahoon BE, Karu AE. The influence of murine macrophage-conditioned medium on cloning efficiency, antibody synthesis, and growth rate of hybridomas. *J Immunol Methods.* 1985;79(2):263-75.

Trevor K. et al. Generation of dendritic cell-tumor cell hybrids by electrofusion for clinical vaccine application. *Cancer Immunol Immunother.* 2004;53(3):705-714.

Vienken J, Zimmermann U. An improved electrofusion technique for production of mouse hybridoma cells. *FEBS Lett.* 1985;182:278-80.

Zhao Q, et. al. A novel function of CXCL10 in mediating monocyte production of proinflammatory cytokines. *J Leukoc Biol.* 2017 Nov;102(5):1271-1280.

Zimmerman U, et al. Generation of a human monoclonal antibody to hepatitis C virus, JRA1 by activation of peripheral blood lymphocytes and hypo-osmolar electrofusion. *Hum Antibodies Hybridomas.* 1995;6:77-80.

Zuchero, YJY, Chen X, Bien-Ly N, Bumback D. Discovery of novel blood-brain barrier targets to enhance brain uptake of therapeutic antibodies. *Neuron.* 2016 Jan;89(1):70-82.

Appendices

Appendix A: Medarex Data

Hybridoma Production: E-Fusion vs. PEG Medarex Results

The data here was produced by Marco Coccia, Ph.D. in the Platform Development Group, Medarex, Inc., Milpitas, CA using the BTX Hybrimune Commercial Electrofusion System. BTX has permission from Medarex to release this information.

In preparation for the fusion, mice were immunized intraperitoneally or subcutaneously every 2 to 4 weeks with an antigen plus Ribi adjuvant. The mice were bled periodically to determine if an adequate antibody titer had developed. Mice were given an intravenous antigen boost 3 to 4 days before harvesting spleens to activate B cells and increase the number of antigen positive cells located in the spleen. Spleens were collected from adequately immunized mice just prior to the electrofusion.

For the fusion, mouse spleen cells and SP2/O mouse myeloma cells were washed twice in Cytofusion medium. The cells were at a concentration of 10 million cells per ml. A volume of 4.5 ml of each cell suspension was mixed then placed into a 9 ml volume electrofusion chamber. Cells were aligned and fused using the following parameters.

Pre-pulse Sine Wave	40 V to 60 V, 1.4 MHz for 15 seconds
Pulse	Amplitude 800 volts, width 40 ms, one pulse
Post pulse Sine Wave	60 V to 5 V, 1.4 MHz for 30 seconds

After electrofusion, cells were left in the fusion chamber for 30 minutes. Samples of cells from wells were then analyzed by Wrights stain of Cytospin preparations of the samples. The bulk of the cells were cultured in HAT medium. The cells were cultured in 96-well plates at 5000 cells/ml.

Total clones were counted by screening the wells in 96-well plates by eye for hybridoma growth on day 7 to 9. The number of clones was mathematically calculated using a poison distribution analysis.

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

Symbols used:

γ	The immunoglobulin heavy chain for IgG antibody (includes IgG 1-3)
k	One of two immunoglobulin light chains
#γ,k	The number of wells that contained IgG antibody of any specificity (the number of wells with hybridoma clones secreting IgG antibody)
#Ag,γ	The number of wells with hybridoma clones secreting IgG antibody with specificity for the antigen of interest
Ag	antigen, the specific antigen used in the screening
TT	Tetanus toxoid

The table shows the results of 12 experiments. For electrofusion, the average number of wells in the 12 experiments that produced IgG-secreting hybridomas was 542. Of those, 91 bound to the antigen of interest. For the PEG fusions, the average number of wells in the 12 experiments that produced IgG-secreting hybridomas was 58 and 12 of those were antigen specific. This means that on the average electrofusion produced approximately 9 times as many IgG producing hybridomas and 8 times as many antigen specific hybridomas. However, there were many cases where E-fusion produced results and PEG did not.

Appendix B: Medarex Data

Experimental Results					
Experiment	**Ag**	**E-fusion**		**PEG Fusion**	
t		**# ϒ,k**	**# Ag,ϒ**	**# ϒ,k**	**# Ag,ϒ**
1	TT	336	96	ND	ND
2	TT	170	40	ND	ND
3	TT	208	20	0	0
4	TT	1400	10	150	0
5	TT	<1100	<400	83	23
6	TT	582	151	69	21
7	Ag 1	456	65	8	1
8	Ag 2	ND	166	ND	18
9	Ag 3	493	101	128	56
10	Ag 4	71	0	0	0
11	Ag 5	323	0	47	0
12	Ag 5	246	0	36	0

Appendix B: MEDCOAT 2000™ Data

MEDCOAT 2000™ is a commercially available proprietary coating developed for stainless steel medical devices. The coating increases resistance to wear, prevents corrosion, and provides a smooth surface to clean.

Physical Properties	
Wear Resistance	Improved 1400%
Crevice Corrosion	Excellent resistance to corrosion
Microhardness	R_c 72
Composition of Coating	100% Chrome
Embrittlement Relief	Meets the 200-hour requirement
Resistivity	Decreased 25%
Magnetic Characteristics	Permeability reduced 20%
Adhesion to Base Metal	No separation occurred

Biological Compatibility	
USP Class VI Certification	
Acute Systemic Toxicity	No systemic toxicity
Intracutaneous Toxicity	No localized tissue reaction
Surgical Muscle Implantation	No irritation to human tissue
ISO/Tripatite Testing	
Cytotoxicity	Non-toxic to living cells
Rabbit Pyrogen	Non-pyrogenic
Hemolysis	Non-hemolytic
Sensitization	Non-sensitizer
Ames Mutagenicity	Non-mutagenic
Sterilization	No evidence of change following repeated autoclave procedures

Contact Us

US & Canadian Customers

phone 508.893.8999
fax 508.429.5732
e-mail support@hbiosci.com
website www.btxonline.com

International Customers

To locate a distributor
near you, please visit our
website at www.btxonline.com

BTX®

a division of **Harvard Bioscience, Inc.**