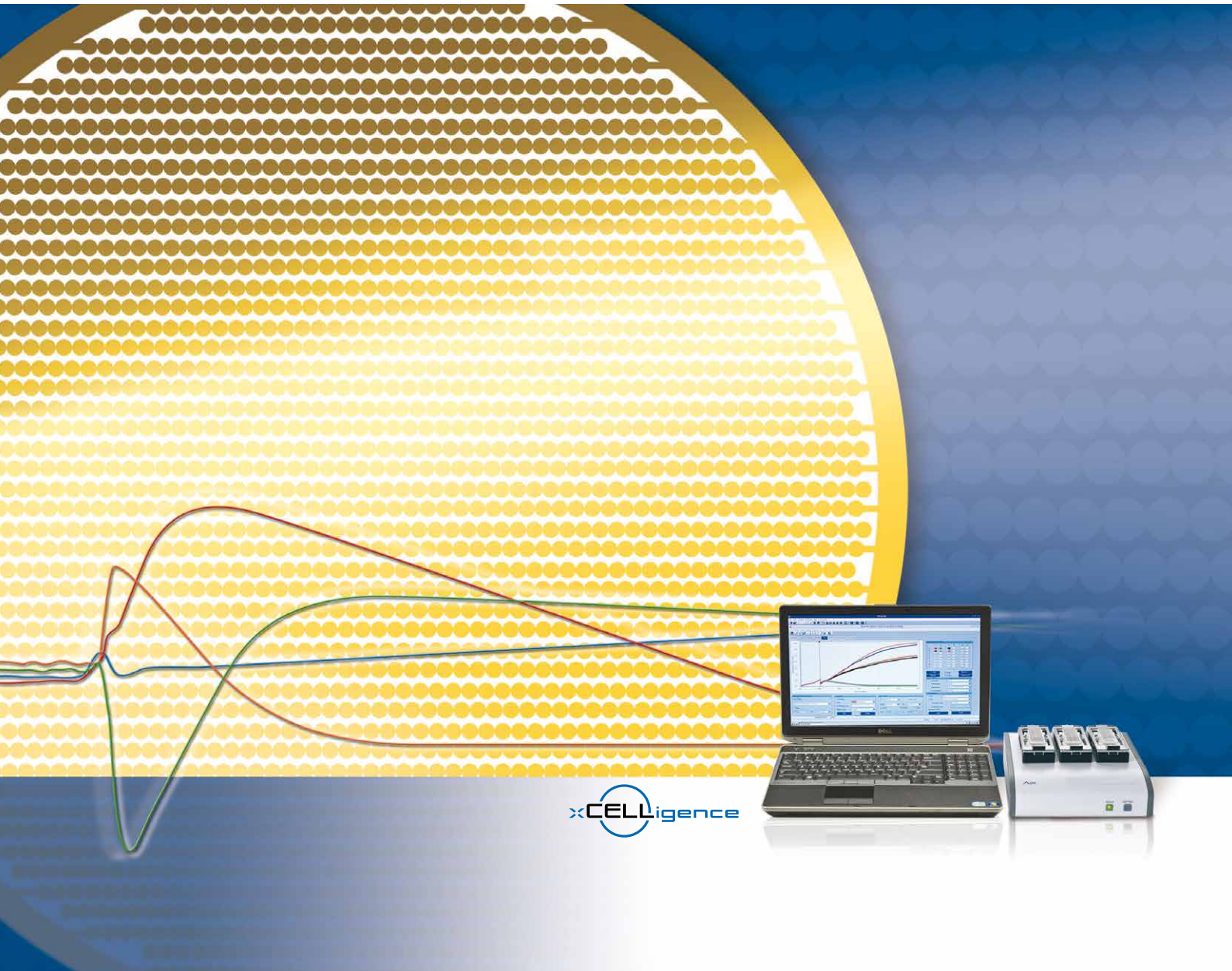
RTCA DP Instrument
Operator's Manual

Version January 2013



Prologue

I. Revision History

Version	Revision Date
1.0	March 2009
2.0 (Inclusion of CIM-Plate 16)	November 2009
3.0	January 2013

Questions or comments regarding the contents of this manual can be directed to the address below or to your ACEA representative.

ACEA Biosciences, Inc.
Customer Support
6779 Mesa Ridge Rd., Suite 100
San Diego, CA 92121
USA

Every effort has been made to ensure that all the information contained in the RTCA DP Instrument Operator's Manual is correct at the time of printing.

However, ACEA Biosciences, Inc. reserves the right to make any changes necessary without notice as part of ongoing product development.

II. Contact Addresses

	Manufacturer	ACEA Bio (Hangzhou) Co, LTD. Building 5, West Lake Technology & Economy Park. 2# Xiyuan 5 Road Sandun, Hangzhou Zhejiang, China, 310030
	Distribution in China	ACEA Bio (Hangzhou) Co, LTD. Building 5, West Lake Technology & Economy Park. 2# Xiyuan 5 Road Sandun, Hangzhou Zhejiang, China, 310030
	Distribution in USA	ACEA Biosciences, Inc. 6779 Mesa Ridge Rd., Suite 100 San Diego, CA 92121 USA
	International Distributors	For a complete listing of international distributors, contact ACEA Biosciences, Inc. or visit www.aceabio.com

III. Declaration of Conformity

CE

The instrument meets the requirements laid down in Council Directive 2004/108/EC relating to "Electromagnetic Compatibility" and Council Directive 2006/95/EC relating to "Low Voltage Equipment". The following standards were applied: IEC/EN 61326 (EMC) and IEC/EN 61010-1 (Safety).

IV. Warranty

Information on warranty conditions are specified in the sales contract. Contact your ACEA representative for further information.

Any unauthorized modification of the instrument invalidates the guarantee and service contract.

V. Trademarks

XCELLIGENCE, E-PLATE and ACEA BIOSCIENCES are registered trademarks of ACEA Biosciences, Inc. in the US.

Other brands or product names are trademarks of their respective holders.

VI. Intended Use

The RTCA (Real-Time Cell Analyzer) DP (Dual Plate) Instrument is intended for label-free, real-time, automated monitoring of cell status in a variety of cell-based assays, using proprietary micro-electronic sensor technology developed by ACEA Biosciences. It can be used for both high throughput screening and research laboratory environments.

The RTCA DP Instrument is intended for life science research and must be used exclusively by laboratory professionals who are trained in laboratory techniques and have studied the instructions for use of this instrument. **The RTCA DP Instrument is not intended for use in diagnostic procedures.**

VII. License Statements for the Instrument

This RTCA DP Instrument is a real-time cell based assay system covered by US patent No.7,192,752, 7,459,303, 7,468,255, 7,470,533, 7,560,269, 7,732,127, 7,867,108, 8,026,080, 8,041,505; European patent applications 03748948.1, 03751801.6 and 04801001.1, 05722991.6, 05786773.1, and other patents and patent applications owned by ACEA Biosciences, Inc..

VIII. Software License Agreement

An envelope containing all license and end user agreements is packaged with the RTCA Control Unit. It contains:

- RTCA Software End User License Agreement
- End User License Agreement for Microsoft Windows 7 Professional
- End User License Agreement for Microsoft Office Home and Business 2010
- Microsoft Office Home and Business 2010 product key
- End User License Agreement for AVG AntiVirus FREE Software

Please comply with all statements made in these documents.

IX. Preamble

The RTCA DP Instrument Operator's Manual has to be used in conjunction with the corresponding RTCA Software Manual. Before setting-up operation of the RTCA DP Instrument, it is important to read this Operator's Manual and the corresponding RTCA Software Manual thoroughly and completely. Failure to observe the instructions contained in this manual could be hazardous.

X. Use of the Instrument Operator's Manual

This Operator's Manual describes the operation of the RTCA DP Instrument and contains the following chapters:

Chapter A Overview contains a short introduction to the operation of the RTCA DP Instrument and describes the instrument's specifications.

Chapter B System Description contains a description of the instrument's components and consumables and instructions on the installation of the RTCA DP Instrument.

Chapter C Operation describes the operating procedures for the RTCA DP Instrument.

Chapter D Maintenance and Care describes the maintenance procedures that are required for the RTCA DP Instrument.

Chapter E Appendix contains trouble shooting and ordering information of the RTCA DP Instrument, accessories and consumables.

XI. Conventions Used in this Manual

Text Conventions

To impart information that is consistent and memorable, the following text conventions are used in this Operator's Manual:

Numbered Listing	Describes the steps in a procedure that must be performed in the order listed.
Italic type, gold	Points to a different related chapter in this Operator's Manual which should be referred to for better understanding.
Italic type	Describes buttons, icons or functions when operating the RTCA Software. In addition, important notes and information notes are shown in italic type.

Symbols

In this Operator's Manual symbols are used as a visual signal to highlight important points.

Symbol	Heading	Description
	WARNING	This symbol is used to indicate that noncompliance with instructions or procedures could lead to physical injury or even death or could cause damage to the instrument.
	BIOHAZARD	This symbol indicates that certain precautions must be taken when working with potentially infectious sample material.
	IMPORTANT NOTE	Information critical to the success of the procedure or use of the product.
	INFORMATION NOTE	Additional information about the current topic or procedure.
▶▶▶		Table continued on next page.
■		End of table.

The following symbols appear on the instrument:

Symbol	Heading	Description
	WEEE	Electrical and electronic equipment marked with this symbol are covered by the European Directive WEEE. The symbol denotes that the equipment must not be disposed of in the municipal waste system.
	BIOHAZARD	This symbol indicates that certain precautions must be taken when working with potentially infectious material.
USB	USB	USB Communications Port

XII. Warnings and Precautions

Handling Requirements

The RTCA DP Instrument must only be used by trained and skilled personnel. It is essential that the following safety information required for installation and operation of the RTCA DP Instrument is carefully read and observed. Please ensure that this safety information is accessible to every employee working with the RTCA DP Instrument.

- Follow all safety instructions printed on or attached to the instrument.
- Observe all general safety precautions applicable to electrical instruments.
- Do not access any electrical parts while the RTCA DP Instrument is connected to the USB port of the RTCA Control Unit.
- Never touch the RTCA USB cable with wet hands.
- Do not open the housing of the RTCA DP Instrument.
- Never clean the instrument first without unplugging the instrument USB cable from a power-on RTCA Control Unit.
- Only authorized service personnel are permitted to perform service operations or repairs required for this instrument.
- Do not open the clamp plate of the RTCA DP Analyzer during measurement as this will cause an open circuit, leading to an error message in the RTCA Software.

For your own safety, please consider all biological material as potentially infectious. Handling and disposal of such material should be performed according to local safety guidelines. Spills should be immediately disinfected with an appropriate disinfectant solution to avoid spreading infection to, and contamination of, laboratory personnel or equipment. When working with potentially infectious material, please do always use protective gloves (powder-free).

- Please refer to the section on *Maintenance and Care* for instructions on cleaning the RTCA DP Instrument.

General Precautions

Microsoft Office and AVG AntiVirus FREE software have been tested and do not interfere with the RTCA Software. No other additional software may be installed on the RTCA Control Unit. Installation of any other additional software on the RTCA Control Unit involves the risk of interference with RTCA Software, and could affect the results or control unit security.

Follow the installation instructions carefully. Keep all potentially flammable or explosive material (for example, anesthetic gas) away from the instrument. Inappropriate location of the instrument can produce incorrect results and damage to the equipment parts. Spraying liquid on electrical parts can cause a short circuit and result in a fire.

Please refer to the section on Maintenance and Care for instructions on cleaning the RTCA DP Instrument.

Keep the cover closed while the instrument is connected to the control unit and do not use sprays in the vicinity of the RTCA DP Instrument. During firefighting operations, disconnect the RTCA DP Instrument from the control unit.

XIII. Disposal of the Instrument

All electrical and electronic products should be disposed of separately from the municipal waste system. Proper disposal of your old appliance prevents potential negative consequences for the environment and human health.

The instrument must be treated as biologically contaminated-hazardous waste. Final disposal must be organized in a way that does not endanger waste handlers. As a rule, such equipment must be sterile before it is passed on for final disposal.

For more information contact your local ACEA Support personnel.

The instrument should also be decontaminated prior to shipping for outside service or repairs.

Components of the RTCA Control Unit such as the computer, monitor, keyboard, etc. which are marked with the crossed-out wheeled bin symbol are covered by the European Directive 2002/96/EC (WEEE).

These items must be disposed of via designated collection facilities appointed by government or local authorities.

For more information about disposal of your old product, please contact your city office, waste disposal service or ACEA Support personnel.

It is left to the discretion of the responsible laboratory organization to determine whether the RTCA Control Unit is contaminated or not. If contaminated, carry out decontamination in the same way as for the RTCA DP Analyzer.

A Overview

1. Introduction

Over 95% of current biological assays are based on optical detection (e.g., fluorescent and optical detection in a fluorescence plate reader, optical microscopy, FACS analyzer, fluorescence scanner). In contrast, the new RTCA DP Instrument developed by ACEA Biosciences is based on electronic detection of biological assay processes, thereby integrating molecular and cell biology with microelectronics.

The presence, absence, or change in properties of cells or molecules affects the passage of electrons and ions on sensor surfaces. Measuring the electronic properties of sensor surfaces provides important information about the biological status of cells (or proteins) present on the sensors. When changes occur in the biological status of cells or proteins, the RTCA DP Instrument automatically measures the corresponding changes in the electronic properties of the sensors near the cells. These analog electronic readout signals are then converted to digital signals for processing and analysis. The major advantages of the RTCA DP Instrument for biological assays over conventional approaches include:

- Label-free detection
- Non-invasive, real-time monitoring
- Automated measurement
- Easy set-up, operation and control
- High information content
- High sensitivity and accuracy
- Broad range of applications

This new microelectronic sensing system represents one of the first integrations of electronic methodology and cell-based assays.

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1.1 RTCA DP Instrument

The new RTCA DP (Dual Plate) Instrument is a proprietary microelectronic biosensor system for cell-based assays. The core of the system is the microelectronic cell sensor arrays that are integrated into the bottom of E-Plate 16 or the bottom of CIM-Plate 16 upper chamber. Measuring the electronic impedance of these sensor electrodes allows changes in cells on the electrodes to be detected and monitored. Cell viability, cell number, cell morphology, and degree of adhesion all affect electrode impedance.

Impedance measured between electrodes in an individual well depends on electrode geometry, ionic concentration in the well and whether cells are attached to the electrodes. In the absence of cells, electrode impedance depends mainly on the ionic environment both at the electrode/solution interface and in the bulk solution. In the presence of cells, cells attached to the electrode sensor surfaces will act as insulators and thereby alter the local ionic environment at the electrode/solution interface, leading to an increase in impedance. Thus, the more the cells there are on the electrodes, the larger the change in electrode impedance (Figure 1).

Furthermore, the impedance change also depends on the extent to which cells attach to the electrodes (Figure 1). For example, cell spreading resulting in a large cell/electrode contact area leads to a large change in impedance.

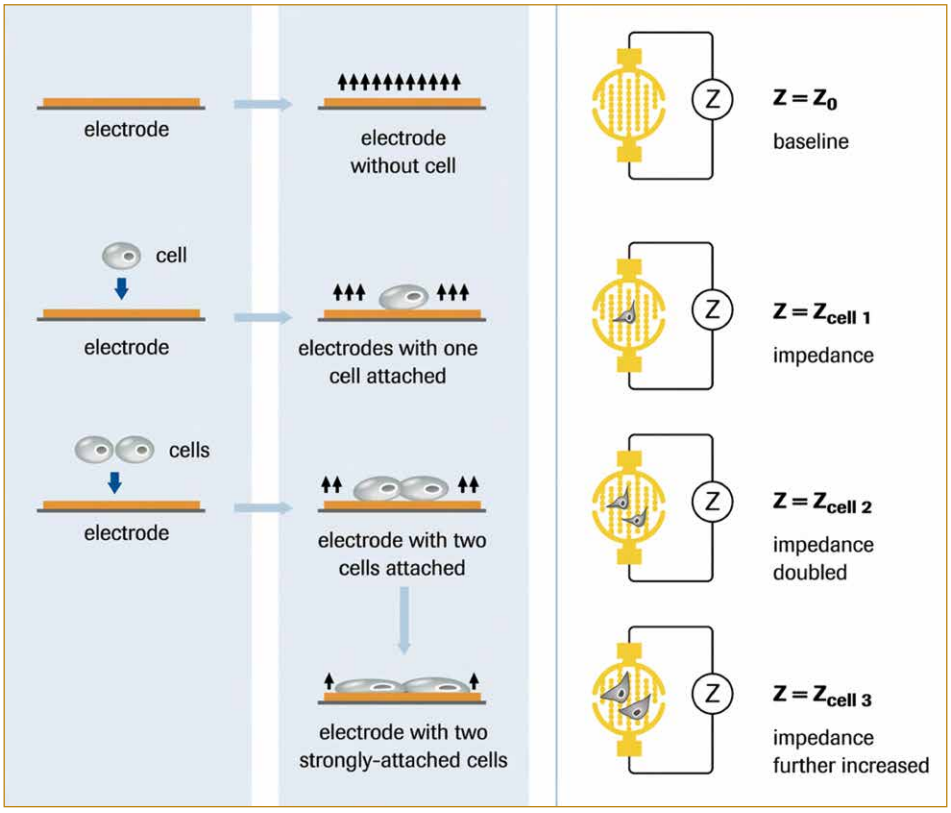

Figure 1: Schematic illustration of the impedance measurement principle.

Derivation of the Cell Index (CI)

A unitless parameter termed Cell Index (CI) is used to measure the relative change in electrical impedance to represent cell status. The CI is a relative and dimensionless value since it represents the impedance change divided by a background value. When there are no cells present in the medium, the sensor's electronic property will not be affected and the impedance will be small as shown in Figure 1 when $Z = Z_0$.

When there are more cells on the electrodes, the impedance will be larger. The CI calculation is based on the following formula: $CI = (Z_i - Z_0)/15\,\varsigma$, where Z_i is the impedance at an individual point of time during the experiment, and Z_0 is the impedance at the start of the experiment. Thus CI is a self-calibrated value derived from the ratio of measured impedances. For more detailed information on how the CI is derived, please refer to the Monitor an Experiment section in the RTCA Software Manual. Several features of the CI are summarized:

- When cells are not present or are not well-adhered on to the electrodes, then the CI is about zero.
- Under the same physiological conditions, when more cells are attached on the electrodes, then the CI values are higher. In this case CI is a quantitative measure of the number of cells present in a well.
- Additionally, a change in cell status, such as cell morphology, cell adhesion or cell viability can lead to a change in CI.

The measurement of impedance is a non-invasive method with respect to the cells, and only a very weak electrical signal is applied to the sensor electrodes. The AC excitation voltage level is in the lower mV range and the resulting current is in the µA range so that individual wells in a plate can be interrogated repeatedly over a wide interval range throughout the experiment. Chemical compounds or other reagents can be added at any time and monitored continuously over hours or days. Unlike "end point" assays, the system allows an investigator to monitor repeatedly over the entire course of the experiment in real time without any interfering with the cell behavior.

The RTCA DP Instrument consists of the following main components (see Figure 2):

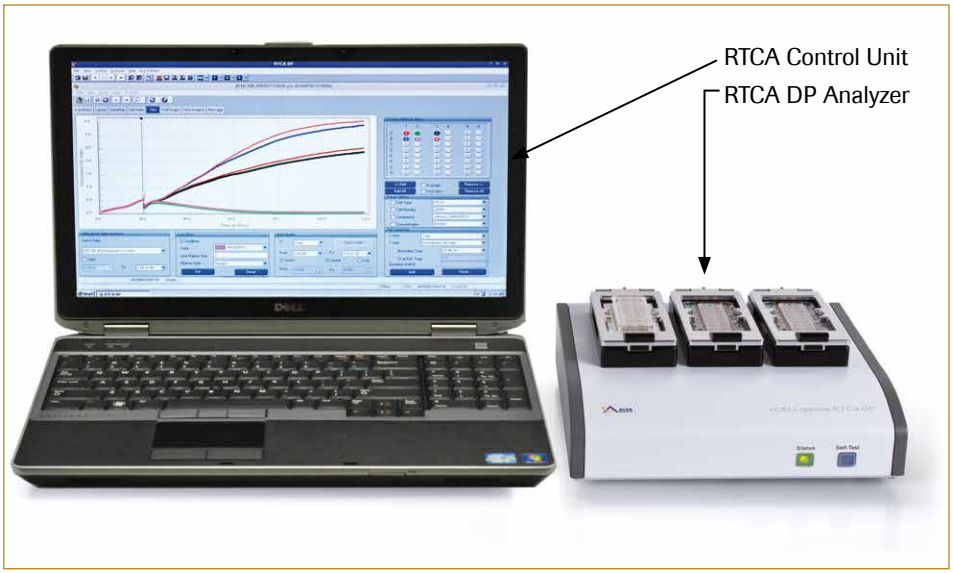

Figure 2: The RTCA DP Instrument with the main components: RTCA DP Analyzer, and RTCA Control Unit.

Cells are introduced into the wells of the E-Plate 16 or CIM-Plate 16, making contact with, and attaching to the sensor-electrode surfaces. The electronic properties of the sensor surfaces are monitored through the RTCA DP Analyzer, which can be placed inside a tissue culture incubator. This is done under the control of the RTCA Software on the RTCA Control Unit, to provide real-time, quantitative information about the biological status of the cells, including cell number, viability, morphology and cytoskeletal dynamics.

The RTCA DP Instrument is applicable for a wide variety of cell-based assay applications, including:

- Cell adhesion and spreading
- Cell proliferation and differentiation
- Compound-mediated cytotoxicity/Apoptosis
- Cell-mediated cytotoxicity
- Receptor-mediated signaling
- Quality control of cells
- Virus-mediated cytopathogenicity
- Cell invasion and migration

The RTCA DP instrument is highly quantitative, with excellent accuracy, precision, and ease of use. The RTCA Software pre-installed in the RTCA Control Unit can be used for simplified experimental design and set-up; it will then direct the instrument to perform the experiment and provide fully automated data acquisition, data analysis, and data presentation.

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1.2 CIM-Plate 16

Cell migration is the orchestrated movement of cells in a particular direction from one area to another generally in response to a chemical signal. Cell migration is a central process in the development and maintenance of multicellular organisms. It plays an important role in diverse physiological and pathological processes including embryonic development, cell differentiation, wound healing, immune response, inflammation, and cancer metastasis. Understanding the cell migration mechanism is of great importance to allow a better understanding of the associated biological and physiological processes. In in vitro experiments, cell migration can be observed as cells migrate from an upper chamber to a lower chamber through a porous membrane.

Cell invasion is a process in which cells invade through a basement membrane. In in vitro cell invasion assays, extracellular membrane protein matrix is prepared and used to coat a microporous membrane. The effective coating procedure occludes the membrane pores and provides a functional barrier similar to the basement membrane in vivo. Such coating blocks the passage of non-invasive cells and allows the invasion and passage of cells with invasive capabilities.

The RTCA DP Instrument employs the CIM-Plate 16 which has an upper chamber with 16 wells, sealed at the bottom with a microporous PET membrane having integrated microelectronic sensors underneath, and one lower chamber with 16 wells, which serves as a reservoir for media and any chemoattractant. Cells migrate through the membrane or through extracellular matrix protein coating and then attach onto the electronic sensors, leading to increase in the impedance signals measured by the electronic sensors. Therefore, cell migration activity can be monitored by using the impedance readouts.

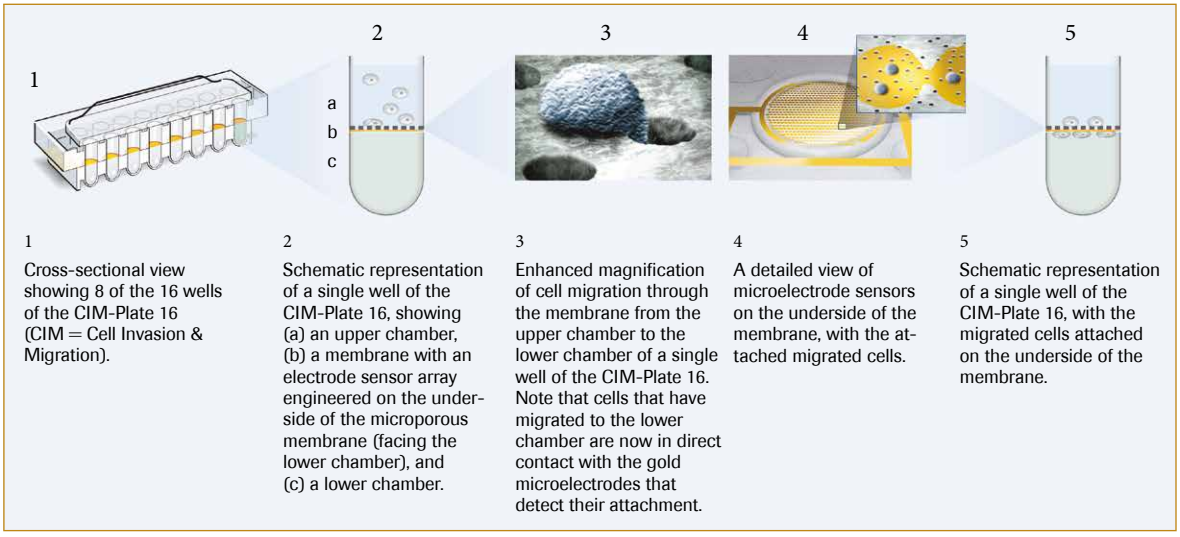

Figure 3. Schematic illustration of the CIM-Plate 16 principle.

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2. Specifications of the Instrument

The RTCA DP Instrument has to be operated at an altitude/pressure of 0 – 2000 m above sea level, 80 – 106 kPa.

2.1 Specifications of the RTCA DP Analyzer

- Dimensions: 24.0 cm x 26.0 cm x 22.5 cm (W x D x H) (fully opened)
- Weight: 4.5 kg
- Electrical Input: +5 VDC, 1 W max
- Test signal: 22 mV rms ± 20% with max. 5 mV DC offset at 10, 25 and 50 kHz
- Impedance Measurement Accuracy: ± (1.5% + 1 Ω)
- Impedance Measurement Repeatability: 0.8%
- Impedance Dynamic Range: 10 Ω to 5 kΩ
- Electronic Switch Resistance: 2 to 5 Ω
- Communication: Virtual RS232 Serial communications at a baud rate of 57600 bits/second
- Environment: Temperature, +15°C to +40°C; relative humidity, 98% maximum without condensation

2.2 Specifications of the RTCA Control Unit

- ≥ 160 GB Hard disk drive
- ≥ 2 GB RAM
- ≥ 256 MB Graphics device

The Windows® Operating System (OS) delivered with the RTCA Control Unit is modified to ensure optimal performance and operation with the RTCA DP Instrument. These modifications restrict certain network capabilities such as sharing folders and printers to the network. However, to facilitate the transport of data to and from the RTCA Control Unit, the following network features remain enabled:

- Mount Network Drives for data exchange
- FTP (File Transfer Protocol)
- Automatically update Virus Definition Files

2.3 Specifications of the E-Plate 16

- Dimension: 4.0 cm × 8.7 cm × 1.96 cm (W × D × H) (with plate cover)
- Spacing: The spacing of the wells for 16-well titer plates is 9 mm center-to-center as per the ANSI/SBS 4-2004 standard
- Volume: 270 µl ± 10 µl
- Bottom Diameter: 5.0 mm ± 0.075 mm
- Electrical Interface: Interface with RTCA DP Analyzer
- Sensor Impedance: 17 Ω ± 5 Ω at 10 kHz, when measured with a 1 × PBS Solution
- Material: Biocompatible surfaces
- UV irradiated
- Environment: Temperature, +15°C to +40°C; relative humidity, 98% maximum without condensation

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2.4 Specifications of the RTCA Resistor Plate 16

- Dimension: 4.0 cm × 8.7 cm × 1.96 cm (W × D × H) (with plate cover)
- Electrical Interface: Interface with RTCA DP Analyzer
- Environment: Temperature, +15°C to +40°C; relative humidity, 98% maximum without condensation
- Resistor values: 37.4, 64.9, 91.0, 115.0 Ω, ± 0.5%

The resistor values specified for the RTCA Resistor Plate 16 are different variables than raw scan data acquired during resistor plate verification. Therefore, they are not comparable to acceptance values listed in chapter Resistor Plate Verification of the RTCA DP Instrument.

2.5 Specifications of the CIM-Plate 16 Upper Chamber

- Dimension: 3.99 cm × 8.69 cm × 0.97 cm (W × D × H) (with membrane)
- Spacing: the spacing of the wells for 16-well titer plates is 9 mm center-to-center per the ANSI/SBS 4-2004 standard
- Volume: 180 µl ± 5 µl
- Membrane: PET membrane with pore size of 8 µm
- Bottom Diameter: 5.0 mm ± 0.075 mm
- Electrical Interface: contact pads with RTCA Contact Pins 16 on RTCA DP Analyzer
- Mechanical Assembly: assembled with CIM-Plate 16 Lower Chamber
- Sensor Impedance: 24 Ω ± 8 Ω at 10 kHz, when measured with a 1× PBS solution
- Material: biocompatible surfaces
- UV irradiated
- Environment: +15˚C to +40˚C, relative humidity: 98% maximum without condensation

2.6 Specifications of the CIM-Plate 16 Lower Chamber

- Dimension: 3.99 cm × 7.50 cm × 1.94 cm (W × D × H)
- Spacing: The spacing of the wells for 16-well titer plates is 9 mm center-to-center per the ANSI/SBS 4-2004 standard
- Volume: 162 µl ± 3 µl
- Top Diameter (O-ring): 6.0 mm ± 0.075 mm
- Mechanical Assembly: assembled with CIM-Plate 16 Upper Chamber
- Material: biocompatible surfaces
- Gamma-ray irradiated
- Environment: +15˚C to +40˚C, relative humidity: 98% maximum without condensation

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2.7 Specifications of the CIM-Plate 16 after Assembly

- Dimension: 3.99 cm × 8.69 cm × 2.60 cm (W × D × H) (with plate cover)

2.8 Specification of the CIM-Plate 16 Assembly Tool

- Dimensions: 18.0 cm × 10.0 cm × 1.5 cm (W × D × H) (with rubber foot)
- Weight: 0.4 kg
- Autoclavable

B System Description

1. System Package

The table below lists all the components required for the RTCA DP Instrument. Check for completeness and inspect packaging prior to installation.

The original shipping boxes must be transferred unopened to the installation site. On delivery, carefully inspect the boxes. It is essential that you report any suspected damage immediately to your local ACEA representative and to the shipping agent before accepting the product.

Use only the original packaging for transportation or relocation of the equipment.

Quantity	Component
1	**RTCA DP Analyzer**
1	RTCA USB Cable Connection between RTCA DP Analyzer and RTCA Control Unit (round, shielded cable)
3	RTCA Resistor Plate 16
10	RTCA Contact Pins 16
1	CIM-Plate 16 Assembly Tool
1	Pliers
1	Brush
1	Dust Blower
1	RTCA Contact Pin Insertion Tool
1	Tweezers
1	**RTCA Control Unit**
1	RTCA Software Package, including: ▶ RTCA DP Instrument Operator's Manual ▶ RTCA DP Instrument Short Guide I & II ▶ RTCA Software Manual ▶ RTCA Software CD
1	Power Adapter and Cables for RTCA Control Unit

B

2. System Description

The RTCA DP Instrument is designed for label-free cell based assays. The instrument integrates microelectronics with cell and molecular biology. The core of the instrument is a microelectronic biosensor array incorporated into each well of standard-sized 16-well microplate devices. This permits the RTCA DP Instrument to measure the activity of living cells in real-time without any labels or reporters. Any number of cellular parameters such as attachment, spreading, growth, death, and even specific morphological changes can be simply and reliably detected with the RTCA DP Instrument.

The RTCA DP Instrument includes the following main components:

- RTCA DP Analyzer
- RTCA Control Unit with RTCA Software preinstalled
- E-Plate 16 or CIM-Plate 16

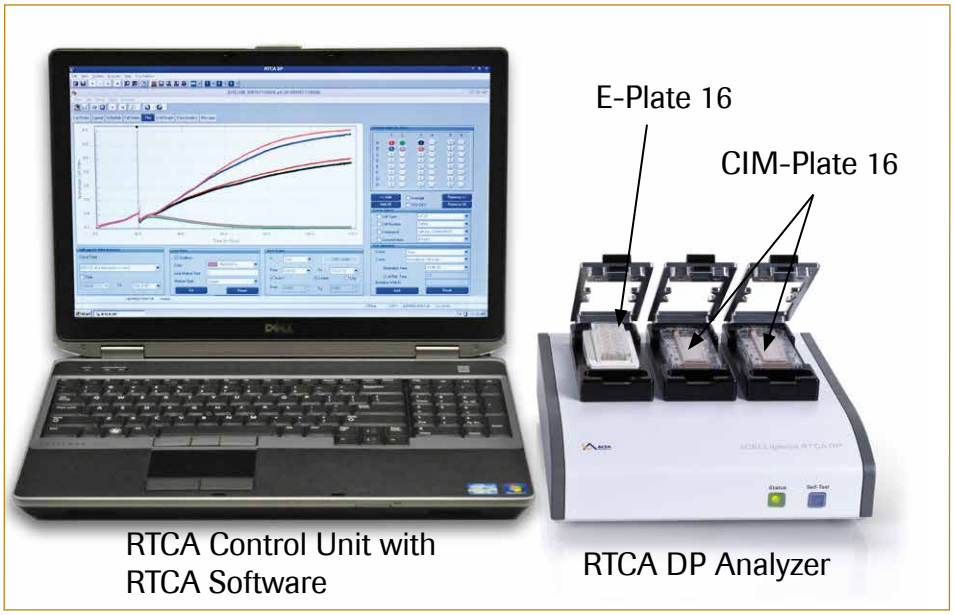

Figure 4: The RTCA DP Instrument.

In addition, several accessories are required to use the RTCA DP Instrument:

- RTCA Resistor Plate 16
- RTCA Contact Pins 16
- Brush and Dust Blower
- Pliers, Insertion Tool and Tweezers
- RTCA USB Cable

2.1 Description of the RTCA DP Analyzer

The RTCA DP Analyzer is an electronic analyzer that can, under the control of RTCA Software, conduct electronic impedance measurement of sensor electrodes at various signal frequencies. The RTCA DP Analyzer is capable of computer-controlled signal generation, processing and analysis, automatic frequency scanning and rapid measurement. The average measurement speed is approx. 4 seconds for an entire 16 well plate, or approx. 250 ms for each individual well.

The RTCA DP Analyzer is placed inside a standard CO_2 incubator and used to connect with up to three different E-Plates 16 or CIM-Plates 16. Appropriately connected precision electronic switches in the RTCA DP Analyzer enable software control or selection of individual electrode sensors within an E-Plate 16 /CIM-Plate 16 to be measured by the RTCA DP Analyzer.

Each cradle pocket (D1, D2, D3) of the RTCA DP Analyzer contains 20 individual, spring-loaded RTCA Contact Pins 16, each for connecting to one of the 20 conductive pads on the back of an E-Plate 16 or CIM-Plate 16.

Figure 5: The RTCA DP Analyzer.

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2.1.1. Interfaces and indicators

The RTCA DP Analyzer communicates with the RTCA Control Unit through one USB port that is designated as a virtual COM port. The communication port is located at the rear panel of the RTCA DP Analyzer.

Figure 6: The RTCA DP Analyzer rear panel.

2.1.2 Keypad and indicators

- Status LED: Solid Green if system is ready, blinks green if communication between RTCA DP Analyzer and RTCA Control Unit is active, and blinks green once if the self-test is passed. Red indicates a system error, such as failed self-test, no device while sweeping error or communication error.
- Self-test button: When pressed, self-test within the RTCA DP Analyzer will be conducted. The status LED will flash green once if the test is passed. The LED is blinking red if the self-test is failed.

Figure 7: The RTCA DP Analyzer front panel.

2.1.3 Cradle Pocket and Clamp Plate

The three cradle pockets and the clamp plates of the RTCA DP Analyzer provide mechanical support and electrical connection for each of the E-Plates 16 or CIM-Plates 16 to the electronic circuits of the RTCA DP Analyzer. Each cradle pocket assembly connects the sensors in each well of the E-Plate 16 or CIM-Plate 16 to the PCB board through the RTCA Contact Pins 16.

2.1.4 RTCA Contact Pins 16

In electronic equipment, a Contact Pin is a device that provides a frequent but temporary electrical connection. Contact Pins are widely used in automatic test equipment (ATEs) for circuit testing. In the RTCA DP Analyzer, the RTCA Contact Pins 16 are essential for ensuring electrical connection between the sensor and the control board.

To ensure good electrical connection, the RTCA Contact Pins 16 need to be cleaned regularly. It is neccessary to clean the RTCA Contact Pins 16 at least every three months. More frequent cleaning may also be required, such as when certain positions show larger contact resistance.

The RTCA Contact Pins can also be replaced. Please refer to the *Maintenance and Care* section of this manual for instructions on the cleaning and replacement of RTCA Contact Pins 16.

Figure 8: RTCA Contact Pins 16.

RTCA DP Analyzer	**Cat. No. 05469759001**
RTCA Contact Pins 16 (20 units)	**Cat. No. 05471575001**

B

2.2 RTCA Control Unit

The RTCA Control Unit consists of a laptop computer (Figure 9). The operating system and all software tools necessary to run the RTCA DP Instrument are already pre-installed.

Figure 9: The RTCA Control Unit.

RTCA Control Unit	Cat. No. 05454417001

2.3 E-Plate 16 and RTCA Resistor Plate 16

E-Plates 16 are single use, disposable devices used for performing cell-based assays on the RTCA DP Instrument. The E-Plate 16 is in many respects similar to commonly used 16-well chamber slide plates. However, each well of the E-Plates 16 has incorporated sensor electrode arrays, so that cells inside each well can be monitored and assayed.

The E-Plate 16 has a low evaporation lid design. The diameter of the bottom of each well is 5.0 mm ± 0.075 mm, permitting a total volume of 270 µl ± 10 µl. Approx. 80% of the bottom surface area of each well are covered by the electrodes. The E-Plate 16 is designed to be used in an environment of +15°C to +40°C, relative humidity 98% maximum without condensation.

Figure 10: The E-Plate 16.

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For system verification purposes, the RTCA Resistor Plate 16 is used with the RTCA DP Instrument as a standard accessory (see section on *Resistor Plate Verification of the RTCA DP Instrument*). The RTCA Resistor Plate 16 has the same size as the E-Plate 16. However, instead of a micro sensor array, an array of resistors is placed at each well position in such a way that it shows resistance values, close to that of an E-Plate 16, when only cell culture media are put into each well.

System functionality can be verified by putting the RTCA Resistor Plate 16 in the RTCA DP Analyzer.

Figure 11: The RTCA Resistor Plate 16.

RTCA Resistor Plate 16		**Cat. No. 05469783001**
E-Plate 16	6 Units	**Cat. No. 05469830001**
	6 × 6 Units	**Cat. No. 05469813001**

2.4 CIM-Plate 16

CIM-Plates 16 are single use, disposable devices used for performing cell invasion and cell migration assays on the RTCA DP Instrument. The CIM-Plate 16 comprises a plate cover, an upper chamber and a lower chamber (Figure 12 and 13). The upper chamber has 16 wells that are sealed at the bottom with a microporous polyethylene terephthalate (PET) membrane containing microfabricated gold electrode arrays on the bottom side of the membrane. The median pore size of this membrane is 8 µm. The lower chamber has 16 wells, each of which serves as a reservoir for media and any chemoattractant for the cells in corresponding upper chamber wells. After addition of media, the lower and upper chamber are easily assembled together with the aid of the CIM-Plate 16 Assembly Tool (Figure 14).

The bottom diameter of each well in both upper and lower chambers is 5.0 mm ± 0.075 mm, permitting a total volume of 162 µl ± 3 µl and 180 µl ± 5 µl (135 µl ± 5 µl are recommended) for lower chamber and upper chamber, respectively. Approximately 80% of the membrane's under-side surface area of each upper chamber well are covered by the electrodes. The CIM-Plate 16 is designed to be used in an environment of +15°C to +40°C, with a relative humidity 98% maximum without condensation.

**Figure 12: Left: CIM-Plate 16 Upper Chamber with a plate cover (with blue dot in the corner of the upper chamber and middle of the plate cover).
Right: Bottom side of the upper chamber.**

Figure 13: CIM-Plate 16 Lower Chamber (with blue dot in the corner).

B

Figure 14: CIM-Plate 16 Assembly Tool.

CIM-Plate 16 Assembly Tool		**Cat. No. 05665841001**
CIM-Plate 16	6 Units	**Cat. No. 05665817001**
	6 × 6 Units	**Cat. No. 05665825001**

2.5 RTCA Software

The RTCA Software provides unparalleled instrument control for flexible experiment setups, data acquisition, and data analysis. All instrument and experiment controls are embedded in the RTCA Software to simplify setup and operation. The Software is used in conjunction with the RTCA DP Analyzer.

For a comprehensive description of the RTCA Software please refer to the RTCA Software Manual.

RTCA Software Package	**Cat. No. 05454433001**

2.6 RTCA Cleaning and Replacement Equipment

The tools for replacing and inserting the RTCA Contact Pins

The Pliers, Tweezers, and RTCA Contact Pin Insertion Tool are used to remove defective Pins from the RTCA DP Analyzer and to insert new Contact Pins. The Contact Pins can be extracted using pliers and then be safely inserted using tweezers and the RTCA Contact Pin Insertion Tool.

Figure 15: The Pliers (upper part), Tweezers and RTCA Contact Pin Insertion Tool (lower part).

RTCA Cleaning Kit

The RTCA Cleaning Kit includes a fiber-free brush to clean the RTCA Contact Pins 16. The dust blower is used to remove dirt particles from the RTCA DP Analyzer or the RTCA Contact Pins 16.

Figure 16: The RTCA Cleaning Kit.

B

3. Installation

3.1 Installation Warnings

Always make sure that the RTCA DP Analyzer is not connected to the RTCA Control Unit through the USB cable before installing or relocating the instrument.

Do not operate the instrument in an environment where potentially damaging liquids or gases are present.

Please do NOT put the RTCA Control Unit in the incubator!

After installing an RTCA DP Analyzer in an incubator, allow at least TWO hours before connecting the RTCA DP Analyzer to the RTCA Control Unit through RTCA USB Cable. This allows the RTCA DP Analyzer to reach equilibrium with the incubator environment and reduces the risk of condensation-related problems.

Do not touch or loosen any screws or parts other than those specifically designated in the instructions. Doing so might affect instrument performance and void the instrument warranty.

3.2 Unpacking

The RTCA DP Analyzer is packed in a specially designed box. Retain the box and packing materials. If the system needs to be returned for repair, it should be returned in the packaging provided. It is essential that you report any suspected damage immediately to ACEA and to the shipping agent before accepting the product.

The RTCA DP Analyzer weighs approximately 4.5 kg and should be lifted with care, taking proper precautions to avoid injury or damage to the system.

After examining the box, place it on a flat surface in the upright position. Open the top of the box and remove the top packing material covering the RTCA DP Analyzer. Lift the RTCA DP Analyzer up and out of the shipping box and remove the other two boxes containing USB Cable/RTCA Resistor Plates and tool sets from the shipping box. Set the instrument down carefully.

3.3 Space and Power Requirements

The RTCA DP Instrument is designed as a very low-power consumption system with USB cable connection. The total power consumption of the RTCA DP Analyzer is generally less than 1 Watt.

The RTCA DP Analyzer should be placed inside a tissue culture incubator in the middle shelf. Do not place anything within 15 cm of the side side panels of the RTCA DP Analyzer. Failure to observe this may result in overheating of the equipment and increase the risk of fire.

To facilitate easy installation and access to the RTCA DP Analyzer, the recommended space requirement inside the incubator is larger than 35 cm × 40 cm × 30 cm (W × D × H).

The shelves inside tissue culture incubator contain holes. Such holes should be left "uncovered". Sealing these holes may affect temperature distribution within the incubator and may also result in overheating of the RTCA DP Analyzer.

3.4 Environmental Requirements

The RTCA DP Analyzer is designed to be used in a tissue/cell culturing incubator with 98% maximum humidity without condensation or in a standard laboratory environment. Failure to follow the environmental requirements may reduce the operating life or cause damage to the instruments.

The humidity in the incubator must be set at such a level that at no time condensation may occur within the incubator. Condensation within the incubator may adversely affect the performance of the RTCA DP Analyzer or cause damage to the sensitive measurement circuitry.

B

3.5 Installation of the RTCA DP Instrument

This chapter describes the installation of the RTCA DP Instrument. A schematic presentation showing how to connect each component is provided in Figure 17.

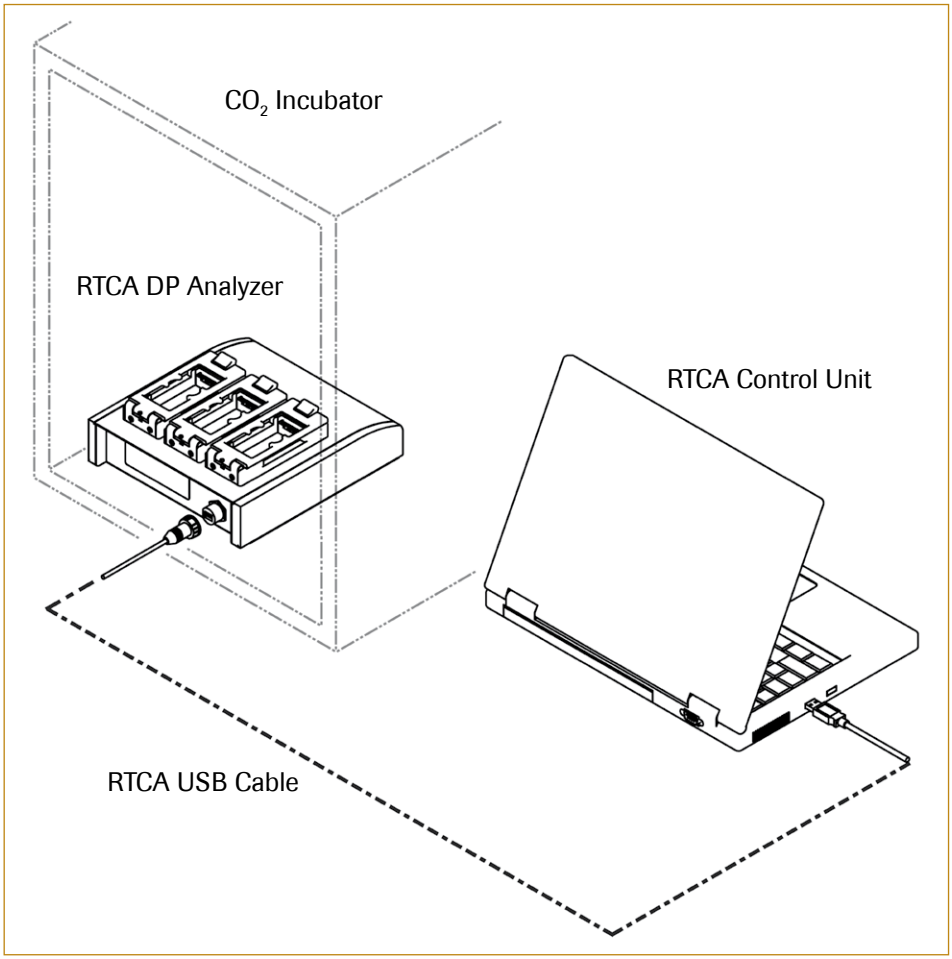


Figure 17: RTCA DP Instrument installation diagram.

B

Remove instruments from boxes

1. Remove RTCA DP Analyzer from the box.
2. Remove RTCA Control Unit and RTCA Software Package from the box.

Attach the power cord to RTCA Control Unit

3. Attach the DC power plug of the AC adaptor (with attached power cord) to the RTCA Control Unit and connect the power cord to an AC outlet.

Place the RTCA DP Analyzer into an incubator

4. Wipe the RTCA DP Analyzer with tissue paper or a soft cloth slightly soaked with 80% ethanol or another sterilizing agent.

   Avoid spraying any sterilizing agent directly on the RTCA DP Analyzer, except Contact Pin cleaning (see Cleaning and Exchanging the RTCA Contact Pins 16)!

   Ideally, before placing the RTCA DP Analyzer inside an incubator, it is recommended to set up the RTCA DP Instrument outside the incubator using a procedure similar to that being described here in section 3.5, and to perform a Resistor Plate Verification as described in section 3.6.

5. Plug the RTCA USB cable into the back of the RTCA DP Analyzer, tighten the screw on the cable, then place the RTCA DP Analyzer in the incubator and run the RTCA USB cable from the RTCA DP Analyzer to the outside of the incubator, making sure that the incubator door still closes.
6. Wait for two hours to let the RTCA DP Analyzer reach an equilibrium temperature without condensation.

Connect the RTCA DP Analyzer to the RTCA Control Unit and Setup RTCA DP Analyzer on the RTCA Control Unit

7. Turn on the RTCA Control Unit.

   *Login as "**ADMINISTRATOR**" as described below:*

   - *Click "Shut Down".*
   - *Select "Log off RTCAOperator".*
   - *Keep the SHIFT key pressed, until the login dialog window is displayed.*
   - *Login as "ADMINSTRATOR" in the login dialog window*

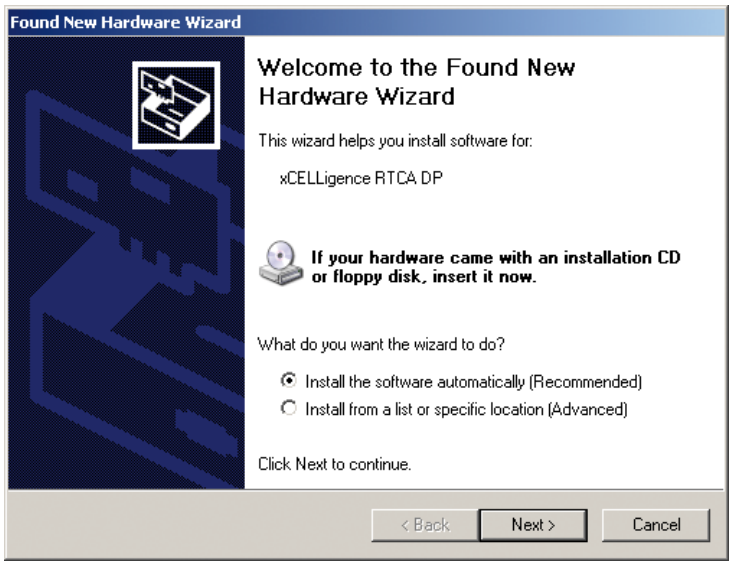
8. Plug the other end of the RTCA USB cable into a USB port on the Control Unit.

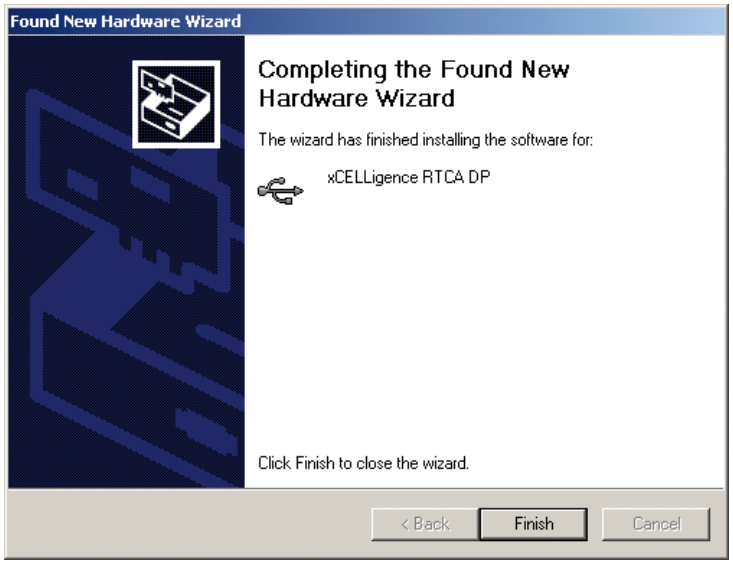
   To avoid condensation, it is important to wait for at least TWO HOURS (after placing RTCA DP Analyzer inside the incubator) before connecting RTCA USB Cable to the RTCA Control Unit or running any experiments.

▶▶▶

B

9 Install the xCELLigence RTCA DP driver software automatically: In the "**Found New Hardware Wizard**" window, click "Next" and then "Finish", as shown in the two screenshots below.

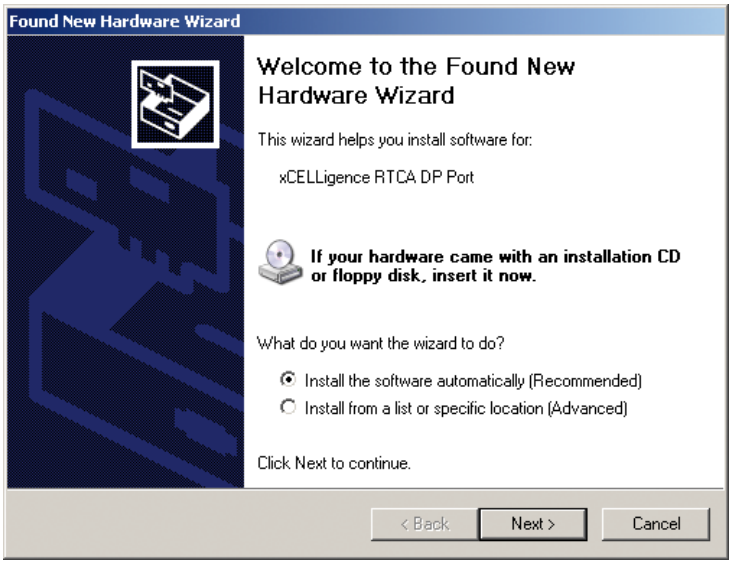


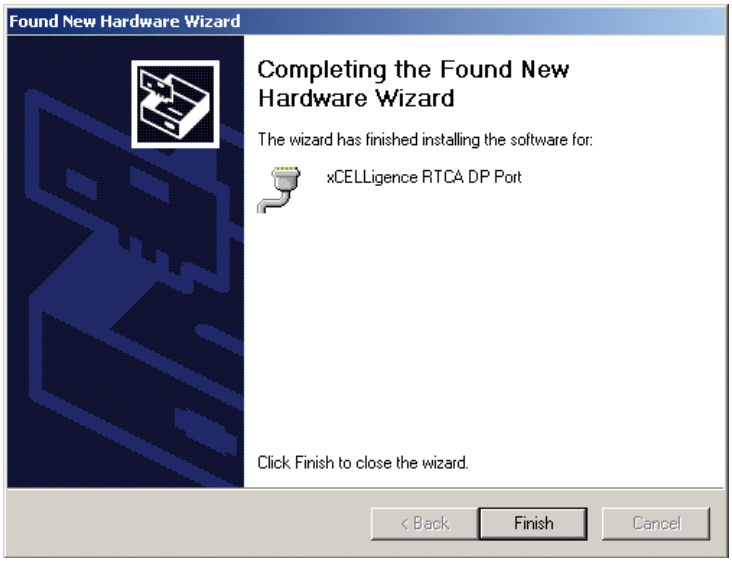


B

10 Next install the xCELLigence RTCA DP Port automatically:

In the “Found New Hardware Wizard” window, click “Next” and then “Finish”, as shown in the two screenshots below.





11 Restart the Windows Operating system to finalize the RTCA Analyzer installation.

Windows will restart and login default to “RTCAOperator” as user.

The LED on the front panel of the RTCA DP Analyzer should be green.

The RTCA DP Analyzer is ready to run an assay.

The RTCA DP Analyzer is pre-programmed and ready to communicate with the RTCA Software.

There is one button on the front panel of the RTCA DP Analyzer which is used to perform a self-test of the instrument. A red LED will blink if the RTCA DP Analyzer fails the self-test. In this case, please contact ACEA support.

B

3.6 Resistor Plate Verification of the RTCA DP Instrument

Resistor Plate Verification is a very important step to verify the installation and the functionality of the RTCA DP Instrument. The verification process can be done outside of the incubator at room temperature. Ideally, a Resistor Plate Verification should be performed before and after the RTCA DP Analyzer is put in an incubator. This ensures that the installation is correct, and that no condensation is affecting the RTCA DP Analyzer after it is placed inside the incubator.

While a TWO hour equilibrium time is needed when the RTCA DP Analyzer is first put into an incubator, resistor plate verification outside the incubator does not require this waiting period.

Start the RTCA Software

1. Having connected the cables and installed the system correctly, start the RTCA Software by double clicking the RTCA Software icon on the desktop.

2. If the program is already open in the OFFLINE mode (as indicated on the bottom status bar on the RTCA program window), or open in the ONLINE mode but no experiment is running, close the program and then reopen it.

   Set up the *Exp Notes, Layout* (Turning on all the wells, right click on the *Layout* page, select all wells, then click *APPLY* button; Figure 18), and *Schedule* Pages (click *Add a Step*, set 10 sweeps with an interval of 30 sec; Figure 19). *Save* the experiment.

   RTCA DP Analyzer can be operated at different experiment patterns varying from a single experiment running three plates simultaneously to three separate experiments each running a single plate. For the Resistor Plate Verification described here, an experiment operating three RTCA Resistor Plates 16 is shown for illustration purposes.

   For more information about setup, data format and storage, please refer to the RTCA Software Manual.

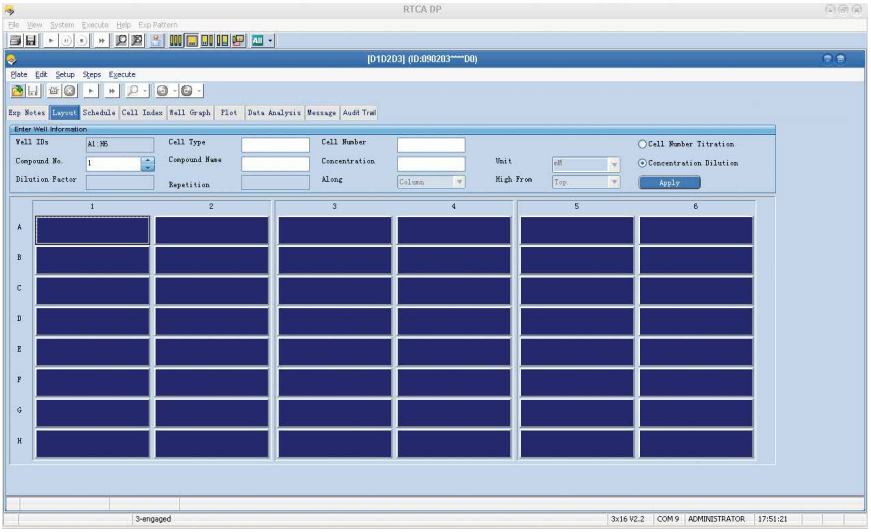


Figure 18: Setup of the *Layout* page for Resistor Plate Verification.

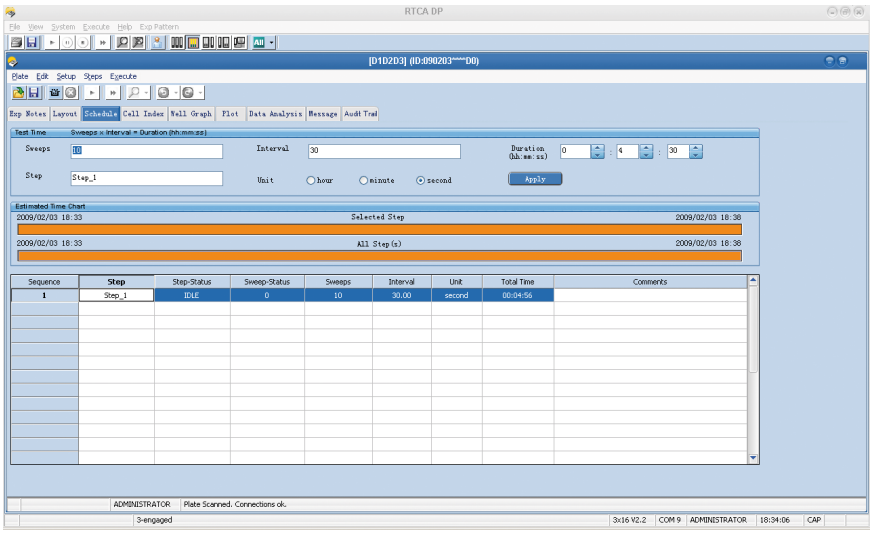


Figure 19: Setup of the *Schedule* page for Resistor Plate Verification.

B

Place the RTCA Resistor Plate 16 in the RTCA DP Analyzer

3 Lift the clamp plate of the RTCA DP Analyzer with one hand, insert one RTCA Resistor Plate 16 (with plate cover) in the cradle pocket of the RTCA DP Analyzer (Figure 20). Ensure that the angled corners are inserted first and that the plate is fully inside the cradle pocket.

Figure 20: Insert the RTCA Resistor Plate 16 and lock the clamp plate in position.

4 With the thumb pressing the lock button, lower down the cradle's clamping plate with other fingers positioning on the grip on the clamping plate until it reaches the horizontal position. Let go of the thumb from the press button and the fingers from the cradle's grip. The clamp plate will lock the RTCA Resistor Plate 16 in the horizontal position.

Please make sure that the clamp plate locks the RTCA Resistor Plate 16 properly. If not, the clamp plate, after all fingers are off the cradle, would pop up slightly. When this occurs, repeat the above process until the clamp plate locks the RTCA Resistor Plate 16.

Make sure that all RTCA Contact Pins 16 and the contact pads of the RTCA Resistor Plate 16 are clean and free from any dust or dirt particles. If not, use the RTCA Cleaning Kit to remove any dust.

When inserting the RTCA Resistor Plate 16 into the cradles of the RTCA DP Analyzer, make sure that the Resistor Plate is FULLY inside the pocket and is aligned properly with the edges of the pocket. It should be flat and without any tilt.

Perform the test

5 Wait until the automatic scan finishes, then click *Start* to begin the test. While the test is running, notice that the bottom of the program window reads "Test Col 1,2,3, ….6", and the LED on the RTCA Analyzer will flash during the measurement.

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Check the data

6 Upon completion of the test, check the *Cell Index* Page. All of the Cell Index values should be less than 0.063. Go to the *Plot* page and click *Add All*. All CI values will be displayed for the 48 wells (Figure 21).

7 In the lower portion of the *Cell Index* page, you can also view the raw scan data.

The data has 8 rows by 6 columns as shown in Figure 21. The raw scan data should exhibit a repeated value pattern of 40.0 ± 2.0 (rows A and H), 67.5 ± 2.5 (rows B and G), 93.6 ± 2.9 (rows C and F) and 117.6 ± 3.2 (rows D and E).

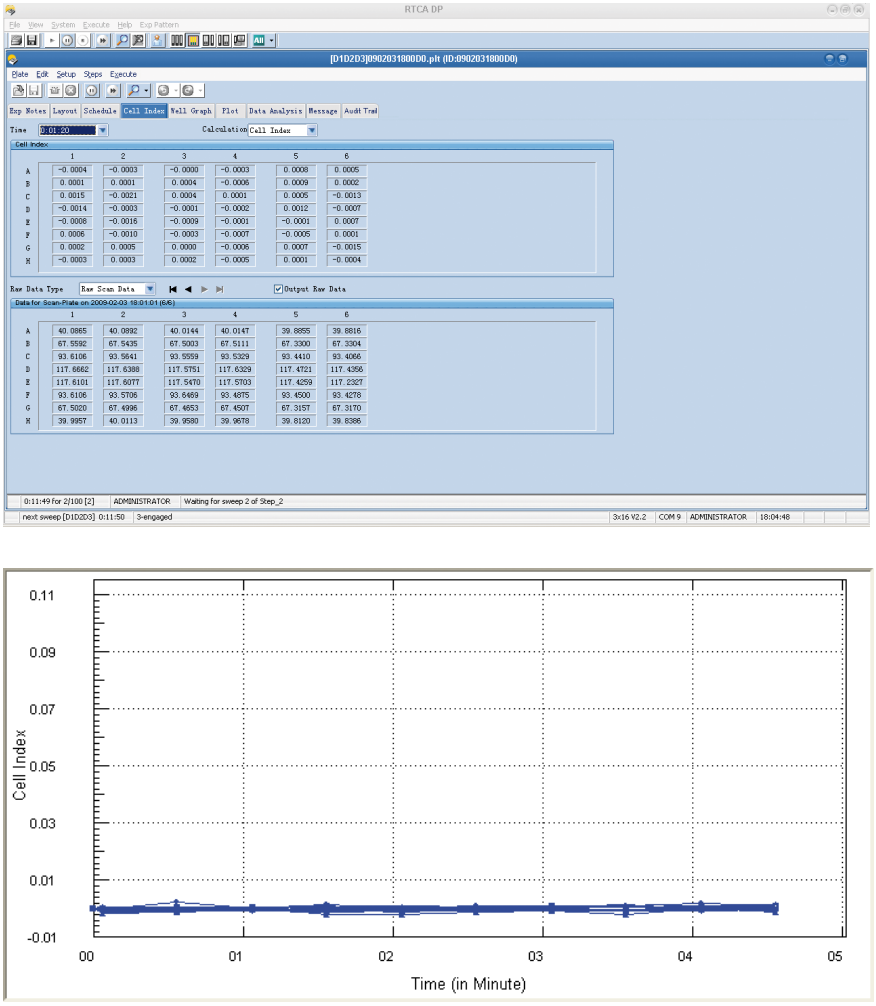

Figure 21: An example of resistor plate verification calibration raw data and Cell Index curves.

8 If the acceptance criteria described in 6 and 7 are fulfilled, the resistor plate verification is passed successfully. If the criteria are not fulfilled, please contact ACEA support

B

Remove the RTCA Resistor Plate 16

9 To remove the RTCA Resistor Plate 16 from the RTCA DP Analyzer, gently press the release button with a thumb whilst holding the cradle grip with index and middle fingers and lift up the clamp plate. Remove the Resistor Plates 16 from the RTCA DP cradle (Figure 22).

Figure 22: Removal of an RTCA Resistor Plate 16 from the RTCA DP Analyzer.

C Operation

1. Introduction

Prior to the operation of the RTCA DP Instrument, please refer to the *Overview* and *System Description* sections in this manual and review the RTCA Software Manual to familiarize yourself with the system.

The operator should have previous experience with tissue/cell culture and be familiar with in vitro techniques for handling cells and media.

2. Instrument Start-Up and Warm-Up

The electrical power for the RTCA DP Analyzer is provided by the RTCA Control Unit through the USB cable, so there is no power switch on the RTCA DP Analyzer. After placing the RTCA DP Analyzer in the incubator, to which one end of the USB cable is connected, plug the other end of the USB cable to a USB port on the RTCA Control Unit.

After placing the RTCA DP Analyzer in the incubator, allow it to warm up for two hours, so the RTCA DP Analyzer reaches the same temperature as the incubator. This ensures a condensation-free analyzer and smooth data recording, since both the electrical properties of the medium and the electronics are temperature sensitive.

The RTCA DP Analyzer has been developed and designed as a Plug-and-Play instrument. This means that after reaching temperature and humidity equilibrium inside a tissue culture incubator, the RTCA DP Analyzer can be connected or disconnected to the USB port of the RTCA Control Unit at any time. Please note, that the RTCA Software must be closed on the RTCA Control Unit before attempting to connect or disconnect the USB cable.

3. Preparing and Starting a Run on the RTCA DP Instrument

3.1 Running a quick experiment using the E-Plate 16

When the RTCA DP Analyzer is connected to the RTCA Control Unit, a self-test is performed and both the Power and Status LED indicators should be green.

If a RED LED lights up, it means that the instrument has failed the self-test, and you should contact ACEA support.

A cell titration experiment being run on the RTCA DP Instrument is used for illustration purpose. Whilst the RTCA DP Analyzer can be used simultaneously by up to three users each running a separate experiment with a single E-Plate 16, the illustration experiment described below is based on a single user using all three E-Plates 16 in a single experiment. The procedure for this experiment includes:

- Adding culture media to three E-Plates 16
- Inserting the E-Plates 16
- Starting the RTCA Software
- Setting up *Exp Notes, Layout*, and *Schedule* pages
- Starting the experiment
- Removing the E-Plates 16, adding cells to the E-Plate 16 wells and adding water to evaporation-control troughs on the E-Plates 16
- Reinserting the E-Plates 16
- Checking and plotting the Cell Index data on the *Plot* page

1 Add Culture Media to three E-Plates 16

Add 100 µl of cell culture media to each of the 16 wells in three E-Plates 16.

For optimal results, leave the E-Plate 16 in the tissue culture hood for 30 minutes at room temperature. This ensures that the culture media and E-Plate surface achieve equilibrium.

Please consider the maximal volume of each well for your experimental setup. It is not recommended to use > 200 µl total volume for each well.

Do NOT touch the E-Plate 16 on the electrical contacts pads and do NOT wear powdered safety gloves. Always bear in mind that the system is especially dependent on a clean, dust-free environment.

Please avoid scratching the gold electrodes on the bottom of the wells with pipette tips. This could influence functionality of the gold electrodes.

The 16 wells within E-Plate 16 are organized in such a way that each column of 8 wells is divided into two 4-well groups. To achieve consistent and accurate data during the experiment it is necessary to use the wells within a group in the same experiment. Examples of such 4-well groups are: A1, B1, C1 and D1 as a group; E3, F3, G3 and H3 as a group, etc.

Please always use the whole E-Plate 16 at one time. Using only part of the wells on a plate is not recommended.

During the experiment in the incubator, the properties of the micro sensor arrays in the unused wells may be affected, thus their performance can no longer be guaranteed. If using only a fraction of the wells on the E-Plate 16 can not be avoided, it is best to fill the remaining unused wells with buffer (*e.g.*, PBS).

2 **Insert the E-Plates 16**

1. Insert the front end of an E-Plate 16 (with corner cut) into a cradle pocket of the RTCA DP Analyzer (Figure 23).

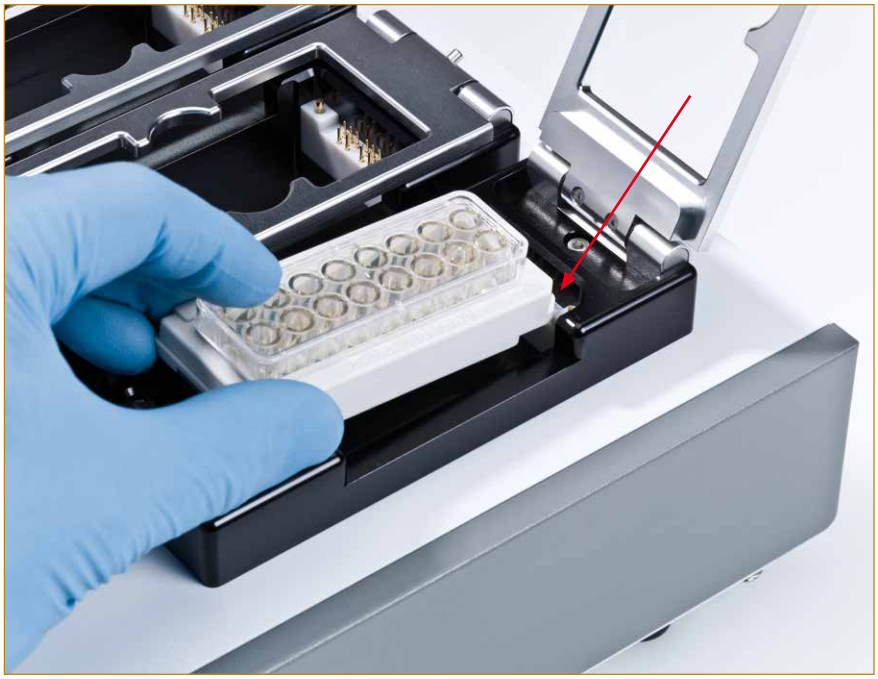

Figure 23: Correct orientation of the E-Plate 16.

Please remember which E-Plate 16 is inserted into which cradle pocket since E-Plates 16 would be removed from the cradle pockets for cell addition or other treatments during the experiment and must be then repositioned back in the cradle pockets in correct order. We recommend to insert E-Plates 16 into the cradle pockets in such an order that the E-Plates 16 inserted into the cradle pockets from left to right should have an increasing serial number.

2. Make sure that the plate is in position without any tilting. With the thumb pressing the lock button, lower down the cradle's clamp plate with other fingers positioning on the grip on the clamp plate until it reaches the horizontal position. Let go off the thumb from the lock button and the fingers from the cradle's grip. The clamp plate will lock the E-Plate 16 in the horizontal position.

   Please make sure that the clamp plate locks the E-Plate16 properly. If not, the clamp plate, after all fingers have been removed from the cradle, pops up slightly. When this occurs, repeat the above process until the clamp plate locks the E-Plate 16 securely.

3. Close the door of the incubator.

   Make sure that all the 20 connector pads of the E-Plate 16 are clean and free of any dust or dirt particles. If not, wipe the connector pads with lint-free lab wipes or a cloth slightly soaked with 80% ethanol and/or dust off the Contact Pins with compressed air.

   Make sure that all the 20 RTCA Contact Pins are clean and straight without any bending. If any damage to the Contact Pins is found, please change the Contact Pins as described in the Maintenance and Care part of this manual.

3 Start the RTCA Software

Start the RTCA Software by double clicking the RTCA Software icon on the desktop. If the program is already open in the OFFLINE mode, (as indicated on the bottom status bar on the RTCA program window), or open in the ONLINE mode but no experiment is running, close the program and then reopen it. Log into the RTCA Software, select the experiment pattern as "three E-Plates in a single experiment".

Please refer to the RTCA Software Manual for more information about logging into the Software and selecting the experiment pattern.

4 Set up the *Exp Notes* page

This page records experiment-related information such as user, experiment date, experiment name, experimental purpose, experimental procedure, experimental conclusion, and the devices used in the experiment. Enter the information in the appropriate spaces. When the experiment starts, this information is automatically saved. You can also click *Browse…* to select a File Directory in which to save the experiment files.

Please refer to the RTCA Software Manual for more information about setting up the Exp Notes page.

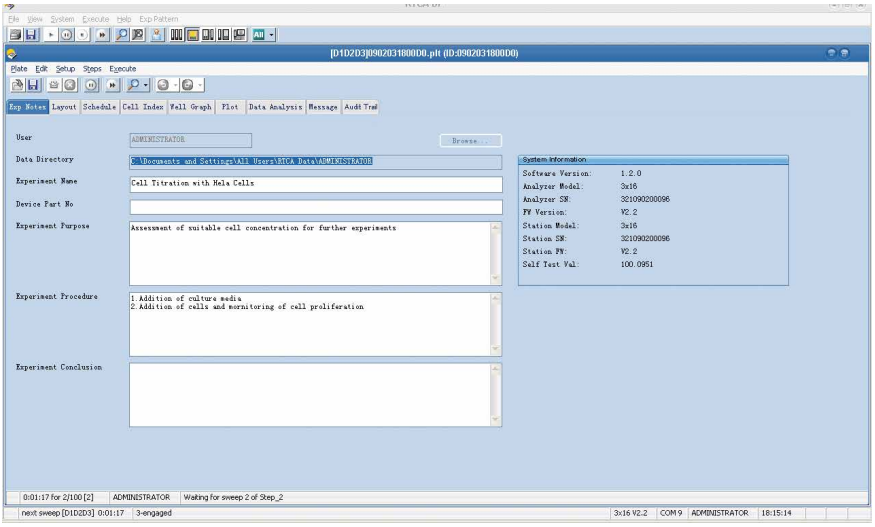

Figure 24: Example of a programmed *Exp Notes* page.

5 Set up the *Layout* page

This page determines the experiment layout and records experimental details about each well, including name and number of cells, name and concentration of compounds added into wells, etc.

Select the wells to be edited:

- To select a single well, move the mouse cursor over the well to be selected, and left-click. The selected well is highlighted. The well ID of the selected well is automatically filled into the *Well IDs* section. If, for example, the well D1 is selected, then the *Well IDs* section reads: "D1:D1".
- To select multiple wells, move the cursor over the first well to be selected, left-click and hold the mouse. Drag the mouse across the other wells, to the last well to be selected. Release the mouse button. All selected wells are highlighted, the well's ID, for example A1:D2, is automatically filled into the *Well IDs* section, if all wells between A1 and D2 are selected.

  Note that the text in the Well IDs section has the format First well ID: Last well ID.

- To select an entire row(s) or column(s), move the mouse to the row ID (*e.g.* A or E) or column ID (*e.g.* 1 or 2), and left-click. To select adjacent multiple-rows or multiple columns, left-click and drag across row IDs or column IDs.

  Enter information into appropriate information edit boxes, and click *Apply*. For example: *Cell Type* – HeLa; *Cell Number* – 20000; tick the *Cell Number Titration*; Titration factor - 2.

  For a detailed description of other fields, including *Compound No.*, *Compound Name*, *Concentration*, *Unit*, *Dilution Factor*, *Repetition*, *High From*, *Along* please refer to the RTCA Software Manual.

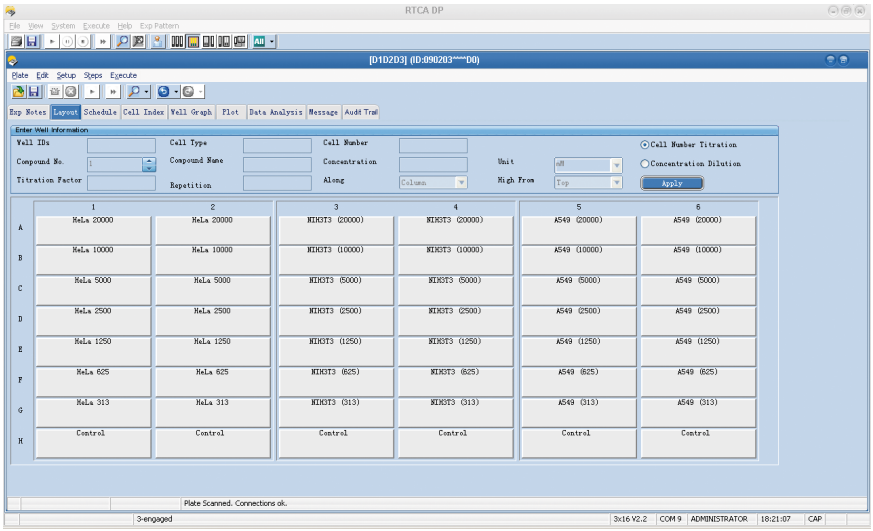

Figure 25: Example of a programmed *Layout* page.

6 **Set up the *Schedule* page**

This page is used to program the experimental procedure. Generally, experiments can be divided into multiple steps, and each step consists of one or multiple sweeps.

Each sweep is one measurement across all the selected wells. There is a predetermined time interval between neighboring sweeps within a step. After starting a current sweep, the RTCA DP Analyzer automatically starts to count down the time interval and starts the next sweep.

After completing all of the sweeps within a step, the RTCA DP Analyzer does not move on to the next step until it is manually instructed to do so.

A manual intervention is always necessary to move from one step to the next one.

Generally, there should be a separate step each time E-Plates 16 are removed from and placed back into the RTCA DP Analyzer. Users can manually stop a current step at any time before the system completes all pre-set sweeps and proceed to the next step. However, a current step cannot be manually stopped in the middle of a measurement sweep. Doing so may cause system instability and data loss.

- *Step 1* is typically used for the background test, where no cells are present in the wells.

  It is set as one sweep, one minute by default. The *Step Status* is *idle*, and all other buttons are blank.

  The sweep number and sweep interval for the Step 1 is preset to be one sweep with an interval of one minute by the software. This step is used for background measurement and should not be changed.

- *Step 2* is typically the first step after cell addition.

  To add Step 2, move the mouse to the *Steps* column on the left side of the page. Left click the *Add a Step* Icon , or go to the pull down *Steps* menu and select *Add a Step*. Step 2 is added. Edit the *Interval* and *Sweeps* section by entering the appropriate numbers. Select the Interval *Units* in hours or in minutes. Click *Apply*, and the appropriate time intervals, with corresponding unit and sweeps are added. The *Sweep Status* is automatically set to 0. *Step Status* displays *Idle* before the start of the step and as *Test* while the experiment is in progress. When the step is completed, *Done* will be displayed. The *Sweep Status* section is updated and displays the number of sweeps that have been completed.

  Depending on the specific experiment, appropriate sweep numbers and time intervals can be set (for example, 48 sweeps, and 30-minutes intervals result in a period of 24 hours).

  It is recommended to program some sweeps in excess rather than to give a precise number, because a step can be stopped before all sweeps are completed.

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▶ *Step 3* typically comes after an action (for example, compound addition) on the cells.

Depending on the specific experiment, appropriate sweep numbers and time intervals can be set as described for *Step 2*. For example, to measure rapid kinetics of cell responses to compound addition, a time interval of one minute can be set. Within a step, substeps with different time intervals can also be used. For example, step three can consist of 200 sweeps having one minute time intervals and another 200 sweeps having 15 minute time intervals.

▶ To remove a step, highlight the step and click the *Delete a Step* icon , or go to the *Steps* menu and select *Delete a Step*.

Please refer to the RTCA Software Manual for more information regarding the Schedule page and generation of substeps.

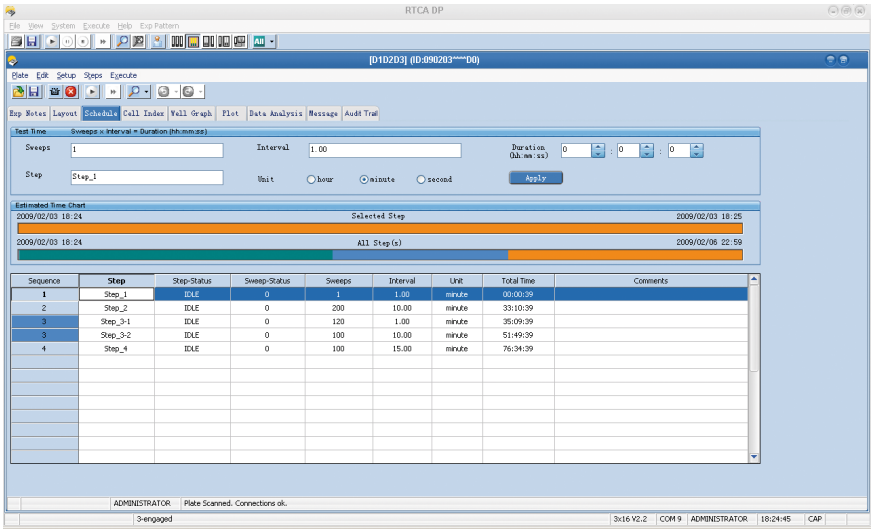

Figure 26: Example of a programmed *Schedule* page that allocates the Test Time.

7

Scan Plate function

Scan is automatically performed each time an E-Plate 16 is inserted into a RTCA DP Cradle. In addition, Scan Plate can be performed manually.

Always use the *Scan Plate* function before you start the experiment to make sure that the connection between the E-Plates 16 and the RTCA DP Analzyer is good. Click *Scan Plate* at the upper right corner. Then the RTCA DP Analyzer will do a measurement scan over the E-Plates 16 for the selected wells. Next, go to the *Message* page to check the status.

If the measured resistance value is within the expected range, a message *Plate Scanned. Connections ok.* will appear. Otherwise, you need to remove the plate and reinsert the plate into the RTCA DP Analyzer or do some cleaning. In the meantime, a data file is also generated to record the scan plate data. By default, it is located at C:\Documents and Settings\All Users\RTCA Data\ScanPlateData\.
The file has the format PlateScanning*yymmdd*D0.txt.

8

Start the experiment

Click *Start* to begin the experiment. This step measures the background impedance of cell culture media. The data is then used as reference impedance for calculating Cell Index values. During the experiment, the bottom of the program window displays *Test Col 1, 2, 3 … 6*, and the RTCA DP Analyzer status LED will blink during the measurement and communication with the RTCA Control Unit. After completion of the measurement the bottom-left of the main program window displays *Ready for Next Step. Please Click Next Step to start.*

▶▶▶

9 **Remove the E-Plates 16, add cells to the E-Plate 16 wells and add water to evaporation-control troughs on the E-Plates 16**

Open the clamp plate by gently pressing the release button with a thumb whilst holding the cradle grip with index and middle fingers and lifting up the clamp plate. Remove the E-Plates 16 from the RTCA DP cradle.

Add 100 µl of cell suspension to each well. The cell suspension should have been properly prepared for the desired cell concentrations for the cell titration experiment.

Keep shaking the container with prepared cells for evenly distributed cell suspension so that each well has the appropriate cell numbers.

Leave the E-Plate 16 in the tissue culture hood for 30 minutes at room temperature, so that the cells settle to the bottom of the well.

Add water to evaporation-control troughs located on the edges on the E-Plates. Water level in the troughs should be at the same vertical position as those in the wells.

For best results, always fill the troughs between the wells of the E-Plate 16 with water. This will create local humidity, thereby reducing evaporation in the wells containing culture media and cells, during the experiments. While this may not be mandatory for short term experiments, it is highly recommended for long-term experiments.

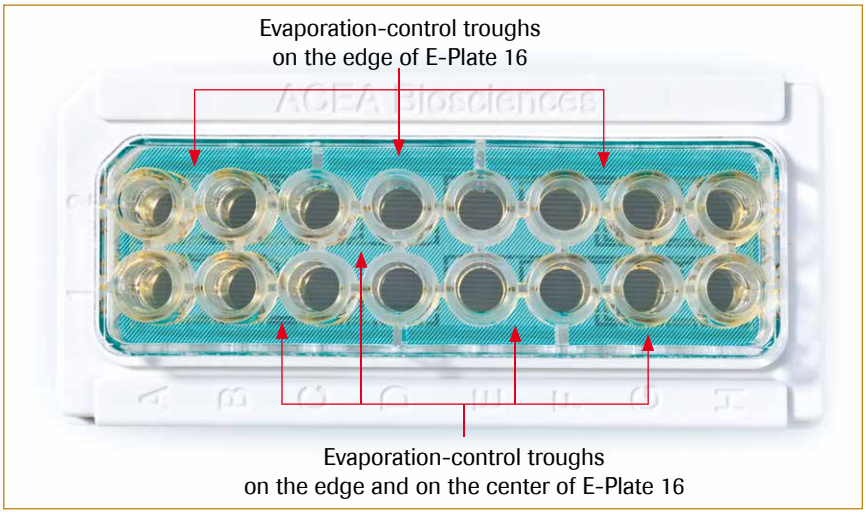

Figure 27: Adding water in evaporation-control troughs on the edge and on the center of E-Plate 16

10 **Reinsert the E-Plates 16 and start step 2**

Insert the E-Plates 16 as described above. Lock the E-Plate 16 to the cradle pocket by lowering down the lock handle to the horizontal position whilst pressing the release button, as described above. Close the incubator door.

A Scan Plate is automatically performed. Go to the Message page to check the status.

Start step 2 by clicking the *Start Step* button. During the experiment, note that the bottom of the program window states *Test Col 1, 2,..., 6*. The bottom-left of the program window displays a countdown to the next sweep start time (for example, "0:10:30 left for 3/100[2]").

11 Check and plot the Cell Index data

During the experiment and between sweeps, check the *Cell Index* or *Plot* page where data is plotted in real-time. In the *Cell Index* page, move to different test time points (the time is the time since the start of the experiment). Cell Index values at the time point are shown in boxes. In the *Plot* page, plot the Cell Index for a well as a function of time, by highlighting the well, and clicking *Add*. To add another well, repeat the process. By clicking *Add All*, data for every well included in the experiment is shown. In the *Well Graph* Page, individual Cell Index curves for all the wells are plotted in individual graphs.

For detailed information on how to plot and analyze experimental data please refer to the RTCA Software Manual.

Typical results of a cell titration experiment generated on the *Plot* page are shown in Figure 28.

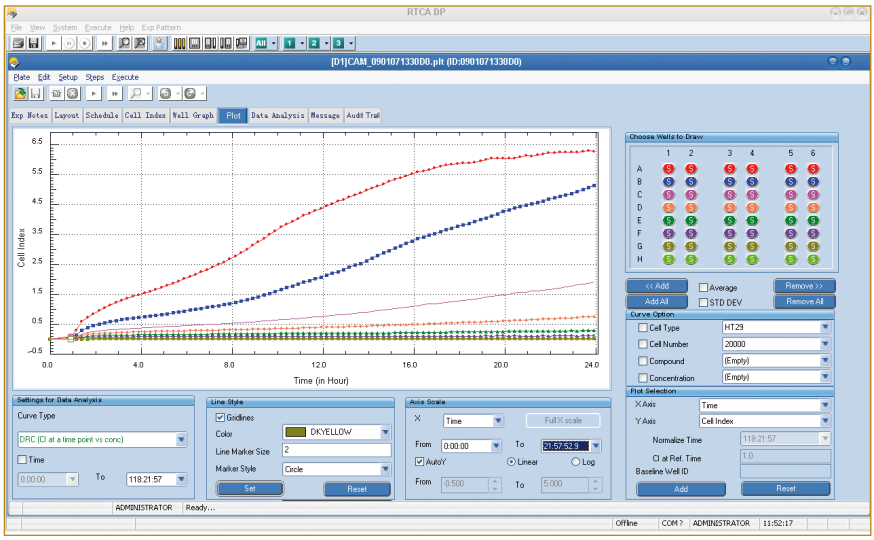

Figure 28: *Plot* **page after completion of a cell titration experiment.**

Charts and data on the charts can be easily exported using a right-click of the mouse. For detailed information on how to export charts and data on the charts, please refer to the RTCA Software Manual.

3.2 Running a quick experiment using the CIM-Plate 16

- When the RTCA DP Analyzer is connected to the RTCA Control Unit, a self-test is performed and both the Power and Status LED indicators should be green.
- If a RED LED lights up, it means that the instrument has failed the self-test, and you should contact ACEA support.

For illustration purposes, this chapter describes a basic cell migration experiment run on the D1 cradle of the RTCA DP Instrument.

The illustration experiment is performed using HeLa cells with 10% Fetal Bovine Serum (FBS) as chemoattractant. The following is a recommended protocol meant to serve as a guideline. It may be modified to fit individual protocols for different cell lines or other chemoattractants.

The procedure for this experiment includes:

- Assembling CIM-Plate 16 and equilibration in the incubator
- Starting the RTCA Software, setting up *Exp Notes*, *Layout*, and *Schedule* pages
- Cell addition to CIM-Plate 16
- Incubation of CIM-Plate 16 at room temperature for cell sedimentation
- Starting the experiment
- Checking and plotting the Cell Index data on the *Plot* page

1 Assemble CIM-Plate 16

I. Remove both upper and lower chamber of CIM-Plate 16 from the package. Place the CIM-Plate 16 Assembly Tool inside the tissue culture hood with blue spots away from you. Load the lower chamber to an individual pocket of the Assembly Tool to ensure the lower chamber sits flat inside.

CIM-Plates 16 should be used on the same day when the sealed package for CIM Plates 16 is opened.

Handling of CIM-Plates 16 should always take place in the sterile tissue culture hood. Minimize the time of CIM-Plates 16 being exposed to a non-sterile environment.

There is only one correct orientation for the lower chamber inside the CIM-Plate 16 Assembly Tool. The blue spot on the lower chamber should be positioned at the corresponding location to the blue spot on the tool.

Figure 29: Correct orientation of the CIM-Plate 16 Lower Chamber inside the CIM-Plate 16 Assembly Tool.

II. Fill each lower chamber well with **160 µl** media (containing 10% FBS or serum-free as control) using an 8 channel pipette.

Ensure that a clearly defined meniscus is formed on each well after the well is filled with media. To minimize the bubble formation in the lower chamber, do not introduce bubbles during pipetting media. 160 µl recommended here is based on a calibrated pipette, while exact volume for pipetting into the lower chamber may need to be optimized by the user, depending on the pipette calibration condition. The optimization should be based on using the lowest volume without bubbles in the lower chamber after the lower chamber is assembled with the upper chamber. (Figure 30).

During handling of the lower chamber, you can put the upper chamber in the neighboring position of the assembly tool.

Figure 30: Adding 160 µl media to CIM-Plate 16 Lower Chamber.

III. Turn the CIM-Plate 16 Assembly Tool 90 degrees counter-clockwise. Place the upper chamber onto the lower chamber with the sensor surface facing down and the blue spot on the upper chamber at the same side as the lower chamber. Push the upper chamber downwards to lock the upper and the lower chambers together (Figure 31).

Two "click-clack" sounds should be heard to ensure that the chambers are locked together properly

Figure 31: Assembling the CIM-Plate 16 Upper Chamber with the Lower Chamber.

IV. Add 25-50 µl serum-free media to each well of the upper chamber to cover the membrane surface (Figure 32).

Figure 32: Adding serum-free media to CIM-Plate 16 Upper Chamber.

During media addition, do not introduce any bubbles and avoid the pipette tips touching the membrane.

The serum-free media volume is not critical here. The key is to ensure that the media covers the entire lower surface of the upper chamber.

▶ The 16 wells of the CIM-Plate 16 are organized in such a way that each column of 8 wells is divided into two 4-well groups. To achieve consistent and accurate data during the experiment it is necessary to use the wells within a group for the same experiment. Examples of such 4-well groups are: A1, B1, C1 and D1 as a group; E3, F3, G3 and H3 as a group, etc.

Please always use the whole CIM-Plate 16 at one time. Using only part of the wells on a plate is not recommended.

▶▶▶

2 Equilibration of the CIM-Plate 16 in the incubator

I. Put the plate cover on the upper chamber with the blue spot on the cover matched to the blue spot on the upper chamber.

II. Load the CIM Plate 16 into the tissue incubator. You may place CIM-Plate 16 in the RTCA DP Analyzer. In this case make sure that the edge-cut corner on the CIM-Plate 16 matches to the edge-cut corner on the RTCA DP Analyzer (Figure 33).

If all the cradles are occupied by other plates, you can also leave the CIM-Plate 16 in the incubator for one hour to allow the membrane surface to reach an equilibrium with media. Then, load the CIM-Plate 16 into the RTCA DP Analyzer inside the incubator to proceed on background measurement.

Figure 33: Loading an CIM-Plate 16 into the D3 Cradle of the RTCA DP Analyzer (as an example).

III. Make sure that the plate is in position without any tilting. With the thumb pressing the lock button, lower down the cradle's clamp plate with other fingers positioning on the grip on the clamp plate until it reaches the horizontal position. Let go off the thumb from the lock button and the fingers from the cradle's grip. The clamp plate will lock the CIM-Plate 16 in the horizontal position. Please make sure that the clamp plate locks the CIM-Plate 16 properly. If not, the clamp plate, after all fingers have been removed from the cradle, pops up slightly. When this occurs, repeat the above process until the clamp plate locks the CIM-Plate 16 securely.

Please remember which CIM-Plate 16 is inserted into which cradle pocket since CIM-Plates 16 will be removed from the cradle pockets for cell addition or other treatments during the experiment and must be then repositioned back in the same cradle pockets in correct order. For this reason, we recommend to insert CIM-Plates 16 into the cradle pockets in such an order that they have an increasing serial number from left to right.

IV. Close the incubator door and wait 1 hour to allow the membrane surface of CIM-Plate 16 to reach an equilibrium with media.

Make sure that all the 20 connector pads of the CIM-Plate 16 are clean and free of any dust or dirt particles. If not, wipe the connector pads with lint-free lab wipes or a cloth slightly soaked with 80% ethanol and/or dust off the connector pads with compressed air.

Make sure that all the 20 RTCA Contact Pins are clean and straight without any bending. If any damage to the Contact Pins is found, please change the Contact Pins as described in the Maintenance and Care part of this manual.

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3 Start the RTCA Software

Start the RTCA Software by double-clicking the *RTCA Software* icon on the desktop. If the program is already open in the OFFLINE mode (as indicated on the bottom status bar on the RTCA program window), or open in the ONLINE mode but no experiment is running, close the program and then reopen it.

Log into the RTCA Software, select the experiment pattern as “one plate in a single experiment”.

Click on the *Plate Window* button to select the D1 Plate Window .

Please refer to the RTCA Software Manual for more information about logging into the Software and selecting the experiment pattern.

4 Set up the Exp Notes Page

Click on the *Exp Notes* tab in the RTCA Software. Enter the information for this experiment (*e.g.*, experiment name, experiment purpose, device part no.) in the appropriate spaces.

Please refer to the RTCA Software Manual for more information about setting up the Exp Notes page.

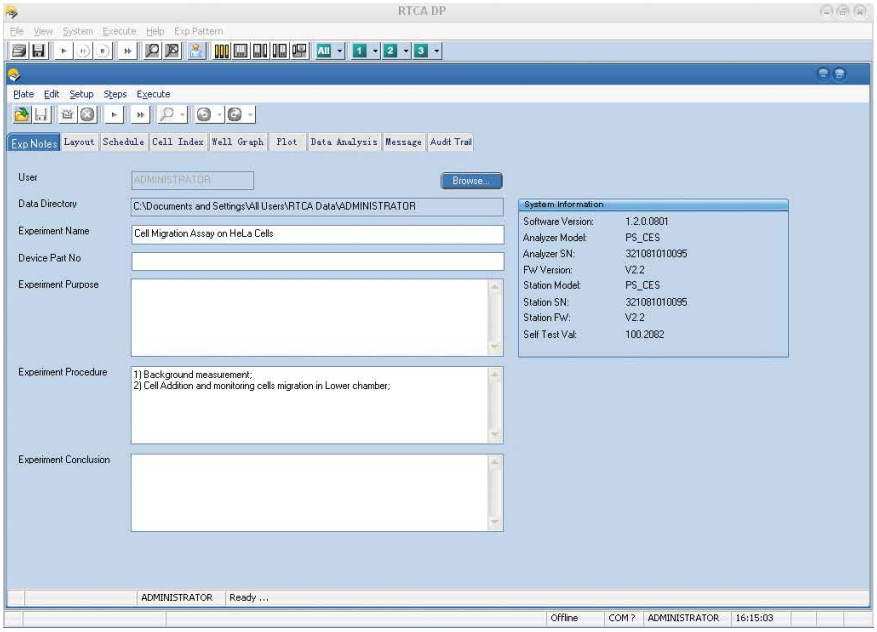

Figure 34: Example of a programmed *Exp Note* Page.

5 Set up the Layout Page

1. Click on the *Layout* tab in the RTCA Software.
2. Enter information into appropriate information edit boxes, and click *Apply*.
   For example: Highlight well A1 to H1, *Cell Type* – HeLa; *Cell Number* – 40,000.
   Then, highlight well A2 to H2, *Cell Type* – HeLa; C*ell Number* – 20,000.
3. Highlight well A1, A2 and H1, H2, *Compound Name* - SF_Control (Serum-free control) and click *Apply*.

For a detailed description of other fields, including Compound No., Compound Name, Concentration, Unit, Dilution Factor, Repetition, High From, Along please refer to the RTCA Software Manual.

Cradle D2 or D3 (well A3 to H6) can be used for other cell lines as described above, or for different experimental purposes.

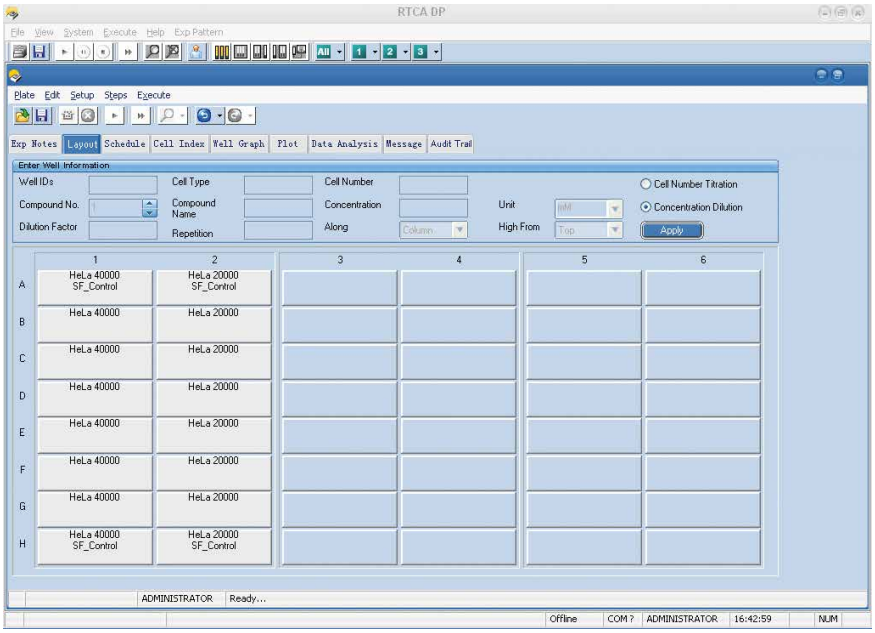

Figure 35: Example of a programmed *Layout* Page.

6 Set up the Schedule Page

I. Click on the *Schedule* tab in the RTCA Software. This page is used to program the experimental procedure. Generally, experiments can be divided into multiple steps, and each step consists of one or multiple sweeps.

- Each sweep is one measurement across all the selected wells. There is a predetermined time interval between subsequent sweeps within a step. After starting a sweep, the RTCA DP Analyzer automatically starts to count down the time interval and starts the next sweep.
- After completing all of the sweeps within a step, the RTCA DP Analyzer does not move on to the next step until it is manually instructed to do so.

A manual intervention is always necessary to move from one step to the next one.

II. Click on the *Add a Step* icon. *Step 1* appears in the *Step* column. Step 1 is the background step, where no cells are present in the wells. It is set as one sweep, one minute by default.

III. The *Step Status* column should state *IDLE* for *step 1*. The *Sweeps* and *Interval* should automatically be set to *1* and *1.00* minute.

IV. Click on the *Add a Step* icon to create *Step 2*.

V. Enter *100* in the Sweeps box and *15.00* in the *Interval* box. Choose *minute* as the unit definition. Click *Apply*.

VI. Once step 2 is initiated, the RTCA DP Instrument will record all the impedance changes for up to **25 hours** (15 minutes interval per sweep, 100 sweeps in total).

It is recommended to program some sweeps in excess rather than to give a precise number, because a step can be stopped before all sweeps are completed.

Please refer to the RTCA Software Manual for more information regarding the Schedule page or generation of substeps.

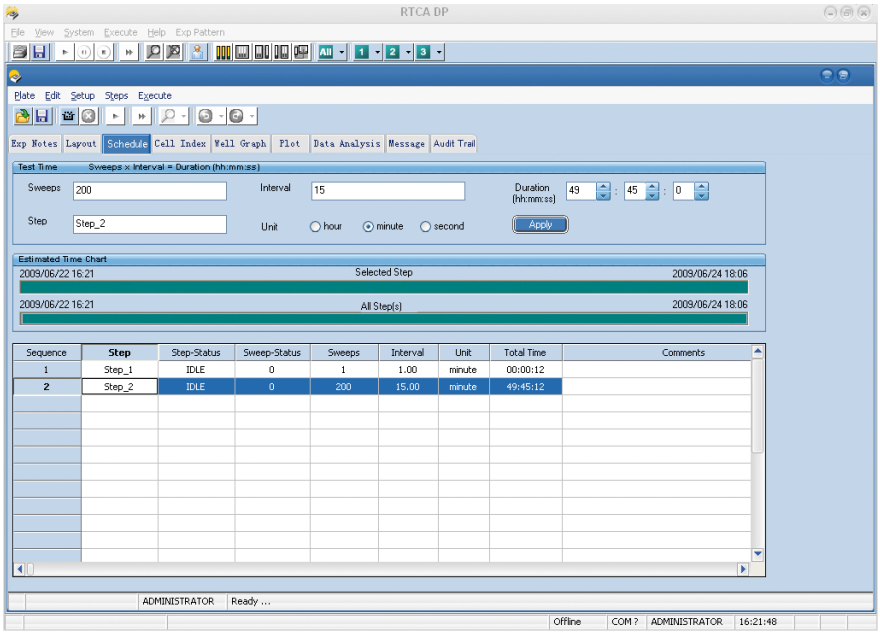

Figure 36: Example of a programmed *Schedule* Page.

7 Background measurement

I. After one hour equilibration of media, start *step 1* (1 minute and 1 sweep) to perform background measurement.

II. Once the measurement is completed, the bottom-left of the main window displays the message *Ready for Next Step. Please Click Next Step to start.* Click on the *Message* tab to read any other messages with respect to background or connection problems.

8 Cell preparation

I. Cells of interest should be passaged one day before the experiment and should reach 60-80% confluence.

It is recommended that cells used for cell invasion/migration assays should be passaged less than 20 times.

II. Remove serum containing media from the flask as much as possible, and gently rinse the cell monolayer with PBS once to wash off the serum containing media.

III. Trypsinize cells by adding 0.5 ml of 0.05% Trypsin solution (with 0.02% EDTA) per T25 flask and leave the flask at room temperature or 37°C for 1-2 minutes.

IV. Stop trypsinization by adding 10% FBS containing media or TNS solution (*e.g.,* from Clonetics) at a 1:1 ratio.

V. Wash trypsinized cells once, resuspend the cells with FBS free medium after centrifugation.

VI. Count cells and prepare cell suspension in FBS free media at the concentration of 4×10^5 cells/ml and 2×10^5 cells/ml, respectively.

9 Cell addition to CIM-Plate 16

I. Unlock the D1 cradle by gently pushing the press-button with the thumb whilst holding the cradle grip with other fingers. Remove the CIM-Plate 16 from the D1 cradle.

II. Remove the cover, then add 100 µl cell suspension of 4×10^5 cells/ml to well A1 to H1 of the upper chamber and cell suspension of 2×10^5 cells/ml to the well A2 to H2. The final cell numbers per well should be 40,000 and 20,000, respectively.

Only if the experiment takes longer than 24 hours, the user should as well add water to evaporation-control troughs located on the edges on the CIM-Plate 16. This will create local humidity, thereby reducing evaporation of the culture media during the experiment. Water level in the troughs should be at the same vertical position as those in the wells of upper chambers.

10 Incubation of the CIM-Plate 16 at room temperature

Put the plate cover on the upper chamber at its appropriate direction, then leave the CIM-Plate 16 in the tissue culture hood at room temperature for 30 min after cell addition to allow the cells to settle down to the upper side of the membrane located in bottom of upper chamber.

11 Start measurement

I. Load the CIM-Plate 16 on the RTCA DP Analyzer inside the incubator as described above (*2 Equilibration of the CIM-Plate 16 in the incubator*). Close the incubator door.

II. A *Scan Plate* is performed automatically. Open the *Message* tab to check whether *Scan Plate* was successful. The following message should be displayed: *Plate scanned. Connections ok.*

If the scan plate was not successful, you need to remove the plate and reinsert the plate into the RTCA DP Instrument or clean the contact pins (see Cleaning and Exchanging the RTCA Contact Pins 16)! In the meantime, a data file is also generated to record the scan plate data. By default, it is located at C:\Documents and settings\All Users\RTCA Data\ScanPlateData\. The file has the format PlateScanningyymmddD0.txt.

III. Start step 2 by clicking the *Start Step* button. The RTCA DP Instrument will now automatically monitor the cells every **15 minutes** with **100 repetitions**.

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C

12 Check and plot the Cell Index data to determine cell migration activity

I. On the second day, (*e.g.*, after 18 hours), check and plot the Cell Index in the *Plot* page of the RTCA Software.

II. Click *Add All* to show data for every well included in the experiment.

III. Activate the *Average* and *STD DEV* boxes to display the averages and corresponding standard deviation error bars of the respective replicates.

IV. Analyze the Cell Index (CI) curves. The positive migration signals should meet the criteria of averaged CI ≥ 2 fold of standard deviation of the Cell Index (Figure 37).

The criteria of average Cell Index as positive result is cell type specific. The user should determine whether a Cell Index signal is positive based on specific assay conditions.

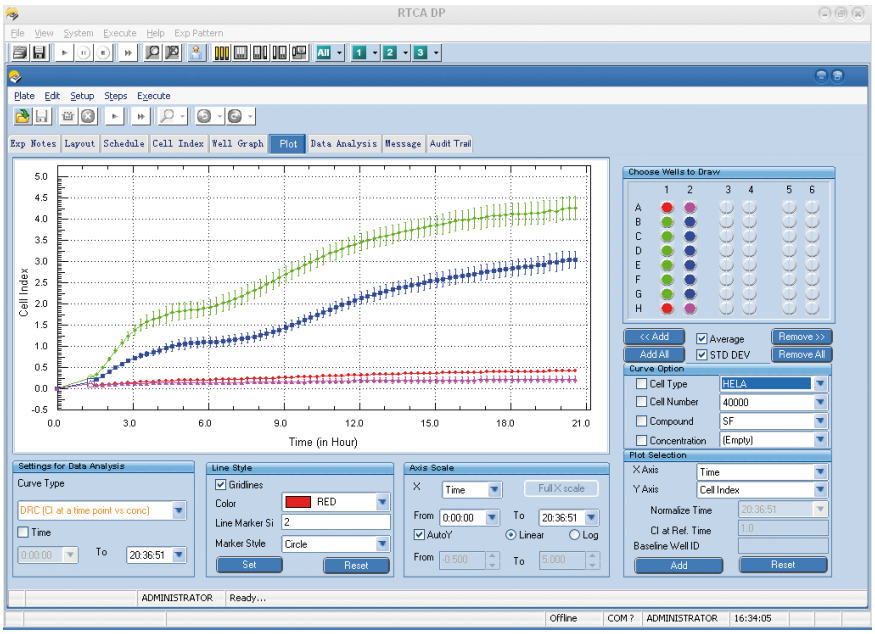


Figure 37: Plot Page after completion of a cell migration experiment.

3.3 Important Tips for Using the RTCA DP Instrument

The RTCA DP Analyzer, the E-Plate 16 and the CIM-Plate 16 are manufactured and tested according to rigorous quality standards. Each well in E-Plate 16 or CIM-Plate 16 is quality controlled prior to packaging and shipment ensuring that every well meets these quality standards.

In order to obtain optimal data and to avoid any problem with the RTCA DP Instrument, it is essential that you observe the following instrument handling procedures:

Always perform background measurement of E-Plate 16 or CIM-Plate 16 with cell-free culture media.

Without cell culture media, background measurement of E-Plate 16 or CIM-Plate 16 cannot be performed since what is being measured is an open circuit. Similarly, the background impedance of the E-Plate 16 or CIM-Plate 16 cannot be measured properly when culture media contains cells, because what is being measured is not a real background.

Do not remove the E-Plate 16 or CIM-Plate 16 from RTCA DP Analyzer before completing the required background measurement.

If the E-Plate 16 or CIM-Plate 16 is removed from the RTCA DP Instrument before completion of the background measurement, then those wells whose background has not been measured would be represented as an open circuit. These wells lack their respective background data and cannot be used to produce valid experimental data. The RTCA Software displays a warning message in such cases.

For the same reason, please do not remove E-Plate 16 or CIM-Plate 16 from the RTCA DP Instrument when the measurement or experiment is in progress. If E-Plate 16 or CIM-Plate 16 are removed, the impedance for these E-Plates 16 or CIM-Plates 16 would show open circuit data which, in turn, will display very high Cell Index values in the *Plot* page of the RTCA Software.

*Always allow the E-Plate 16 or CIM-Plate 16 to sit outside the incubator for **30 minutes** after cell addition.*

To ensure consistent data, cells need to settle down uniformly on the bottom surfaces of the E-Plate 16 or CIM-Plate 16 wells before they are put in the incubator. Otherwise, temperature-gradient related convection in the E-Plate 16 or CIM-Plate 16 wells would cause the cells to be distributed nonuniformly over the bottom surface of the wells, leading to substantial variation in the experiment data.

Always add water to the evaporation-control troughs of E-Plates 16.

To minimize the evaporation of culture media from the wells of E-Plate 16, the evaporation control troughs were incorporated into the edges of E-Plates 16 for water addition and for producing a saturated humidity underneath the E-Plate 16 cover. Adding water to these troughs greatly reduces evaporation in especially the corners wells and minimizes assay variation associated with such evaporation.

To ensure a high level even distribution of humidity inside the incubator, we recommend the placement of a tray of water at the bottom of the incubator, in addition to making sure that the troughs between the wells of the E-Plate 16 are filled with water. Please take care to regularly check your incubator for proper humidity. Changes in humidity, and excessive evaporation from the wells with culture media and cells, are major factors influencing variation and reproducibility of experimental results.

Minimize the handling time during compound addition.

When an E-Plate 16 or CIM-Plate 16 is taken out of the RTCA DP Analyzer for compound addition or other treatment, please minimize the handling time (preferably less than 5 minutes) because cooling of the media and the cells to room temperature will have an impact on cellular behaviour and the corresponding CI values.

Add compounds gently and in as low volume as possible to avoid disturbing the cells. Use a compound solution which has been pre-equilibrated to room temperature. Addition of cold compound solution may impact cellular behavior and the corresponding CI values.

Do not interrupt an experiment or a particular step while the measurement sweep is in progress.

Always wait until the current measurement sweep finishes and then stop or pause an experiment or a particular step. Otherwise, the experiment data file may be corrupt and data cannot be retrieved.

Each step must have at least one sweep.

For the RTCA Software to run properly, at least one sweep is needed for any given step.

Otherwise, the data file format may not be correct. The RTCA Software will not continue to the following steps without measurement sweep numbers from the previous step.

***Do not re-use** the E-Plate 16 and CIM-Plate 16.*

The E-Plate 16 or CIM-Plate 16 devices are designed for single use only. Data quality cannot be guaranteed for re-used plates since sensor surface properties of re-used plates would differ from the tissue-culture-compatible properties of fresh plates. Furthermore, residual contamination in the wells will most likely generate unreliable results.

Seed appropriate number of cells into the wells of the E-Plate 16 or CIM-Plate 16.

Before running any assay on the RTCA DP Instrument, a cell titration experiment is necessary to test cell proliferation for a range of cell numbers. This approach helps to determine the appropriate number of the cells for the assay. If the number of cells is too high or too low, it would affect the quality of the data generated from the RTCA DP Instrument.

Thoroughly mix the cell suspension when seeding cells into the wells.

Cells will settle to the bottom of the cell-suspension tube unless the tube is repeatedly mixed.

In order to add a consistent number of cells to each well, it is essential to have cells uniformly (and randomly) suspended in the tube. Thus it is important to thoroughly mix the cell suspension in the tube before adding cells to each well.

Please be aware that under some circumstances (e.g., extremely low cell numbers) experimental data might vary between wells in the center and wells on the edge of E-Plates 16 or CIM-Plate 16 (so-called "Edge Effect"), a commonly known phenomenon of conventional microtiter plates.

Make sure that all the 20 connector pads on the backside of the E-Plate 16 or CIM-Plate 16 are clean and free of dust/dirt particles.

Clean connector pads (located on small, green PCB boards on the bottom of the E-Plate 16) are essential for reliable contacts between E-Plate 16 and RTCA DP Instrument. If connector pads are dirty, wipe the pads gently with 80% ethanol-soaked tissue (paper/cloth). Do not press too hard on the contact pads of E-Plate 16 to avoid damaging the connection between the small PCB boards and the plate.

For CIM-Plate 16, the connector pads are located on the bottom of the CIM-Plate 16 Upper Chamber. If connector pads are dirty, wipe the pads with 80% ethanol-soaked tissue (paper/cloth) with special care. Do not press too hard on the pads to avoid damaging the connection between the small PCB boards and the plates, or damaging the membrane.

Always check the Scan Plate data when the E-Plate 16 or CIM-Plate 16 is re-positioned on the RTCA DP Instrument after being removed. "Connections ok" will be displayed if there is no connection problem after each Scan Plate operation, including auto-scan after plate repositioning.

After the Scan Plate operation, please always check the *Message* page for scan results to see whether some wells have connection problems. When some wells show a problem, first try either of two steps to resolve the issue:

1. With the E-Plate 16 or CIM-Plate 16 on the RTCA DP Instrument and in the locked position, you may want to press the plate down a couple of times. This action can sometimes remove the dust between the RTCA Contact Pins 16 and the connector pads, or reset the RTCA Contact Pin 16 connection to allow better contact. You can then do a Scan Plate which may show that the problematic well is now OK.
2. The second option is to remove the E-Plate 16 or CIM-Plate 16 and re-position it on the RTCA DP Instrument. Before you re-position the E-Plate 16 or CIM-Plate 16, please check to see whether the connector pads are clean. Alternatively, you may just clean the connector pads of the E-Plate 16 or CIM-Plate 16 before re-positioning it for another Scan Plate test.

For a comprehensive description of Contact Pin cleaning see Cleaning and Exchanging the RTCA Contact Pins 16.

If the problem persists you may want to continue the experiment with this E-Plate 16 or CIM-Plate 16, or you may want to try again with a new E-Plate 16 or CIM-Plate 16 (assuming that the cells have not been added). You should report the problem to ACEA support and provide the serial number of the E-Plate 16 or CIM-Plate 16 and the appropriate data file (for details, see *Troubleshooting*).

Disconnect the RTCA DP Analyzer from the RTCA Control Unit when the system will not be used for several days or longer.

The RTCA DP Analyzer is designed and developed as a Plug-and-Play instrument. When not in use, the RTCA DP Instrument should be disconnected from the USB port of the RTCA Control Unit. This will prolong the operating life of the equipment and save electricity.

CIM-Plates 16 should be used on the day when the sealed CIM-Plate 16 package is opened.

Handling of CIM-Plates 16 should always be in the sterile tissue culture hood. Minimize the time of CIM-Plates 16 being exposed to a non-sterile environment.

While pipetting media or any solution into CIM-Plate 16 Upper Chamber wells, avoid touching the electrode-containing membrane surface with pipette tips.

Always follow recommended protocols in handling and assembling CIM-Plates 16.

D Maintenance and Care

1. General Maintenance

The RTCA DP Instrument is maintenance-free.

There are no user serviceable parts inside the RTCA DP Analyzer.

If the system will not be used for a long period of time (a month or more), pack the RTCA DP Analyzer into the original plastic bags inside the shipment boxes.

When you are ready to use the instrument again, please refer to the *Installation* section for details on unpacking and installation.

*The surface of the RTCA DP Analyzer should be cleaned and wiped with a towel or tissue paper that has been **lightly** soaked in 80% ethanol. Ensure that this is done for the RTCA DP Analyzer to be placed inside an incubator.*

Avoid spraying any sterilizing agent directly on the RTCA DP Analyzer, except Contact Pin cleaning (see Cleaning and Exchanging the RTCA Contact Pins 16)!

2. Cleaning Instructions

2.1 General Cleaning

General cleaning of the RTCA DP Instrument is simple. Use some soft cloth or fiber free tissue paper moistened slightly with 80% ethanol, gently wipe off any dust or contaminants from the surface of the RTCA DP Analyzer. A small air blower can also be used to help remove any dust.

Be careful not to touch the RTCA Contact Pins 16. They must be cleaned with a special brush.

Do not soak the cloth or tissue paper excessively. In particular, do not let any liquid drip on to the RTCA Contact Pin contacts. Clean up any liquid spilled on the RTCA Contact Pin cradle pocket surface immediately. Any liquid spilled on the RTCA Contact Pin contacts may affect the reliability of the electrical connection.

Decontamination of the RTCA DP Analyzer and RTCA Control Unit

For decontamination of the RTCA DP Analyzer and RTCA Control Unit the user has to follow the governmental guidelines for the inactivation of the organisms used in the experimental environment.

The following compositions have been tested for being compatible with the surfaces of the RTCA DP Analyzer and the RTCA Control Unit and cables:

- 80% Ethanol
- A mixture containing propan-1-ol 450 mg/g, propan-2-ol 250 mg/g, ethanol 47 mg/g

This mixture is available for instance from Bode Chemie under the tradename Bacillol® AF.

This mixture is fungicidal, tuberculocidal, mycobactericidal, virucidal against enveloped viruses (including HBV, HIV, HCV), Adeno virus, FCV, Papova virus and Rota virus when used as described in the supplier's instructions.

Other sterilizing agents, such as oxidizing agents or strongly alkaline or acidic substances used for decontamination may damage the surfaces of the RTCA DP Analyzer and the RTCA Control Unit and/or may interfere with their function.

2.2 Further Recommendations

The RTCA DP Instrument is a precision instrument that requires care to enable it to perform consistently at optimum level. General decontamination is highly recommended by wiping away any dust or mold growing on RTCA DP Analyzer surfaces.

This cleaning and care also applies to the tissue culture incubator that holds the RTCA DP Analyzer. It is important to prevent any mold from growing inside the incubator, because this will probably affect the performance of the RTCA DP Analyzer. We recommend the use of copper alloy based incubators and regular cleaning to prevent any mold growth inside the incubator.

3. Cleaning and Exchanging the RTCA Contact Pins 16

The RTCA Contact Pin 16 contacts provide an important signal path from the microsensor array in the E-Plate 16 to the control and measurement circuits. To guarantee the proper function of the RTCA DP Analyzer, clean the RTCA Contact Pins regularly. In addition, whenever a particular RTCA Contact Pin shows significantly higher contact resistance compared to the others, that pin should be cleaned. If one of the CI values during RTCA Resistor Plate 16 verification tests is higher than 0.063, the RTCA Contact Pins 16 should either be cleaned or replaced.

The RTCA Contact Pin 16 cleaning procedure takes 10 to 15 minutes and is helpful in obtaining consistent optimum experimental data. Regular cleaning every three months, or whenever contaminants are present on the RTCA Contact Pins produces the most reproducible and accurate measurements.

Clean the Contact Pins 16 of one cradle of the RTCA DP Analyzer at a time. While cleaning Contact Pins of one cradle, cover all the other cradles with a clean cloth, or a clean fibre-free paper towel, or a clean plastic sheet.

For illustration, the steps for cleaning Contact Pins of D3 cradle are described below.

1. Press the release button on the front of D3 cradle, and lift the clamp plate from the cradle pocket. Blow the entire surface of D3 cradle using the small dust blower which comes with the RTCA DP Instrument. If compressed air and nozzle are available, it can also be used. Use a pressure less than less than 15 psi and blow air gently.

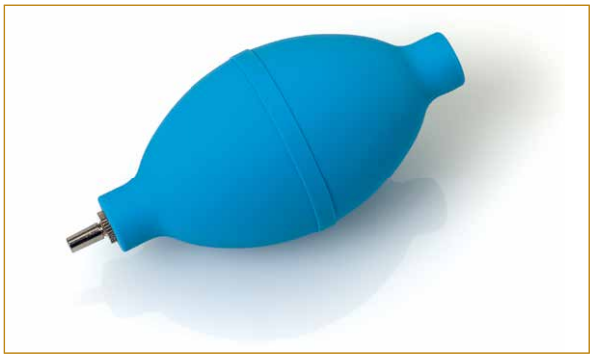


Figure 38: Dust Blower.

2. Brush the RTCA Contact Pin field with the nylon brush. There are two rows of the Contact Pins and each row contains 1 group (5 pcs) of Contact Pins. The procedure for cleaning one row of Contact Pins is as follows:

   (a) Position the nylon brush to cover 1 group of Contact Pins.

   (b) Brush across the tips of the Contact Pins gently in one direction towards the center of the cradle pocket, as shown in Figure 39.

   (c) Repeat the motion in (b) at least ten times.

   (d) Position the brush to cover and clean the other group of Contact Pins.

   (e) Use the air blower to remove any dust from the surface close to this row of Contact Pins.

   When using the blower to remove dust, always remember to blow the air towards the outside of the cradle area.

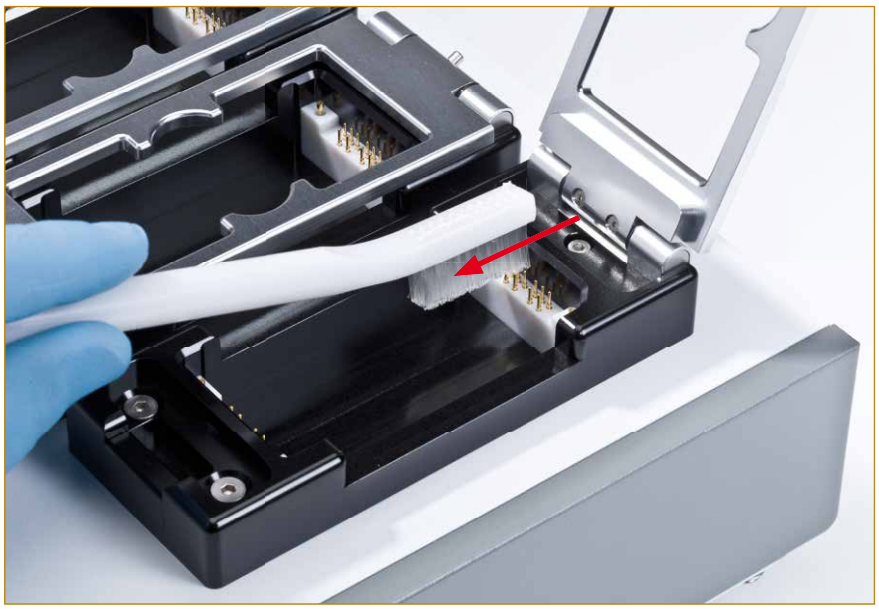


Figure 39: Using the Nylon brush to clean the RTCA Contact Pins 16 in only one direction at a time.

Before using the nylon brush to clean the Contact Pins, please make sure the brush is clean. If you are not sure, please use the blower to blow the brush from each side for at least 5 times.

3. Before covering the clamp plate of D3 cradle, be sure that none of the RTCA Contact Pins have come loose. They should all be straight and at the same height.

Decontamination of RTCA Contact Pins 16

1. Press the release button on the front of D1 cradle, and lift the clamp plate from the cradle pocket. Clean the RTCA Contact Pins 16 with the brush and use the dust blower (from RTCA Cleaning Kit) to remove any dust particles from the Contact Pins, following the protocol described in this section.

2. Spray 80% ethanol with a spraying bottle on all Contact Pins, two times on each location. Make sure that all the Contact Pins are covered with ethanol. The total volume of ethanol for spraying the two columns of Contact Pins should not exceed 3 ml.

3. Wait at least 30 minutes at room temperature for the ethanol to evaporate and let the RTCA Contact Pins 16 dry completely.

   Since the brush is used for cleaning the dust particles from the Contact Pins, the brush should also be decontaminated with ethanol. Fill a container with 80% ethanol. Then soak the brush in the container for 5 minutes. Take the brush out, shake the excess ethanol from the bristles and wait for the brush to dry.

Exchange the RTCA Contact Pins 16

In case of a damaged or failed RTCA Contact Pin contact, the respective pin can be exchanged with a new one using an easy and quick process.

Defective RTCA Contact Pins 16 must be replaced according to the instructions described in this manual.

Spare RTCA Contact Pins 16 are included with the RTCA DP Instrument. If more RTCA Contact Pins 16 are needed, please contact ACEA support

The well(s) affected by the failed Contact Pin(s) (*e.g.*, G1) can be pinpointed with the aid of Figure 40:

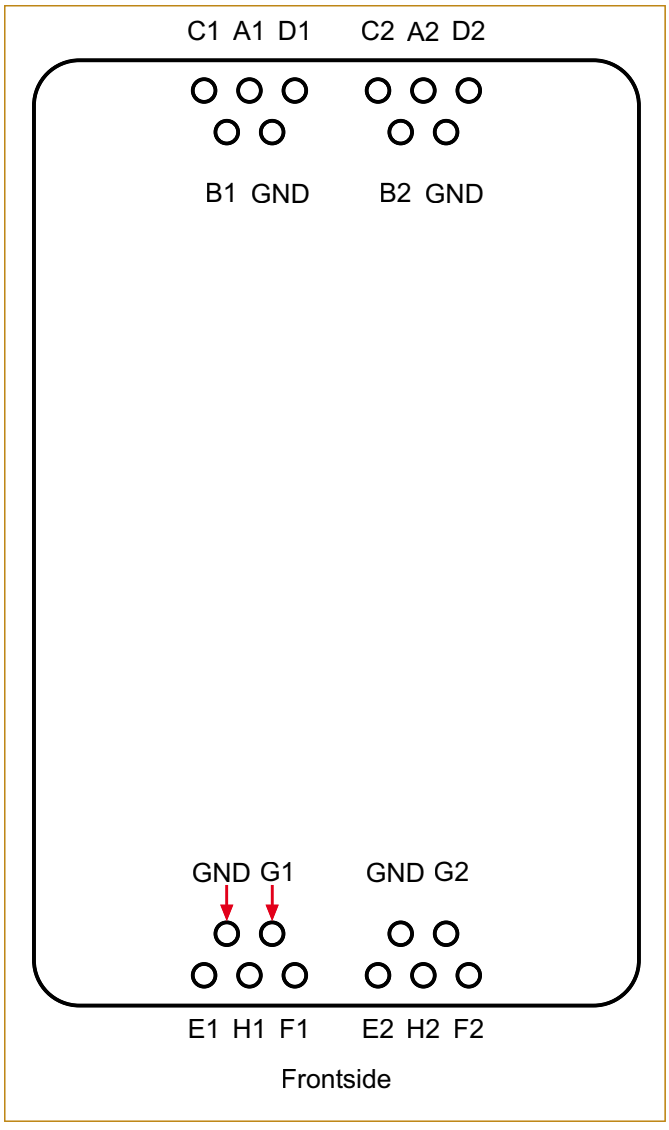


Figure 40: RTCA Contact Pin fields. The front side of the RTCA DP Analyzer is where the LED indicator is located.

For example, if well G1 would have a contact problem, the RTCA Contact Pin for G1 and/or the corresponding Pin for ground (GND) would have to be exchanged. The positions are marked by arrows in Figure 40.

If well G1 has a contact problem but wells E1, F1 and H1 do not, then replace the RTCA Contact Pin for G1.

If well G1 has a contact problem and wells E1, F1 and H1 do also, then replace the GND Pin for the group E1 to H1.

The following procedure is suggested for exchanging the RTCA Contact Pins 16:

1. Before replacing RTCA Contact Pins, please close the clamp plates of the RTCA DP Analyzer first, and make sure the clamp plates are locked well. Then blow any dust away from the entire surface of the RTCA DP Analyzer.

2. Use the pliers (Figure 41) to clamp and fasten the RTCA Contact Pin gently. Then vertically extract the defective RTCA Contact Pin as shown in Figure 42. Be careful not to press other adjacent contact pins.

Figure 41: Pliers.

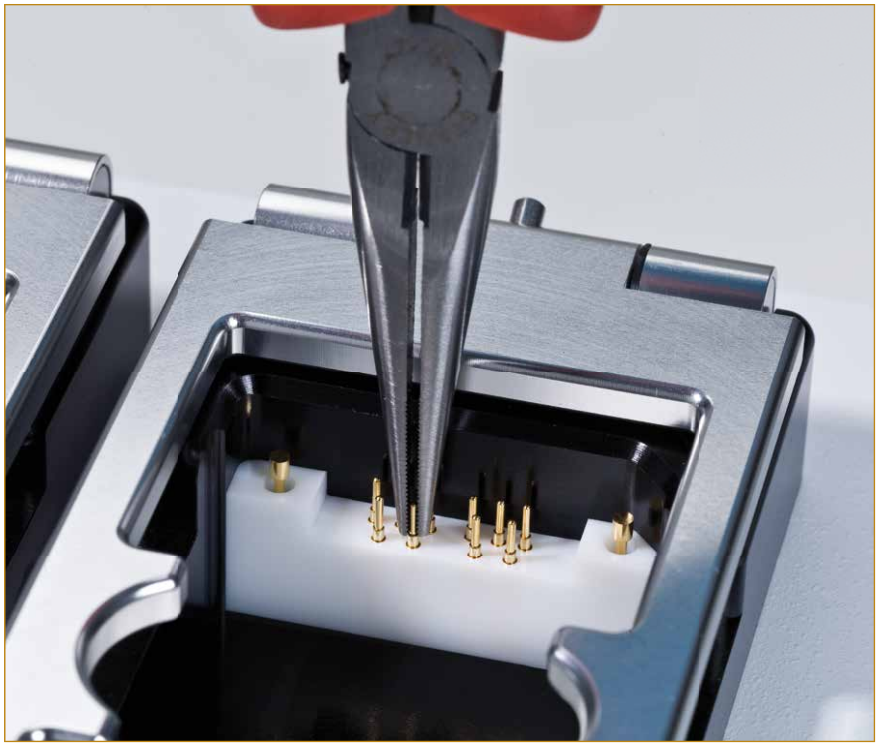


Figure 42: Extraction of an RTCA Contact Pin 16 (Pin GND on Cradle D3).

3. Carefully insert a new RTCA Contact Pin 16 vertically into the receptacle hole, without bending, then use the RTCA Contact Pin Insertion Tool to push down the RTCA Contact Pin 16 vertically down close to the bottom surface. The RTCA Contact Pin 16 should be at the same height as the other adjacent RTCA Contact Pins 16 (Figure 43).

Figure 43: Replacing RTCA Contact Pins 16 (Pin GND on Cradle D3).

4. Use the dust blower to blow the surface around the RTCA Contact Pins 16 to remove any dust.

E Appendix

1. Troubleshooting

1.1 I started an experiment and there was an error message, “Cannot find instrument”, what happened?

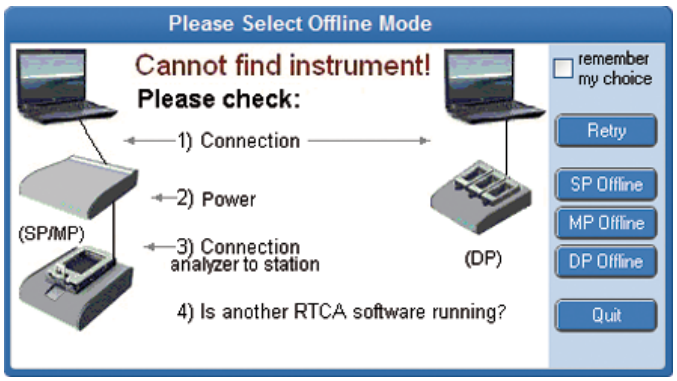


Figure 44: “Cannot find instrument” error message.

The RTCA Control Unit failed to communicate with the RTCA DP Analyzer. This message occurs right after one clicks the RTCA Software icon.

First, make sure that the RTCA DP Analyzer is powered-on and that the RTCA USB Cable, linking the RTCA Control Unit to the RTCA DP Analyzer (USB port) is securely connected. If not sure, please unplug and reconnect the RTCA USB Cable.

Secondly, make sure that two drivers, one for xCELLigence RTCA DP and one for xCELLigence RTCA DP Port, are installed.

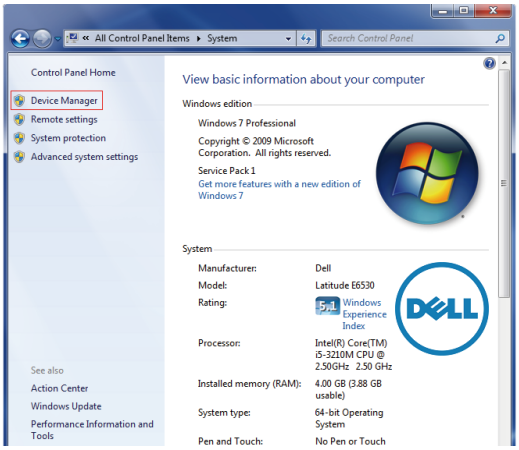
In order for the RTCA DP Analyzer to communicate with the RTCA Control Unit, the two drivers must be installed on the Control Unit. These drivers are provided as part of the Control Unit recovery image, and are installed only in the following cases:

- The RTCA DP Analyzer is connected to the RTCA Control Unit for the first time, *e.g.*, after the Control Unit was unpacked upon receipt.
- The RTCA DP Analyzer is connected to the RTCA Control Unit after the Operating System (OS) was restored, using the RTCA Recovery DVD.

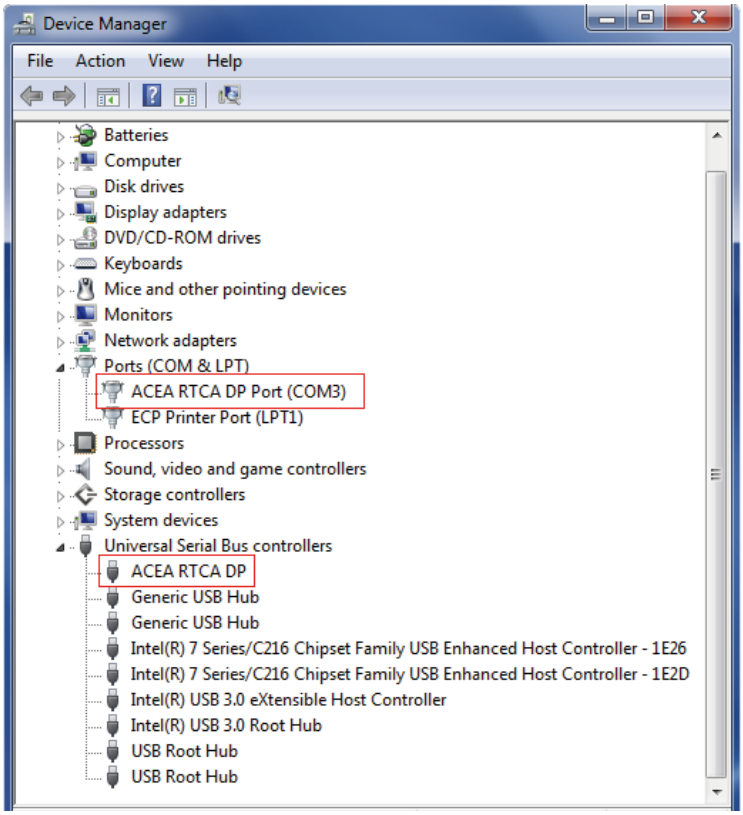
The installation of the two drivers is described in *Chapter B3.5*.

To verify the correct installation of the two drivers, follow the steps described below:

1. Open the Device Manager in the Software of the RTCA Control Unit:



2. In the list of Ports (COM & LPT1) and Universal Serial Bus controllers, the following devices must be present:
   a. *Ports (COM & LPT)* must contain *ACEA RTCA DP Port (COM3)*. Check that the virtual COM is listed and make sure the COM number of *ACEA RTCA DP Port* is less than 10 (COM1~COM9), as shown in the screenshot below.
   b. If the COMn is larger than COM9, please double click the *ACEA RTCA DP Port* and enter into *Port Settings* page, click the *Advanced* button and reassign the COM number to less than COM10 (for instance, change from COM11 to COM3).
   c. *Universal Serial Bus controllers* must contain *ACEA RTCA DP*.



3. Close all the dialogue boxes and retry the RTCA Software.

1.2 I started an experiment. There was an error message, "ACK communication lost." What happened?

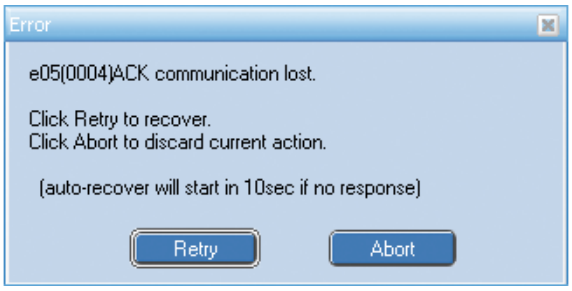


Figure 45: "ACK communication lost" error message.

The RTCA Control Unit failed to communicate with the RTCA DP Analyzer.

First, make sure that the RTCA DP Analyzer is powered-on and that the RTCA USB Cable, linking the RTCA Control Unit to the RTCA DP Analyzer (USB port) is securely connected. If not sure, please unplug and reconnect the RTCA USB Cables.

Secondly, make sure that two drivers, one for xCELLigence RTCA DP and one for xCELLigence RTCA DP Port, are installed (see *Troubleshooting, Chapter 1.1*).

1.3 How do I reinitialize the RTCA DP Instrument after a power failure?

1. When the power is restored, restart the RTCA Control Unit. Do not open the RTCA Software, and do not connect the RTCA DP Analyzer to the RTCA Control Unit at this time.
2. Connect the RTCA USB Cable to the RTCA Control Unit.
3. Open the RTCA Software and locate the data file that was generated before and during the power failure. Open the file.
4. Click the *Continue* button in the top left corner of the RTCA Software. The experiment starts where it left off when power was interrupted.

1.4 The Cell Index signal is significantly lower than usual. Why?

You should take the E-Plate 16 out of the incubator and check the cell status (cell number, cell distribution and morphology) under a microscope. The following are possible reasons:

- Maybe the initial seeding cell number was not correct.
- Maybe you forgot to leave the E-Plate 16 at room temperature for about 30 minutes after the cells were seeded. If that step was not done, then cell distribution might not be uniform over the well surface. The Cell Index signals would be low, especially for the corner wells.
- There could be some type of contamination and cells are dying.
- The cells may have been harvested from an over-confluent flask. Our experience is that if the cells in the flask reached a confluence of over 80%, then the cells may not be in the Log phase and the cell status may not be ideal. Such cells may give a low Cell Index signal when seeded into an E-Plate 16.
- There could be something wrong with the tissue culture incubator. One likely symptom of incubator problems is that the Cell Index is lower on edge wells than on the center wells, or the Cell Index drops to nearly zero faster on the edges than the center wells. The problems with incubators may include CO_2 level, temperature control, humidity control and certain chemicals released from the incubator. We have encountered several instances where the incubator itself was "toxic" or affecting cell growth. Sometimes a simple but thorough cleaning of the incubator interior may be sufficient to solve the problems. Alternatively, simply running the experiment in another incubator may also be a solution.

Please make sure, when removing the E-Plate 16 from the incubator, that the instrument is not performing a measurement. If the instrument is *waiting for next sweep*, make sure that you have enough time to check the cells under a microscope. If you do not have time, you may have to pause the current step and then take the device out of the incubator for assessment. Then *Resume* the experiment after putting the E-Plate 16 back in.

1.5 The Cell Index signal decreases with time, but there was no compound added. Why?

Remove the E-Plate 16 from the incubator and check the cell status (cell number, cell distribution and morphology) under a microscope. Check for the following:

- Contamination resulting in cells dying.
- Culture media that is too old to support the cell growth, because the experiment is too long.
- Culture media quantity is insufficient to support cell growth, due to too much evaporation.
- Maybe there is something wrong with the incubator. One likely symptom of incubator problems is that the cell index is lower on edge wells than on the center wells, or the cell index drops to nearly zero faster on the edges than the center wells. The problems with incubators may include CO_2 level, temperature control, humidity control and certain chemicals released from the incubator. We have encountered several instances where the incubator itself was "toxic" or affecting cell growth. Sometimes a simple but thorough cleaning of the incubator interior may be sufficient to solve the problems. Alternatively, simply running the experiment in another incubator may solve the problems.

Please make sure, when removing the E-Plate 16 from the incubator, that the instrument is not performing a measurement. If the instrument is *waiting for next sweep*, make sure that you have enough time to check the cells under a microscope. If you do not have time, you may have to pause the current step and then take the device out of the incubator for assessment. Then *Continue* the experiment after putting the E-Plate 16 back in.

1.6 The Cell Index signal is significantly higher than usual. Why?

Remove the E-Plate 16 from the incubator and check the cell status (cell number, cell distribution and morphology) under a microscope. It is possible that the initial seeding number was high.

Please make sure, when removing the E-Plate 16 from the incubator, that the instrument is not performing a measurement. If the instrument is *waiting for next sweep*, make sure that you have enough time to check the cells under a microscope. If you do not have time, you may have to pause the current step and then take the device out of the incubator for assessment. Then *Continue* the experiment after putting the E-Plate 16 back in.

1.7 The Cell Index signal in CIM experiments is significantly lower than usual. Why?

Here are some possible reasons for lower Cell Index signals in a CIM experiment.

- The initial cell-seeding number may be incorrect, due to a cell counting error.
- Cells may be unhealthy, due to improper trypsinization, or due to confluence of cells, or due to high passage number of the cells.
- CO_2 and temperature of the CO_2 incubator may be inappropriately set.
- Wells of the CIM-Plate 16 may be contaminated.
- Large bubbles may become trapped in the upper chamber wells or the lower chamber wells.
- Medium may have leaked due to improper assembly of the upper and lower chambers.

Most importantly, please make sure that the recommended protocols are followed and the cells are in good conditions (i.e., <20 passage number for cell lines, and that cells were grown to 60%-80% confluence with one-day culture).

1.8 The well-to-well Cell Index variation is larger than usual when I am using the CIM-Plate 16. Why ?

Here are some possible reasons for a large variation in Cell Index signals when using the CIM-Plate 16.

- Seeded cell numbers may be inconsistent due to insufficient trypsinization (*e.g.* cell clumps), or due to poorly mixed cell suspension, or pipetting error.
- Membranes may have been damaged by pipette tips during cell or medium addition.
- Maybe you forgot to leave the CIM-Plate 16 at room temperature for about 30 minutes after the cells were seeded. If that step was not done, then cell distribution in the upper chamber wells would not be uniform, leading to a lower Cell Index, especially for the corner wells.
- Bubbles may have been trapped in some of the lower chamber wells.
- Media leakage may have occurred due to improper assembly of the upper and lower chamber.

1.9 There was a noticeable delay in some Cell Index curves. Why?

The Cell Index curve fluctuated up and down when performing a cell migration or invasion experiment on CIM-Plate 16. Below are possible reasons for the fluctuation or delay of the Cell Index curves:

- The contact pads may be short circuited by media due to over filled lower chamber wells.
- The CIM-Plate 16 was not engaged properly within the RTCA DP Analyzer cradle during the experiment.
- There were dust particles or other materials on the Contact Pins 16 on the RTCA DP Analyzer, providing inconsistent contacts to CIM-Plate 16. Follow the procedure to clean the Contact Pins 16 on the RTCA DP Analyzer.
- Media leakage may have occurred during the measurement.
- The user did not put the CIM-Plate 16 at 37°C in the incubator for equilibration after assembly for one hour.

1.10 I did a background measurement. The RTCA Software indicated that many wells may have background problems. What should I do?

Please remove the E-Plate 16 or CIM-Plate 16 from the RTCA DP Analyzer, ensure that the contact pads on the E-Plate 16/CIM-Plate 16 are clean and re-insert the plate. Then perform *Scan Plate*. Check the *Message* page for results to see whether you have many wells showing connection problems. You can also check the plate scanning data shown on the *Cell Index* Page. You can check whether any measured well values show an open circuit (*i.e.*, the resistance values are abnormally high, for example, more than 500 Ω) or are negative. If the *Message* page shows *Plate Scanned. Connections ok*, then you should be able to re-start the experiment. Note that the RTCA Software may still indicate that a few wells may have background problems since the software has strict criteria for determining whether a well has a good background. A well that is indicated as "may have background problems" does not always cause an experimental problem.

If the message indicates connection problems with many wells or if the plate-scanning data file shows many wells having open circuits, there may be problems with the E-Plate 16/CIM-Plate 16 or RTCA DP Analyzer or all. Please perform an additional *Scan Plate* after removing the E-Plate 16 or CIM-Plate 16 in question from the RTCA DP Analyzer and then re-inserting/repositioning it in the instrument. Check the *Scan Plate* results again, as described above, to see whether the results for the wells concerned are similar to previous results. If problems do not occur in the 2nd *Scan Plate*, experiments can be continued. The initial issue may be related to the insertion or positioning of the E-Plate 16 or CIM-Plate 16 into the RTCA DP Analyzer.

If the results for wells with connection problems or open circuit wells persist, then either the E-Plate 16, the CIM-Plate 16, and/or the RTCA DP Analyzer are the reason.

To test this, first check to see whether the E-Plate 16/CIM-Plate 16 or RTCA DP Analyzer is damaged. If so, contact ACEA support.

Second, if there is no visible damage, verify that all connector pads on the E-Plate 16/CIM-Plate 16 are clean and free of dust/dirt particles. If not, clean the connector pads on the E-Plate 16/CIM-Plate 16 with a tissue wetted with 80% Ethanol. After this cleaning, re-insert and reposition the E-Plate 16/CIM-Plate 16 for another *Scan Plate* test. If the number of open-circuit wells is the same as before, then please report the problems to ACEA support

1.11 I am contacting ACEA support. What data files should be sent?

In case of inquiries or complaints, please ALWAYS SEND the experiment file (*.plt), including the experiment data and information. Alternatively, please use the Problems Report function, which is available under the *File* pull-down menu in the RTCA Software, to create a Problem Report. The RTCA Software will provide an option that allows you to remove any confidential information from your experiment. Please send this Problem Report file (Err_YYMMDD****.zip) to ACEA support.

2. Ordering Information

ACEA Biosciences Service and Support

At ACEA we are committed to providing innovative, high-quality products and excellent customer service. For a complete overview of our products or to find a local representative, visit www.aceabio.com.

RTCA DP Analyzer		**Cat. No. 05469759001**
RTCA Control Unit		**Cat. No. 05454417001**
RTCA Resistor Plate 16		**Cat. No. 05469783001**
E-Plate 16	6 Units	**Cat. No. 05469830001**
	6 × 6 Units	**Cat. No. 05469813001**
RTCA Contact Pins 16 (20 units)		**Cat. No. 05471575001**
RTCA Software Package		**Cat. No. 05454433001**
CIM-Plate 16 Assembly Tool		**Cat. No. 05665841001**
CIM-Plate 16	6 Units	**Cat. No. 05665817001**
	6 × 6 Units	**Cat. No. 05665825001**
E-Plate Insert 16	1 × 6 Units (6 16well inserts)	**Cat. No. 06465382001**

E

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