XF96 Extracellular Flux Analyzer and Prep Station

Installation and Operation Manual

General Assay Flow Chart for XF96 Assays

The Seahorse Bioscience logo appears at the top.

Day Prior to Assay (materials preparation)

- Prepare stock compounds to be injected
- XF Sensor Cartridge Hydration
- Seed cells at proper density on XF96 TC plate
- Prepare XF Assay Media

Day of Assay

- Thaw and warm to 37°C
- Remove growth media. Wash cells with XF Assay medium, add XF Assay media to proper volume using Prep Station
- 1X XF Assay Medium pH 7.4 @ 37°C
- Incubate 37°C, 60 min NO CO_2
- Add stock solution(s) with initial assay conditions to wells (if applicable)
- Add to injection ports of cartridge
- Prepare stock compounds (if needed freshly made)
- Load Ports and Calibrate Cartridge
- Transfer plate to XF96

100900-400_MANUAL XF96 W/ PREP STATION_RA

Seahorse Bioscience

Quick Reference
XF96 Analyzer Protocol

Please refer to the XF96 Manual for detailed instructions

1. Pre-incubate sensor cartridges overnight in XF96 Calibrant solution in a non-CO_2 incubator at 37ºC.

2. Leave XF96 Analyzer on overnight with XF96 software running and logged in to ensure temperature is at 37ºC on the day of the assay.

3. Warm all media and compounds to 37ºC and adjust pH to 7.4. Filter to ensure sterility.

4. Using the Prep Station, perform XF Assay media change on cell plate and incubate in a non-CO_2 incubator at 37ºC for ~20 minutes before running on XF96 Analyzer. Final volume per well should be 150-200 µl.

5. Load XF Assay template into XF96 software or make a new template using the Assay Wizard. Use the table below to program the optimal mix, wait and measure cycle times. When working with a new or unknown cell line, start with a 2 minute mix and 5 minute measure cycle. Verify instrument protocol commands, export preferences and any instrument settings that may have changed.

6. Load 25 µl pre-warmed compounds (diluted in <u>XF Assay</u> media) at pH 7.4 into appropriate ports on the sensor cartridge. Optimal injection volume is 25 µl. All wells of each port used need to be loaded with either compound or media control, including temperature correction wells. If the sensor cartridge is allowed to cool down (more than 5 minutes), return to incubator for 10 minutes to allow it to heat back up before starting the calibration.

7. Load sensor cartridge and Utility plate into the right side of the instrument tray.

8. After calibration is complete, replace Utility plate with the pre-incubated cell plate.

Table of Contents

Introduction

1. Seahorse Bioscience XF96 Instrument Overview and Intended Use

The Seahorse Bioscience XF96 instrument measures the rate of change of analytes dissolved oxygen and pH in the media immediately surrounding living cells cultured in a microplate. Changes in the extracellular media are caused by the consumption or production of analytes by the cells. Therefore, a sensitive measurement of the media flux can be used to determine rates of cellular metabolism with great precision and in a totally non-invasive, label-free manner.

A unique feature of the XF technology is its ability to make accurate and repeatable measurements in as little as five minutes. This is accomplished by isolating an extremely small volume (less than 3 μl) of media above the cell monolayer. Cellular metabolism causes rapid, easily measured changes to the "microenvironment" in this small volume.

Typically, a measurement cycle is performed for 5-8 minutes. The media is gently mixed and the analyte levels are then measured until the oxygen concentration drops approximately 20-30% and media pH declines approximately 0.1-0.2 pH units. The measurement is performed using 96 optical fluorescent biosensors embedded in a sterile disposable cartridge that is placed into the Seahorse 96 well tissue culture microplate.

Baseline metabolic rates are typically measured 3-4 times, and are reported in pmol/min for OCR (and PPR) and in mpH/min for ECAR. Compound is then added to the media and mixed, and then the post-treatment OCR and ECAR measurements are made and repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR (PPR) changes.

Because XF measurements are non-destructive, cells can be profiled over a period of minutes, hours or days.

The XF96 system include a bench top analyzer and touch screen controller, a plate preparation station (PS) for exchanging media in the tissue culture plate, disposable measurement / compound delivery cartridges, and microplates for cell culture, calibration media and XF96 software.

Consumable cartridges, microplates and calibration reagents are packaged and sold in sets named "Fluxpaks". One Fluxpak contains the materials for 18 assays.

2. Technical Specifications

Model	XF96	Prep Station
Dimensions: Controller: Analyzer:	Width x height x depth 18″ x 22 (max)″ x 14″ 46 cm x56 cm x 36 cm 16″ x 24″x 17″ 41 cm x 61 cm x 43 cm	Width x height x depth 14″ x 19″ x 20″ 36 cm x 41 cm x 51 cm
Weight:	Controller: 20 lbs. / 9 Kg Analyzer: 35 lbs, / 14 Kg	35 lbs. / 16 Kg
Power Requirements	100-120V AC 50/60Hz (220-240VAC 50/60Hz export) 6A (3A) Analyzer 1.8A (0.9A) Controller	100-150V AC 50/60Hz, (220-240VAC 50/60Hz export)
Power cord rating	3- wire (grounded) AC power cord rated 10A or greater	3- wire (grounded) AC power cord rated 10A or greater
Power fuse ratings	10A (5A export) in each of Analyzer and Controller	5A
Environmental Conditions	"Normal" Environmental conditions- indoor use, altitude to 2000 m	"Normal" Environmental conditions- indoor use
Room Temperature Range	+60˚ to 86˚F / +15˚C to +30˚C No direct sunlight Humidity less than 80% for temp up to 31˚C, decreasing linearly to 50% at 40˚C	+60˚ to 86˚F / +15˚C to +30˚C No Direct Sunlight
Internal Temperature and Environment	Controlled to user selected temperature not less than 10 ˚C above ambient temp No CO_2 control	Incubator and bottle holder calibrated and set to 37°C, (user may change both) No CO_2 control
Software OS	Windows Vista	Windows Vista
Data Interface	RS232c (internal) TCP/IP (external)	RS232c > USB (1 cable, with separate Serial to USB adaptor)

XF96 MICROPLATE DIMENSIONS

MATERIAL: PET

CELL CULTURE COMPATIBLE: PROPRIETARY TISSUE CULTURE TREATMENT

STERILITY: RADIATION TREATED

WELL VOLUME: 450µL OR LESS (200µL = TYPICAL WORKING VOLUME)

MEASUREMENT VOLUME: 2.28µL

ALL DIMENSIONS IN MILLIMETERS UNLESS OTHERWISE NOTED
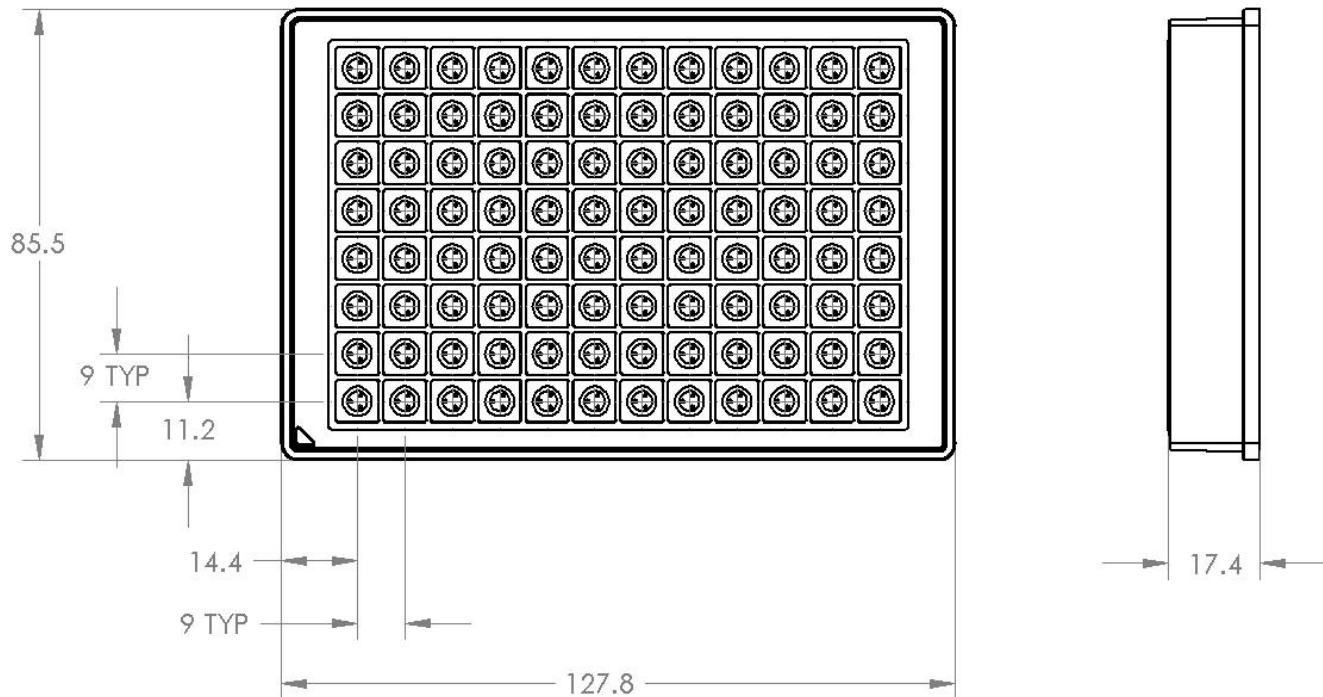
3. XF96 Instrument, Controller and PS Installation Procedure

Unpacking and Component Identification

The XF96 system is packaged in three boxes.

On receipt, immediately check each box for damage. Shipping damage must be reported to the transportation company and Seahorse Bioscience using the contact information on page 2 of this document.

The following items are included in an XF96 system:

XF96 Analyzer- The analyzer is a temperature controlled instrument that contains all optical and electronic measurement components to measure oxygen and proton flux of cells grown in XF96 cell culture plates. The analyzer is used in conjunction with XF96 sensor cartridges.

Plate Preparation Station (PS) – The PS is required for accurately exchanging growth media for assay media in the tissue culture plate wells. It also contains a temperature controlled oven for incubating the cell culture plates in a 37°C/non-CO_2 environment after the media exchange and a temperature controlled dry bath for warming and incubating the assay media.

Controller. Operation of the Analyzer is done through a high resolution color LCD touch screen that may be located in front or beside the XF96 analyzer (a separate keyboard and mouse (included) may also be used). The controller communicates with the analyzer using a single digital communication (RS232) cable and a single Serial > USB cable and communicates with the PS via a single Serial > USB cable.

Prep Station (PS) and XF96 Flux Analyzer

The XF96 and Prep Station require two people to lift and handle safely.

Each person should firmly grasp the base of the unit at opposite ends to one another. Use OSHA standards for lifting techniques.

The following items are included in the XF96 instrument and controller boxes

Instrument/Controller	Number	
XF96 Instrument	1	
XF Controller	1	
XF Controller Stand	1	
Power Cord (instrument)	1	
Power Cord (controller)	1	
Power Supply (controller)	1	
RS232 Cable	1	

RS232 > USB Cable	1	
Wireless Mouse/Keyboard	1 set	
USB Extension Cable	1	
Extra Fan Filters	2	
Excel 2007 CD	1	
USB drive	1	

The following items are included in the Prep Station Box:

Prep Station	Number	
Prep Station	1	
8 channel Manifold		

XF Plate Tray		
Power Cord	1	
RS232 Cable	1	
RS232>USB adaptor and USB cable	1	
Reagent Bottles	5	
Reagent Bottle Top/Tube (with white check valve)	2	
Reagent Bottle Top/Tube (without check valve)	2	

Waste Bottle	1	
Bottle Hose Assembly (with red check valve)	1	
Media Hose Assembly (with red check valve)	1	
Waste Hose Assembly (with waste bottle top)	1	
Vacuum Filter/muffler	1	

Suitable Locations for the XF96 system

The XF96 is designed for laboratory use. The internal environment of the analyzer is controlled to a preset temperature by the user, and therefore, laboratory room temperature must be maintained within the range listed in the specification table. Analyzer temperature control performance can be monitored using the status display on the right side of the analyzer.

The XF96 uses optical detection technology to measure extremely low levels of fluorescent emission from analyte sensors. While the analyzer has been designed to

shield room light, excessive light such as direct sunlight is likely to influence measurements and should be avoided.

The XF96 system is intended to be used with a non-CO_2, 37°C incubator that is a built in component of the Prep Station. This allows cell culture plates to remain warm during transport from the incubator to the analyzer.

Removing Shipping Protection Components from the XF Analyzer

The analyzer is shipped with protection components that must be removed prior to use. To prevent damage during shipping, the instrument is shipped with a cartridge loaded onto the probe head and lowered onto a plate on the tray. These items must be removed prior to running your first assay by doing the following:

1) Power on both the controller and instrument. Launch the XF96 Analysis software. The software will initialize the instrument and raise the probe head.

2) Click on the "Instrument" button, and then the "plate manager" button as shown to the right.

3) Click on the "strip cartridge" button. The tray on the instrument will open and you will be prompted to put a plate in the tray. Since there is already one present, just click "OK".

4) The shipping cartridge will be stripped from the probe head and then the cartridge and plate will be ejected from the instrument. When the tray opens, you will be prompted to remove the cartridge and plate. Once removed, click OK.
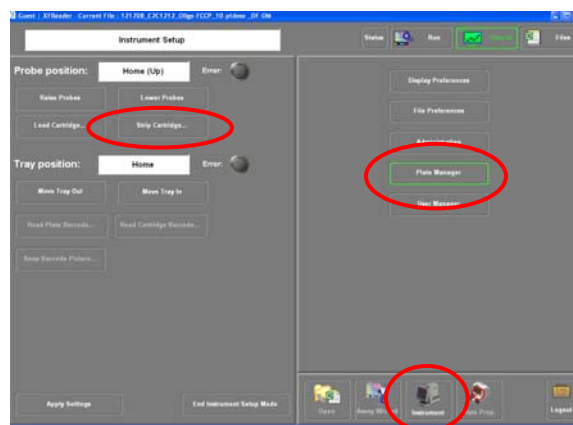
Internal components of the XF96 Analyzer

The measurement enclosure is the heart of the XF96 Analyzer. Removing the side doors reveals the measurement chamber in which the assay is conducted. The electro-optics hardware is enclosed in a card cage in the rear chamber and this is connected to the probe head through a set of bundles of fiber optic cables. The base of the enclosure contains the primary controller board and heater assembly. The following describes each of the hardware features pointed out in the images below.

1 LCD Message screen—Displays current instrument action and internal temperature.
2 Cover latches—Push buttons to lift gull wing doors (not shown) up to expose the internal components of the instrument
3 Tray and Heat Sink
4 Probe Head and Injection Manifold—The probe head consists of 96 "light pipes" to carry the optical signals to and from the sensors. The Injection Manifold uses compressed air to inject compounds loaded into Sensor Cartridge ports
5 Fiber Bundles
6 Front Stripper Arm—Used for Sensor Cartridge removal at the end of assay runs, there are 4 total Stripper Arms, 2 in front and 2 in rear.
7 Bar Code Reader—Reads bar code on Sensor Cartridge and cell plate
8 Circulating Fan—Helps to stabilize environment inside XF96
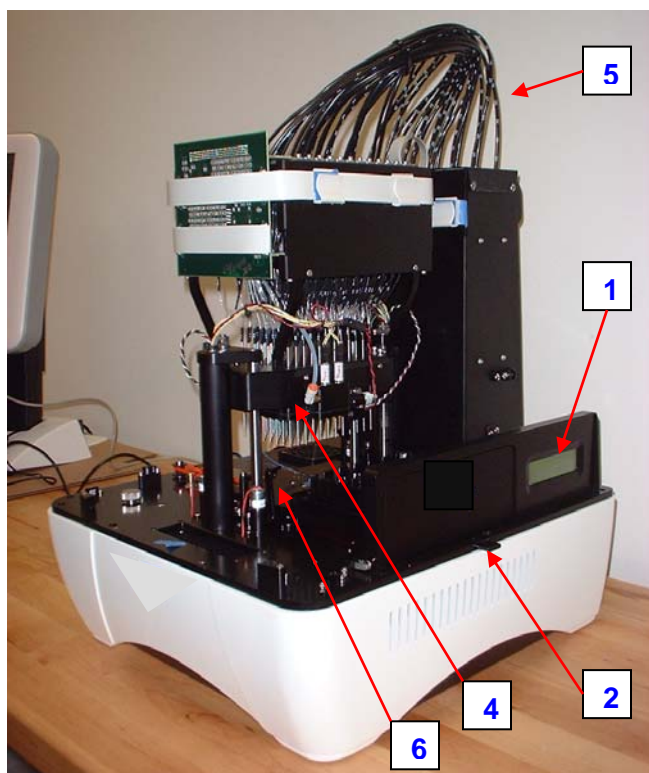
XF96 Front/Side View   XF96 Front View
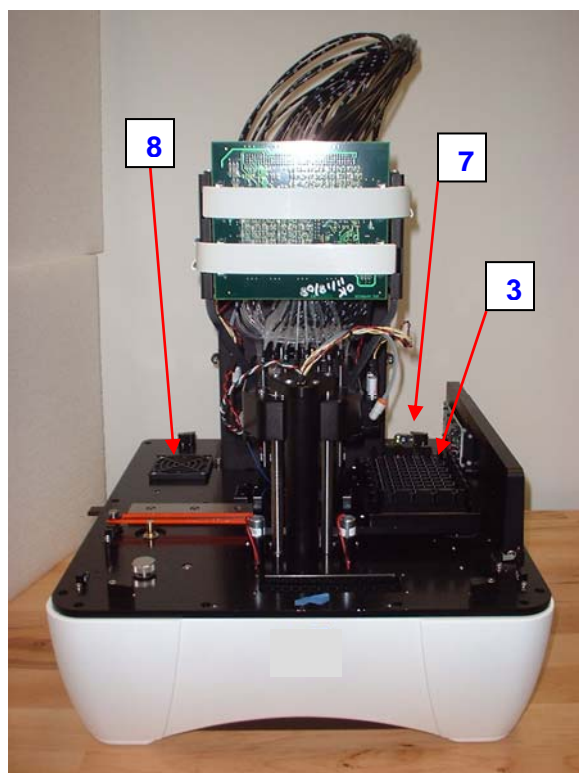
Setup and Interconnects: Cable Installation

The XF96 Analyzer is operated from a touch screen computer mounted to a stand that fits up against the front of the instrument. Two RS-232 cables handle the communication of commands and data between the instrument and the computer.

The controller may be connected to an external network. A mouse and keyboard have been provided to allow the use of software other than the XF96 instrument control application.

Refer to the list and pictures on pages 13-16 to identify each cord, and refer the diagrams on the right to identify the sockets.

Controller Ports (underside)

1. Connect power cords:

   One power cord is used to connect the instrument to a grounded AC (mains) outlet. A second power cord is used to connect the controller power supply module to the AC supply. The power supply module is then connected to the socket at the bottom of the controller (lay the controller on a flat surface to expose the connectors). Repeat this procedure to connect the PS using a power cord and power supply module.

XF96 Rear Panel

2. Connect the data cables

   One RS 232 cable connects the controller serial port to the analyzer socket labeled "COM 1"

   A second cable (serial to USB, *no separate adaptor*) connects the analyzer socket labeled "COM 2" to the USB port on the

PS Rear Panel

controller *directly adjacent to the network (Ethernet) port.* This port must be used for proper functioning of the instrument and barcode reader (see diagram to right).

A third cable (RS232 serial cable with a *separate USB adaptor*) connects the PS serial port to the USB port (Com 3) which is directly below the Com 2 USB port on the controller. This port must be used for proper functioning of the instrument and Prep Station (see diagram to right).

Network Port

USB Port for Com 3 (Prep Station)

USB Port for Com 2 (Barcode Reader)
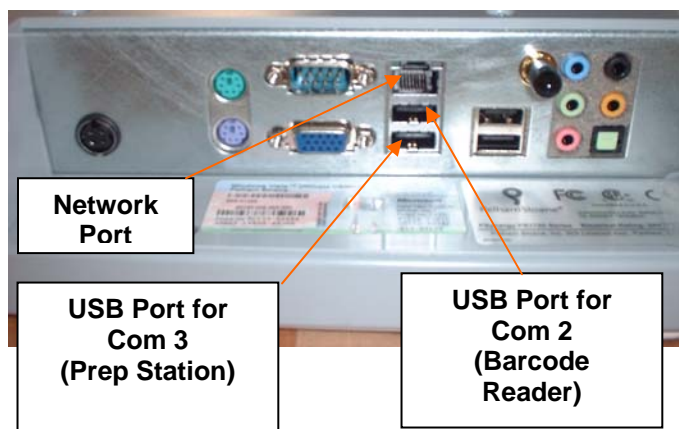
Controller USB Ports for Com 2

and Com 3 connections

Connect the USB extension cable to one of the USB ports on the controller to allow for easy access to the USB port for data transfer to a USB memory device.

3. Connect external network cables. The controller may be networked via the Ethernet port on the controller.

4. Connect the Mouse and Keyboard: Open the mouse and keyboard set and insert the batteries properly. Insert the wireless USB module into one of the USB ports on the controller. (Note: the mouse and keyboard should function properly without inserting the accompanying CD into the controller.)

Note that you may be required to install the Keyspan USB Serial Adapter software in order for the controller and PS to communicate with each other. Please see below for detailed instructions on installation and set up of the Keyspan adapter.

Keyspan USB Serial Adapter Installation and Setup

The Keyspan USB serial adapter is designed to connect the Plate Preparation station to the controller or separate computer. It is a communications pass through that is required for the Plate Preparation station to be operated by a computer.

Installation

Install the drivers for the Keyspan adapter BEFORE plugging the cord into the computer.

1. Insert the CD with the Keyspan label that came with the instrument.
2. At the prompt, follow the installation instructions.
3. If you do not have a CD with the drivers, you can download the driver here:
   http://www.tripplite.com/en/products/model.cfm?txtModelID=3914
4. Plug the USB end of the adapter into the computer. The installation will now complete.

Assigning a Communications Port to the Keyspan Driver

Once the driver is installed, it is necessary to check the communications, and if necessary assign a communications port to the device.

1. Access the device manager. For windows XP, go to "Start -> Control Panel" and click on "System." Next click the "Hardware" tab and then click "Device Manager." For Vista, click on the Windows icon, then select "Control Panel." Switch to "Classic View" then follow the above instructions.
2. In the device manager, expand the "Ports & LPT" tree.
3. Next to "Keyspan" should be (COM3). If not, right click on Keyspan and select "properties."
4. Click on "Port Settings."
5. In the COM Port settings drop down, select COM port 3.
6. Click "OK" then close the windows.

Setting up Communications in the XF Software

After driver installations and once the communications port has been set correctly, you may need to let the XF software know which port to use to communicate with the Plate Preparation Station.

1. Start the XF96 software.
2. Login to the software.
3. Click on "Instrument" in the lower right corner of the screen.
4. Click on "Administration" on the right side of the screen.
5. Click on "Communications" on the left side of the screen.
6. Under "Communications Settings: Prep Station" make sure that Comm3 is selected and that the light is GREEN.
7. If "none" is displayed, use the drop down to select "Comm3."
8. Click "Connect."
9. After a moment the light should turn green.

5. Preparing and installing the Prep Station (PS)

Unpacking the Prep Station

PS in shipping carton

PS accessory tray and boxes

PS with foam end caps

Remove the accessory tray and accessory boxes from the shipping carton. These contain the tubing, valves, bottles, etc.)

Tip the shipping crate on its side and slide out the instrument and its foam end caps.

Tip the instrument upright and remove the foam end caps.

Place the instrument on the bench with the spray shield facing you (see diagram to right).

PS ready for packaging removal

Remove the tape holding the incubator door closed.

Open the incubator door and remove the packing foam.

Remove the plate shelf assembly and remove its packing sleeve.

Replace the plate shelf and close the incubator door.

Installing the PS Manifold and connecting the Hose Systems

Slide the incubator to the right of the instrument to clear the spray shield. It will slide far enough so the left side of the incubator is in the center of the instrument (see left diagram below).

Loosen the two thumb screws on the left side of the instrument and remove the spray shield. Lift the left side of the spray shield while pulling away from the PS.

Remove the packing foam from underneath the manifold mount and remove the two thumb screws from the top of the manifold mount (see right diagram below).

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Unpack and install the manifold on the top of the manifold mount using the two thumb screws removed in the previous step. The manifold is installed on the front underside of the mount with the metal nozzles facing down and the three plastic hose couplings facing towards the rear of the instrument.
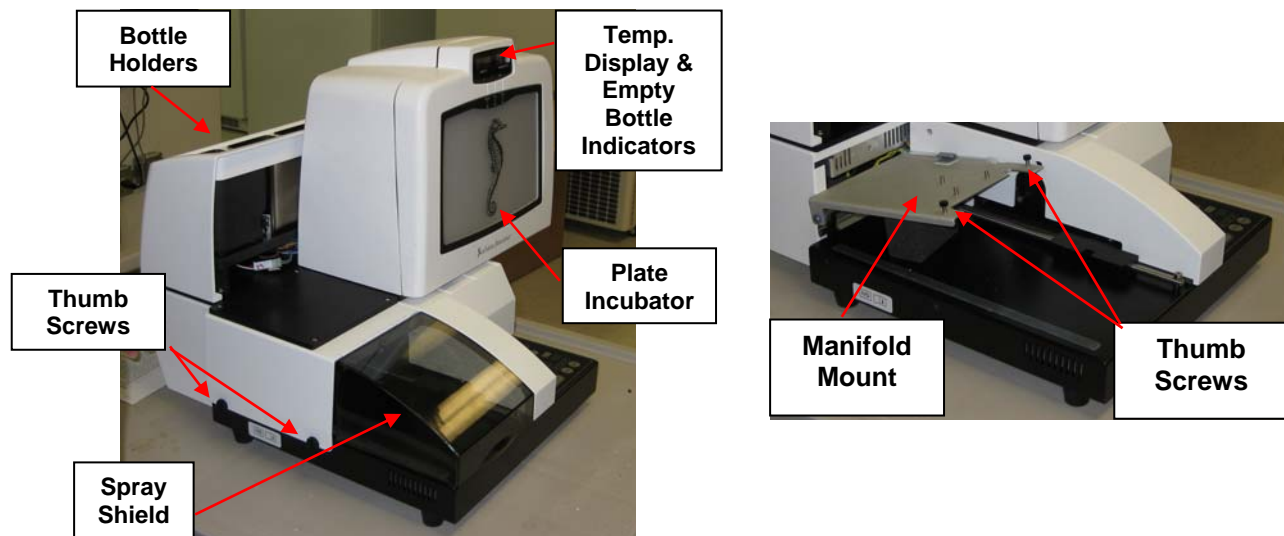
Please refer to list and pictures on pages 14-16 to assist with hose, bottle and valve identification. Use the diagrams below and pictures on the following pages as well as the diagrams on the PS itself for connecting the hoses, valves and bottles. Note that the hoses and connectors are coded with different color O-rings to facilitate the connection process. Often it is easier to install the hoses if the instrument is placed on the bench with the rear side facing out.
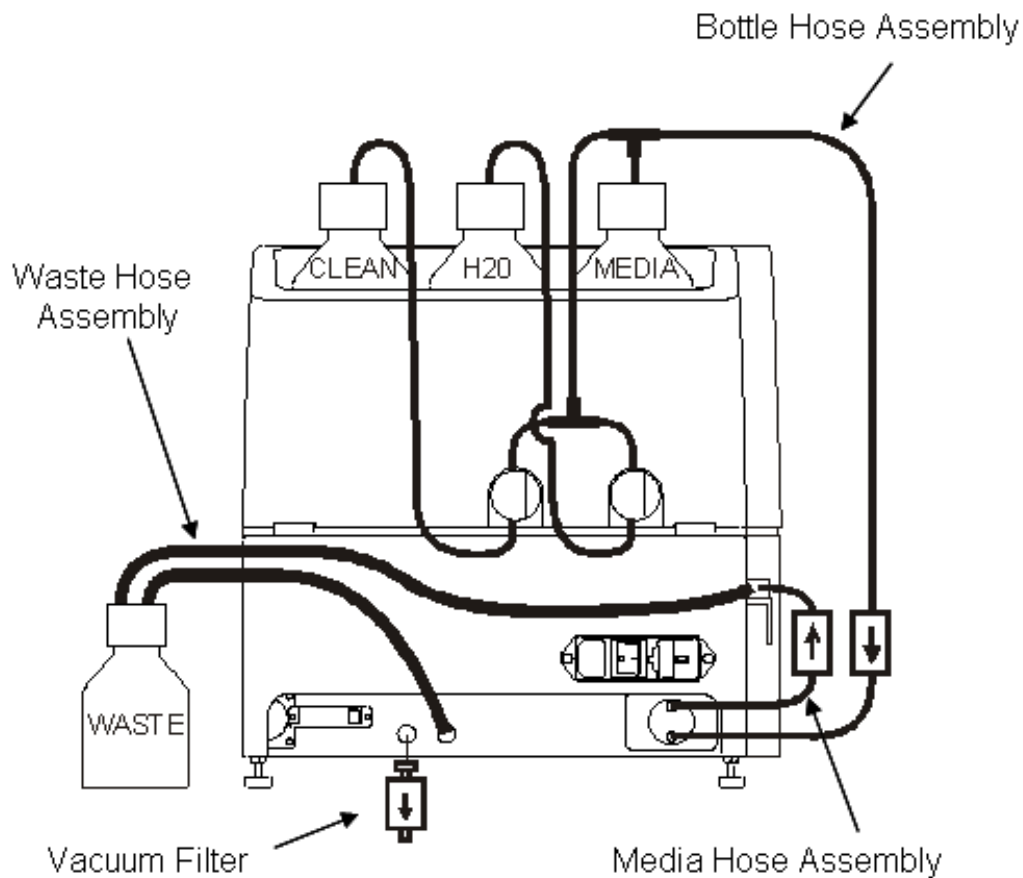
Schematic Diagram of the PS Hose Connections (rear view)

Schematic Diagram of the Waste Hose Assembly and Media Hose Assembly to the manifold (Top view).
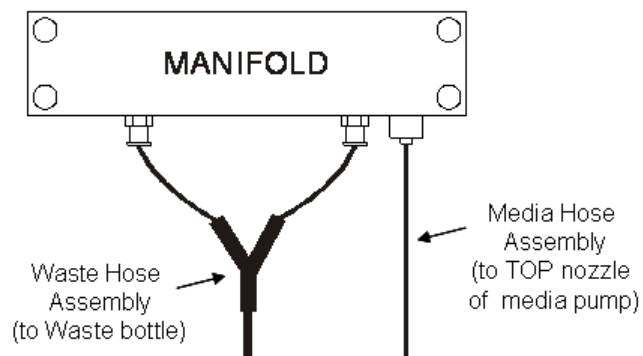
Identify the waste hose assembly and locate the Y-shaped waste manifold hoses portion.

Connect the two waste manifold hose connectors to the two connectors in the *center* of the manifold. They screw in with a half turn.

Twist the 2 hose ends ½ turn to the left to facilitate hose attachment
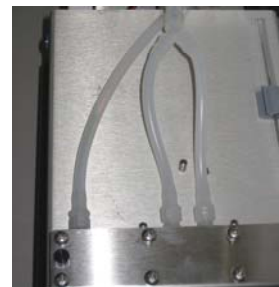
Media Hose & Waste hose assembly connected to manifold

Connect the single end of the waste hose assembly to the vacuum pump connector on the rear of the instrument (match black O-rings).

Identify the media hose assembly. Attach the end with the twist fitting to the connector on the end of the manifold (match black O rings).

Attachment of the media hose to the manifold. Note orientation of manifold.

Attachment of media hose to the output of the media pump (top nozzle, white O-rings). Note waste hose connected to vacuum pump connector (left side)

Attach the other end of the media hose assembly (with red check valve) to the UPPER (output) nozzle of the media pump (match white O rings). Ensure the arrows on the red check valve indicate that the flow direction is out of the pump and into the manifold

Next, unpack and install the three bottles and caps into the bottle holders. The media bottle (with white check valve under cap and brown O ring) should be placed in the right bottle position (facing the back of the PS). The H_2O bottle and cap (no check valve and blue O ring) should be placed in the center position. The clean/sterilize solution bottle (no check valve and red O-ring) should be placed in the left position (facing the back of the PS).

Locate the bottle hose assembly and install using the pictures and diagrams as a guide. The end with the red check valve is fitted onto the LOWER (input) media pump nozzle (match green O-rings). Ensure the arrows on the valve indicate that the flow direction is out of the bottles and into the pump.

Connect the 3 bottle connectors to the three bottles. Use the color coding on the O rings and the diagram on the rear of the PS to facilitate accurate connections.

The insertion of the hoses into the pinch valves, (which are the round white valves on the rear of the PS) requires some force. The hoses may be stretched to facilitate insertion into the pinch valves (see diagram to right). The end of a wooden Q-tip also may be used to facilitate insertion.
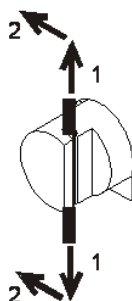
Fitting tubing into a pinch valve. The tube must be stretched, then slid into the pinch valve completely.

Note this is critical step. If the hoses are not completely engaged in the valves, the instrument will allow the flow of clean/disinfectant solution or water when commanded to pump media.

Ensure that the media bottle contains the intake hose with the white check valve and be sure that the bottle intake hoses do not occlude the paths of the empty bottle sensors.

Blue vacuum filter connected to the vacuum intake port

Install the vacuum filter (blue) on the vacuum intake (see diagram to right).

Insert the large stopper (Waste Hose Assembly) into the large white waste bottle and place next to the PS.

Finally, place the XF Plate Tray onto the PS as shown in the figure to the right. Ensure that the rinse basin is toward the rear of the instrument and that the tray is square and level, with the guidance tabs on the bottom aligned properly onto the tray track.
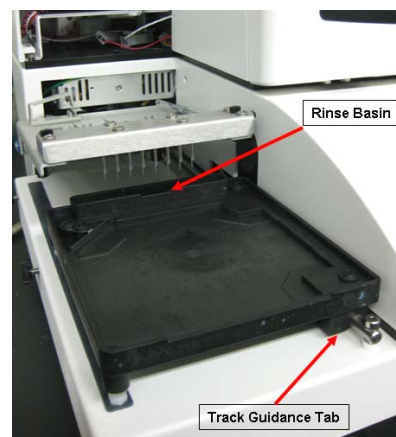
Tray Plate in PS

Testing the Prep Station

Connect the USB cable to the serial adaptor. Connect the USB end of the assembly to the XF controller in the designated port (see page 19) and the serial end to the PS.

Place an XF96 cell plate into the plate holder.

Turn on the power of the instrument, PS and the XF controller (see page 28). Check that temperature displays are on and the bottle empty indicators are lit. Note that they are only lit with empty bottles, nearly empty bottles, or when no bottles are installed.

Loading the cell plate on the PS

Fill all bottles with water and check that the bottle empty indicators are now off. To ensure that the hoses and valves are assembled correctly and connected to the proper bottles, add a few drops of food coloring to each of the bottles (use a different color for each bottle).

Launch the XF96 Software and log in (see page 29) Click on the "Plate Prep." button in the lower right of the screen. The PS Operations screen will appear.
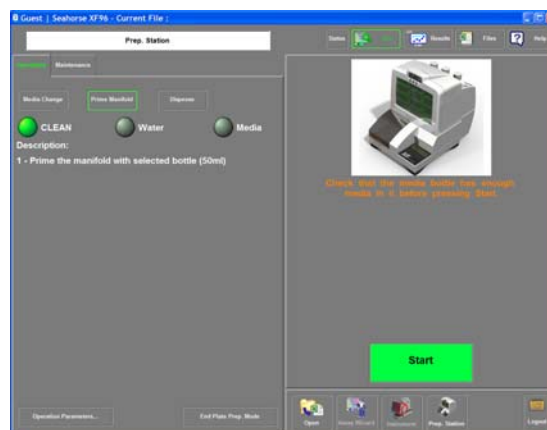
PS: Prime Manifold Screen

Click on the "Prime Manifold" Tab. Click on the CLEAN button, and press the green Start button. Check the liquid color and flow in the tubing to ensure that the manifold is being primed from the correct bottle. Repeat the test priming for the H$_2$O and Media bottles.

Next, perform a mock media change to test the system. Click on the "Media Change" button. Engage the "Do Prime" and "Do Rinse" buttons. Load an XF96 culture plate onto the PS. Press the green start button. The PS will prime the manifold from Media bottle, then perform 2 aspiration and
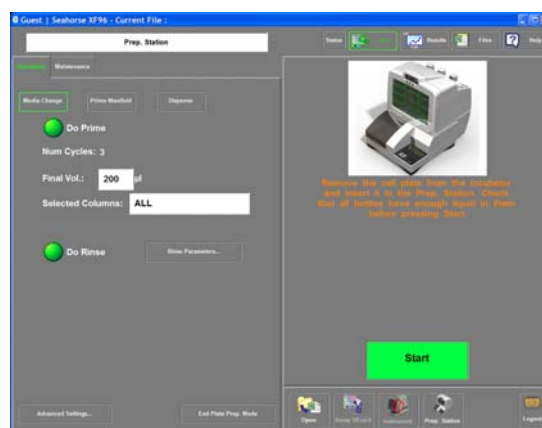
PS: Change Media Screen

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dispense cycles. There will be a pause in the operation, then the PS will perform a third aspiration and dispense cycle. *Do not remove the cell plate until all 3 cycles are complete.* After the 3rd wash/dispense cycle is complete, the PS will automatically enter into a self cleaning mode that consists of washing the manifold with H$_2$O, clean/disinfectant solution and H$_2$O again.

Once proper assembly has been confirmed, rinse the bottles with water.

Install the spray shield and tighten the thumb screws. To install the spray shield ensure that the metal tabs on the right side of the shield are aligned with the holes in the middle of the PS where the spray shield inserted. Slide the incubator back to its normal position.

Ensure that the temperature controllers both reach 37°C.

When an experiment is to be performed, fill the bottles with the appropriate solutions.

Important Note: All fluid lines need to be primed for the unit to dispense properly*. (e.g. If water line is empty, media will flow back in the wrong direction.)* To prime the lines, enter the Prime Manifold screen (see above) and select the bottle/reagent to prime in the following order.

- o A: Clean
- o B: H$_2$O
- o C: Media

Temperature Settings for the PS Incubator

The media and incubator temperature controllers are factory set to 37°C and calibrated for this temperature. Changing the temperature set point does not guarantee that the temperature of the media or incubator is at the set point and it is recommended that a thermometer be put into the incubator to obtain an accurate reading if the temperature of the incubator is changed.

Green:	OFF -	PV < SP
	ON -	PV = SP
	Flashing -	PV > SP

Yellow:	OFF -	Normal Operation
	ON -	Control Setup
	Flashing -	Instrument Configuration

| Red: | Flashes when alarm(s) active |
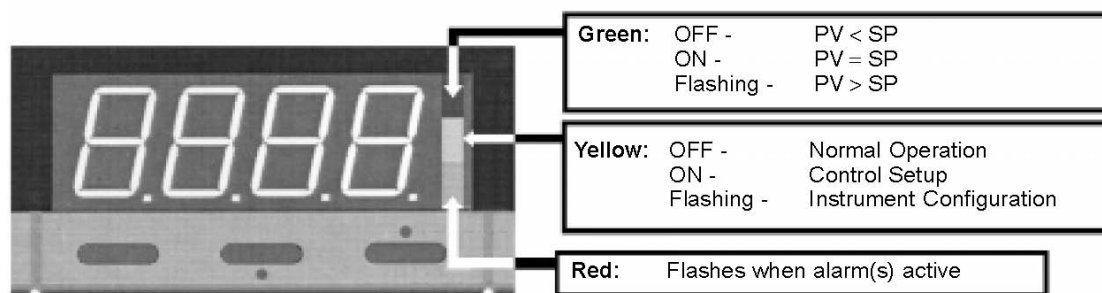
Scroll   Up   Down

Note that the displayed temperatures are the temperatures measured by a sensor on the outside of the media bottle holder and a sensor in the incubator. It does not necessarily reflect the temperature of the plates in the incubator or the media in the media bottle. Note that the typical warm up time for 500 ml of medium from 4°C to 35°C is approximately 90 minutes (compared to ~2 hours in regular water bath).

Changing the Temperature Settings (see diagram previous page)

Open the incubator door and press the scroll key twice until SP is displayed.
In several seconds, the current set point will show on the display.
Use the up and down keys to change the setting. Note that the range of available set points is from ambient to 50°C.
Press the scroll key twice until PROC is displayed. The controller will enter normal operation in several seconds where the temperature of the controller feedback sensor is displayed.

Safety Considerations

Safe operation of the instrument requires that the cover be securely attached and plate tray door is closed. This also prevents heat loss and system cooling which can affect data quality.

The door opens automatically when the tray is extended, allowing the operator to insert or remove the assay consumables.

When the cover is securely attached and tray door is closed, a physical switch is engaged to monitor the system. An optical sensor is used to determine the status of the door.

The XF96 has a heater that maintains a stable internal system temperature. Typically, the temperature will be maintained at 37°C, as monitored by temperature sensors and controllers embedded in the tray and above the tray. Two fans circulate air through the

plenum containing the heater. A thermal fuse will disable the heater should it reach an abnormally high temperature.

The plenum also provides additional spill protection. Should a spill occur outside the tray, the fluid will fall through the air circulation grates and into the plenum. A drain tube leads from the bottom of the plenum to the exterior of the instrument to prevent accumulation.

4. Basic Operation of the XF96 Instrument

Power and Warm up

To power on the touch screen controller, press the power switch on the top of the controller. To prevent accidental power down of the controller, the switch may be disengaged in the "Power Options" menu of the Windows OS control panel under the "Advanced" tab.
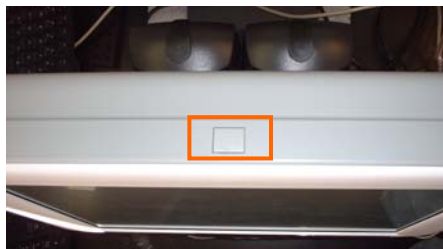
Turn the instrument on using the power switch on the rear panel

On the right side of the instrument near the access door, there is an LCD message screen. When the instrument is powered up the LCD message screen will show:

When the XF96 software is launched, the LCD message screen will update and show the following display. Note that it is recommended to allow at least 2 hours for the instrument to fully warm and equilibrate to the set temperature.
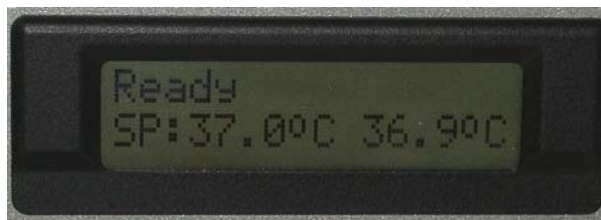
Measurements are taken using the XF96 Sensor Cartridge. It has two analyte sensor spots on the bottom of each probe that are sensitive to oxygen and proton concentration, respectively. By measuring the concentration of each analyte over time, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) may be calculated. Please see Sections 7 and 8 below for details on cell plate and cartridge preparation.

XF Software and Login

Launch the XF96 software and allow the XF96 Analyzer to warm to 37°C for at least two hours. Ideally, the instrument should be left on overnight to ensure consistent and accurate temperature. Note that the instrument will NOT warm to 37°C unless the XF96 software is launched (simply turning on the instrument only will NOT engage the heater). The following screen is displayed upon launching the software
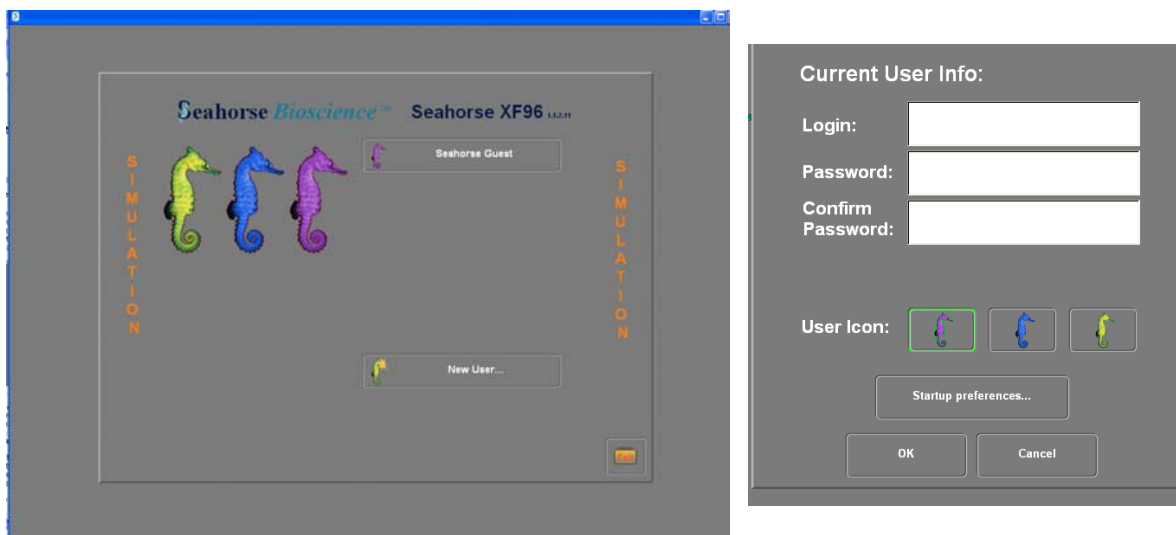
The XF96 Start Up Screen and User Preference Dialogue

Clicking on the "Seahorse Guest" button will take the user directly to the XF96 "Run" Screen. Clicking on the "New User" Button will open a dialogue to allow users to choose a preferred start screen, assay template directory, and default assay template. It also allows for password protected templates, files and data. Please see Appendix V (page 138) for more details on setting up individual user preferences.

Set up an XF assay template by using the Assay Wizard. Appendix I of this manual details the steps to use the Assay Wizard to make a template. This template can be prepared in advance (either on the controller or on a separate laptop or desktop PC) so it is ready to be loaded into the XF96 controller.

Note that the XF Software is MS Windows based, and may be loaded and used any Windows compatible computer

Introduction to the XF Software and Graphic User Interface

The "Run" and "Files" screens look like this:
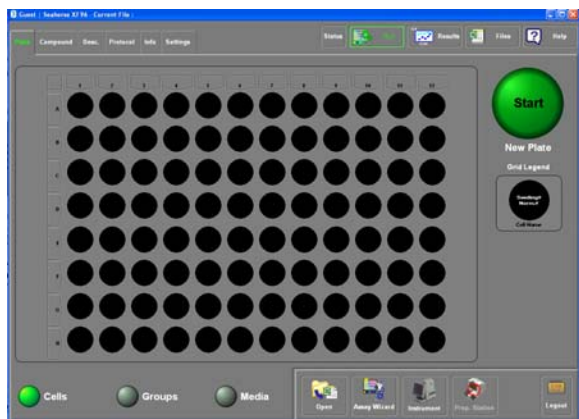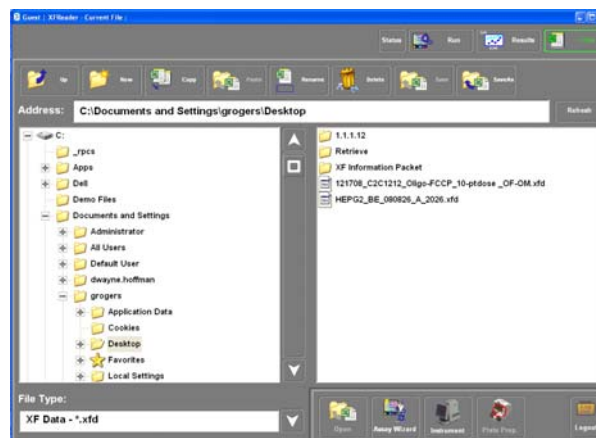
The XF96 Run Screen   The XF96 Files Screen

The user may open a previously constructed template by clicking the "Open" or "Files" icon and choosing the desired template. Choose the proper folder on the left side of the screen and choose the file on the right side of the screen. Alternatively, use the "Assay Wizard" icon to launch the wizard which will take the user step by step through the assay creation process.

Navigating through the buttons and tabs across the top (right): 4 buttons

1. The **Run** button/screen is for viewing the XF assay file that is currently open. It has several tabs for information and user preferences.

   a. **Plate**: shows experimental set-up for the cell plate. Displays information well by well for cells, well labels, and media conditions.
   b. **Compound**: Displays injected compound information for all wells; note ports A-D may be different.
   c. **Desc**.: Provides user information and details for the project, assay and plating.
   d. **Protocol**: Lists the user defined protocol for calibration, mix, wait, measure and injection commands.
   e. **Info**: Bookkeeping info for the user detailing cells, medium, compounds, and consumables. Note the fields under the consumables tab for the plate and cartridge will be automatically populated when the assay is run.
   f. **Settings**: Instrument settings for temperature correction, buffer capacity, and rate calculation settings for OCR and ECAR (see Appendix II for more detailed information on buffer capacity and rate calculation settings.)

2. The **Status** button shows the status of the assay in real time, including the current command, current temperature, time remaining for the current step and for the entire

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assay. You can position the dialog by touching and dragging it by the top bar. Touch the Status button again to hide the dialog.

3. The **Results** button is used to review and analyze data after an experiment; this will be discussed in greater detail in Section 12.

4. The **Files** button (see diagram below): Used to navigate the file menu to open, close, save, rename, and delete files. Use the left side of the screen to select and open folders, use the right side of the screen to select and open individual files.

A menu bar across the top of the Files screen provides several standard file and folder management features. They all behave similarly to their standard Windows counterparts. See Appendix V for more information about the menu bar and file management.

Bottom Row of Buttons (right side): Open, Assay Wizard and Instrument Set-up

Open button: prompts user to open a template or data file, similar to Files button above. Note templates and files may be opened with the "open" button or by double clicking on the template file itself.

Assay Wizard: Used to generate new assay templates or modify existing templates for assay design. A thorough discussion of the Assay Wizard is presented in Appendix I.

Instrument: Interface for instrument set-up, preferences, troubleshooting and Seahorse instrument and software maintenance.

Prep Station.: provides a series of screen for interface and operation of the Prep Station.

The **Instrument Set-up** commands:

Display preferences: defines user preferences for units and graph options, **Units** are relatively self-explanatory, note that some fields are fixed at this time.
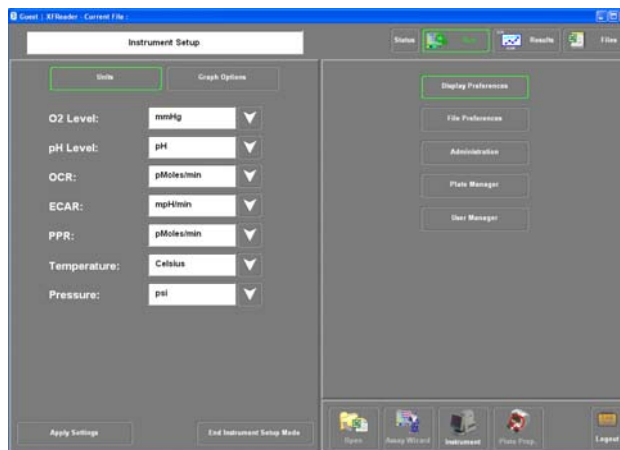
Display Preferences Screen

The **Graph Options** screen:
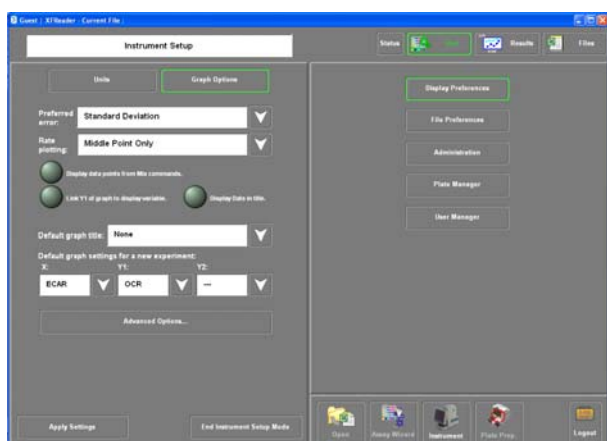
Graph Options Main Screen

The Graph Options Sub-Menu

This dialogue allows for user defined graphing preferences including preferred error display (Standard Deviation or Standard Error of the Mean), rate plotting, and various default graphing displays including graph title, and graph setting (X , Y1 and Y2 axes).

This screen has a sub-menu indicated by the "Graph Appearance and Well Group Mode display options" button, clicking this button brings up a dialogue with more user defined preferences for the graph appearance including font size, line width, grid options, and graph legend position.

File Preferences:

The **Assay** button allows user defined preferences for an **assay template directory.** This is useful for opening templates and specifying a **default template file** and a default **Protocol** (the protocol consists of the commands used to drive the instrument).
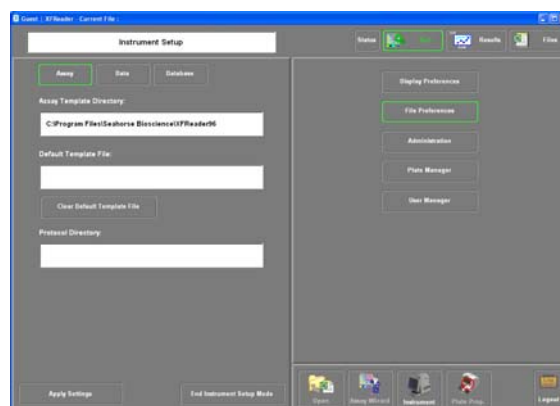
Instrument Setup: Assay Screen

The **Data** button allows user defined preferences for exporting data files to a default location and associated file information, including auto-naming options for resulting data files.

This function is especially useful if the results are to be saved on a common computer server or if the instrument is available to the local area network.

Instrument Setup: Data Screen

The **Database** button allows the user to specify a location for cell type, media type, compound, info and user databases These databases are accessible through the Assay Wizard.
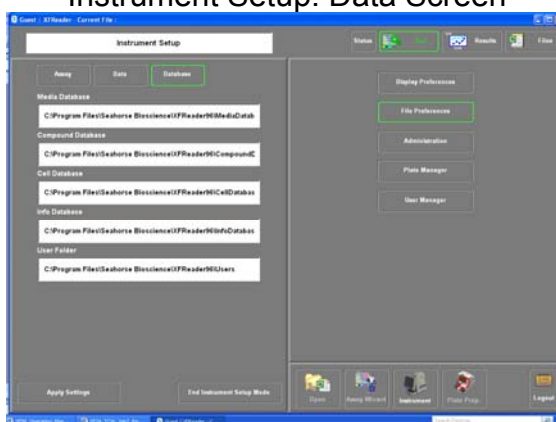
Instrument Setup: Database Screen

Administration:

These are settings and subsystems typically used by Seahorse Bioscience for instrument set-up. **Hardware** settings are fixed by Seahorse Bioscience and cannot be accessed without a password.

The **Subsystem Buttons** allow users to modify certain hardware and software settings. **Instrument ID** displays the serial number of the instrument
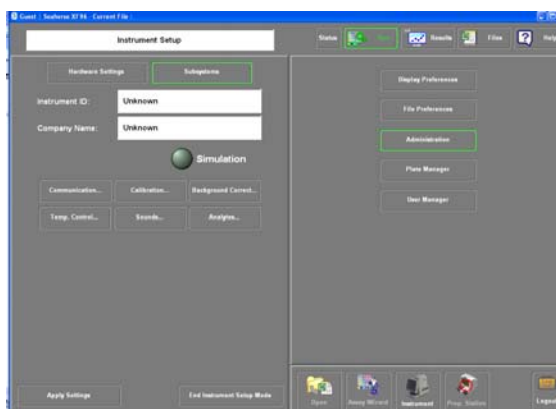
Instrument Setup:
Administration Screen

Communication….: There are three buttons indicating the communication status of the controller with the instrument, barcode reader, and PS (if used). All buttons should be green. This indicates that the controller is communicating properly with the other components of the system.
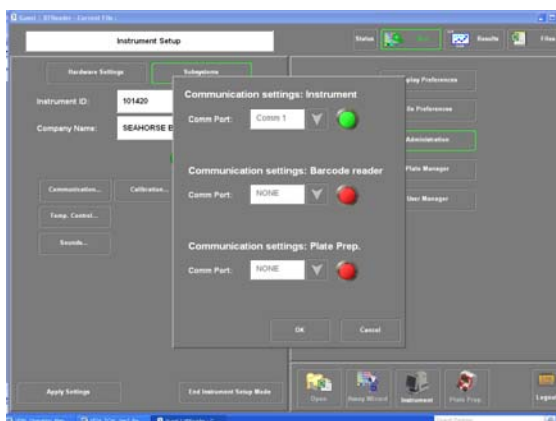
Subsystems: Communication

The **Calibration Screen** should look like the screen on the right. The settings are set by Seahorse Bioscience for optimal instrument and consumable performance. Changing these default settings may affect the quality of the sensor cartridge calibration. The "Parameters" button is only used for troubleshooting calibrations.

Subsystems: Calibration
Subsystems: Background Correction

Background Correction: Clicking the button will show a dialogue that allows the user to assign wells for temperature correction. Highlight the wells and click "OK." The current recommendation for Background Correction wells is **A1, A12, H1,** and **H12**. This information will already be set if the Assay Wizard Template is set up correctly.
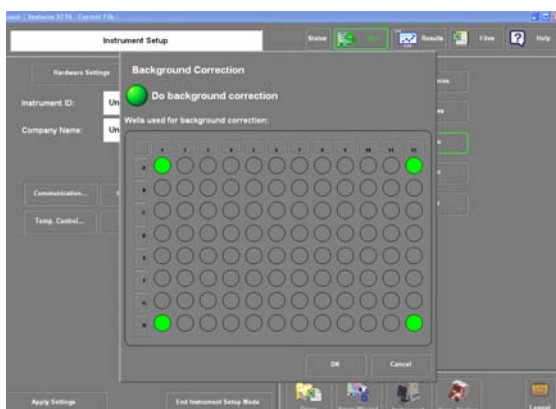
Subsystems: Background Correction

Temperature Control: A submenu for control over the temperature of the instrument. Currently this may be set from ~33°C – 37°C. Note there are two temperature control settings, 1) the instrument and 2) the plate tray. The instrument should be set to 36.0°C and the plate tray to 37.0°C, both must be engaged for proper temperature control. The instrument temperature information is also displayed on the LCD screen on the side of the analyzer.

Analyte settings: Allows user to gather information of both (O_2 and pH) or just one analyte. Looking at only one analyte will allow the instrument to gather more data points per unit time.

Sounds: You may download your favorite sound (a .wav file) to notify you when the instrument has completed a step and needs attention from the user (e.g. plate change from calibration to cell plate)

Plate Manager: Allows the user to control the probe and tray positions manually should there be an issue or problem with the instrument. SHB may ask you to access these settings should you experience issues with barcode reading, stripping the cartridge, calibration, or similar issues, otherwise you will likely not need to use these commands.
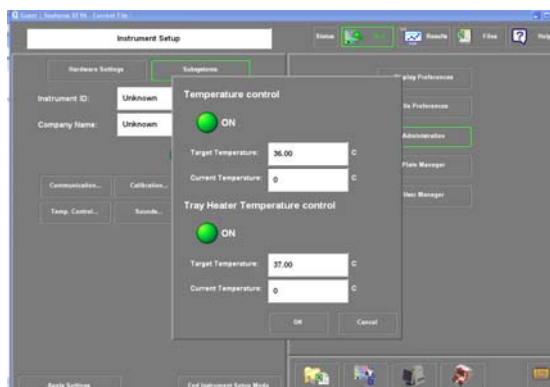
Subsystems: Temperature Control

Subsystems: Analyte Settings
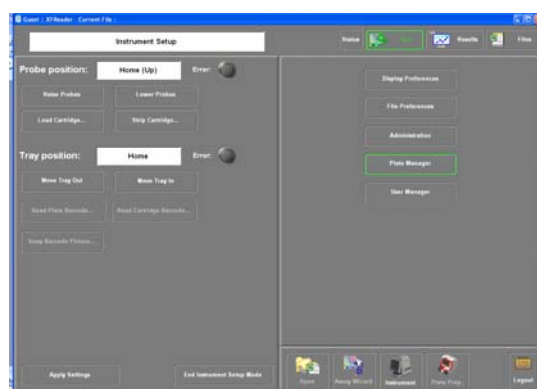
Plate Manager Screen

User Manager: Allows the user to edit the User List and User Info. Please see Appendix V for more information on user preferences.

After completion of instrument setup, click "End Instrument Setup Mode."
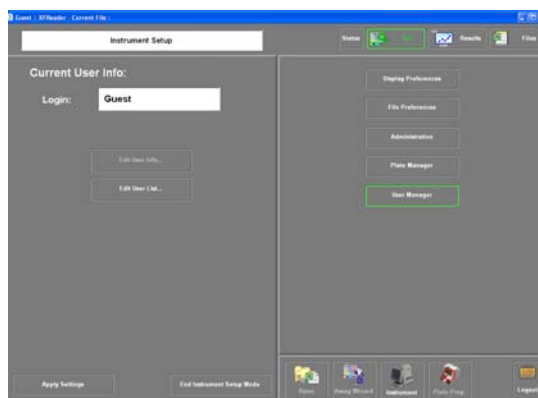
User Manager Screen

Cleaning and Routine Maintenance

The XF96 instrument was designed for minimal cleaning and maintenance by the user. Note all consumables are disposable and none of the instrument components come into contact with the cell plate at any time, preventing cross-contamination of biological or chemical materials. However, there are some minor tasks that can be performed by the user if necessary.

Probe Cleaning:

If cell culture plate wells are over-filled, or over a long period of time, the optical surface on the bottom of the measurement probes may require cleaning. Use a cotton-tipped stick and 95% ethanol to clean these optical windows beneath each probe. Take care not to scratch the surface and do not use a cleaner that leaves a residue.

Air filter replacement:

At approximately one year intervals, the air filter at the rear of the analyzer should be replaced. The filter retainer snaps away from the housing- use a screwdriver or wooden stick to remove it.

Performing the XF Assay

5. Seahorse Bioscience Cell Culture Overview

In order to obtain accurate and reproducible data from XF experiments, it is imperative that good cell culture techniques are established and practiced in a consistent and reliable manner. The techniques for thawing, passaging, and seeding cells, washing and changing media, and making reagents have been employed and optimized for use with the XF instrument system. Most of these techniques do not differ significantly from what most cell culturists practice, however, we have found that following these protocols in a strict manner allows for tight control of experimental variables associated with cell biology studies and assists with troubleshooting when problems do arise. Most important, adherence to these protocols usually ensures success of the XF experiment. In other words: the better one practices upstream cell culture, the more reliable, reproducible and accurate the downstream XF data. Please refer to the following sections to familiarize yourself with these cell culture techniques.

XF Assay-Day 1

6. Seeding Cells in XF96 Cell Culture Plates

Assays are performed in a Seahorse 96-well cell culture plate in conjunction with an XF96 sensor cartridge. Both are formatted in a typical 96-well format, with rows designated A-H and columns 1-12. The seeding surface of each well is smaller than a typical 96-well plate, but larger than a typical 384 well plate.
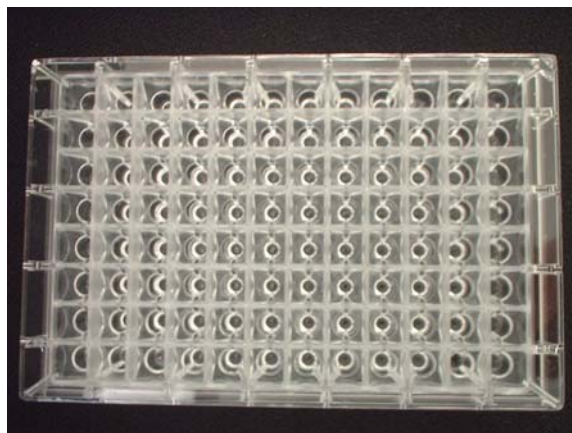
XF96 Cell Culture Plate

A single-step seeding process is strongly recommended when seeding XF96 cell culture plates. This procedure produces consistent and even monolayers of cells.
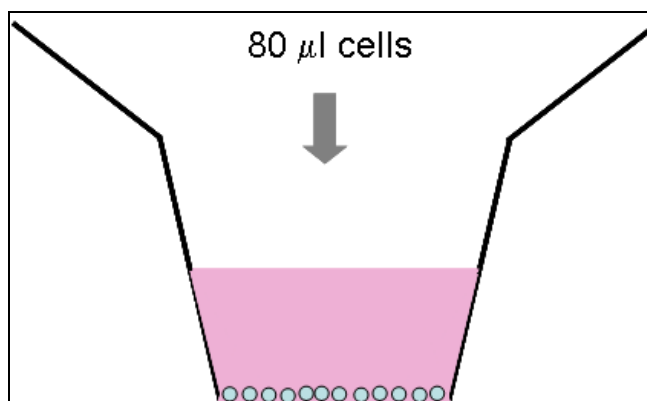
A. Resuspend cells to obtain desired final concentration to seed in 80 μL of growth media. Typical seeding densities vary from 5,000 to 50,000 cells per well.

B. Seed 80 μL of cell suspension per well, **leaving the appropriate background correction wells unseeded (A1, A12, H1, H12). Be sure to put media only (no cells) in the background correction wells.**

   Hint: Hold the pipette tip at an angle and rest the tip just below the circular rim at the top of the well for best technique and homogenous cell layer.

C. Place plates in incubator and allow cells to adhere. This generally takes approximately 1 hour for strongly adherent cells, but may take up to 5-6 hours for less adherent cell types.

D. Allow the cells to grow overnight (37°C, 5-10% CO_2).

7. Preparing Sensor Cartridge for the XF96 Assay

For optimum data quality and consistency, Seahorse Bioscience recommends hydrating XF96 Sensor Cartridges overnight using the following procedure:

A. Add 200 μl of Seahorse Bioscience XF96 Calibrant pH 7.4 to each well of a Seahorse Bioscience 96-well Utility plate.

B. Place sensor cartridge on top of plate and store at 37°C **without** CO_2 overnight or for up to 72 hours. If cartridge is being hydrated for more than 24 hours, wrap parafilm around the edges to prevent evaporation.

C. Turn on instrument and start XF software to allow instrument to stabilize at 37°C.

XF Assay-Day 2

8. Preparing Cell Plate for XF96 Assay

A. Warm XF Assay media (unbuffered DMEM, RPMI, KHB or other assay media) to 37˚C.
B. Inspect cells in the XF96 cell culture plate under microscope to assure confluence and even seeding.
C. Use the Prep Station (PS) to remove the growth media, wash with XF assay media and dispense XF assay media to the final specified volume. *See detailed instructions below for using the PS.*
 D. Incubate the XF96 cell culture plate in a 37˚C incubator **without** CO_2 until ready for use or at least 60 minutes prior to loading plate in XF96 instrument to allow cells to equilibrate to the new media.

Media Changes for Differentiation in XF96 Cell Culture Plates

1. After 24 hours, perform a full media change into differentiation media.
2. Every other day after the initial change, partial media changes are necessary.
3. Aspirate media, leaving 50-100 µl behind in each well. Add 100-150 µl of fresh media to each well using the side of the well to avoid disturbing the monolayer.

Maintenance of Cells Growing in XF96 Culture Plates

1. Media can be fully changed up to 48 hours after initial seeding.
2. After 48 hours, a partial media change is suggested for monolayer integrity, following the same recommendations detailed above.

9. Operation of the Prep Station for Media Exchange

1. Turn on the Prep Station (PS) and allow the incubator and bottle holder to warm to 37°C. It is recommended that the PS be turned on the day before the XF assay is to be performed or allow at least 2-3 hours for the unit to warm to 37°C.

2. Fill the PS bottles with the appropriate solutions (see diagram to right): Clean/disinfectant solution; dH_2O; and XF Assay Media*. If the assay media is cold (4°C), allow at least 2 hours for the media (500 ml) to reach 37°C.*
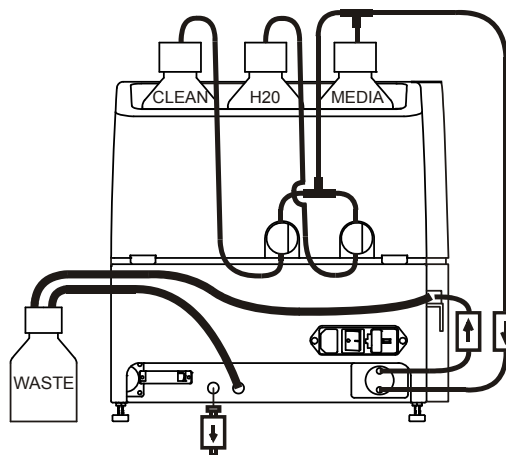
Orientation of Prep Station Bottles

3. Launch the XF96 software, log in, and press the "Prep Station." Button in the lower right portion of the screen.

4. If the Clean and dH$_2$O lines (tubes) are dry, prime the lines with Clean solution and dH$_2$O, respectively. Use the prime function screen as described on page 25 and see diagram to right.

PS: Prime screen

5. After Clean and dH$_2$O lines (tubes) are primed, click on the Media Change Tab. The screen should look similar to the screen diagram on the right (bottom). Engage the "Do Prime" and "Do Rinse" buttons (green), set the desired final volume (typically 150 µl), and be sure that "ALL" appears in the "Selected Columns" bar.
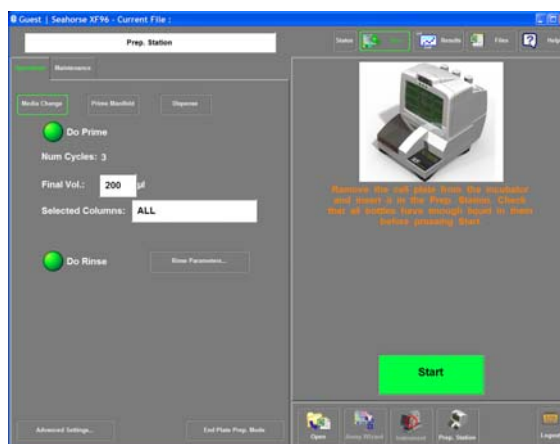
6. Place the cell plate into the PS and ensure proper positioning. Press the green "Start" button.

7. The PS will prime the manifold with XF Assay Media, then perform 2 aspiration and dispense cycles. There will be a pause in the operation, then the PS will perform a third aspiration and dispense cycle. **Do not remove the cell plate until all 3 cycles are complete!**

PS: Media Change Screen

8. Remove the cell plate and place in the incubator until calibration of the sensor cartridge is complete.

9. After the 3rd wash/dispense cycle is complete, the PS will automatically enter into a self cleaning mode that consists of washing the manifold with dH$_2$O, Clean/disinfectant solution and dH$_2$O again.

10. If more XF assays are to be performed, simply repeat steps 4-7 above, ensuring that adequate volumes of media, dH$_2$O and clean/disinfectant solution are in the appropriate bottles.

11. Note that the PS will enter into auto maintenance mode 8 hours after the most recent use of the PS. There will be a warning before the maintenance mode is automatically engaged (see diagram to right).

    This window will be displayed with a 30 second count-down. If cancel is selected, the auto maintenance is delayed by 1 hour. If start is selected or if the count-down reaches 0, the software will switch to the maintenance tab and a rinse and soak command is started. When it is completed, the software will return to the screen before the warning.

    The auto maintenance feature will engage every 8 hours and is recommended. *Note that it is OK to allow the PS tubing and dH$_2$O and Clean bottles to run dry, there will be no damage to the PS, just ensure that all lines are primed before performing a media change.*

WARNING! WARNING!

The auto maintenance will be done on the Prep. Station. Press Cancel to delay the maintenance cycle or press Start to do it immediately.

In 26 Seconds

Start   Cancel
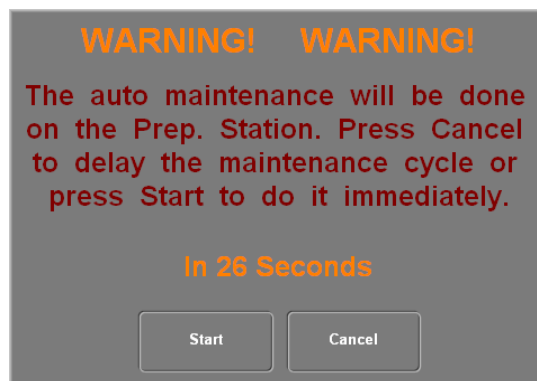
PS: Auto maintenance warning
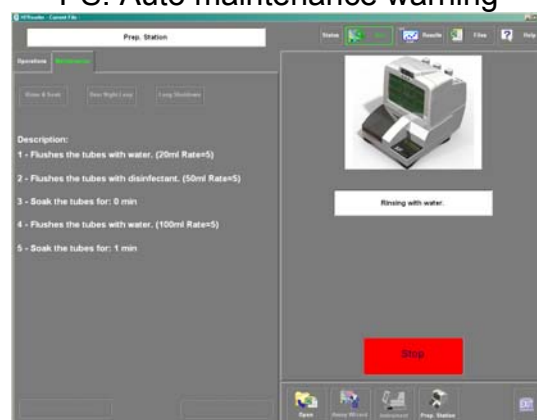
PS Maintenance: Rinse & Soak

12. After the final XF experiment for the day is complete, *the tubing and cap that goes directly to the media bottle should be cleaned by removing it, rinsing with dH$_2$O, clean/disinfectant solution and dH$_2$O.*

13. Should the user wish to perform PS maintenance manually, click on the maintenance tab in the PS screen and choose the appropriate option for your needs (Rinse and Soak, Overnight Loop, or Long Shutdown).

10. Loading the XF Sensor Cartridge with Compounds

The XF96 Sensor Cartridge has a single Analyte Sensor Spot on the bottom of each probe that measures oxygen consumption and extracellular acidification. Each well has two injection ports, designated Port A and Port B. The Cartridge Lot Number is on the front of the cartridge and the Bar Code is on the back. The sensor cartridge is loaded into the instrument with the Bar Code facing the back (lot number facing front).

The notch in the plate and cartridge denotes the LOWER LEFT corner of the plate/cartridge (see red circle below).
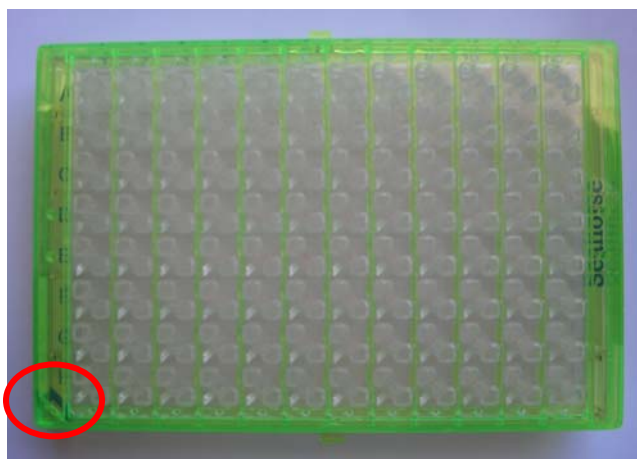
XF96 Cartridge - Top View
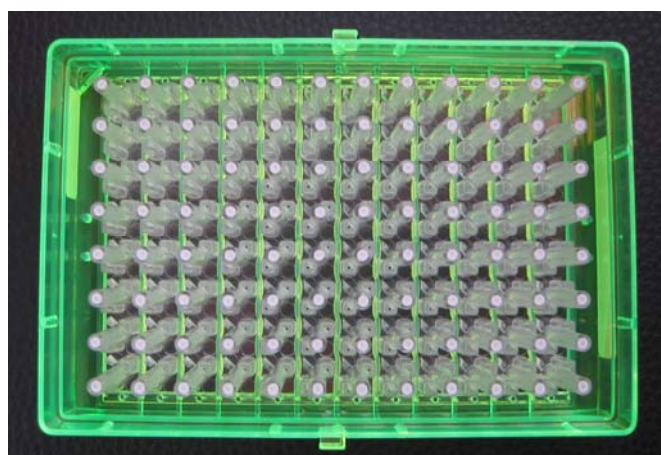
XF96 Cartridge – Bottom View

When preparing for compound injections, it's important to know the layout of the ports.

The ports are indexed as Port A and B are arranged as shown to the right.

Compressed air is used to force the compounds through the ports and into the wells.

Injections are performed on a port basis. All A ports are injected at once, as well as all B ports.
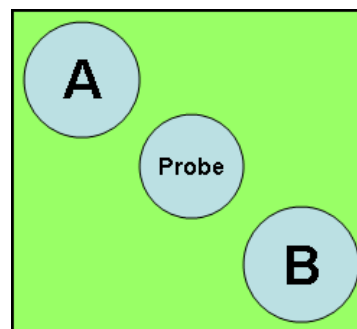
A drug Loading Guide is provided with the XF96 cartridges. It allows access to either all A ports OR all B ports while covering the sensor hole to prevent accidentally dispensing compounds into the sensor hole. The guide was designed for using Rainin 250 µl pipette tips (Rainin product # RT-20S). Tips should be inserted vertically with no angle, and the solution dispensed using gentle pressure.
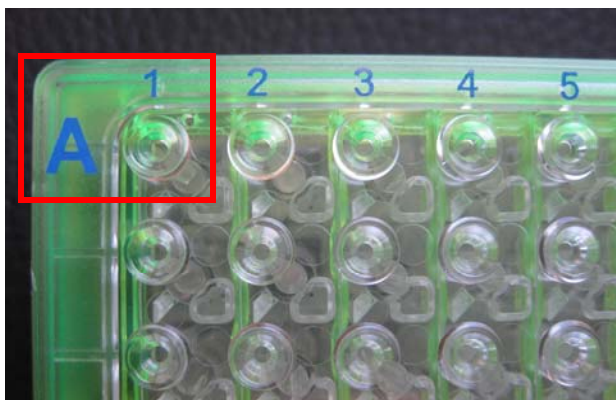
Close up view of the drug loading guide. Note the A ports are accessible but the B ports and probe channels are occluded.

Using the Loading Guide, load pre-warmed (37°C) injection compound into desired sensor cartridge port(s) prior to beginning calibration of the sensor cartridge. The ports are loaded by placing the loading guide onto the cartridge in the correct orientation (see below) and inserting the tips into drug loading guide holes and gently loading, making sure not to cause any leakage into the well below. Do NOT insert the pipette tip completely into the port/guide to avoid pushing the volume through the bottom of the injection reservoir.

The recommended injection volume is 25 µl, but injections can range from 20 µl to 25 µl. All wells, including Background Correction or blank wells, need to have a control, media or compound loaded in the port being used to ensure proper injection in all wells.

Note: all compounds should be diluted with the appropriate <u>running media</u> (DMEM, KHB or RPMI) before being loaded into the sensor cartridge.
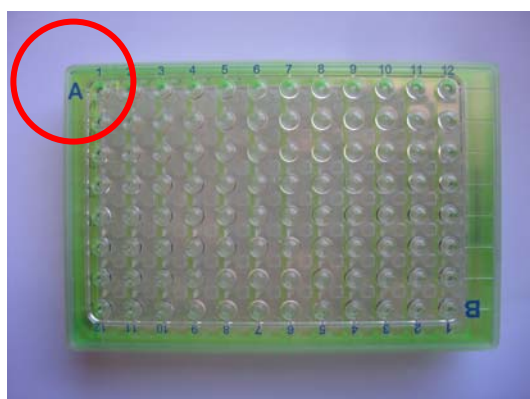
Loading A ports using the loading guide   Flip 180°   Loading B ports using the loading guide
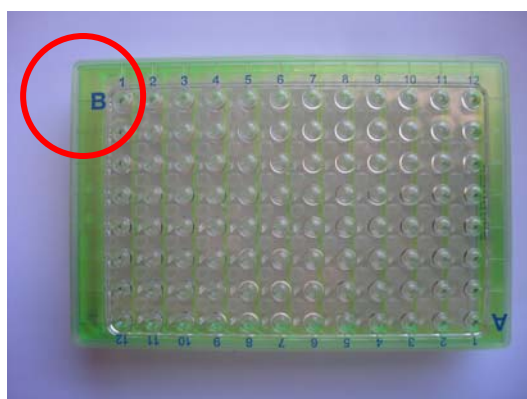
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11. Calibrating the sensors and running the experiment

Login to the XF96 software and allow the XF96 Analyzer to warm to 37°C. The XF96 Analyzer should be left on overnight so the instrument is at the proper temperature.

Set up an experimental template by using the Assay Wizard (see Appendix I). This template can be prepared in advance so it is ready to be loaded into the XF96 software.

Open the appropriate assay (.xls) template by clicking the "Open" icon on the bottom of the screen and choosing the desired template. If using the Assay Wizard to design an XF experiment just before running, then this template will already be loaded.

When finished with loading the compounds into the injection ports (if applicable), press the large green START button and you will see a screen similar to that shown below.

Verify the current protocol. If necessary, change any commands using the icons located on the bottom left of the screen. To change any of the information located on the right side of the screen, touch the box you wish to change and a keyboard will appear to type any additional information.

Note to make sure the <u>Save Directory</u> and <u>Save Name</u> fields contain the appropriate information!

Experiment Ready to Start Screen

Once the assay has started, it is still possible to change commands that have not yet been executed. To do this, simply modify, delete, or add commands as desired using the action icons.

Click the "Start" button and follow the prompts on the screen for sensor cartridge calibration. When the loading door opens, place the sensor cartridge and calibrant plate on the tray as shown below:
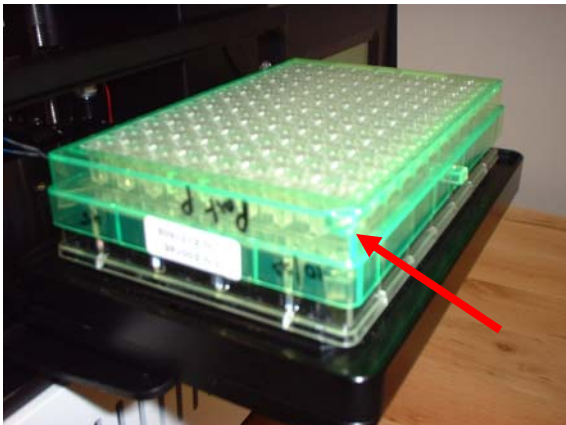
NOTE that the notch in the cartridge should be located to the front and right side (lower right corner in the photo above) and the bar code should face towards the rear of the instrument.

At the end of the pre-programmed calibration procedure, the user will be prompted to replace the calibrant plate with the cell plate (which should be incubating at 37°C without CO_2).

Remember to keep cell plate, sensor cartridge, and calibrant plate at 37°C before running the assay.

The calibration will take approximately 30 minutes.

After calibration is complete, continue to follow the instructions on the screen. When prompted, remove the calibration plate from the instrument (when door is open) and replace with the experimental (cell) plate. Note that the sensor cartridge remains in the instrument during this exchange.

Once calibration is complete and the assay has begun, the "Results" button can be clicked to view the data while it is being collected.

When the run is complete, the user will be prompted to remove the cell plate and sensor cartridge from the XF96 Analyzer. All data will be automatically exported to the export file set up at the beginning of the assay.

12. Basic Operation of the XF Data Viewer and Graphic Analysis

This section introduces the basic graphing, data display and data analysis features of the XF96 Analyzer software, both in the XF Reader application and the Excel-based Data Viewer. Note that these formats function in a very similar manner and either can be used for data display and data analysis. The Excel version supports additional functionality (e.g. statistics, multiple file viewing and averaging, other additional data display and information features).

Results screen in XF Reader	Data Viewer Screen in Excel
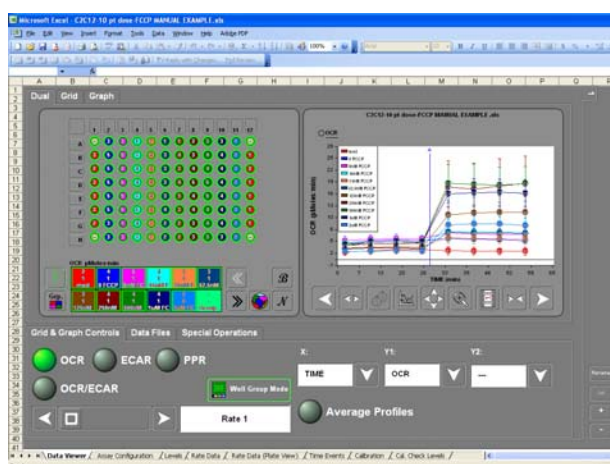
The first part of this section briefly discusses the Results screen tabs in the XF Reader, then focuses on basic graphing and analysis tools in the Excel format. More detailed information on advanced graphing and data analysis tools may be found in the Appendices.

Results Screen Tabs in the XF Reader:

On the **Results** screen in the XF Reader, screen, there are **tabs in the upper left: Dual, Grid, Graph, Protocol and Settings**. The Protocol and Setting tabs are identical to the corresponding tabs on the Run screen. During an assay, the contents of these new tabs can be displayed side-by-side with the graph. The Protocol tab allows for monitoring assay progress while viewing the graph. However, to edit the protocol during the assay, the Run screen must be used.

Tab Controls on Results Screen

The **Settings tab** provides access to the rate calculation settings. Typically, a user might adjust these settings while analyzing the data after the assay has completed.

To make changes to a currently loaded data file, the Settings tab in the **Results** screen must be used. Changes made to the Settings tab on the **Run** screen are not applied to the currently loaded data. Those changes will only apply to the next assay.

During an assay, the settings tab on the Run screen is not accessible. Changes can only be made to the Settings tab on the Results screen.
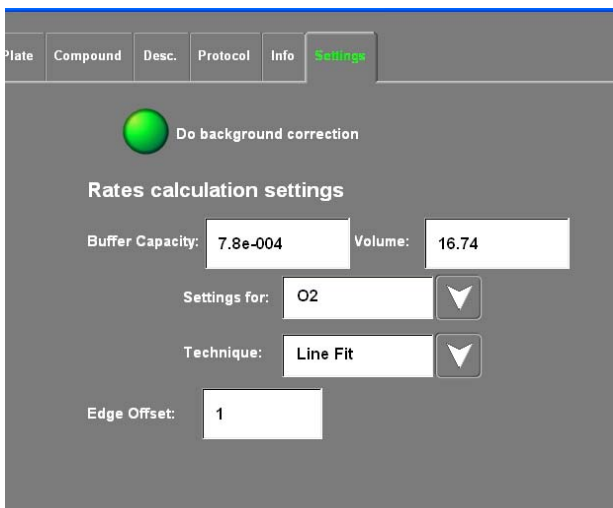
Settings Tab on Results Screen

Viewing results and basic graphing and analysis features

Note that XF result files will be automatically saved as a MS Excel (.xls) file that may be opened using MS Excel. The following pages focus on the Excel presentation of the data.

Note there are 7 tabs along the bottom.   A brief description of each follows:

Data Viewer: Displays the plate layout and graphic presentation of the XF results. Features will be discussed in detail below.

Assay Configuration: exported from the XF Assay Wizard. This tab contains all the information about experiment that was entered into the Assay Wizard (Cell, media, compound info, groups and labels, protocol, etc.)

Level Data: the raw data from the experiment in terms of pH and partial pressure O_2. This data cannot be changed, manipulated, or otherwise corrupted.

Rate Data (Plate view): all of the rate data for each analyte is shown in a "plate" view" by measurement.

Rate Data: all of the data for each analyte by column.

Time Events: Lists all of the time events associated with the experiment, including Calibration, mix, wait measure, and injection commands.

100900-400_MANUAL XF96 W/ PREP STATION_RA

Calibration: Used to determine if the sensors passed the calibration requirements and provides calibration information and statistics about each individual sensor.

Well Grid Features and Options
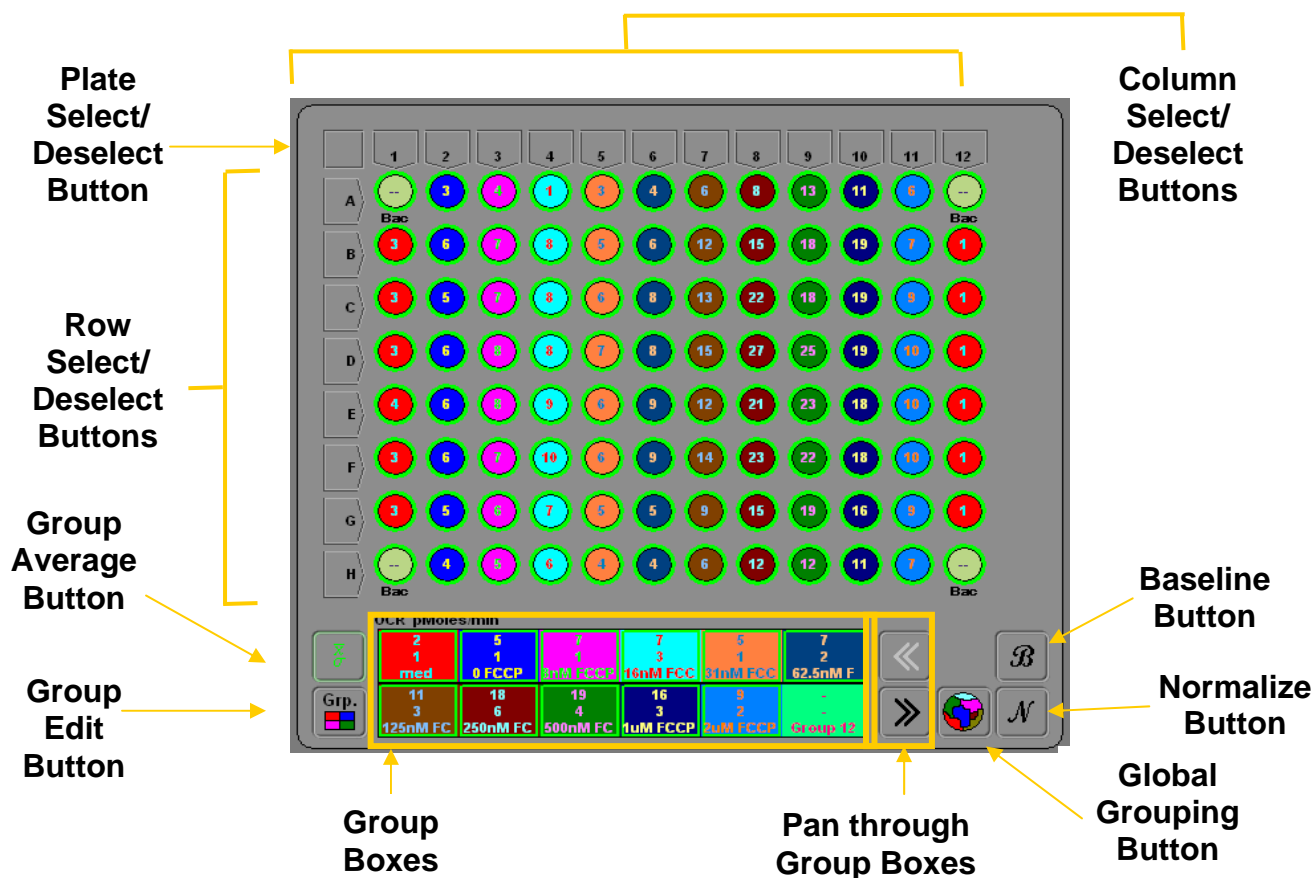
Individual wells may be selected or deselected by clicking on the appropriate well. Columns or rows may be selected or deselected by clicking on appropriate column number or row letter. The entire plate (all wells) may be selected or deselected by clicking on the square in the upper left corner of the plate layout.

Group Average Button: Calculates the mean of the selected wells in each group and displays the mean (top number) and the error (bottom number). The error is reported at either SD or SEM (user defined). These values appear in the **Group Boxes** below the plate layout.

Group Boxes display the mean rate value and error associated with each group. The Group Boxes may be selected and deselected, with corresponding rate data displayed or removed from the graph, respectively. Note that only the selected wells of a group are factored into the mean and error values for the entire group. This allows the user to remove outlier values or view a set of wells within a group.

Group Edit Button:
Brings up a dialogue that allows the user to edit the group names and color designations after the assay has been performed (see diagram to right). To edit the
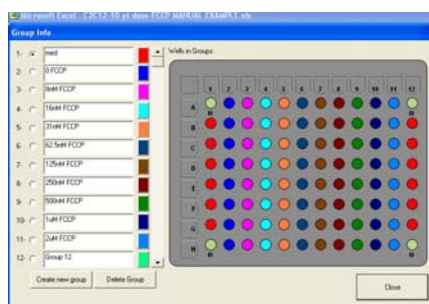
Group and Color Edit Dialogues (Excel)

group name, click on the desired field and re-enter the new group name. To reassign the wells, select the desired group on the left side, then select the wells you wish to reassign. To change the color of the group, click on the colored square adjacent to the group name. A color palate dialogue will appear. Select the desired color from the existing palate or create a customized color.

Global Grouping Button: Changes the plate view from individual wells to show grouping of wells. (see diagram to right). Groups are colored and labeled as defined by the user in the assay wizard (or via the group edit button). The average rates and associated error is displayed and will change as the user scrolls through the rate control bar. This is a convenient way to view the group data in the well plate format.

Global Grouping Plate View

Baseline Button: Baselines the data to a user defined point (typically the measurement just before compound injection), and presents the data and graph in a "**%**" format. For

more complex Baseline methods, the point used for baseline reference can be changed and/or multiple points may be used for Baseline subtraction. To access more Baseline options, click on the Graph options dialogue button. A dialogue will appear that will contain more settings for Baseline options.
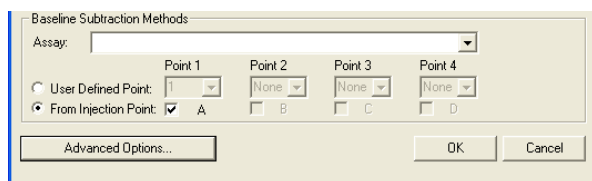
Baseline Subtraction Methods (Excel)

Normalization Button: Allows users to normalize rate data after the assay has been performed. Data may be normalized to cell count, protein or DNA content, cell viability, etc. This is especially useful when working with primary and/or differentiated cells where the user may have difficulty determining an accurate cell number before the XF assay.

Normalization data can be entered interactively into the data file. This can be done using the **Set Normalization Count** button in the **Special Operations** tab.

The **Set Normalization Count** button brings up the entry dialog shown in the diagram to the right. If multiple data sets are loaded, you can select the data set from the drop down box at the top.

Click on the desired well to select it and then click on the **Set normalization cell count…** button at the lower left. This brings up a text entry box. Enter the value and click **OK**. Repeat this for all the wells and then close the dialog.

Then click on the $\mathcal{N}$ button on the well grid to view the normalized data.
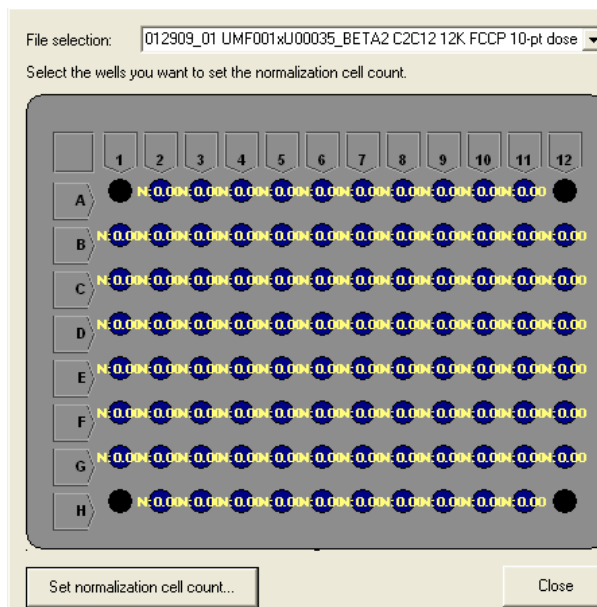
Normalization count entry dialog

If normalization values have not been entered and the Normalization button is selected, a warning message appears providing an option to edit the values. Selecting **Yes** brings up the same entry dialog. The warning message is shown below.
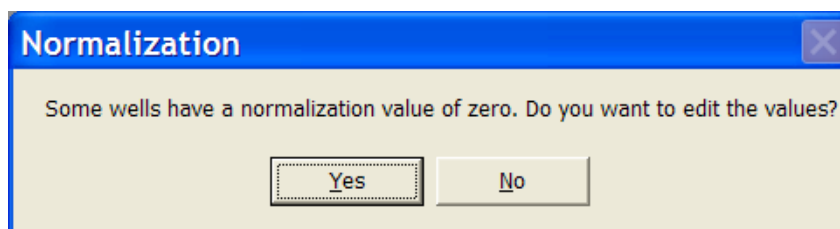
Warning if Normalization values have not been added

Basic Graphing Features and Options

Analyte Buttons and Rate control

Use the appropriate button to choose which data is displayed in the plate layout (OCR, ECAR, PPR, OCR/ECAR, O_2 or pH). Use the scroll bar to change which rate measurement data appears in the wells and group boxes (Rate 1, Rate 2, etc.).
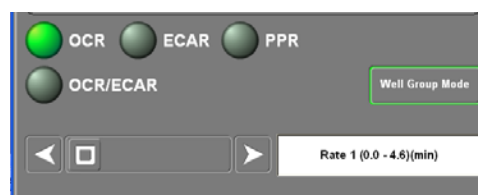
Analyte Buttons and Rate control

Graph Axis Drop Down Menus

Use the drop down menus below the graph to specify which data is to be plotted on which axis (OCR, ECAR, PPR, OCR/ECAR, O2, pH or --). Note only the X axis has options for Time and Group. When selected, the group curve shows the difference in rates between the well groupings. The x-axis tick marks are labeled with each group name as specified in your assay configuration. The groups can represent injected compounds, treatment dosage, cell type, or any other factor.

Click on the **Well Group Mode** Button to engage the Well Groups and color scheme defined during template set-up in the Assay Wizard. If this box is not highlighted, the plate layout will shift to a "histogram" style of data presentation (see diagram to right), with a colored gradient scheme representing the spectrum of data values for each parameter.
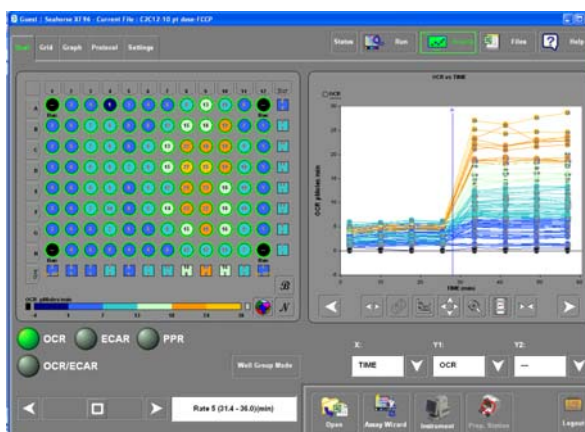
Example Assay Results: Non well group mode

Using the graphing buttons:

 Pans the X-axis to the right or left**.**

 Expands or contracts the X-axis of the graph.

 Auto-scale button: fits data to the graph window.

 Zoom Feature: engages the zoom feature of the data viewer

The Zoom Feature opens a window displaying the graph in a larger viewing format. Note that Y-axis now has panning, expanding and contracting features similar to the X-axis. Also note the tab in the upper right of the window that allows the user to toggle

between the larger graph view and a larger plate view. All functions in the Zoom Window are the same as in the normal viewing mode (see figures below).

Zoom Mode: Grid
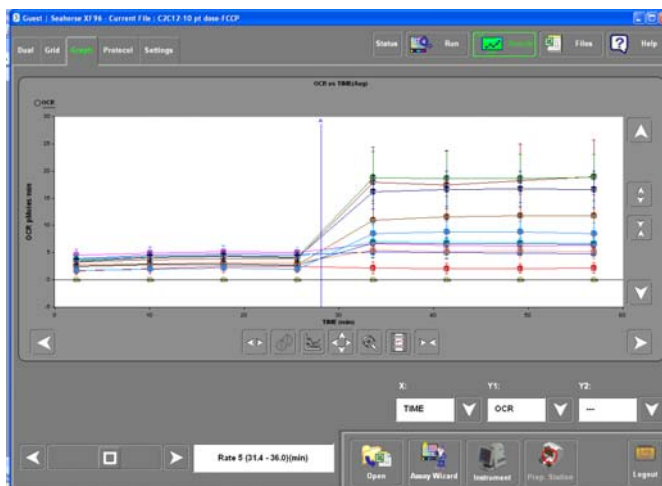
Zoom Mode: Graph

Graph Options Dialogue Button

 Opens a dialogue for multiple graphing options and features including: customizing graph axes, threshold options, error bar format, Baseline options, Time Zero options, and Background Correction.

Graph Options Dialogue

Advanced Graph Options sub-menu

There is a sub-menu that may be accessed via the "Advanced graph options" button that offers more options for customizing the graph, including: Font size, Line width, Point size, graph title, Grids, custom Zero Time Point, Injection marker options, Bar graph option, and graph legend options. Many of the features are explained in more detail in Appendices IV and V.

Rate Options Dialogue Button:

Opens the Rate Options dialogue which allows the user to specify which rate data is displayed on the graph when the X-axis is a parameter other than "Time". Either the current rate or selected rates may be displayed. It also allows display options such as labels, and linking points with directional lines.
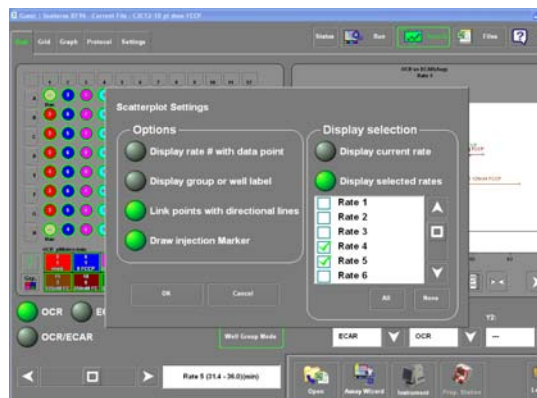
Rate Options Dialogue

For example, if OCR is plotted vs. PPR (ECAR), it may be useful to display only the basal rate data immediately before injection and the perturbed rate data immediately after injection, then to link the shifts in OCR and/or PPR using directional lines.
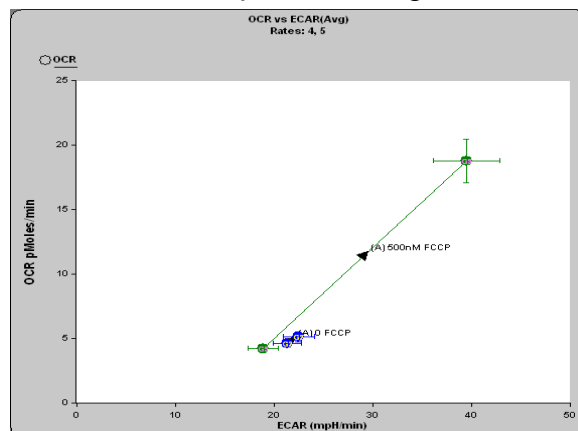
OCR vs. ECAR of Example Assay

Movie Function Button:

Opens a window that shows the data in an animated format. You may play, pause, alter the speed, etc.
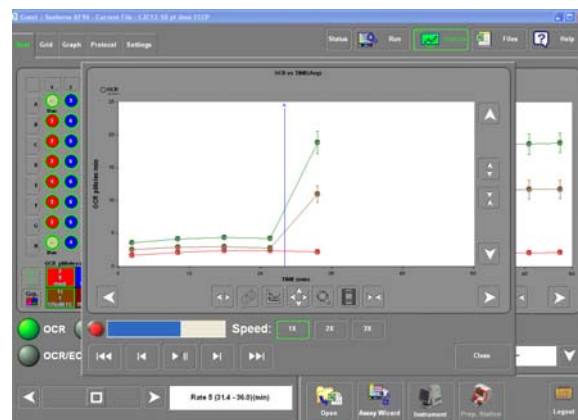
Movie Function

Note that the data viewer and analysis features described above are based on the XFreader software. The XF data files may also be opened in Excel (the XF reader SW needs to be installed on the computer). In addition to features listed above, the Excel data file also includes extra data viewing, analysis and statistical features. These features are presented in detail in Appendix III.

13. Analysis of XF Experimental Data – General Guidelines

Now that you are familiar with the XF analysis tools and have produced an experimental data set, the next question becomes: "How do I analyze and interpret my XF data". Obviously, data analysis will be dependent upon the design and ultimate goal of the XF experiment, however a few general guidelines and a simple methodology are provided to facilitate the analysis process.

A. **Before Analysis**: Begin with a well designed experiment, practice good tissue and cell culture techniques, and optimize the XF assay for the particular cell type you are investigating.

   a) Obtaining a consistent monolayer (but not necessarily confluent) of cells is vital to obtaining consistent and accurate data. Using the recommended 1-step seeding method (Section 6) will facilitate this process. Look for even spacing of cells, with no large cell clumps or blank patch
   b) A well designed experiment typically begins with a specific question or goal to the investigation. Further, a well designed experiment contains the appropriate controls that will facilitate and guide downstream analysis.
   c) Optimization upstream will greatly enhance the ability to obtain meaningful and reproducible results downstream. *Seahorse Bioscience always recommends optimizing the XF system for any new cell type that you will use in XF assays.* Typically, one would optimize the following parameters: cell seeding density, response to metabolic compounds (e.g. Oligomycin, FCCP, 2-DG, rotenone, etc.), mix and measure cycle timing, and the concentration of injected compounds.

B. **Basic Analysis**: To simplify the initial analysis , begin with the following settings:

   Select all wells and ensure that all the group boxes assigned are highlighted.
   Check "Enable Well Groups".
   Engage Group Average Function.
   Disengage Baseline and Normalization Functions.
   Set graph to plot OCR vs. Time or ECAR (PPR) vs. Time.
   In the "Graph Options Dialogue":
   - ✓ Select the preferred statistical parameter (SD or SEM).
   - ✓ Set Rate Data Display As: Middle Point Only
   - ✓ Check "Link Y1 of graph to display variables"
   - ✓ Check "Do Temperature Correction"
   - ✓ Check "Show Graph Legend"
   - ✓ Check "Draw Vertical Markers

Now, looking at the OCR and ECAR data, begin your analysis as follows:

How do the basal OCR and ECAR (PPR) data appear? Do the results indicate a steady basal or baseline rates before injection?

Does the OCR and ECAR (PPR) data make sense, i.e. what are the absolute rate values, do the cells respond injected compounds as predicted, and are there any obvious trends or changes in the rates?

Panning through the rate data, what is the magnitude of the error (SD or SEM)? Are they in an acceptable range (< 20%) or are the errors larger?

Is there an individual well (or wells) that show deviation from the rest of the group (e.g. obvious outliers that might be due to errors in seeding cells or changing media). This may be facilitated by disengaging the average function and looking at the OCR and ECAR by group or by individual well, or turning off the well-group mode. (Note that this may be easier by deselecting the wells and/or entire groups that you are not reviewing).

C. Intermediate Analysis: Once you have reviewed the basic parameters described above, proceed to an intermediate analysis if needed.

Baseline Function: Recall that this baselines the data to a user defined point (typically the measurement just before a compound injection), and presents the data and graph in a "**%**" format. This is useful for knowing the fold enhancement or decrease in OCR and/or ECAR (PPR) in response to an injected compound or chronic drug treatment.

For example, the % change in OCR may be viewed instead of the absolute rates. This is useful when comparing data from multiple plates or experiments run at different times (see below).

Absolute OCR   Baselined OCR (% change)
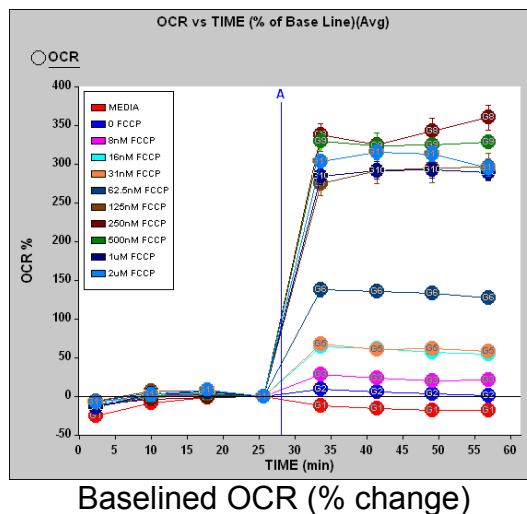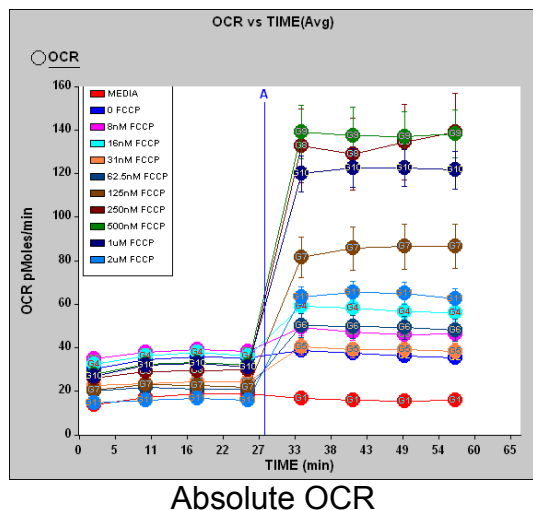
Normalization Function: To use the normalization tool, some method to normalize cell count or cell activity must be used (typically after the XF assay has been performed). This could be an assay to ascertain cell number, total protein content, total DNA content, or total mitochondrial number. This could also be an ATP assay or cell viability assay.

Once the additional assay has been performed, enter the normalization constants into either the XF reader or Excel software and click on the $\wedge$ button to view the normalized results. For more detailed information on AUC and statistical analyses, please see Appendix III (Special Operations).

AUC (Area Under the Curve) and Statistical Analyses Functions: For more detailed information on AUC and statistical analyses, please see Appendix III (Special Operations).

Graph Appearance: The XF viewer allows the data to be displayed in several graphic formats. For example, one may view the kinetic data by plotting OCR and/or ECAR (PPR) vs. time. Other displays include plotting OCR and ECAR (PPR) data by group (see Appendix IV for more detailed information on the Group feature), or plotting OCR vs. ECAR (PPR). The advantages of using the group feature or OCR vs. ECAR (PPR) graph is that total bioenergetic profile of the cells may be displayed on a single graph that is simple to view and interpret. For example, we begin with separate kinetic graphs of the OCR and ECAR (PPR) data:
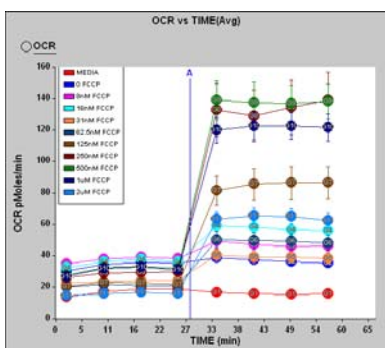
OCR only
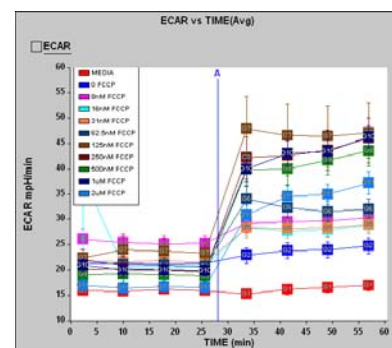
ECAR only
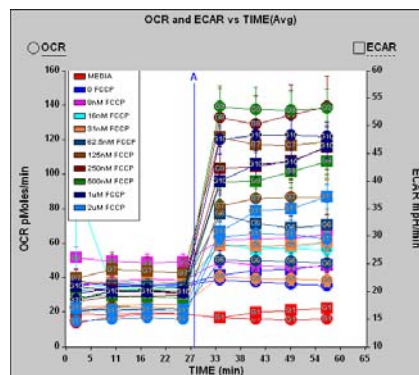
OCR + ECAR

By using the Y2 axis for ECAR, we can now put all the kinetic data on a single graph (filled circles for OCR, filled squares for ECAR), however the graph is more difficult to interpret.

By using the Group Feature along the X-axis, the data is displayed by rate measurement. One loses the kinetic nature of the data, but gains the ability to look at both OCR and ECAR responses together in an organized and easy-to-read bar graph format.
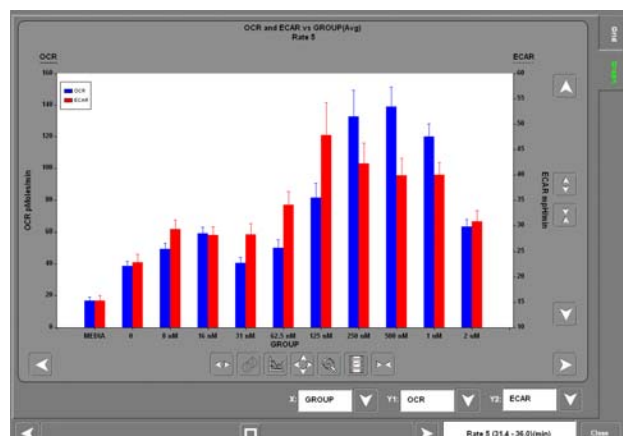
Bar Chart of OCR and ECAR at Rate 5

One may also wish to plot OCR vs. ECAR (PPR), which allows individual rate measurements to selected/deselected. In the example, rates 4 and 5 are displayed to reflect the OCR and ECAR rate data immediately before and immediately after injection, respectively. Note that this format shows the shift from basal rates to enhanced/diminished rates after injection and provides information as to how the aerobic and glycolytic pathways of the cell respond to compounds that alter the metabolism of the cell.
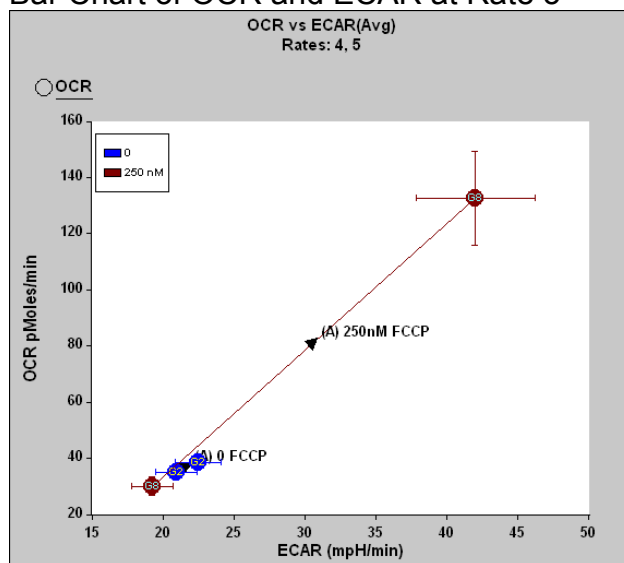
OCR vs. ECAR, 0 and 250 nM FCCP

D. Advanced Analysis: Viewing and interpreting the Level Data

One can probe deeper into the data by using the various graphing options to display data in accordance with the goal of the assay and the user's preferences. One common way to troubleshoot unexpected results is to look at the "level data". Level data is the raw data corresponding to the changes in mm O_2 vs. time or pH vs. time for OCR and ECAR, respectively.

Perform the following actions:

1. Set the graphing options for X axis = time,
Y axis = O_2 (or pH)

2. Pick a single well to analyze (e.g. C2)

3. Turn OFF the averaging function
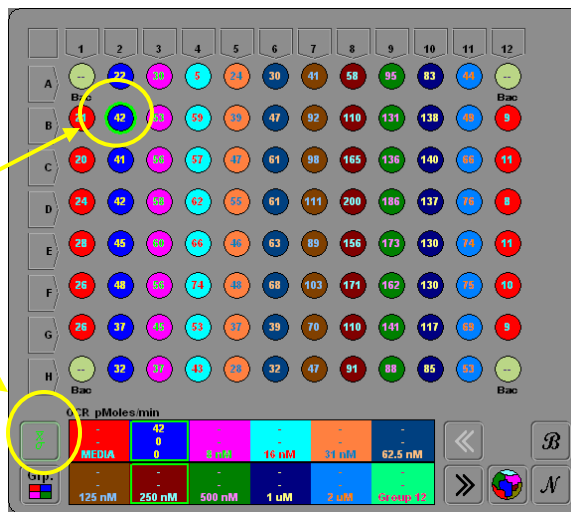
You should now see a graph that looks similar to this:
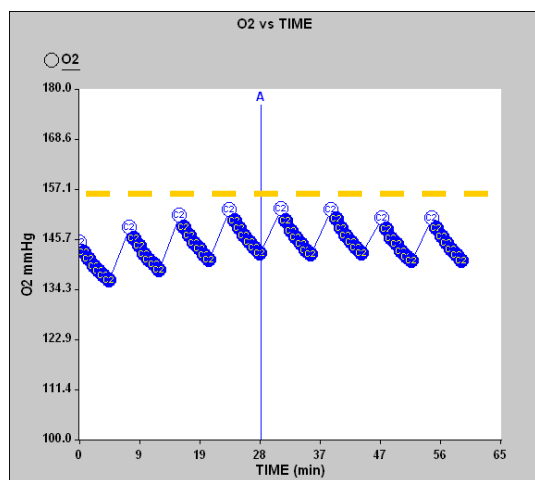
O2 level data: ideal
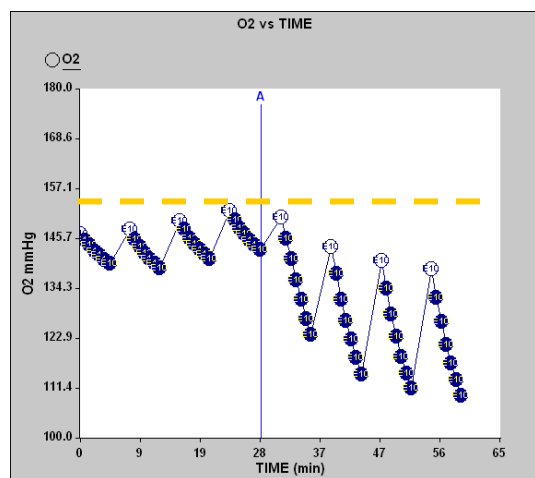
O2 level data: non-ideal

This graph represents the change in oxygen tension (mm Hg of O_2) with respect to time in a single well. Ideally, one would expect to see little variation in the starting point of each measurement (see yellow dashed line), indicating that the O_2 sensors have an adequate amount of time during mixing and waiting to re-equilibrate to ambient O_2 concentration in the media. Inspection of the Y axis scale indicates that variation in the starting O_2 tension among measurements is minor, about ±5 mm O_2, which is within the error specifications of the instrument. Note that the starting O_2 level is approximately 158 mm Hg, which is the accurate value of O_2 partial pressure. Also note that injections do not affect the O_2 levels.

Larger changes would indicate a potential problem, likely an inadequate amount of time for the sensors to re-equilibrate during the mix and wait cycles. In such a case, the O_2 level data may look more similar to the graph to the left (bottom). Note that before injection, the O2 level is returning to ambient levels, however, after an injection of DNP, the starting O2 levels decrease with each successive measurement. This is due to the fact that the cells are now

consuming oxygen at an increased rate, and that the sensors are unable to recover during the mix and wait times. An increase of mixing time by 1-2 minutes would be sufficient to alleviate this problem.

Analysis of the level data for pH is similar to that for O2, simply choose pH for the Y-axis. Again, one would expect to see little variation in the starting point of each measurement (see yellow dashed line), indicating that the pH sensors have an adequate amount of time during mixing and waiting to re-equilibrate to the ambient pH value of the media. In this example note that the starting pH values for each measurement vary within < 0.05 pH units, well within the acceptable "noise" of the instrument.
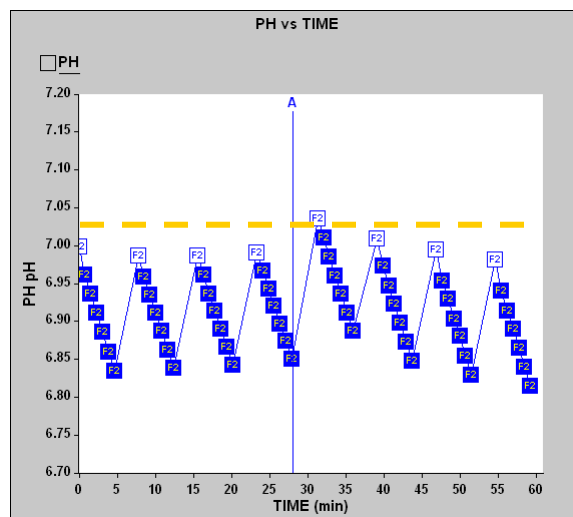
pH level data: ideal

A large increase or decrease (> 0.2 pH units) AFTER injections would indicate that the pH of the injected compound is significantly different from the running media. Care must be taken to ensure the pH of the running media and injected compounds are equal. Again, failure of pH measurements to return to the ambient pH of the media after each rate measurement would indicate a potential problem, likely an inadequate amount of time for the sensors to re-equilibrate during the mix and wait cycles. Below are subtle and extreme examples (left and right, respectively) showing the pH of the injected compound altering the pH of the media in the well.
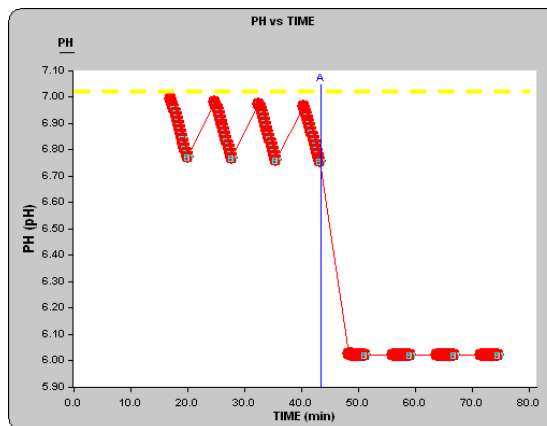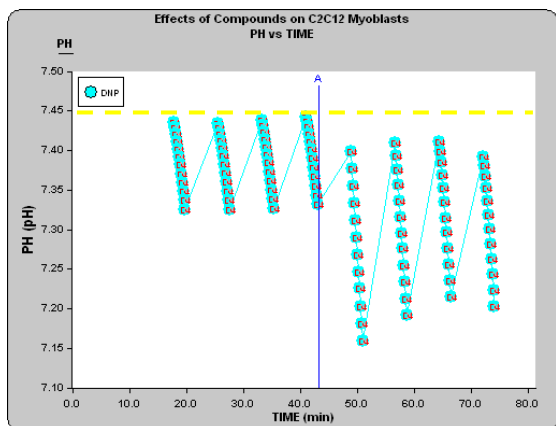
Sample Experimental Protocols for the XF96 analyzer

This section contains the following experimental protocols that have been optimized by Seahorse Bioscience for data reproducibility and consistency:

Dose Response Experiment using C2C12 Myoblast cells

This experiment consists of injecting increasing concentrations of FCCP, and illustrates calculating an EC_{50} value for this compound.

Metformin-Enhanced Fatty Acid Oxidation (FAO) Experiment using C2C12 myoblast cells

This experiment demonstrates addition of the fatty acid, palmitate, to increase the OCR of the cells.

(Note that more example protocols for the XF96 will be added in the near future.)

The purposes of these protocols are several-fold:

1) Introduces new users of the XF96 to the experimental methods used to obtain cellular metabolic measurements, including:

    -proper cell culture techniques
    -optimal experimental design
    -preparation of reagents and solutions
    -gathering data using the XF96 instrument
    -interpreting data using XF Reader software

2) Provides a set of reference or "control" experiments to access experimental (user) technique, biological systems, instrument performance, and software performance. These experiments can thus be used for troubleshooting.

3) Offers a starting point of methods to study and investigate cellular bioenergetics, including: mitochondrial function, metabolic pathways, identifying mechanisms of action, drug effects and toxicity, etc.

14. Dose Response Experiment using C2C12 myoblast cells

In this experiment, C2C12 cells are seeded at a given density in Seahorse cell culture plates. The basal oxygen consumption (OCR) and extracellular acidification (ECAR) rates are measured to establish baseline rates. The cells will then be treated with increasing concentrations of Carbonylcyanide-p-trifluourmethoxyphenylhydrazone (FCCP). Several wells will serve as the control, with running media added as a control "compound".

FCCP is a proton ionophore. It is an uncoupling agent because it disrupts ATP synthesis by transporting hydrogen ions across the mitochondrial membrane instead of the proton channel of ATP synthase (Complex V). This leads to a rapid consumption of energy and oxygen without the generation of ATP. In this case, both OCR and ECAR will increase, OCR due to uncoupling, and ECAR as the cells attempt to maintain their energy balance by using glycolysis to generate ATP.

Note: All assay media and compounds need to be warmed up to 37°C and to have their pH adjusted to 7.4 prior to start of assay.

1. Materials and reagents:

   1. XF Assay DMEM (Seahorse Bio, 100965-000)
   2. Glucose (Sigma, G8270, dry, or Sigma G8769, 45% solution)
   3. 0.1 N NaOH
   4. Glutamax (Gibco, 35050-061)
   5. FCCP (Sigma, C2920)
   6. V-bottom 96 well drug-loading plate (VWR, 80080-048)
   7. Multichannel Pipette
   8. 24 Column low profile reagent reservoir (Seahorse Bio, S30039)
   9. DMSO (Sigma, D2650)
   10. C2C12 cells (ATCC)
   11. Seahorse Calibrant (Seahorse Bio, 100840-000)
   12. Sodium Pyruvate (Sigma, P4562)

2. Growth Medium

   a. 500 mL DMEM (Gibco 11965-092)
   b. 10% FBS (Hyclone SH90070.03)
   c. 5 mL Penn/Strep (Gibco 15140-122)
   d. 5 mL Sodium Pyruvate (Sigma S8636)
   e. 5 mL Glutamax (Gibco 35050-061)

Seahorse *Bioscience*

3. Cell Seeding in XF96 Cell Culture plates

Note: A one-step seeding procedure is used for XF96 plates

a. The culture surface of the XF96 plate is 40% the area of a typical 96 well plate. For XF96 assays, C2C12 cells are seeded at 12,000 cells per well in a final volume of 80 µl. Therefore resuspend stock cell solution to obtain 1.5×10^5 cells/ml (1.2×10^4 cells per 80 µl , final concentration) in growth media. For one XF96 plate: 92 wells* x 80 µl ≈ 8.0 ml of cells.

b. Seed 80 µL of cell suspension per well with multiple channel pipette**, leaving appropriate control wells blank (A1, H1, A12, H12).**

c. Allow the cells to grow overnight in 37°C/10% CO_2 incubator. It is recommended that the plate be placed in the rear portion of the incubator to minimize edge effects of cell growth caused by changes in humidity, temperature and CO_2 level due to opening and closing of the incubator door.

Note that cell seeding number will depend on cell type and cell growth characteristics. It is strongly recommended that the user perform initial cell seeding titration experiments to determine the optimal cells to seed per well for a given application.)

4. Preparation of Assay Template.

Using the Assay Wizard (Appendix I), generate a template with the following group layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	blank
B	DMSO	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	DMSO
C	DMSO	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	DMSO
D	DMSO	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	DMSO
E	DMSO	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	DMSO
F	DMSO	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	DMSO
G	DMSO	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	DMSO
H	blank	0 nM FCCP	8 nM FCCP	16 nM FCCP	31 nM FCCP	62.5 nM FCCP	125 nM FCCP	250 nM FCCP	500 nM FCCP	1 µm FCCP	2µm FCCP	blank

5. Compound Preparation

Make the following serial dilutions of FCCP in DMSO, beginning with diluting a 10 mM stock to 2 mM. Then dilute each solution of FCCP in XF <u>assay</u> media as outlined in the table below.

DMSO stock (mM)	DMSO (uL)	stock (µl)	total µl	Final in assay (µm)	7x stock for ports (µm)	Fold Dilution	Stock (µl)	Media (mL)
2	40	10	50	2	16	8X	8	1
1	20	20	40	1	8	8X	8	1
0.5	20	20	40	0.5	4	8X	8	1
0.25	20	20	40	0.25	2	8X	8	1
0.125	20	20	40	0.125	1	8X	8	1
0.0625	20	20	40	0.0625	0.5	8X	8	1
0.03125	20	20	40	0.03125	0.25	8X	8	1
0.015625	20	20	40	0.015625	0.125	8X	8	1
0.007813	20	20	40	0.0078125	0.0625	8X	8	1
	0	0.5% DMSO	10µl DMSO					

6. Cell Preparation and Media Change

a. Use the Prep Station for changing the media from growth to assay media. Program the PS for a final XF assay media volume of 175 µl. (see Section 9, pages 39-41).
b. Incubate in 37˚C incubator **without** CO_2 for 60 minutes to allow cells to pre-equilibrate with the assay medium.

7. Loading sensor cartridge (Proper orientation: notch at lower left)

a. Warm all compounds to 37°C prior to loading sensor cartridge.
b. Load 25 µl of each FCCP concentration in the proper column with a multi-channel pipette according to the assay layout into ports A , using a multi-channel reservoir.
c. Incubate the loaded cartridge at 37°C non CO_2 incubator until ready for use.

8. Assay Protocol

Protocol Steps		
Command	Time (min)	Port
Calibrate		
Equilibrate		
Loop Start	4	
Mix	2	

Measure	5	
Loop End		
Inject		A
Loop Start	4	
Mix	2	
Measure	5	
Loop End		
End		

9. Results and Discussion

As discussed above, FCCP has a predictable effect on the bioenergetic state of C2C12 cells. The goal of this experiment is to determine the concentration FCCP that will produce maximal OCR and ECAR response. *Note that this method may be used for any acute acting compound to be tested.* Typical results are presented in OCR vs. time and ECAR vs. time graphs below:
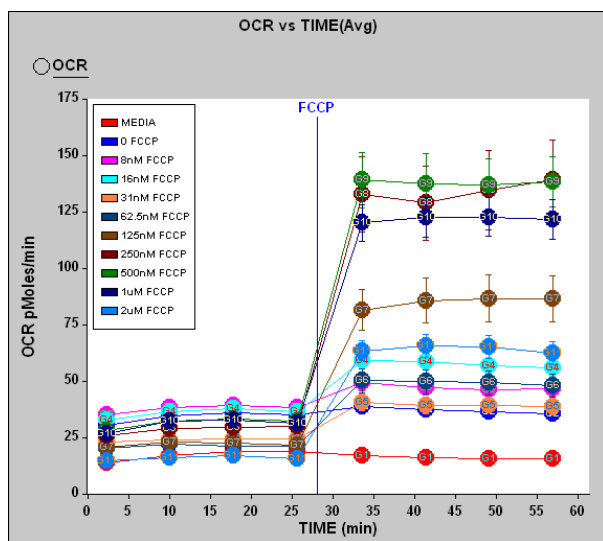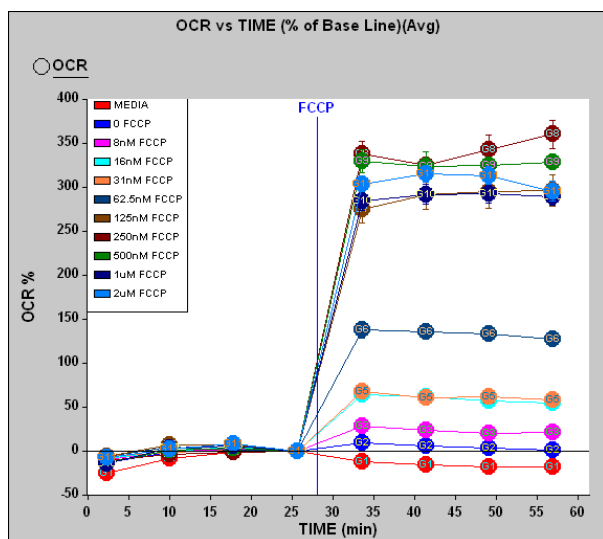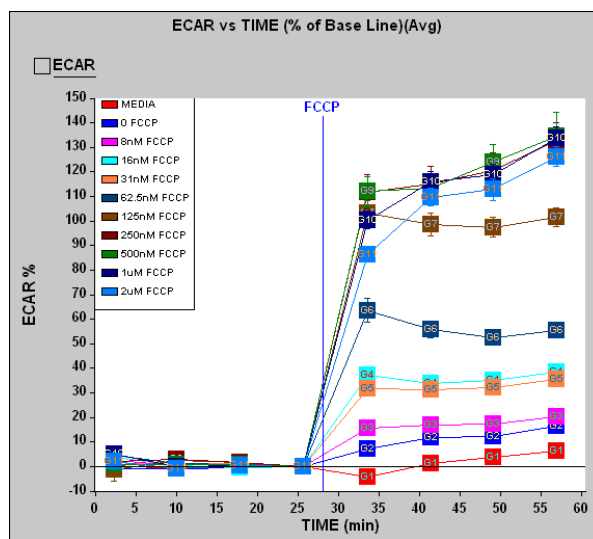

OCR vs. Time
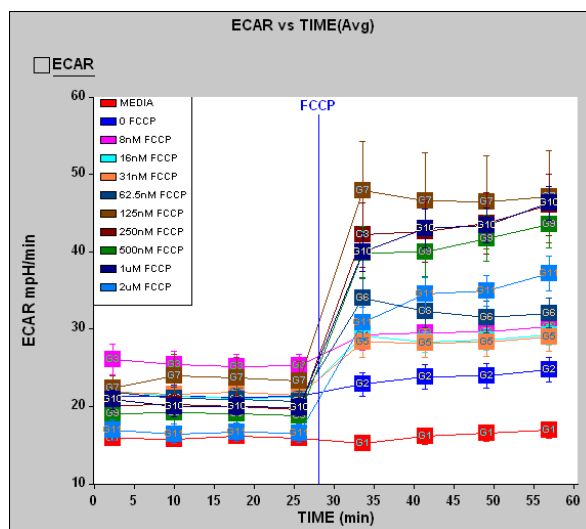
ECAR vs. Time

Note that due to slight variations in basal rates (measurements 1-4), it is often helpful to "baseline" the data to % response to obtain information from the graph. This is performed by using the $\mathcal{B}$ button, and setting the baseline option for "baseline subtraction just prior to injection A (default option). The "baselined" graphs are shown below.
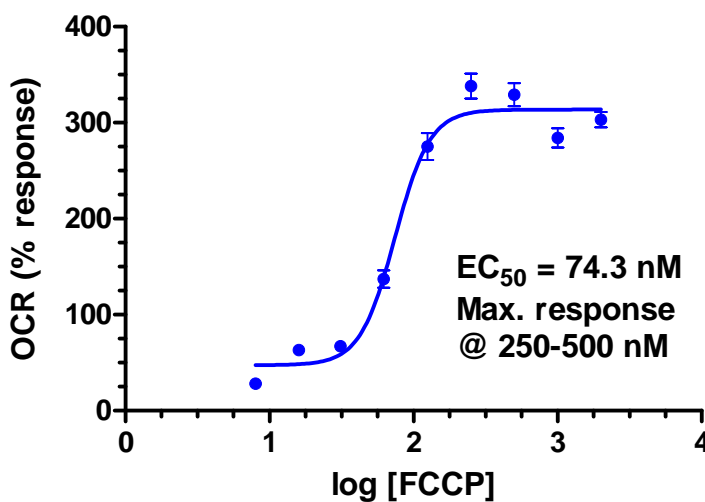
% response OCR vs. Time

% response ECAR vs. Time

By plotting the % response vs. the log [FCCP] (in nM units), one may obtain a typical dose response curve.

**Dose reponse curve
for C2C12 cells with FCCP**

EC_{50} = 74.3 nM
Max. response
@ 250-500 nM

Dose response for % OCR

Qualitatively, one should expect similar responses as those shown above when using C2C12 cells. It is important to note that absolute values of OCR and ECAR may vary due to technical proficiency, what type of and how many cells were seeded, the degree of confluence at time of assay, whether a consistent monolayer was obtained, the exact pH of solutions and added compounds, etc.

15. Metformin-Enhanced Fatty Acid Oxidation (FAO) Experiment using C2C12 myoblast cells

Mitochondria oxidize a variety of fuels to generate ATP through oxidative phosphorylation. Cells can utilize fatty acid, glucose and amino acids as their fuels. Traditionally, fatty acid oxidation (FAO) assay is performed by quantifying radioactive byproducts, H_2O or CO_2 after incubating cells with radio-labeled fatty acids such as palmitate or oleate.

In this protocol, increased oxygen consumption rate (OCR) immediately following exposing partially-starved cells to palmitate substrate is used as an indicator for FAO in XF96 Analyzer. This offers a rapid, easy and non-radioactive alternative assay for fatty acid oxidation. In addition, both kinetic and accumulative data can be obtained in a single assay.

$$\text{Palmitate} + O_2 \longrightarrow CO_2 + H_2O$$

In this experiment, oxidation of palmitate in the presence of increasing concentrations of metformin will be investigated. Metformin is an oral anti-diabetic drug from the biguanide class and improves hyperglycemia primarily through its suppression of hepatic glucose production (hepatic gluconeogenesis). In addition to suppressing hepatic glucose production, metformin increases insulin sensitivity, enhances peripheral glucose uptake, and increases fatty acid oxidation. Metformin is frequently used in research along with AICAR as an AMPK agonist. The mechanism by which biguanides increase the activity of AMPK remains uncertain; however, research suggests that metformin increases the amount of cytosolic AMP (as opposed to a change in total AMP or total AMP/ATP).

1. Reagents and Materials

      a. Krebs Henseleit Buffer (KHB, see Appendix VI)
      b. Palmitate-BSA conjugate and BSA vehicle control (Appendix VI)
      c. XF96 plate of C2C12 cells
      d. V-bottom 96 well drug-loading plate (VWR, 80080-048)
      e. Multichannel Pipette
      f. 24 Column low profile reagent reservoir (Seahorse Bio, S30039)
      g. Seahorse Calibrant (Seahorse Bio, 100840-000)
      h. Metformin (Sigma D5035)

2. Growth Medium

      a. 500 mL DMEM (Gibco 11965-092)
      b. 10% FBS (Hyclone SH90070.03)
      c. 5 mL Penn/Strep (Gibco 15140-122)
      d. 5 mL Sodium Pyruvate (Sigma S8636)
      e. 5 mL Glutamax (Gibco 35050-061)

3. Cell Seeding in XF96 Cell Culture plates

Note: A one-step seeding procedure is used for XF96 plates

a. The culture surface of the XF96 plate is 40% the area of a typical 96 well plate. For XF96 assays, C2C12 cells are seeded at 12,000 cells per well in a final volume of 80 µl. Therefore resuspend stock cell solution to obtain 1.5×10^5 cells/ml (1.2×10^4 cells per 80 µl , final concentration) in growth media. For one XF96 plate: 92 wells* x 80 µl ≈ 8.0 ml of cells.

b. Seed 80 µL of cell suspension per well with multiple channel pipette**, leaving appropriate control wells blank (A1, H1, A12, H12).**

c. Allow the cells to grow overnight in 37°C/**10%** CO_2 incubator.   It is recommended that the plate be placed in the rear portion of the incubator to minimize edge effects of cell growth caused by changes in humidity, temperature and CO_2 level due to opening and closing of the incubator door.

Note that cell seeding number will depend on cell type and cell growth characteristics. It is strongly recommended that the user perform initial cell seeding titration experiments to determine the optimal cells to seed per well for a given application.)

4. Treatment of cells with Metformin

The day after seeding the C2C12 cells, carefully change the growth media and add fresh growth media supplemented with metformin to the final concentration indicated for each column. Metformin is soluble in aqueuous solutions up to 50mg/ml (~300 mM).

Columns 2-3:	0.00 mM Metformin
Columns 4-5:	0.25 mM Metformin
Columns 2-3:	0.50 mM Metformin
Columns 2-3:	0.75 mM Metformin
Columns 2-3:	1.00 mM Metformin

Allow the cells to grow overnight in 37°C/**10%** CO_2 incubator in the presence of metformin.

5. Preparation of Assay Template.

Using the Assay Wizard (Appendix I), generate a template with the following group layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bkg. Cor.	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Bkg. Cor.
B	Blank	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Blank
C	Blank	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Blank
D	Blank	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Blank
E	Blank	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Blank
F	Blank	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Blank
G	Blank	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Blank
H	Bkg. Cor.	0 mM Met + BSA	0 mM Met + PA	0.25 mM Met + BSA	0.25 mM Met + PA	0.5 mM Met + BSA	0.5 mM Met + PA	0.75 mM Met + BSA	0.75 mM Met + PA	1.0 mM Met + BSA	1.0 mM Met + PA	Bkg. Cor.

6. Compound Preparation

Prepare Palmitate-BSA and BSA control according to the protocol in Appendix VI. The palmitate preparation protocol yields a 1 mM stock which is used as a 5X stock solution (200 µM final). ~1.2 mL of a 5x stock is needed for the FAO assay described here.

7. Cell Preparation and Media Change:

a. Use the Prep Station for changing the media from growth to assay media. Program the PS for a final XF assay media volume of 160 µl. (see Section 9, pages 39-41).
b. Incubate in 37°C incubator **without** CO_2 for 60 minutes to allow cells to pre-equilibrate with the assay medium.

8. Loading Sensor cartridge (Proper orientation: notch at lower left)

a. Warm BSA and BSA-Palmitate to 37°C prior to loading sensor cartridge.
b. Load 20 µl of BSA control both ports per well (e.g. ports A & B) for columns 2, 4, 6, 8 and 10 (40 ul total per well).
c. Load 20 µl of BSA-Palmitate into both ports per well (e.g. ports A & B) for columns 3, 5, 7, 9, and 11 (40 ul total per well).

Note: optimal palmitate concentration for different cell types can vary. Palmitate concentration titration is recommended for a specific cell type/line

9. Protocol Commands

| Command	Time (min)	Port
Calibrate		
Equilibrate		
Loop Start	4X	
Mix	2	
Measure	5	
Loop End		
Inject		A
Inject		B
Loop Start	4X	
Mix	2	
Measure	5	
End		

Notes:
1) To assay for the effect of FAO agonists such as metformin, cells can be treated for the desired amount of time before performing the fatty acid oxidation assay. Alternatively, rapidly permeating compounds (e.g. phenformin) can be injected and rate measured before palmitate-BSA injection.

2) C2C12 myoblasts may be differentiated into myotubes by replacing 10% FBS in the growth media with 2% Horse serum and allowing growth for an additional 4-6 days., Myotubes typically show higher OCRs and have a more robust response to added palmitate, thus the Measurement time should be decreased to 1.5 minutes.

9. Results and discussion

As discussed above, addition of palmitate to C2C12 cells has a predictable effect on the bioenergetic state of the cells. Note that injection of palmitate results in an increase in OCR above baseline, while the ECAR is relatively unaffected (the decrease in ECAR during the time course of the assay is likely due to the shift from 25 mM glucose in the growth media to 2.5 mM glucose in the KHB buffer employed for the FAO assay).

Typical results are presented in the % OCR vs. time and % ECAR vs. time graphs below showing the minimal (0.0 mM) and maximal (1.0 mM) doses of metformin and includes the BSA (control) and Palmitate injections. % response to baseline analysis is used in this case since metformin will affect the basal OCR and ECAR values of the cells (increasing concentrations tend to decrease the basal OCR rates, see graph below), thus in order to make a direct comparison among groups, the % response is used.
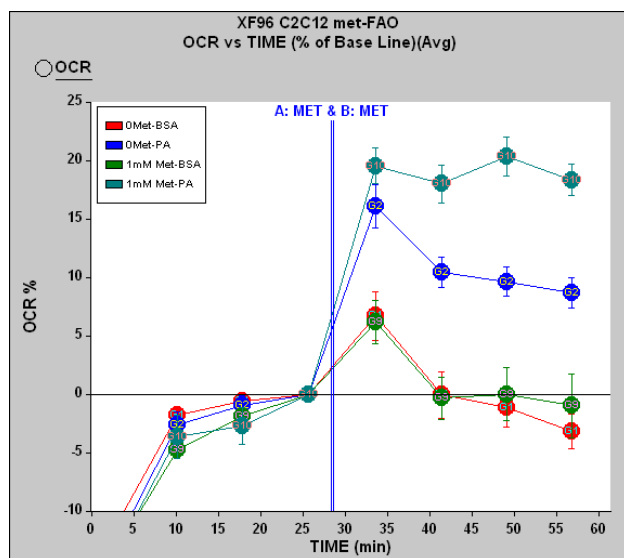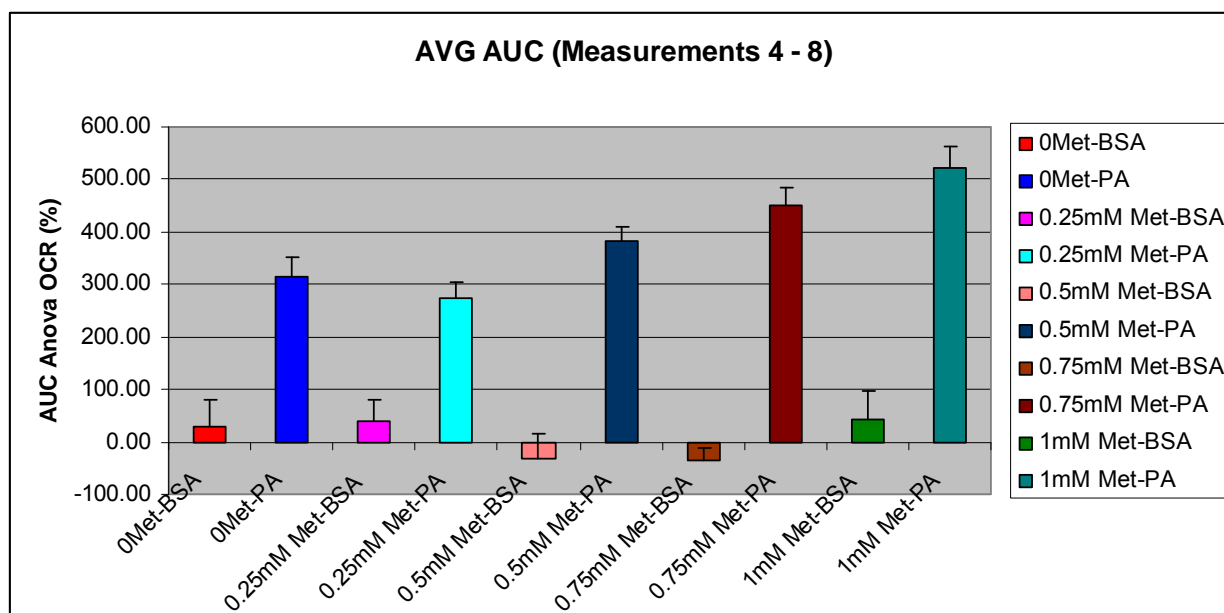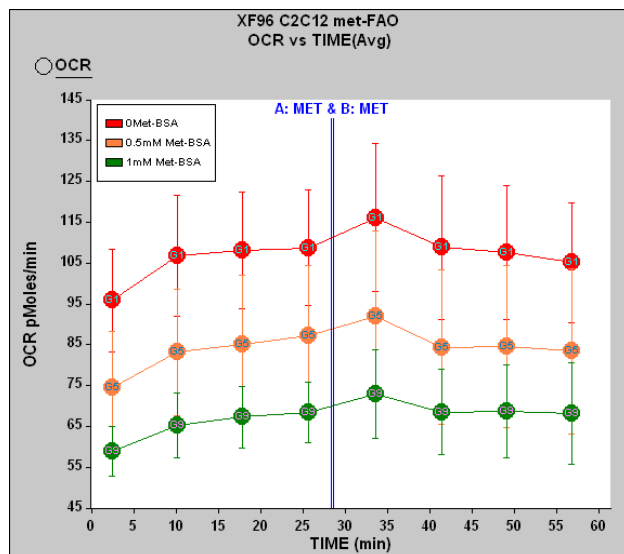
% OCR vs. Time
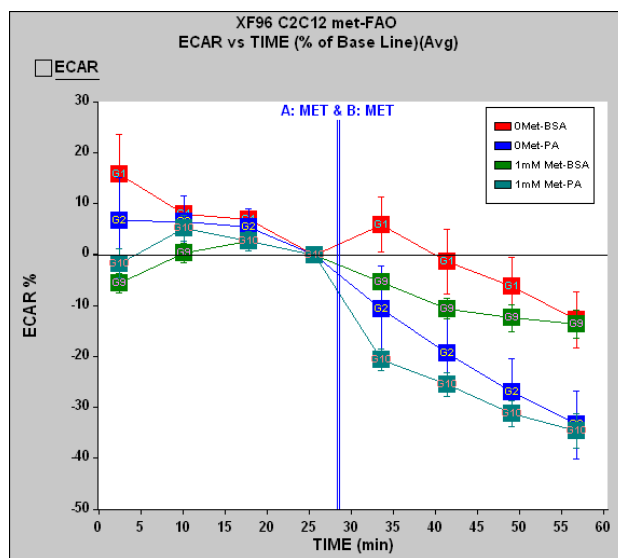
%ECAR vs. Time

Qualitatively, one should expect similar responses as those shown above when using C2C12 cells. It is important to note that absolute values of OCR and ECAR may vary due to technical proficiency, what type of and how many cells were seeded, the degree of confluence at time of assay, whether a consistent monolayer was obtained, the exact pH of solutions and added compounds, etc.

By plotting the data in the AUC format (Appendix III), one can observe the effects of increasing the metformin concentration. Below is a graph generated using the ANOVA AUC function. Here one may observe that there is a significant increase in OCR as the concentration of metformin is increased. Note that the BSA only controls show no significant increase of FAO activity.

By looking at the basal OCR rates, it may be observed that metformin decreases the basal OCR in a dose dependent manner, while there is a corresponding dose dependent increase in the basal ECAR.

OCR vs. Time

ECAR vs. Time
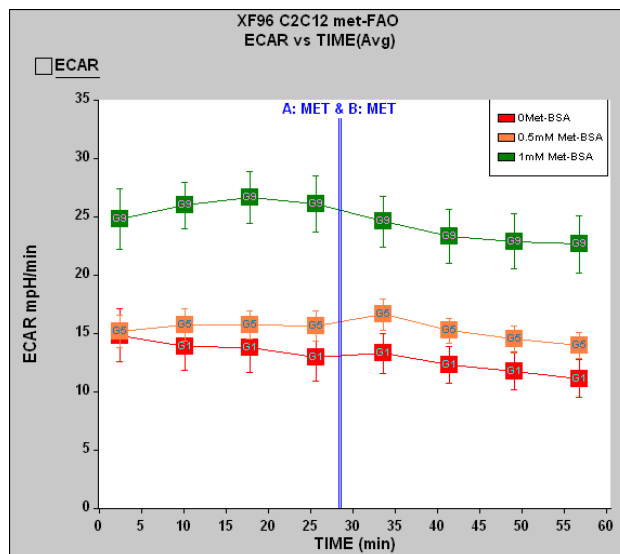
Group Graph: OCR and ECAR

The data above can be summarized conveniently by using the group feature on the X axis and using the left and right Y axes to plot OCR and ECAR respectively. See Appendix IV for using the grouping feature and other graphing options.
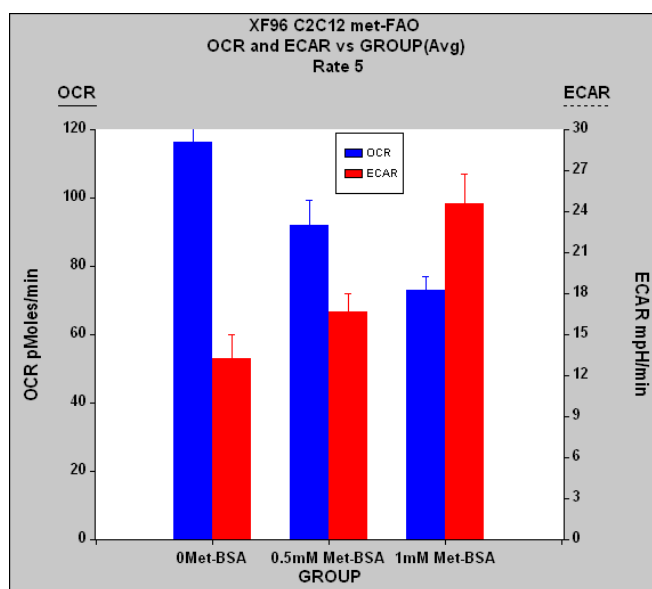
Finally, it should be noted that not all cell lines or cell types may not respond to exogenous fatty acids (palmitate). E.g. HepG2 cells appear to have little to no response in an FAO assay. Further, other cell types may require additional components (e.g. insulin, metformin, etc.) to show an acute response in OCR to exogenous fatty acid injection.

Appendices

Appendix I - The XF Assay Wizard and Creating XF Assay Templates

The **Assay Wizard** is used for creating an XF experimental template This may be performed on the instrument or on a PC, but must be done using the XF Reader software. The different sections of the wizard can be accessed at any time using the tabs. Each section may be broken down into one or more sub-sections that can be selected via buttons across the top of the section. Navigation of the wizard can also be performed using the Next and Back buttons at the bottom. These buttons will take you through the wizard in sequential order. When starting the Assay Wizard, the following should appear:

You may open a previously constructed template using the **Load Template** button and modify the information in the fields as desired, or you may choose to begin with an empty template by choosing the **Clear Assay** button. The Load template button will access the template directory specified in the instrument set-up. When using the Wizard, a **reset button** is available in the lower left corner. In any given section of the Wizard, use the Reset button to clear out that particular section.
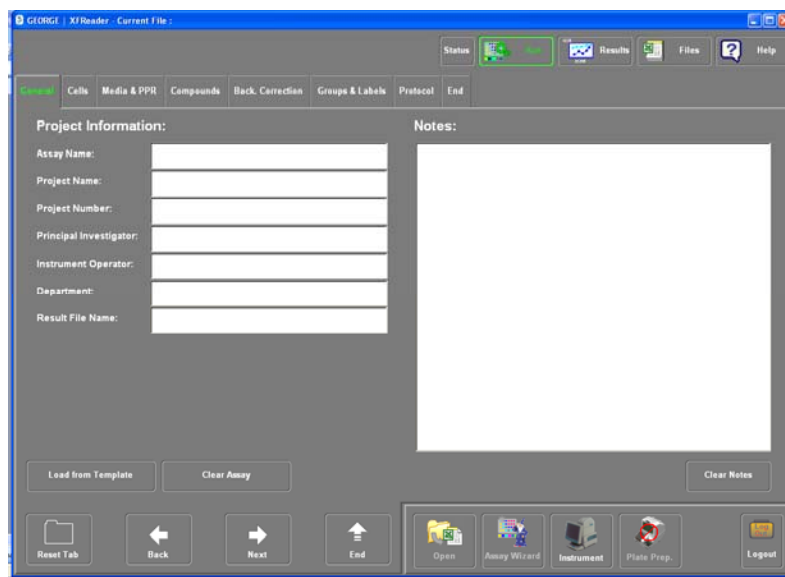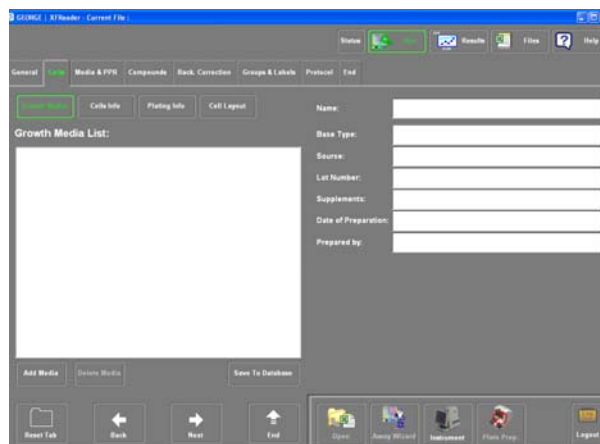

XF96 Assay Wizard Screen: General

Description of the Assay Wizard Tabs and building an XF Assay Template

General Tab: This is to input project info, such as assay name, project name, etc. Add desired information into the tab. The "**Result File Name**" will be the save name for the file. This name cannot contain any illegal characters. If illegal characters are present, the file will not be saved properly and will need to be manually saved after completion of the run.

Cells Tab: This is to input or select info about cell type, growth media, plating info and cell layout.

Growth media: To document Information about the growth media used. It allows one to track lot numbers, dates of preparation, etc. Note that several media compositions may be listed here. Add or delete medias by using the **add media** or **delete media** buttons, respectively. The **add media button** will bring up a dialogue allowing the user to choose to create a **new growth media** or **load from database**. Once the information of a growth media is added, you may press the **Save to Database** button to log this information into the database.

Assay Wizard: Cells/Growth Media Screen

(Note this button will flash green to show that the information is added.) You may also add new growth medias directly into the data base by pressing "new" if the load from database button is selected.

Cells Info: Similar to growth media tab above. It allows one to track lot numbers, dates of preparation, etc. Note that several cell types may be listed here.

Plating info: Information may be entered about the plating of cells for an assay.

Cell Layout: Allows the user to assign specific information about the cells in each well. Note that one could use more than one type of cell per plate. This is where this info would be assigned. To assign information to the wells perform the following steps

a) Highlight cell type to be used (e.g. C2C12).
b) Enter the seeding count per well (e.g. 10000).
c) Select all wells by pressing the upper left corner box of the plate layout.
d) Deselect individual cells to be used for Background Correction (e.g. Blank) wells (A1, A12, H1, and H12).
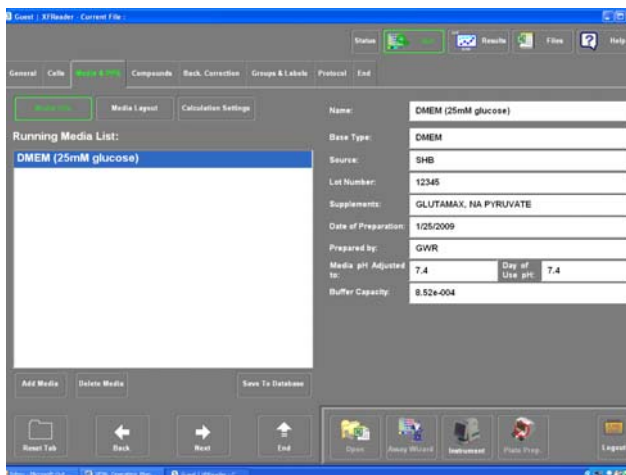e) Click on the **assign to wells** button.
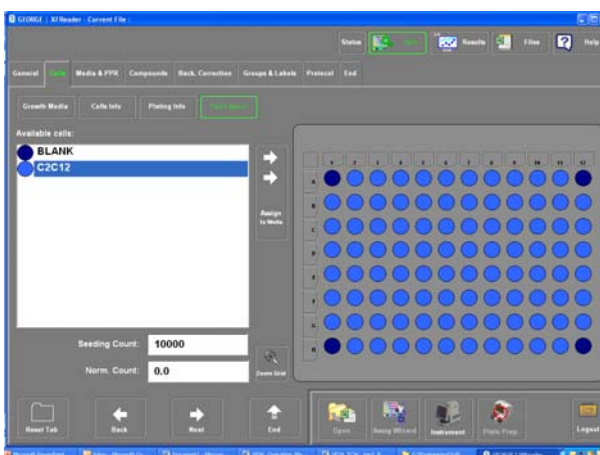
Assay Wizard: Cells/Cell Layout Screen

Repeat steps a-e to assign "Blank" to the reference wells. (Hint: simply select the four wells to be used, then press "assign". Be sure to enter a seeding count of "0" here. You may use the **Zoom Grid button** to view the Cell Layout in full screen.
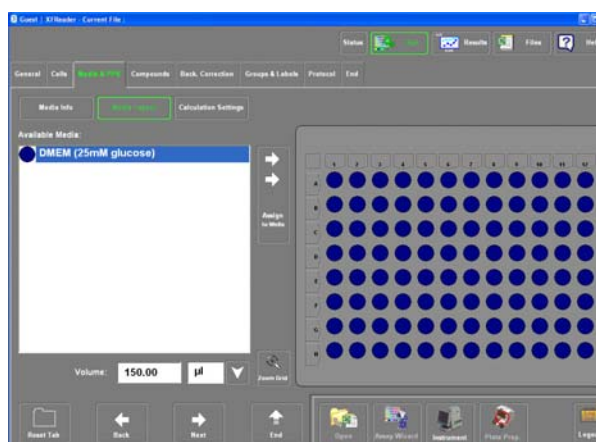
Media and PPR Tab:

Media Info: This tab is for documenting information about the assay (running) media and functions similarly to the growth media tab (above). Different assays may use different running media, e.g. mitochondrial studies may use a DMEM, while FAO experiments will use a KHB buffer. Note that if the "Load from Database" button is used, four (4) pre-defined media choices will be listed (DMEM, 25 mM glucose; DMEM, 11 mM glucose; KHB, and RPMI). Note that these media have their respective buffer capacity values defined (see below for more info on buffer capacity).

Assay Wizard: Media Info Screen

Media layout: Assigns running media to individual wells. Enter the information the same way as assigning cell information to wells (described above). Be sure to specify the volume of running media used. Typically this will be 150-200 µl and be dependent on the number and volume of compound injections.

Assay Wizard: Media Layout Screen

PPR: Proton Production Rate (PPR is an alternative measurement of Extracellular Acidification Rate (ECAR). It is reported in pmol/min (same as OCR) and requires that the buffer capacity of the media be defined and entered into the software for accurate calculations. When defining the running media in the assay configuration (media info tab), a field is available for specifying the buffer capacity of the media at pH 7.4. This value is then used in calculating Proton Production Rate (PPR). Note that if no Buffer Capacity value is assigned to a new assay media, then a default value will be used for the PPR calculation.
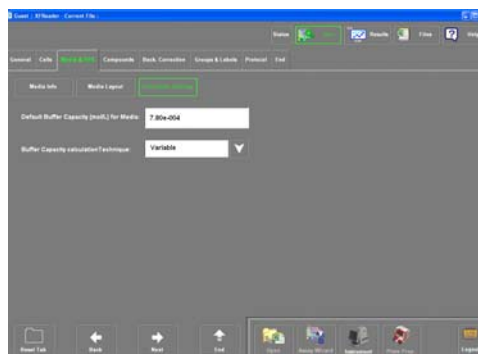
The **Calculation Settings** section is to support additional options for calculating PPR. Note that if a Buffer Capacity value is defined in the Media screen, this value will be used for calculations even though the default value will appear in the calculations tab. (Please see Appendix II for buffer capacity determination and rate calculation settings.)


Media and PPR: Calculations Tab

Setting the PPR

1. In the **Media Info** screen of the **Media & PPR** tab, click on and highlight an existing media name in the list. If no names are listed, click on the **Add Media** button to add one to the assay configuration.

2. Click on the **Buffer Capacity** field located on the right side of the screen and enter a value.

3. If a new Running Media definition was created, go to the **Media Layout** screen. Click on and highlight media name in the **Available Media** list. Select the wells that will be using the media. Enter the volume and set the volume units. Then click on the **Assign to Wells** button. Multiple media with differing buffer capacities can be assigned to the plate.

The XF software will default to using the **Variable** technique, as shown in Appendix II. This is the recommended option as it assumes there will be changes in the buffer capacity of the system during the time course of the experiment This is especially important if using highly glycolytic cell lines.

Compounds Tab:

Compound Info: provides a working list of compounds, substrates, agonists, antagonists, drugs, etc. that you may use for your experiments. You may specify the stock and working concentrations here. To add a compound to the list, click "New", then enter the compound Name and concentration information. Repeat for multiple compounds. Note that the user may also store compound information in the database by clicking "add to database".
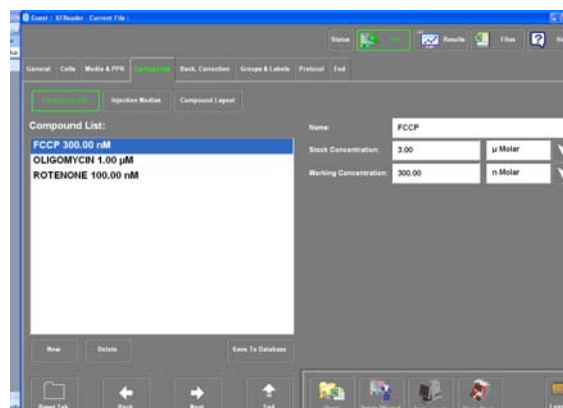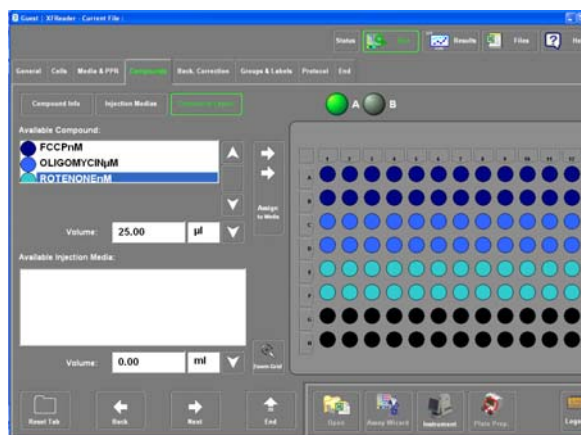

Assay Wizard:
Compound Info Screen

Typically, the working concentration (WC) is the final concentration of the compound after injection. The stock concentration (SC) is the concentration of the compound PRIOR to injection that is loaded into the ports.

Injection Media: This is the description of the media that the compound is diluted in, typically this would be the same as the running media. Add compound injection media by clicking the "Add Media" button and filling in the information on the right. Repeat for multiple injection medias. Note that it is very important to pH the injection solutions to match as closely as possible to the pH of the running media to ensure accurate ECAR values.

Compound Layout: Assigns compounds to individual wells. Highlight desired compound on the left, enter the compound volume; highlight the Injection Media for that compound. Choose port A or B above the plate layout, then highlight which wells will be receiving that compound. Click "Assign to Wells". Repeat for multiple compounds. If multiple ports are being used for compound injection, each port needs to be assigned separately. Be sure to specify the volume of the compound to be injected.

Assay Wizard: Compound Layout Screen

Available injection media: Typically, the injection media matches the running media (e.g. XF assay media), and thus it not necessary to assign the injection media. However, if the injectate is prepared in a media other than the assay running media, it may be assigned here.

Background Correction Tab

This screen is used for choosing and defining the Background Correction parameters for an XF assay template.

Do Background Correction: this button engages the background correction function and should always be ON.

Label for background wells: Typically "Bkg. Cor." is used, but the label may be changed by the user if desired.
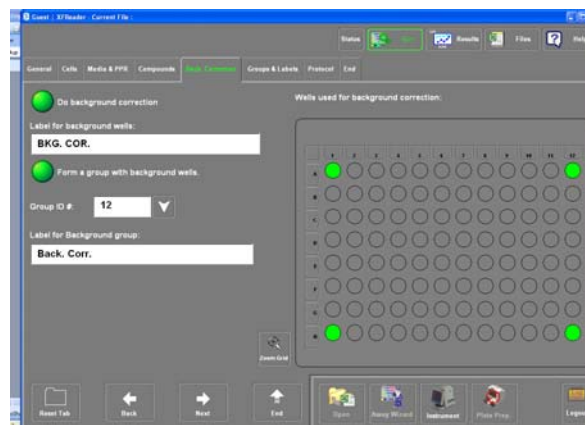
Assay Wizard: Back. Corr. Tab

Form a group with background correction wells: When engaged (default), this instructs the software to automatically form a group using the Background Correction wells chosen. Note that even if a group is not automatically formed, the data and calculations will be performed properly.

Group ID #: User may choose any group number.

Label for Background group: defines a label for the Background Correction. group.
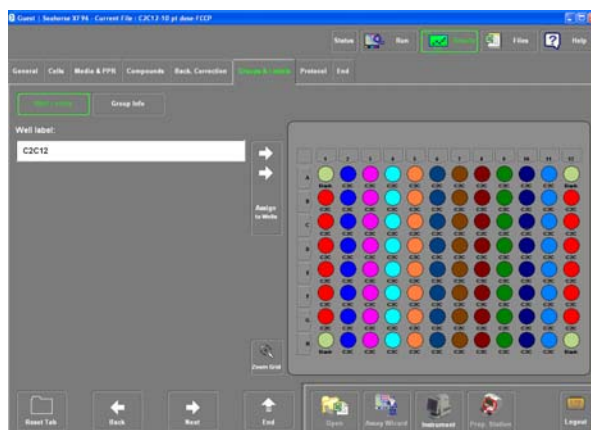
Groups and Labels Tab: Used to assign groupings of cells, compounds, conditions, etc.

Well Labels: Assign specific info to wells e.g., cell type, seeding number, WT or KO, etc. Enter the desired label and highlight the wells to the right. Click "Assign to Wells." This label will appear under each well.

Note this does not have to be the same as the info provided in cells, media, or compounds. This can be used for simple and quick identification of the condition for each group and will be displayed in this manner in the results graph at the end of the experiment. (e.g. control vs. compound).
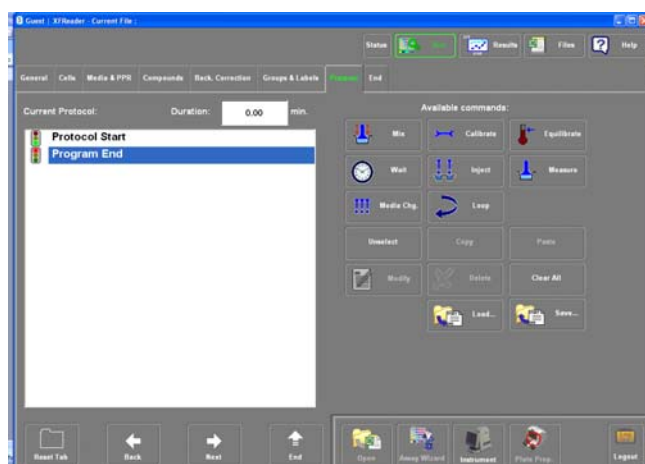

Assay Wizard: Well Labels Screen

Group Info: Assigns group information to wells (e.g. Control vs. test compound, escalating dose series, etc). You can also customize the colors for each group. There are 24 predefined colors. To assign groups to wells:

a. Click on "Create New Group" button
b. Enter a group name in the "label" space.
c. The color of any group may be changed by clicking in the colored box to the right of the "Label" space.

Assay Wizard: Group Labels Screen

d. Click the wells in the plate layout to add them to a specific group.
e. Repeat to assign each well to a group if desired.
f. Up to 96 groups can be identified for each plate. Wells that are not assigned to a group will be black.
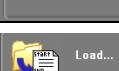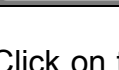
Protocol Tab:

This is an especially important tab as it programs the instrument for the experiment with respect to timing of the calibration, mix, measure and inject commands. Before entering any commands, the screen will look like the screen diagram to the right. There are several command buttons that may be utilized to build a protocol. To create a protocol, use the buttons to insert defined steps, steps are inserted only *above* the currently highlighted step (blue bar).
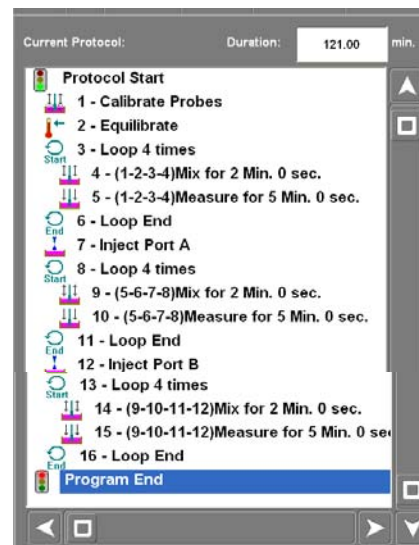
Assay Wizard: Protocol Screen

Command	Description/Comments
Calibrate	Calibrate: The first command for every XF assay, calibrates the sensors for the experiment.
Equilibrate	Equilibrate: used immediately after calibration. Default command consists of a 2 minute mix and a 2 minute wait repeated 3X. This equilibration period allows the temperature of the culture plate and instrument to stabilize after the transfer of the culture plate to the instrument.
Mix	Mix: mixes the volume of the wells by a gentle up/down motion of the sensors, used to oxygenate the cells and mix injected compounds.
Wait	Wait: instructs the instrument to wait between steps.
Measure	Measure: used for the measurement of OCR and ECAR. Time of measurement is dependent on the basal OCR value of cells, typically 4-8 minutes*.
Inject	Inject: instructs the instrument to inject compounds loaded in the ports into the wells. The user may choose port A, B or both.
Loop	Loop: used to repeat a set of commands multiple times. Used before and after the series to build a loop, e.g. "loop 3X: mix 2 min, measure 3 min" will repeat the mix and measure commands 3 times. Since most protocols consist of repeated cycles of **Mix** and **Measure** commands, protocol construction can be simplified by using a loop to repeat the same command cycle. This will also shorten the length of the protocol command structure. One drawback is that once a loop has started, you will not be able to edit the contents of the loop.
Media Chg.	Media Change: This button is not yet functional in the current version of software.
Copy	Copy: Copies the highlighted commands, can be used in conjunction with the Paste command to facilitate protocol set up.

Paste	Paste: Pastes the copied commands into the protocol ABOVE the command highlighted in blue.
Unselect	Unselect: unselects the highlighted command
Modify	Modify: allows the user to modify the highlighted command with respect to time or port injected
Delete	Delete: Removes the selected command(s) from the protocol
Clear All	Clear All: Removes ALL commands from the protocol
Load...	Load: Loads an entire saved protocol into the screen. Useful if a number of protocols will be used for various assays
Save...	Save: Saves an entire protocol in the database for future use.

A. Click on the desired command on the right to enter it into the protocol on the left of the screen. When the command on the right is selected, a pop up screen will appear to enter the time of each command (or which injection port is to be used, etc.)

B. Every protocol must start with the "Calibrate" command. Seahorse Bioscience recommends then performing an Equilibrate command to allow the cell plate to thermally equilibrate after being loaded into the instrument.

C. After calibrate and equilibrate commands are entered, the assay cycle can be entered. A typical assay cycle consists of a mix and measure command. This 2 step cycle can be copied and pasted by highlighting the desired commands on the left, clicking "Copy", and then "Paste" or by using the "Loop" command as shown in the example above (entire Loops may be copied and pasted as well).

Assay Wizard: Protocol Screen Example

D. Note that OCR and ECAR values are typically not stable until the second or third measurement cycle, thus it is strongly recommended that 4 basal measurements are performed prior to injection of compounds in order to establish the baseline metabolic rates for the experiment.

About Mix and Measure Times:

The length of Mix and Measure cycles depends on the particular **baseline OCR** of the cells one is using in the experiment. For most purposes, the recommended times for Mix and Measure are as follows:

> Mix: 2-3 minutes
> Measure: 3-8 minutes.

The length of time will depend on particular cell type and the basal OCR rate. For higher OCRs, use a 3-4 minute measure, while for lower OCRs, use a longer measure time (e.g. 7-8 minutes).

End Tab:

Ends the Assay Wizard. The information entered is applied as the "Current File" and the assay may be started. At the end of the Assay Wizard, you also have the option of saving your configuration to an assay template file, which is an excel worksheet containing the assay information.

Select the directory in which the template will be saved, then enter a save name when prompted at the bottom of the screen. The saved template can be modified at any time by opening it back up in the Assay Wizard and changing the desired fields.

To end the Assay Wizard, press the End Wizard button.

Improved Text Input in the Assay Wizard: Quick-pick Text

When using the Assay Wizard, text entry can be repetitive and tedious. The same text is often used in multiple assays. To make it easier to fill in text fields, entries will now be logged and then made available in future sessions for selection via a quick-pick feature in the virtual keyboard.


The Virtual Keyboard

Use the quick-pick feature by clicking on the down arrow button found to the right of the entry box on the virtual keyboard (red square). If the button is disabled, then there are no prior recorded entries available to select from.

You will only be able to select from prior text entered into that specific field. So it will take time to build up a catalog of entries for each field.

Logged text will be saved to the file, **TextDatabase.tdb**. This file will be stored in the location specified by **Info Database** in the **Database** section of **File Preferences** found under Instrument setup. If this file is deleted, all recorded entries will be lost and the file will be started again.

Protocol Save Feature
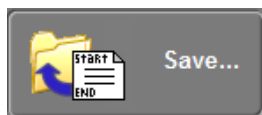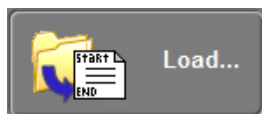
When constructing protocols in the Assay Wizard, **Load…** and **Save…** buttons will be available to load and save protocol files. Protocols can be saved separately as *.ptc files for later recall. This feature is only available in the **Assay Wizard**.

100900-400_MANUAL XF96 W/ PREP STATION_RA

Appendix II - OCR, ECAR and PPR: Information on Rate Calculations

Oxygen Consumption Rate (OCR) Calculation methods and Settings

The "(Level) Direct (Ksv)" method

In the XF Reader software versions 1.1.2.11, the oxygen consumption rate (OCR) is calculated using the Stern-Volmer relationship and implementing a "Fixed Delta" technique for determining the slope. The parameters, Edge Offset and Delta, define the boundaries of the data set used to calculate OCR. Edge Offset defines the number of data points omitted from the beginning of the measurement period. Delta, set in mmHg, defines the range of values to use from the starting point. Seahorse Bioscience recommended that the Edge Offset equal/be set to 1 and Delta to 50 mmHg in order to achieve the most accurate measurements. These are the default settings in the software.
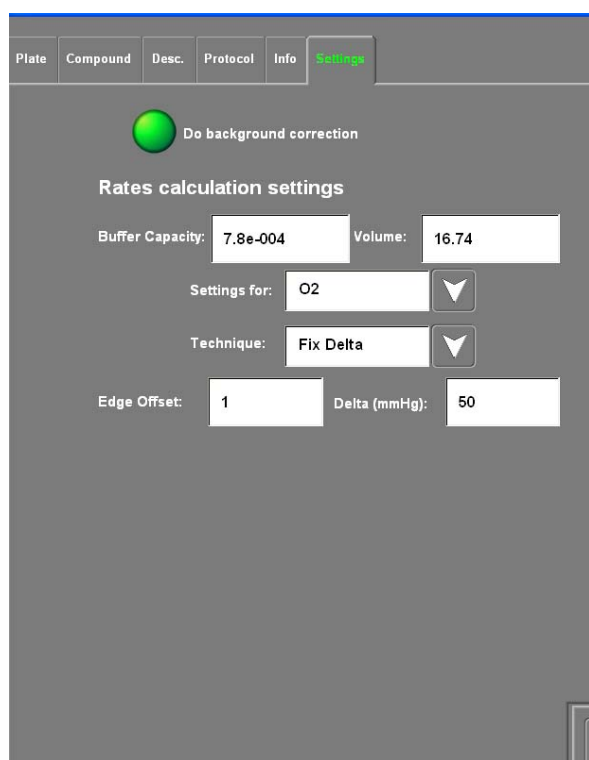

Settings for OCR
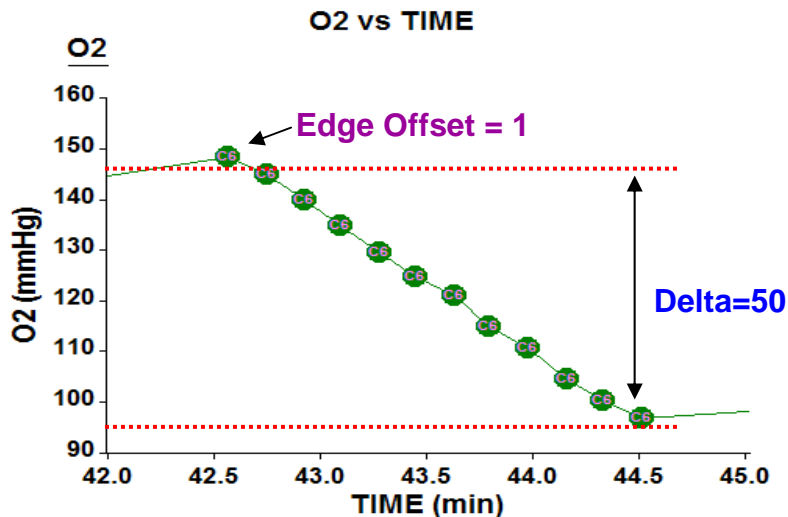
Why a "Fixed Delta"?

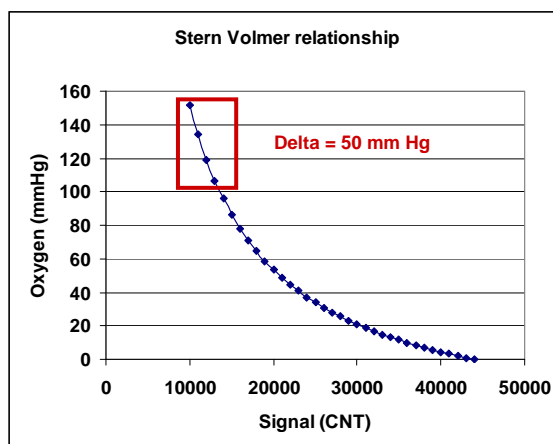The Stern Volmer Constant (K) defines the 1st order relationship between the fluorescent signal (F) and concentration (O2).

Stern Volmer relationship

Delta = 50 mm Hg

$$[O_2] = \frac{1}{K_{SV}}\left(\frac{F_0}{F} - 1\right)$$

Stern-Volmer Equation
F_0 = maximum fluorescence at zero [O2],
F = fluorescence at [O2],
Ksv = Stern-Volmer constant.

Stern-Volmer Relationship
Note that the response is nearly linear between 158 mmHg (ambient O2 tension) and 100 mmHg.

By using only the data points within the first 50 mmHg drop (Delta) in O2 tension, the rate can be modeled using a linear relationship as the Stern-Volmer relationship shows near linearity at O2 tension > 100 mmHg.

Improved Level Data Graph

When looking at rate data and level data, it is not clear where in the measurement the rate was calculated. Depending on the rate calculation parameters, some amount of level data is typically omitted from the calculation.

The graphs of the level data will now consist of solid and outlined points. The solid points will indicate those that are used to calculate the rate. If you modify the rate calculation parameters, the level data graphs will update to reflect any changes in the points used.

In the top figure, the O_2 level data is shown for two measurements. When calculating OCR, there is an edge offset of 1, so the first point of level data is omitted, and is displayed in the graph as an outline.
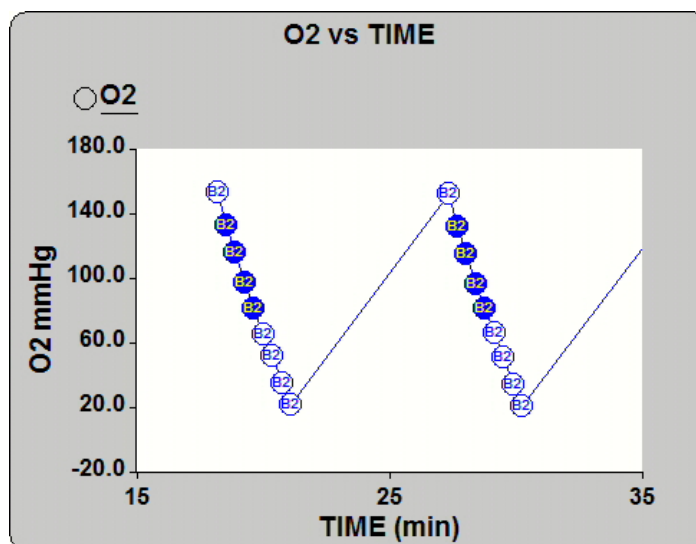
The next four points are solid. They

Level Data

fall within the 50 mmHg drop in O_2 level that is the range used for the OCR calculation.

The last four points, being beyond the 50 mmHg range, are also outlined and omitted from the calculation.

In the figure to the right, the rates corresponding to the level graphs are displayed.

Note this configuration of level data only applies to level data captured using the 1.4 version of XF Reader software that uses the (Gain) Fixed algorithm. This configuration does NOT apply to data captured with the 1.5 software using the (Level) Direct (AKOS) algorithm (see below).
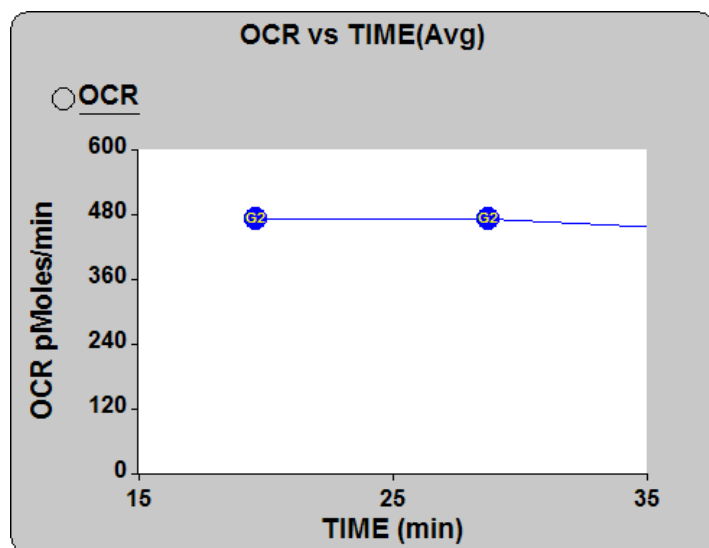
Corresponding Rates
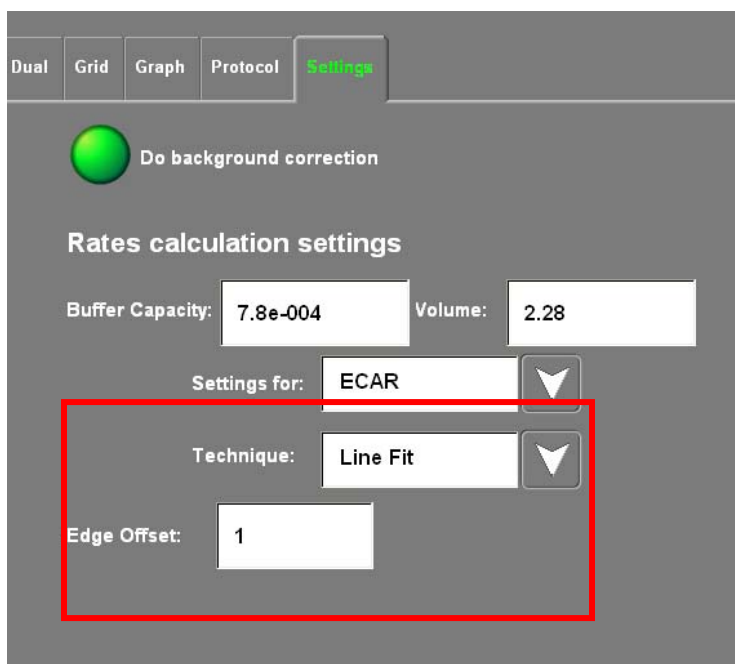
Benefits of this Setting

By discarding the first data point, more linear, reproducible measurements with less bias between columns are achieved. High rates introduce non-linearity to the data set. This is normally found in the first two data points during which time the gradient across the sensor is changing. The degree of nonlinearity is worse for higher rates (above 500 pmols/min) and changes as the gradient become stable. Also, increasing the delta, or the drop across which we measure the slope, provides more data points to calculate the rate. Additional information on how to apply different OCR calculations to your existing data files can be found in the next section.

Volume Setting

A default value of 16.74 µl for the chamber volume is based on calculations comparing rate data between the XF24 and XF96 instruments and knowledge of O2 diffusion and storage properties of the materials used for the XF96 plates and cartridges. It is recommended that this setting NOT be changed. Please contact your local Seahorse Bioscience for further information about this setting.

Extracellular Acidification Rate (ECAR) Calculation and Settings

Since the introduction of the XF96, extra-cellular acidification rate (ECAR) has been calculated using the **2 Points** technique for determining the slope. The slope between the average of three points at the beginning and end of the measurement period determined the rate. As shown in the figure below, the calculation technique has been changed to **Line Fit** with an **Edge Offset** of 1. The **Line Fit** technique uses the same line fitting algorithm as the **Fix Delta** technique to calculate the slope. However, all the points in the measurement, excluding those omitted by the **Edge Offset**, are used.

Recommended Settings for Calculating ECAR

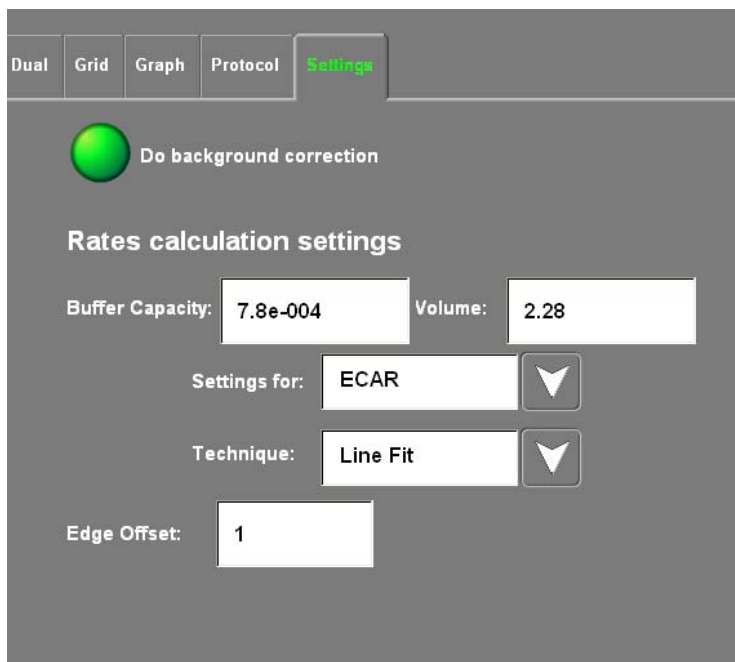### Modifying Instrument Settings

The new recommended values for the ECAR calculation settings are automatically set after installing and running the new software (XF Reader software version 1.4 and higher). They will be the default values for future assays.

Modifying Existing Data Files

The following procedure shows how to apply the new ECAR calculation parameters to your existing data files.

1. In the **Files** screen, browse for and load an XLS or XFD data file (display XFD files by changing the file display filter in the lower left corner of the **Files** screen). After loading the file, go to the **Results** screen to confirm the data was loaded.

2. In the **Run** screen, select the **Settings** tab in the left hand panel (see Figure 21).

3. Display the settings for ECAR. Modify the **Technique** and **Edge Offset**.

New Settings for ECAR

The ECAR data is automatically recalculated. Go to the **Results** screen to view the data. To save the data, go to the **Files** screen and click on **Save As** to generate a new data file.

Proton Production Rate (PPR) Calculations and Settings

Proton Production Rate (PPR) was introduced as an alternative measurement of acidification to Extra-Cellular Acidification Rate (ECAR). Unlike ECAR, PPR accounts for variations in buffer capacity and volume. Even when using the same cells, these differences could theoretically produce different ECARs. The relationship between PPR and ECAR is highly non-linear and BC increases exponentially as pH decreases.

Buffer capacity can be defined in the running media definition of the assay configuration. In addition, a default value can be specified if running media is left undefined. Two options are available for calculating PPR: Variable and Fixed. These options refer to how buffer capacity is treated in the calculation.
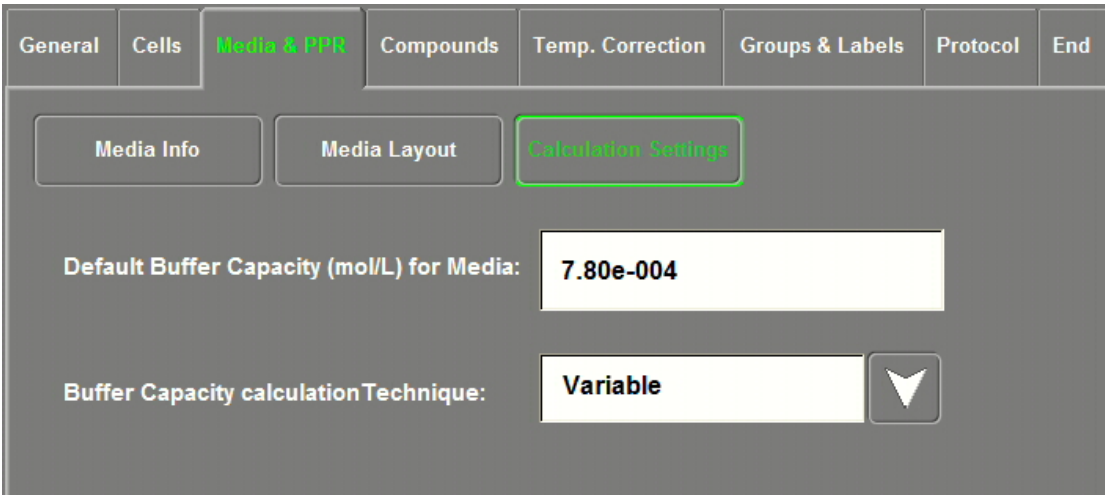
Variable vs. Fixed

The Variable technique assumes that buffer capacity will change appreciably during assay measurements. An exponential function is used to calculate buffer capacity at any measured pH level. This function is derived from a table of pH values and their corresponding buffer capacity. The pH values are in 0.1 pH increments starting at 7.4.

The buffer capacity values in the table are initially calculated assuming zero buffer. The actual buffer capacity at pH 7.4 is specified in the assay configuration as part of the running media definition. With this value as a reference, the ratio of the reference and zero buffer values at pH 7.4 is then used to scale the buffer capacity at the subsequent pH levels. During an assay, the exponential function derived from this table is used to calculate the buffer capacity at any measured pH level. Knowing the well volume, total number of protons can be calculated. As the number of protons increase over a measurement period, the PPR can be calculated.

The **Fixed** technique assumes the pH changes are small and that a fixed buffer capacity value is sufficiently accurate for calculating PPR over the range of pH values seen in the assay. In this case, ECAR will be directly proportional to PPR, since no variation in buffer capacity is assumed.

The software defaults to using the **Variable** technique, as shown in the next figure. This is the recommended option, and is especially important if using highly glycolytic cell lines.

PPR Calculation Settings in the Media & PPR tab

Configuring PPR

The **Media & PPR** tab of the **Assay Wizard** contains the settings for configuring how PPR will be calculated. To calculate PPR, the buffer capacity for the running media used in the assay must be specified. This can be done using the Media Info and Media Layout sections as discussed earlier in this document. The value specified in the **Default Buffer Capacity** field is used if no running media has been defined or assigned to the plate. The **Buffer Capacity calculation Technique** determines whether buffer capacity will be treated as a fluctuating factor in the assay or a constant.

The following is a guide to configuring the settings that affect the PPR calculation. To avoid redundancy, references are made to other sections of this document for specific instructions.

1. Specify the buffer capacity of the assay's running media. This can be done several ways:

   a. With the **Database** feature introduced in Appendix V (page 135), the software comes with four common pre-defined running media. When opting to load a running media from the database, the listing will include **DMEM (25 mM glucose)**, **DMEM (11 mM glucose)**, **MDC RPMI**, and **KHB.** These definitions have pre-set buffer capacity values. Follow the steps for **Recalling a Running Media Definition from the Database** on page 135 (Appendix V).

   b. Alternatively, follow the steps for creating a new assay media definition on page 81 (Appendix I).

   c. To specify multiple buffer capacities in different wells or groups of wells on the plate, assign multiple media to the plate from the **Media Layout** section of the **Media & PPR** tab.

   d. Lastly, instead of using running media in the assay configuration, specify a value in the **Default Buffer Capacity** field in the Calculation Settings section. If no media has been designated as described in the above steps, this default value will be used instead for all wells.

   Steps a, b, or c can be performed on a computer other than the instrument controller. If doing so, save the modified assay configuration to an excel file using the **Save Template** button at the end of the wizard. Load the assay configuration template into the software on the instrument controller before proceeding to step 2. Step d must be performed on the instrument controller.

2. Select the Buffer Capacity calculation Technique. The default value of **Variable** is recommended. This step must be performed on the instrument controller prior to starting the assay. If using the default **Variable** technique, this step can be skipped since no change is required.

Measuring the Experimental Buffer Capacity of XF Assay Media

Buffer capacity (BC) will change as a function of the amount of buffering agent in the media. For the XF assay media this includes the low concentration of phosphate, as well as the presence of any added compounds that may accept or donate protons (H+), including (but not limited to): glucose, pyruvate, glutamine (Glutamax), albumin or protein (including serum). Thus each media composition will have a unique buffer capacity value (expressed in mol/L = M).

For example, the BC of XF DMEM with 25 mM glucose, 1 mM pyruvate, and 2 mM GlutaMax-1 (Gibco 35050-061) = 8.52×10^{-4} M.

Note that BC will also change as a function of temperature of the media, thus, ensure that when calculating the BC you perform at 37°C.

The formula to determine the experimental BC is:

BC = moles of OH- (or H+) added / (pH change)*(volume of buffer tested), so

$$BC = [NaOH]*L / \Delta pH*L$$

Example: You wish to determine the BC of your XF Assay media

Materials and Reagents:
- Accurately calibrated pH meter
- 1.0 M NaOH solution
- Aliquot of XF Assay media warmed to 37°C (e.g. 25 mL)

Volume of media tested   = 25 ml = 25×10^{-3} L

NaOH added   = 27 µl = 27×10^{-6} L

Δ pH   = 1.18 (from 7.40 to 8.58, recall pH is a unit-less value)

Buffer Capacity   = (1.0 M * 27×10^{-6} L) / (1.18 * 25×10^{-3} L)

                       = 27 x 10-6 moles / 29.5×10^{-3} L

                       = 9.152×10^{-4} moles/L

                       = 9.152×10^{-4} M

## Appendix III - Group Statistics and AUC analysis

Statistics, CVs and Data quality

Because the XF96 is a non-invasive instrument, multiple measurements may be made within a well and thus remove biological variability. From measurement to measurement within a well, the CV is +/-5% and can be considered instrument noise only. Further, because basal metabolic measurements are taken before addition of any compounds, each well may serve as its own control.

Variability between wells that includes instrument plus biology for the average trained user is <20%. This is composed of <10% for well-to-well variation, and overall <20% from plate to plate or day to day. Thus the majority of CV is inherent in the cells and operator. By careful experimental design, pipetting technique and proper cell culture and seeding methods, this error can be minimized to give extremely consistent and accurate data.

Group Statistics Feature

The Group Statistics button is located in the Special Operations Tab in the Excel Data Viewer.
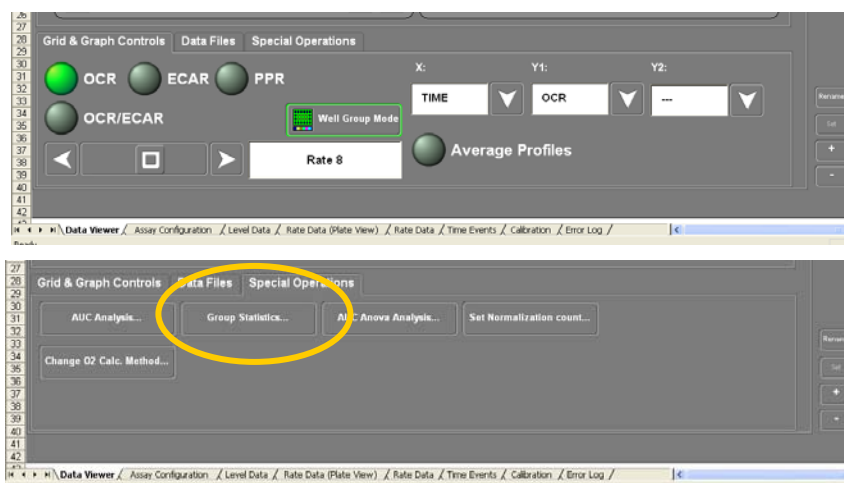
View of the Special Operation Tab Options

Clicking on the button generates another tab in the spreadsheet labeled "User Data". This sheet lists the statistics associated with each group for rate measurement. Listed is the Group Name, Mean, Standard Error (or Standard Deviation, which ever is preferred by the user), Number of Wells in the group, Rate #, Group #, the wells in the group and the compound(s) added. For example, the statistics sheet for the Bioenergetic Profile using C2C12 myoblasts (OCR data) would look like this:

Group StatisticsOCR	C2C12-10 pt dose-FCCP MANUAL EXAMPLE .xls						
Project Name							
Assay Name							
User Manipulated Data	Well Group Averages						
Experiment Date							
Baseline Mode	Off						
Normalization Mode	Off						

Group Name	Mean	Standard Dev	Num Wells	Rate #	Time	Group #	Wells
med	2.536820073	0.729504702	12	1	1:02:57 AM	1	....
0 FCCP	4.802361134	0.906486092	8	1	1:02:57 AM	2	....
8nM FCCP	5.394413013	1.179347352	8	1	1:02:57 AM	3	....
16nM FCCP	4.661777517	1.495065163	8	1	1:02:57 AM	4	....
31nM FCCP	3.771785072	0.813620983	8	1	1:02:57 AM	5	....
62.5nM FCCP	3.386226444	0.894948496	8	1	1:02:57 AM	6	....
125nM FCCP	3.459821493	0.946008657	8	1	1:02:57 AM	7	....
250nM FCCP	4.194249603	0.998264597	8	1	1:02:57 AM	8	....
500nM FCCP	4.465433857	0.930436992	8	1	1:02:57 AM	9	....
1uM FCCP	4.297003142	0.679956108	8	1	1:02:57 AM	10	....
2uM FCCP	2.629029377	0.406470248	8	1	1:02:57 AM	11	....
Temp	0.663916079	0.508204136	4	1	1:02:57 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	3.072888766	0.966426423	12	2	1:10:43 AM	1	....
0 FCCP	5.456960658	0.865533515	8	2	1:10:43 AM	2	....
8nM FCCP	5.937963049	1.064824629	8	2	1:10:43 AM	3	....
16nM FCCP	5.165275738	1.707228078	8	2	1:10:43 AM	4	....
31nM FCCP	3.975652189	0.755066482	8	2	1:10:43 AM	5	....
62.5nM FCCP	3.715725189	0.765356564	8	2	1:10:43 AM	6	....
125nM FCCP	3.068923296	0.833478551	8	2	1:10:43 AM	7	....
250nM FCCP	4.684187943	1.212519205	8	2	1:10:43 AM	8	....
500nM FCCP	5.13755418	0.774753928	8	2	1:10:43 AM	9	....
1uM FCCP	5.120598034	0.863832232	8	2	1:10:43 AM	10	....
2uM FCCP	2.918215875	0.36356516	8	2	1:10:43 AM	11	....
Temp	0.750567745	0.491926655	4	2	1:10:43 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	3.35985346	1.126308349	12	3	1:18:30 AM	1	....
0 FCCP	5.6621168	0.937088531	8	3	1:18:30 AM	2	....
8nM FCCP	6.116029489	1.044079078	8	3	1:18:30 AM	3	....
16nM FCCP	5.335840484	1.982643833	8	3	1:18:30 AM	4	....
31nM FCCP	4.101658899	0.725165515	8	3	1:18:30 AM	5	....
62.5nM FCCP	3.676368036	0.742766266	8	3	1:18:30 AM	6	....
125nM FCCP	3.889547781	0.777811548	8	3	1:18:30 AM	7	....
250nM FCCP	4.816713221	1.164715176	8	3	1:18:30 AM	8	....
500nM FCCP	5.303972885	0.813178084	8	3	1:18:30 AM	9	....
1uM FCCP	5.270655407	0.852636495	8	3	1:18:30 AM	10	....
2uM FCCP	3.093875141	0.417635471	8	3	1:18:30 AM	11	....
Temp	0.806750507	0.585196252	4	3	1:18:30 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	3.551360875	1.068697363	12	4	1:26:17 AM	1	....
0 FCCP	5.772507165	0.778541861	8	4	1:26:17 AM	2	....
8nM FCCP	6.207153683	1.01841422	8	4	1:26:17 AM	3	....
16nM FCCP	5.329530333	1.873000154	8	4	1:26:17 AM	4	....
31nM FCCP	4.276510367	0.752112364	8	4	1:26:17 AM	5	....
62.5nM FCCP	3.833844649	0.709988139	8	4	1:26:17 AM	6	....
125nM FCCP	3.92800095	0.842462959	8	4	1:26:17 AM	7	....
250nM FCCP	5.072166261	1.238704372	8	4	1:26:17 AM	8	....
500nM FCCP	5.347622513	0.816224066	8	4	1:26:17 AM	9	....
1uM FCCP	5.243007867	0.809571603	8	4	1:26:17 AM	10	....
2uM FCCP	3.127011186	0.414719673	8	4	1:26:17 AM	11	....
Temp	0.985022798	0.439464897	4	4	1:26:17 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	3.039141796	1.061400999	12	5	1:34:17 AM	1	....
0 FCCP	6.008435091	1.104001149	8	5	1:34:17 AM	2	....
8nM FCCP	7.468549021	1.462585583	8	5	1:34:17 AM	3	....
16nM FCCP	7.8047575	2.868970478	8	5	1:34:17 AM	4	....
31nM FCCP	6.263174787	1.439587492	8	5	1:34:17 AM	5	....
62.5nM FCCP	7.567691467	2.066513613	8	5	1:34:17 AM	6	....
125nM FCCP	11.83836719	3.535127885	8	5	1:34:17 AM	7	....
250nM FCCP	18.81590222	6.466710852	8	5	1:34:17 AM	8	....
500nM FCCP	19.66688676	4.763833679	8	5	1:34:17 AM	9	....
1uM FCCP	17.0773713	3.182161186	8	5	1:34:17 AM	10	....
2uM FCCP	9.367436942	1.721847142	8	5	1:34:17 AM	11	....
Temp	0.74353008	0.425863289	4	5	1:34:17 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	2.940936253	0.951598884	12	6	1:42:04 AM	1	....
0 FCCP	5.850930305	1.098666147	8	6	1:42:04 AM	2	....
8nM FCCP	7.199562247	1.413163447	8	6	1:42:04 AM	3	....
16nM FCCP	7.795579488	2.894025076	8	6	1:42:04 AM	4	....
31nM FCCP	6.074332657	1.388995884	8	6	1:42:04 AM	5	....
62.5nM FCCP	7.528724271	2.031551061	8	6	1:42:04 AM	6	....
125nM FCCP	12.4208986	3.866915959	8	6	1:42:04 AM	7	....
250nM FCCP	18.32085202	6.356697955	8	6	1:42:04 AM	8	....
500nM FCCP	19.48074657	5.04150187	8	6	1:42:04 AM	9	....
1uM FCCP	17.45369219	3.426356223	8	6	1:42:04 AM	10	....
2uM FCCP	9.683978053	1.953578969	8	6	1:42:04 AM	11	....
Temp	0.76937304	0.429599445	4	6	1:42:04 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	2.8226689	0.868813402	12	7	1:49:52 AM	1	....
0 FCCP	5.679098035	0.992048743	8	7	1:49:52 AM	2	....
8nM FCCP	7.010480209	1.379816293	8	7	1:49:52 AM	3	....
16nM FCCP	7.556147024	2.809179702	8	7	1:49:52 AM	4	....
31nM FCCP	6.065989723	1.363601162	8	7	1:49:52 AM	5	....
62.5nM FCCP	7.434536953	2.038258089	8	7	1:49:52 AM	6	....
125nM FCCP	12.49440976	4.02663925	8	7	1:49:52 AM	7	....
250nM FCCP	19.03388292	6.735843085	8	7	1:49:52 AM	8	....
500nM FCCP	19.37283034	4.410230927	8	7	1:49:52 AM	9	....
1uM FCCP	17.43275787	3.280225044	8	7	1:49:52 AM	10	....
2uM FCCP	9.594720977	1.942042217	8	7	1:49:52 AM	11	....
Temp	0.726516874	0.374874878	4	7	1:49:52 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;
med	2.924798875	0.993200454	12	8	1:57:38 AM	1	....
0 FCCP	5.60080826	0.946891473	8	8	1:57:38 AM	2	....
8nM FCCP	7.109039374	1.255207205	8	8	1:57:38 AM	3	....
16nM FCCP	7.507806603	2.785151823	8	8	1:57:38 AM	4	....
31nM FCCP	6.017033119	1.355351076	8	8	1:57:38 AM	5	....
62.5nM FCCP	7.317834078	1.970563683	8	8	1:57:38 AM	6	....
125nM FCCP	12.55278141	3.914292561	8	8	1:57:38 AM	7	....
250nM FCCP	19.71709997	6.782297234	8	8	1:57:38 AM	8	....
500nM FCCP	19.60261837	4.284996267	8	8	1:57:38 AM	9	....
1uM FCCP	17.34054486	3.366924765	8	8	1:57:38 AM	10	....
2uM FCCP	9.268420717	1.90204666	8	8	1:57:38 AM	11	....
Temp	0.783178479	0.518551692	4	8	1:57:38 AM	14	Back. Cor.;Back. Cor.;Back. Cor.;Back. Cor.;

Note that statistical information will be listed only for wells (and analytes) that are selected. This feature is useful for reviewing the statistics associated with the entire experiment and may be useful for identifying trends and troubleshooting the experiment. These values may also be copied to other graphing programs if desired.

AUC Analysis using Multiple t-Tests

A tool button exists in the data file viewer for AUC (Area Under the Curve) analysis. This is also located under the Special Operations Tab in the Excel Data Viewer. Similar to the Group Statistics tool, the AUC tool will add a worksheet to the file and perform its analysis based on your well groupings. In addition to calculating the area under the curve for the specified analyte, t-tests are performed to compare the groups and a table of P-values is generated.

AUC Analysis: Excel configuration

The AUC tool uses Excel's *Analysis ToolPak –VBA* add-in. Before you can use the tool, you must first enable the add-in. If this feature was not included in the original installation of Excel, you will be prompted to insert the Office installation CD.

To enable the add-in, go to the *Tools* menu and select *Add-Ins…*
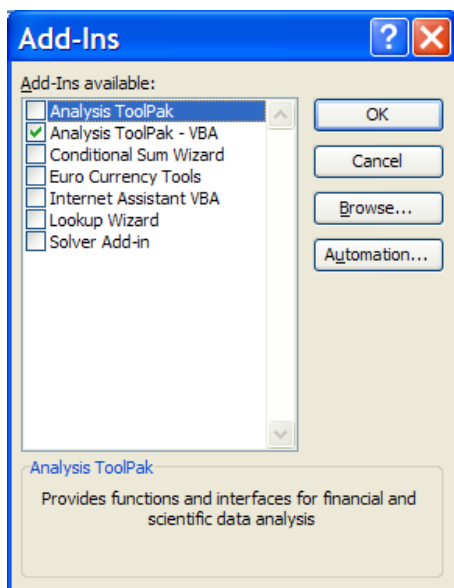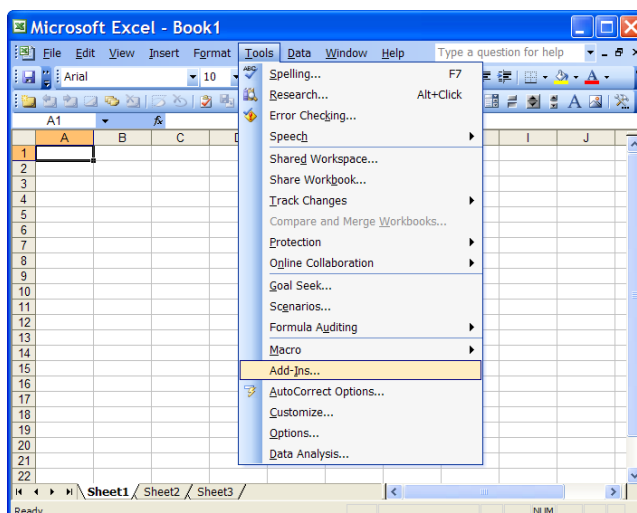
The *Add-Ins* dialog will appear. Select the checkbox for *Analysis ToolPak – VBA*. Click OK.

Using the Tool

To use the AUC tool, click on the *AUC Group T Analysis…* button below the graph in the excel data file.
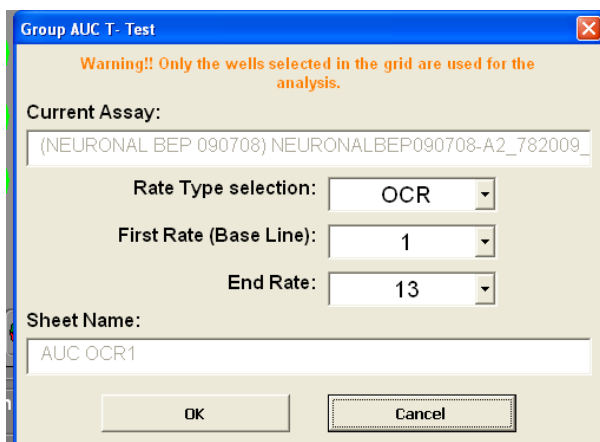
Select the analyte, starting rate, and end rate for the AUC calculations. Click OK after making your selections.

A new worksheet will be added containing the results. The dialog automatically provides a default name for the new worksheet. You can change the name only by modifying the name of the newly created sheet.
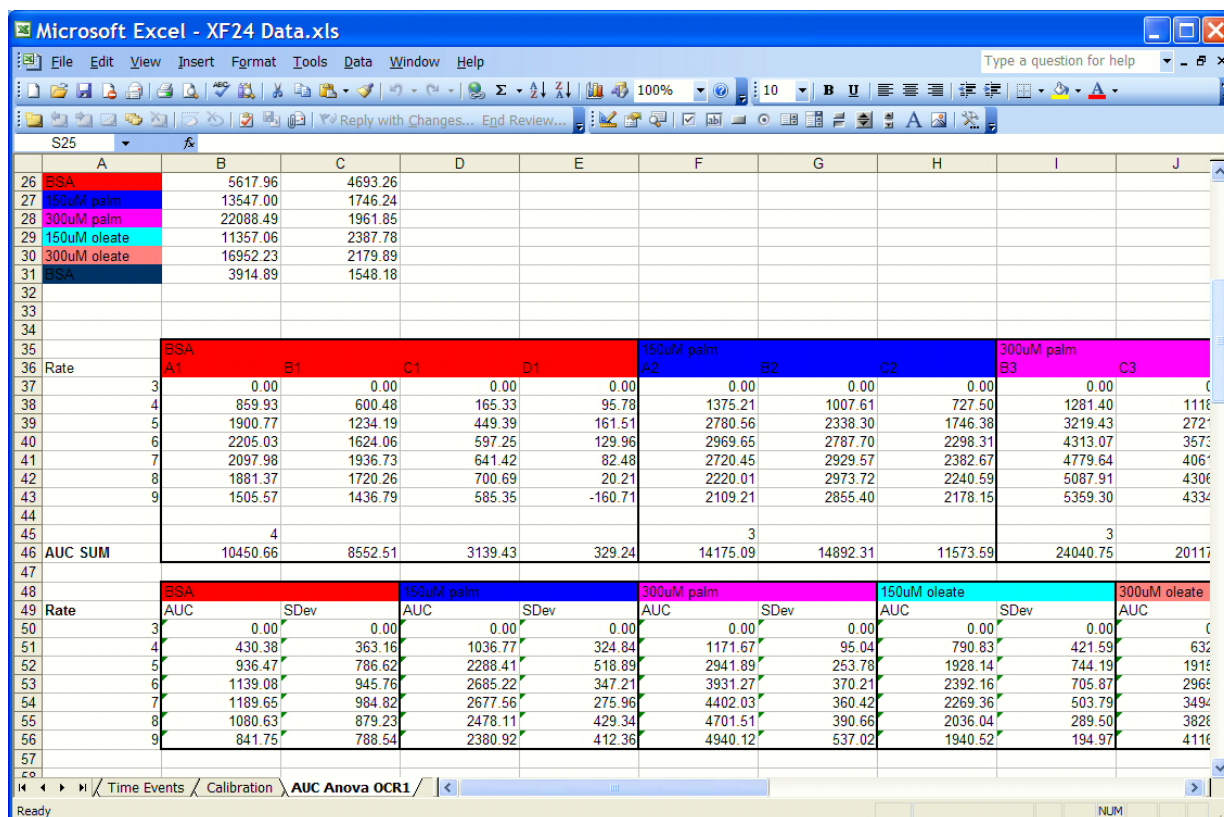

Analysis Results

The AUC worksheet contains several elements.

At the top left is a bar chart of the average AUC for each group.

To the right is a copy of the Data Viewer graph.
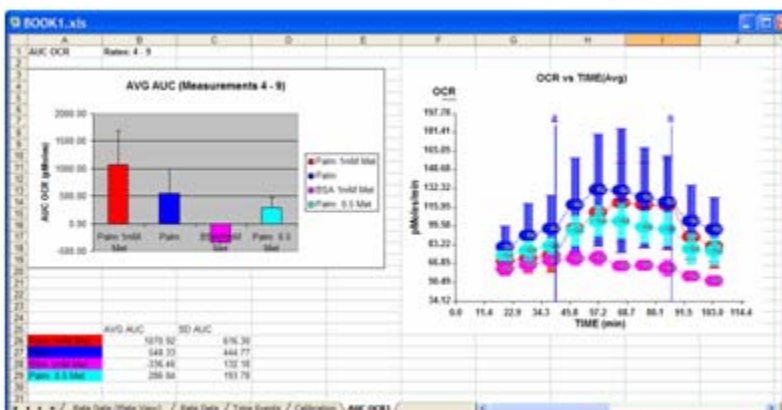
Below the chart are tables of the area information on an individual well and rate basis.

Each section is color coded by group color.

And below that is a table of P-values, followed by summaries of the t-test calculations for each combination of groups.
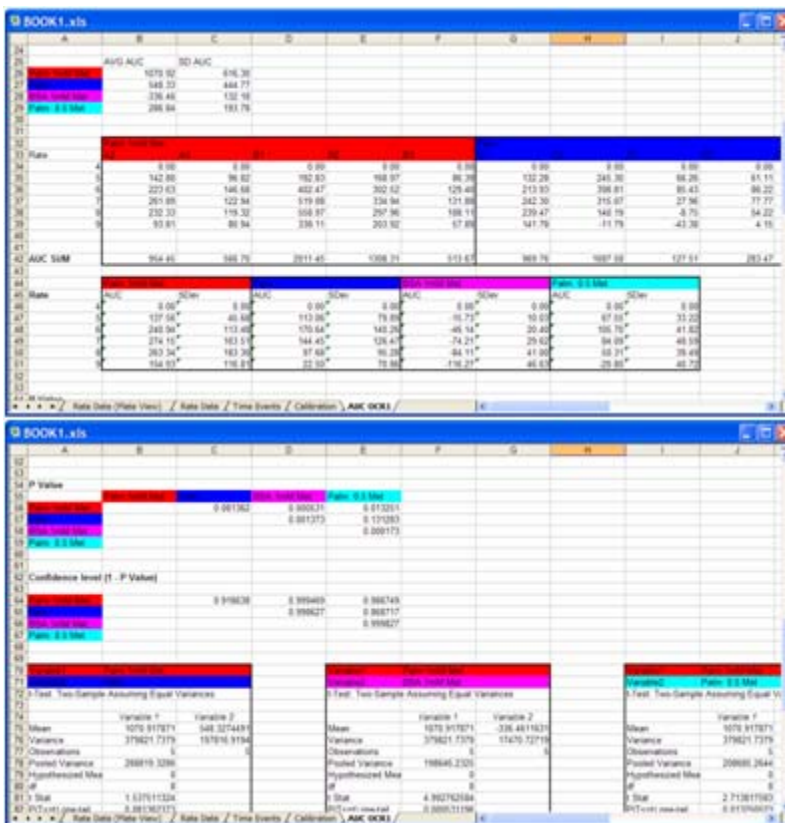
Calculations

To calculate the area under the curve, each rate is multiplied by its time interval to determine the area under that part of the curve. The area for the rate chosen as the baseline for the AUC calculation is then subtracted from the area of each subsequent rate. The results are then summed to provide the total baseline-subtracted area of the curve.

If working with the absolute rate data, the area will be in units of pmols for OCR and mpH for ECAR. However, if the Data Viewer is configured to display the data in percent baseline mode, then the AUC is calculated using the percent values and not the raw pmol/min or mpH/min rate data. In this case, the area would be in units of % and would represent a total percentage change from the baseline. In addition, if the normalization option is enabled and the data is normalized to cell count or some other factor, then the AUC is calculated using the normalized data.
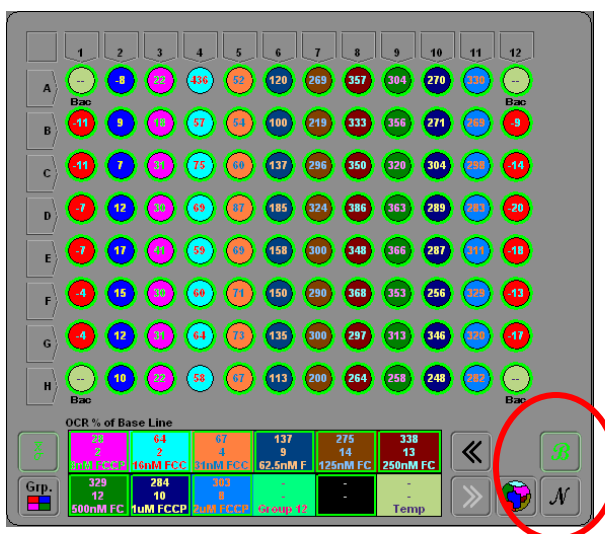
ANOVA AUC Analysis

Another AUC option is AUC ANOVA. Similar to the AUC button, the AUC ANOVA tool will add a new worksheet to the file. The area under the curve is calculated for each group and then the groups are compared using ANOVA, resulting in a table of P-values.

Why ANOVA?

ANOVA provides a more rigorous statistical method for comparing three or more means. The original AUC analysis tool uses multiple t-tests to compare each pair of groups. Using a single t-test to compare two groups is valid. But with more than two groups, misleading P values can result when performing multiple t-tests. The more groups you compare, the greater the chances of getting a significant P value by coincidence.

The new tool performs a one-way ANOVA, which compares the variation among the group means with the variation from the subjects within each group. The null hypothesis is that all populations have identical means and a significant P value indicates that one or more of the population means differ from the rest. To determine which groups are significantly different from the others, the Tukey method has been implemented for determining HSD (Honest Significant Different) to generate a table of P values comparing each pair of groups.

Using the Tool

1. Click on the *AUC Group Anova Analysis...* button.

2. In the dialog, select the analyte and starting and ending rates for the analysis. Note: You must also select which groups to include in the ANOVA analysis. It's important to exclude unrelated groups, such as the Temp Corr wells.

3. Highlight groups in the left column and click Add to move them to the right.

The alpha variable is the significance threshold for P. It defaults to the standard value of 0.05.

Also, one-way ANOVA assumes groups are categorized based on one factor. If there are multiple factors among the groups, such as treatment and cell type, or treatment and seeding number, it is not valid to include both types of groups in the same analysis.

4. Once the groups have been selected, click OK.
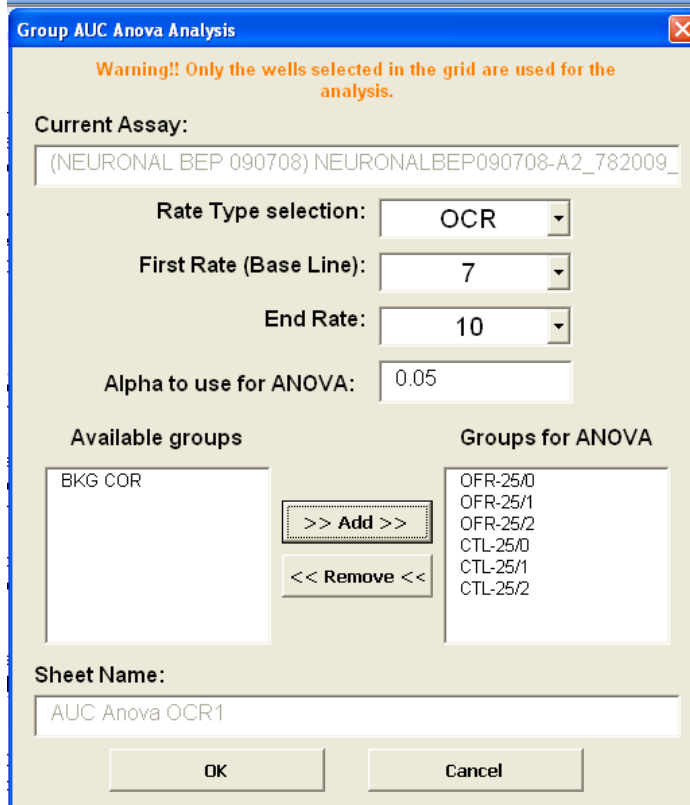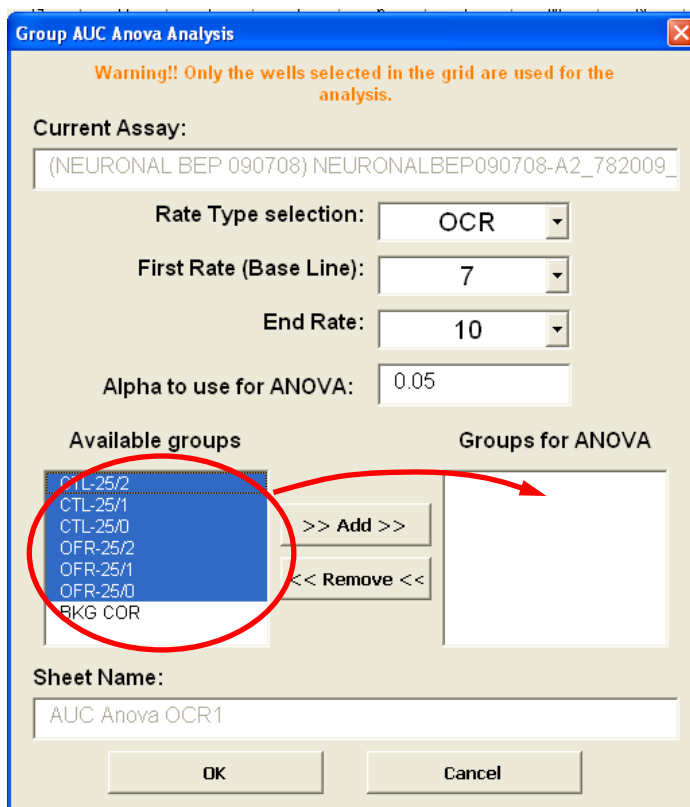
A new worksheet will be added To the workbook containing the results. A default name for the new worksheet is assigned. You can modify the name of the newly created sheet.
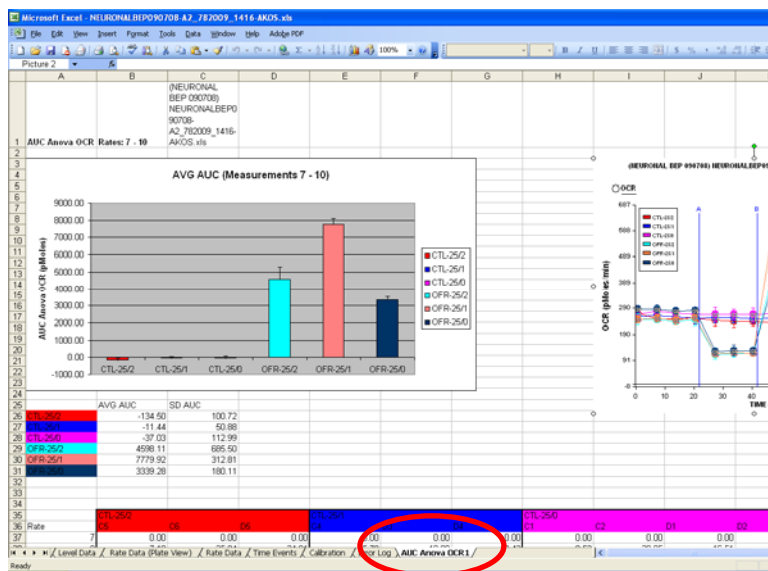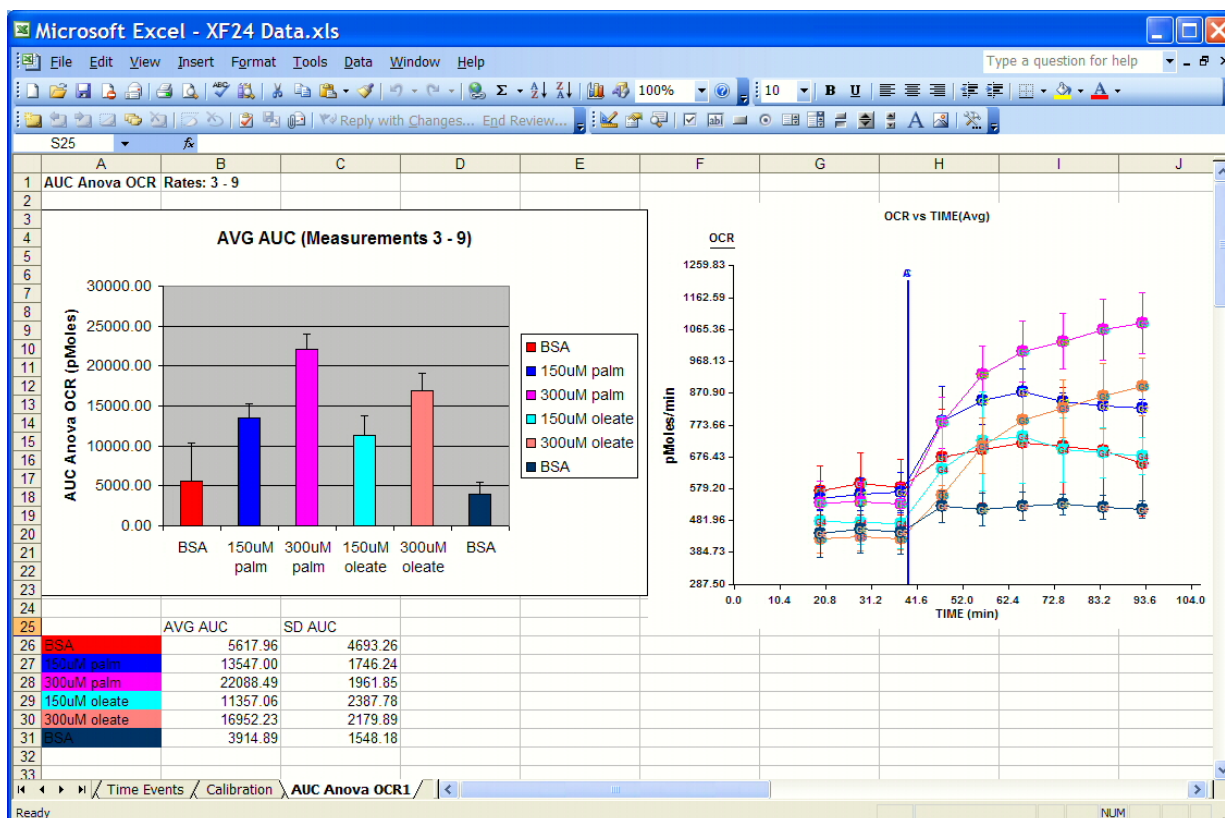
Analysis Results

The AUC worksheet contains several elements. At the top left is a bar chart of the average AUC for each group. The error bars indicate Standard Deviation or Standard Error, depending on your selection in the graph settings dialog.

To the right is a copy of the Data Viewer graph. Configure the graph before running the AUC. Also, make sure it is not scrolled off the edge of the Microsoft Excel application window. You can also go back to the Data Viewer Worksheet and manually copy the graph to paste in this worksheet.

Directly below the chart is the table of data displayed in the chart.

The area data is broken down per well and per rate, as well as the average per group and per rate. The areas are normalized to the starting rate, which is why the starting rate is zero.

This graphic demonstrates how the AUC is calculated. Assuming the third point is the starting rate, the yellow region, representing the baseline area, is subtracted from the total area. The remainder, the red region, is the AUC used in the analysis.
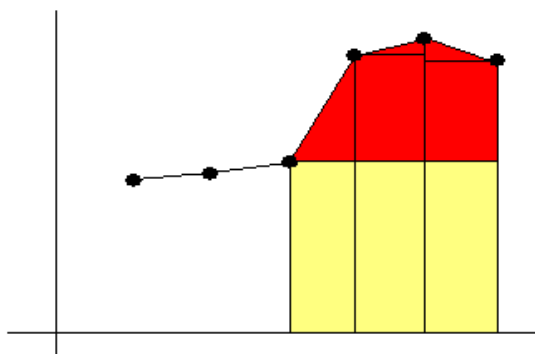
Note that if the β (baseline) function is enabled in the Data Viewer, the yellow region will have zero value, assuming the β function is using the third point as the baseline point.

The P value for the ANOVA test is presented in column A. If the P is significant, use the table of P values starting in column D to compare specific groups. These P values are generated using Tukey's HSD post test. Note that this table should be disregarded if the P value from the ANOVA test is not significant.

To the right of the table of P values is a table of Q values. These values are from the Studentized Range distribution. Tukey's HSD test is based on this distribution.

Microsoft Excel - XF24 Data.xls

	A	B	C	D	E	F	G
79	BSA	10450.66	8552.51	3139.43	329.24		
80	150uM palm	14475.09	14392.31	11573.59			
81	300uM palm	24040.75	20117.19	22107.53			
82	150uM oleate	13528.05	11743.50	8799.63			
83	300uM oleate	15477.12	15923.44	19456.13			
84	BSA	2308.69	4496.24	5796.19	3058.44		
85	Anova: Single Factor						
86							
87	SUMMARY						
88	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
89	BSA	4	22471.83368	5617.95842	22026734.91		
90	150uM palm	3	40640.98514	13546.99505	3049351.723		
91	300uM palm	3	66265.46801	22088.48934	3848842.772		
92	150uM oleate	3	34071.17491	11357.0583	5701508.495		
93	300uM oleate	3	50856.6922	16952.23073	4751928.525		
94	BSA	4	15659.56341	3914.890853	2396875.78		
95							
96							
97	ANOVA						
98	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
99	Between Groups	806689759.3	5	161337951.9	20.91919663	4.83018E-06	2.958248913
100	Within Groups	107974095.1	14	7712435.364			
101							
102	Total	914663854.4	19				
103							
104	Notes on AUC Calculations						
105							
106	The area under the curve is calculated as follows.						
107	For each rate, the group's average is multiplied						
108	by the time interval for that rate. This represents						
109	the area under that part of the curve. The area for						

At the bottom is the output from the Microsoft Excel ANOVA function. Displayed are the P value and F value, as well as the critical F value required to meet the specified alpha.

The F value is the ratio of the mean squares of the two sources of variation. If the null hypothesis is true, F is likely to be close to 1. Larger F values correspond to smaller P values.

Again, note that a significant P from the ANOVA test only indicates that one or more groups may be different from the rest. It does not tell you which group or groups are different. We use Tukey's HSD post-test to determine which groups are significantly different from the others.

Appendix IV - Advanced Graphing Options and Features

Selecting Group for the X-axis

The x-axis drop-down box has a **GROUP** option. As shown in the right graph below, when selected, the group curve shows the difference in rates between the well groupings. The x-axis tick marks are labeled with each group name as specified in your assay configuration. The groups can represent injected compounds, treatment dosage, cell type, or any other factor.
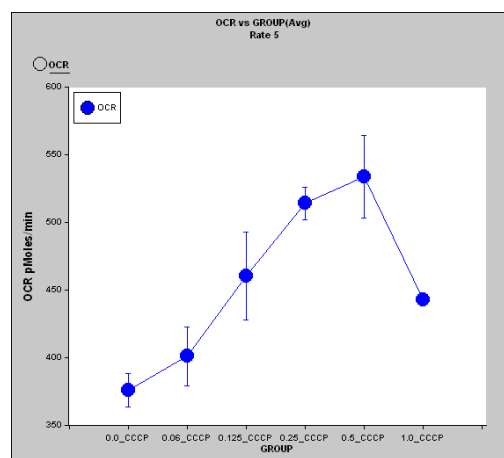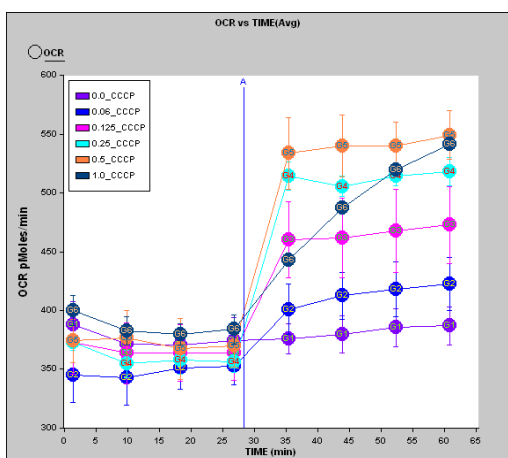
Changing X axis from Time to Group. Example is CCCP titration (µM) with HepG2 cells

Note that the group curve is a snapshot of a *single* measurement of the assay. Use the scroll bar in the lower left of the Results screen to scroll through the rates. The rate number is shown above the graph. Also, this feature is only available when **Well Group Mode** is enabled.

The examples in figure above displayed a single analyte. Use the Y2 axis to display a second analyte on the graph as shown in the figures below, comparing rates 4 and 5.
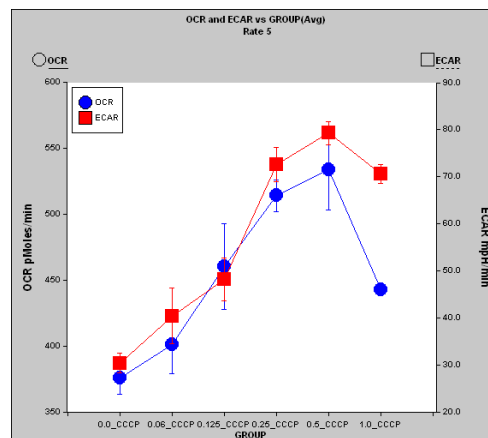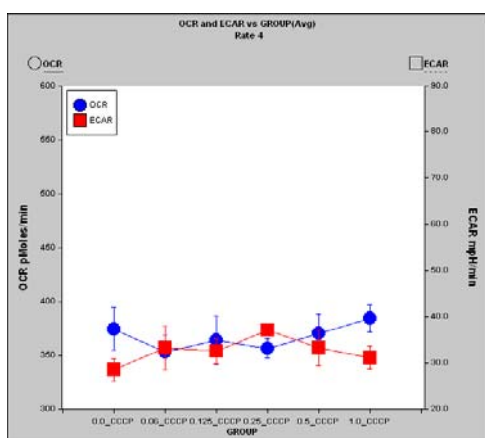
Group Display of OCR & ECAR at Rate 4 (before injection) and Rate 5 (after injection)

A bar graph option is also available as shown in the figure to the right. To enable,

1. Click on the [icon] button to open the graph settings dialog.

2. Click on the **Advanced Graph Options…** button.

3. Enable the option to: **Use Bar Graph when "GROUP" is selected on X axis**.
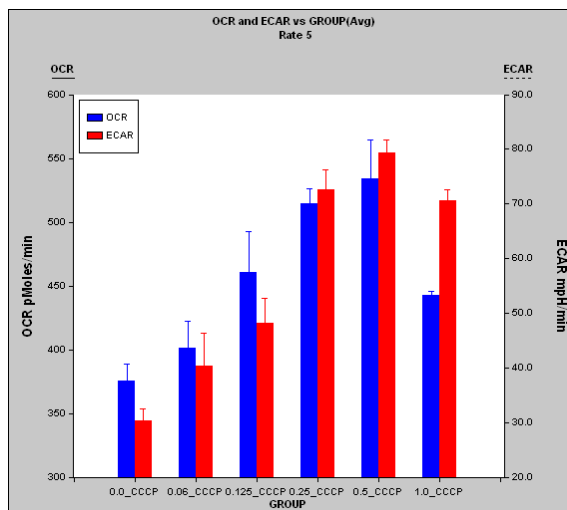
Note: when in non-bar graph mode, the groups are displayed with connecting line making this a convenient way to look at trends. **However, the X axis is scale is fixed by group only and does NOT represent a true linear scale** (i.e. it does not correspond to the listed concentration, cell seeding, or other increasing/decreasing values.)

More Features in the Graph Setting Dialogue

Aligning the Y1 and Y2 origins for Base-lined Data

When displaying the base-lined data for two analytes, it may be useful to scale the Y1 and Y2 axes such that the origins are aligned. The graphs in the figure below show the results of pressing the [icon] (auto-scale) button before and after enabling this option. To enable it,

1. Click on the [icon] button to open the graph settings dialog.

2. Enable the option to **Match Y2 zero to Y1** (This feature is under the Advanced Graph Options in the XF Reader Software).

3. Click **Close.**

4. Click on the [icon] button

100900-400_MANUAL XF96 W/ PREP STATION_RA
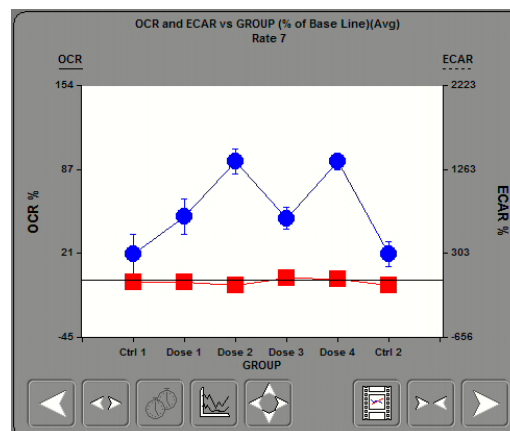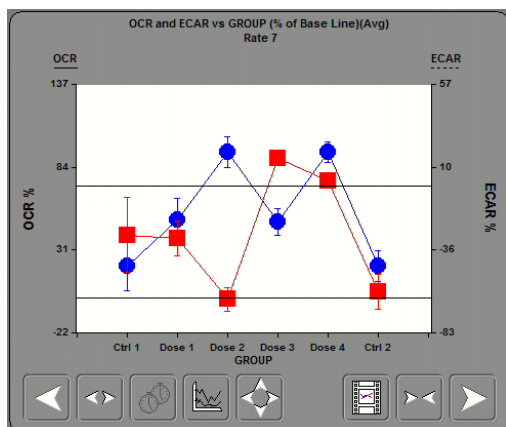
On the left, Autoscaling with **Match Y2 zero to Y1** disabled.
On the right, Autoscaling with the option enabled.

Re-sizing Graphing Elements

The size of the font, thickness of lines, and size of data points can be adjusted individually. To modify these settings,

1. Click on the [icon] button to open the graph settings dialog and then on **Advanced Graph Options**….

2. For **Font Size for Graph**, **Line Width**, and **Point Size**, choose either **Small**, **Medium**, or **Large**.

Displaying the Assay Date

The date on which the assay was performed can be displayed above the graph. To display it as shown in the figure to the right,

1. Click on the [icon] button to open the graph settings dialog and then on **Advanced Graph Options**….

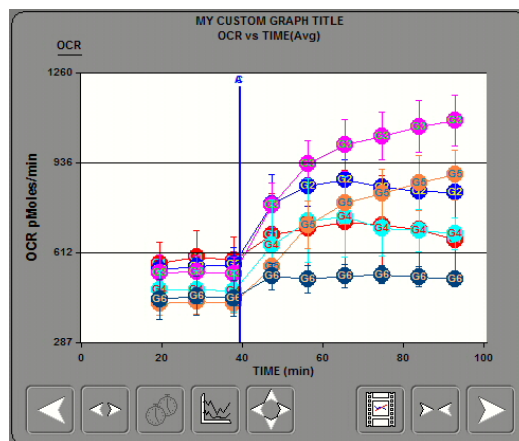2. Enable the option to **Display Date in Title.**
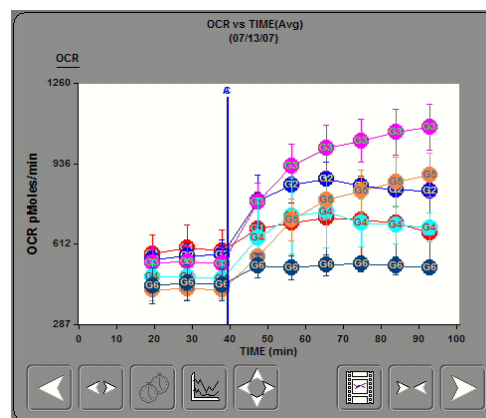
Graph with Assay Date displayed.

![Seahorse Bioscience logo]

Displaying a Custom Graph Title

A customized title can be added to the graph as shown in the figure to the right. To add it,

1. Click on the [graph button] button to open the graph settings dialog and then on **Advanced Graph Options**….

2. Edit the **Graph Title** field.

Graph with a customized Title

Displaying Grid Lines

Grid lines can be displayed on the graphs in several different configurations. To display them,

1. Click on the [graph button] button to open the graph settings dialog and then on **Advanced Graph Options**….

2. Configure the **Grid** settings. Options include single axis lines or lines in both axes. The lines can be either solid or dotted.

The figures below show four examples with grid line options:
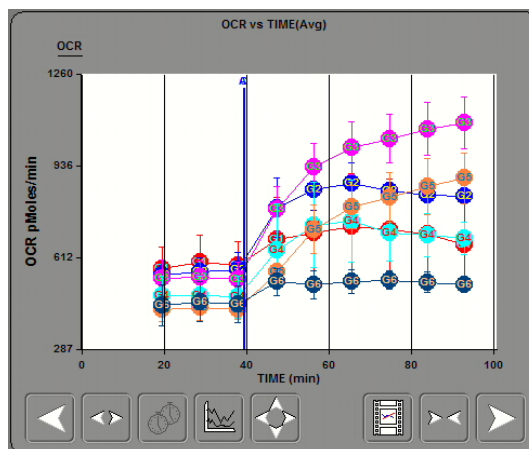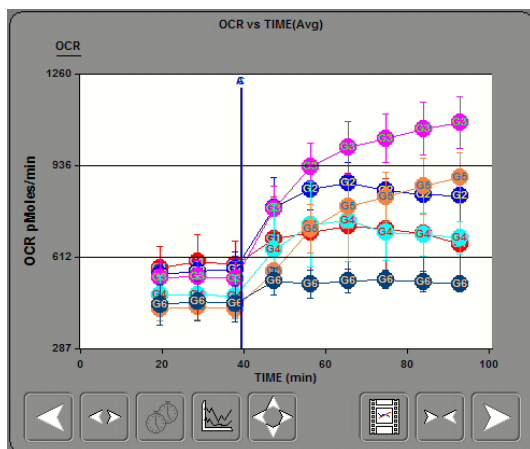
Graph Examples showing Grid Lines in only one axis
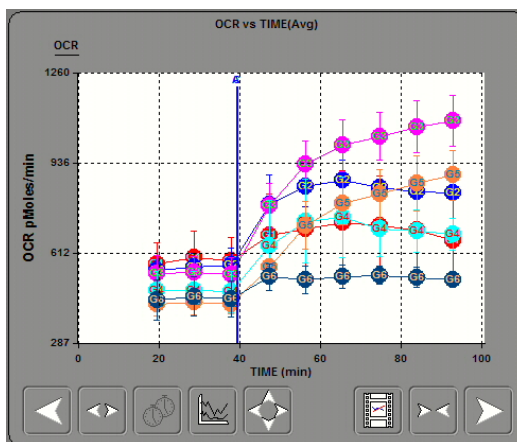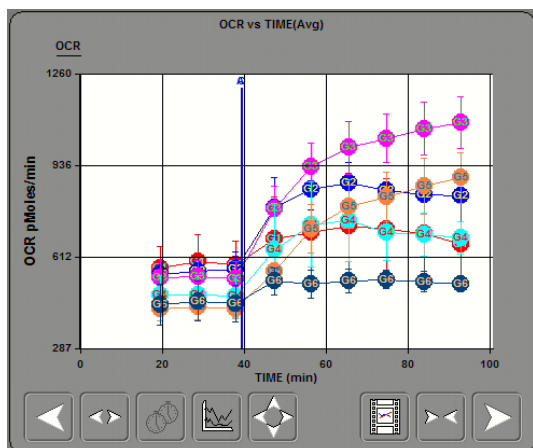
Graph Examples showing Solid and Dotted Grid Lines

Displaying a Graph Legend

A legend can be displayed on the graph as shown in figure below. To display the legend,

1. Click on the [graph icon] button to open the graph settings dialog and then on **Advanced Graph Options**….

2. Enable the **Display Legend when in Well Group Mode** options. Also select the default location for the legend.

3. Once displayed, adjust the position of the legend by clicking and dragging.
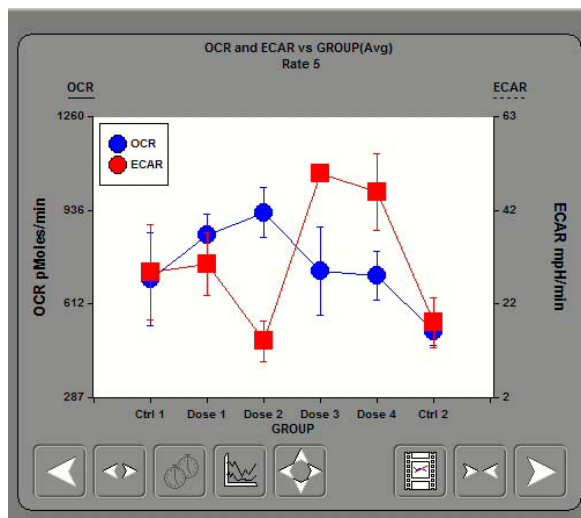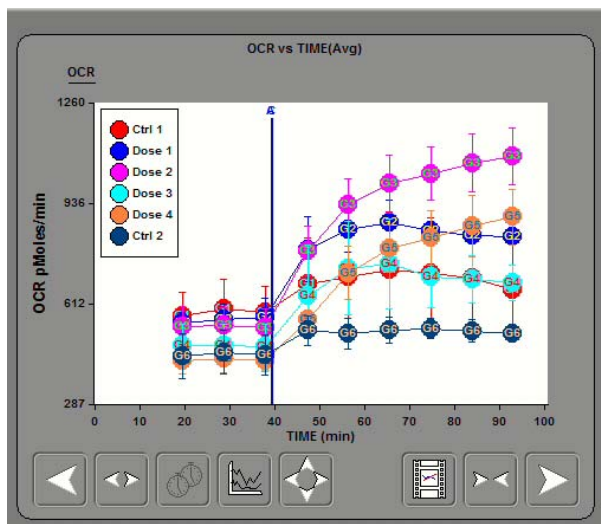
Graph Legend Examples

Displaying Injection Markers and Custom Label Options for Injection Markers

The vertical injection markers can be hidden on the graphs as shown on the right in the figure below. To hide them,

1. Click on the [graph icon] button to open the graph settings dialog and then on **Advanced Graph Options**….

2. Disable the **Display Vertical Markers** option.
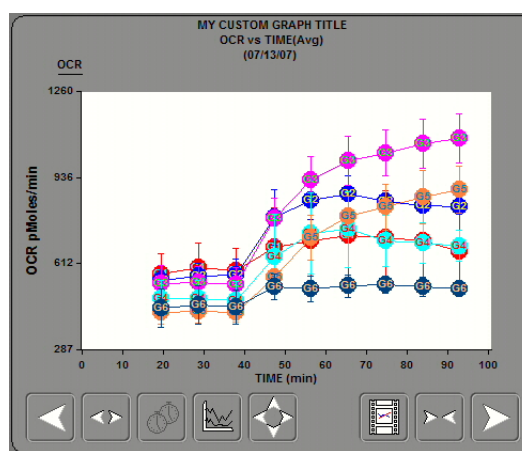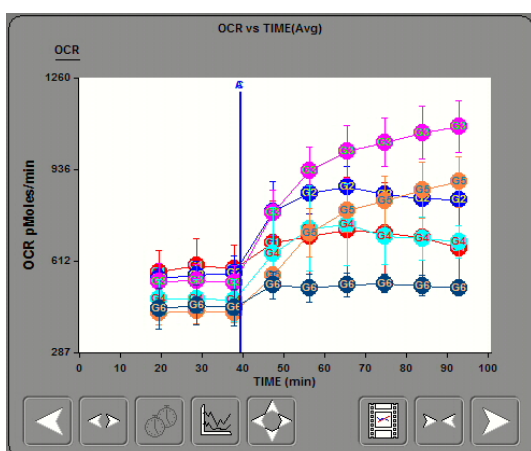
Graph Examples with the Injection Markers displayed and hidden.

The injection markers in the graphs are labeled with the port letters corresponding to the source of the injection. The port letters can now be replaced with customized labels. The figure below shows a graph with the standard port labels and with custom labels.
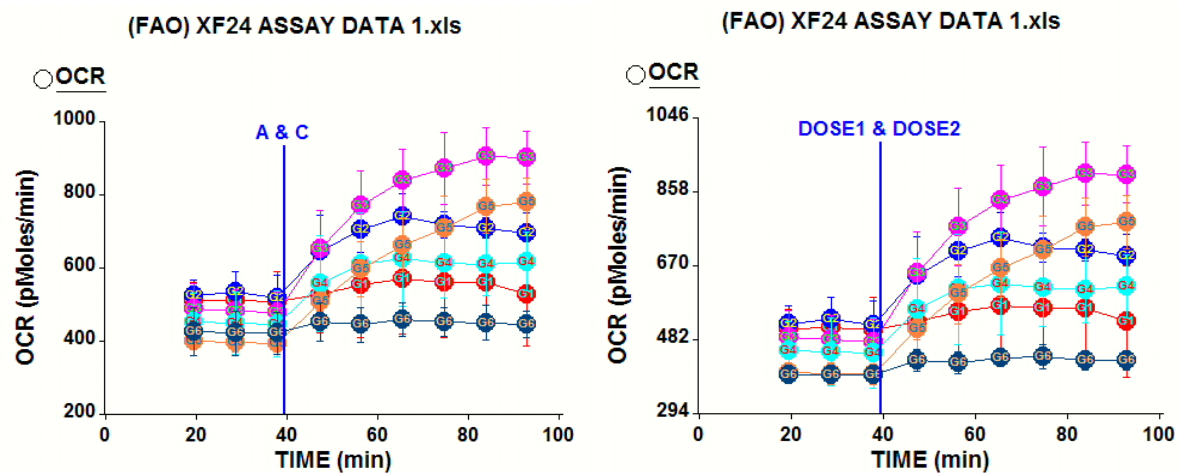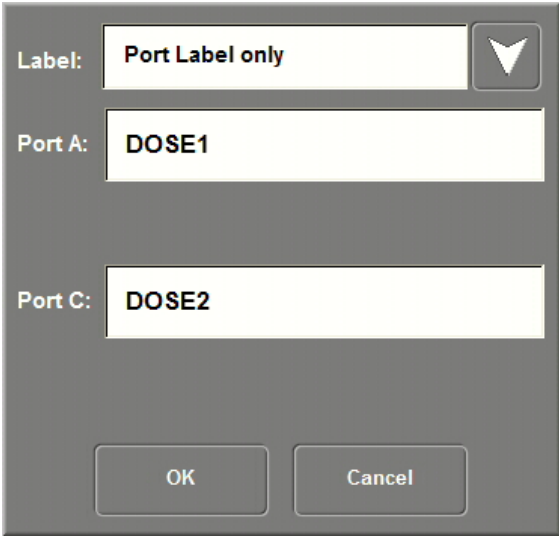
Standard and Custom Injection Marker Labels

Modifying the Labels

To customize the labels,

1. Click on the [icon] button to open the graph settings dialog and then on **Advanced Graph Options**….

2. Click on the **Injection Markers Options** button to show the dialog seen in the figure to the right

3. Select the **Label** option. Port letters or labels can be displayed, or both.

4. Edit the fields with the custom labels. Fields will only be displayed for those ports used in the assay.

Edit Injection Marker Labels

Options for Time Axis Origin

The origin of the time axis defaults to the first recorded tick of data. Options are now available to set the origin to some other point in the assay, such as a particular protocol command or measurement.

To change the origin,

1. Click on the [icon] button to open the graph settings dialog.

2. Click on the **Time Zero Options** button.

3. As shown in the figure below, an injection point or protocol command can be selected as the new time origin. Click **OK** after making a selection.

4. Close the graph settings dialog

Time Zero Options

In the figure below, two graphs are shown with different origins for the time axis. The left graph shows the default time origin. In the right graph, the time origin has been shifted to the beginning of the first measurement.
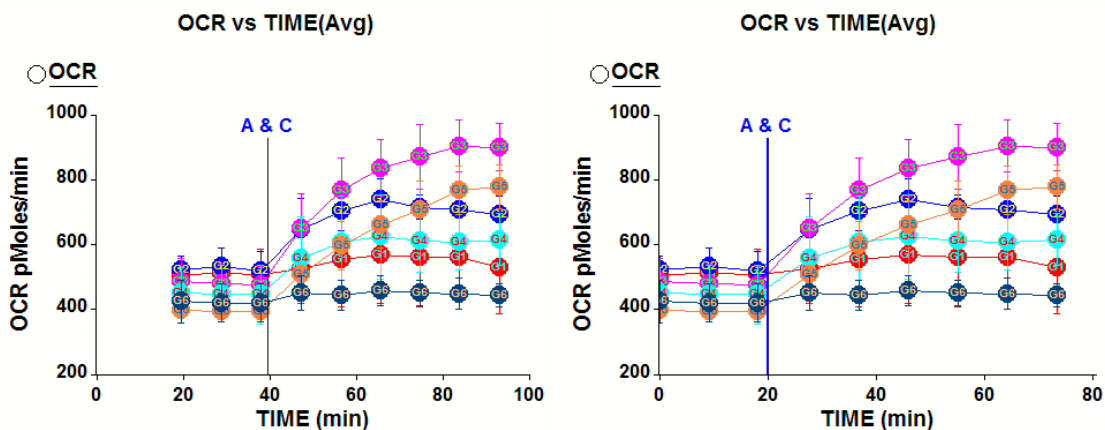
Graphs with two different time origins.

Adjusting Axes Interactively in the Data Viewer Graph

When analyzing data, it's often necessary to manually adjust the min and max values of the graph axes to focus in on a subset of data. In the Excel-based Data Viewer, this can now be done interactively.

1. As shown in the figure to the right, click and drag to create a square region in the graph.
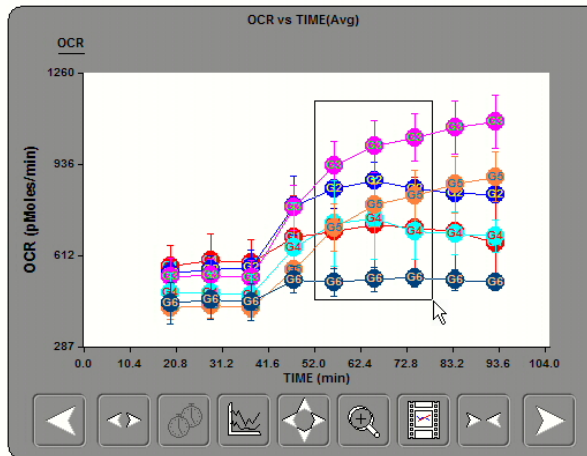
Defining a region for Axis Scaling

2. As shown in the next figure, the graph will magnify the defined region by automatically adjusting the **Min** and **Max** values of the X, Y1, and Y2 (if used) axes.

3. To reset the graph axes, click on the [button icon] button.

Note: This feature is only supported in the Data Viewer and is not available in the XFReader96 application.
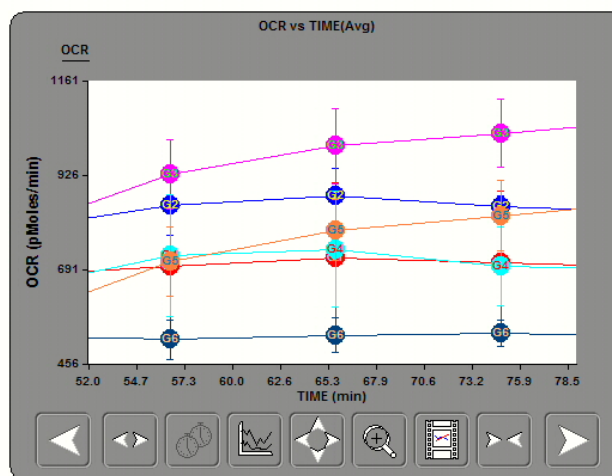
Graph Display after Scaling

Note that one may also explicitly define the X and Y axes with respect to minimal and maximal values, number of divisions and sub-divisions, number of decimal places, units and error bar options. These may be found in the Graph options dialogue (see figure to right).
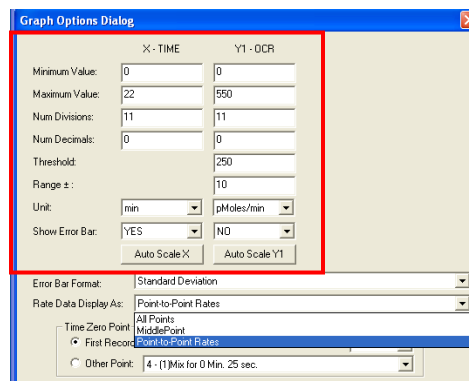
Graph Options Dialogue:
Point to Point Rates mode

Adding Custom Text Labels to the Graph

There is an additional new option on the right-click popup menu for the graph object that allows for the addition of custom text labels to the graph's display.

To create or edit graph labels,

1. Right-click on the graph and select **Edit Graph Labels** from the popup menu.

2. The **Labels** dialog will appear, as shown in the figure to the right. Click on the **Add Label** button to add a new label.
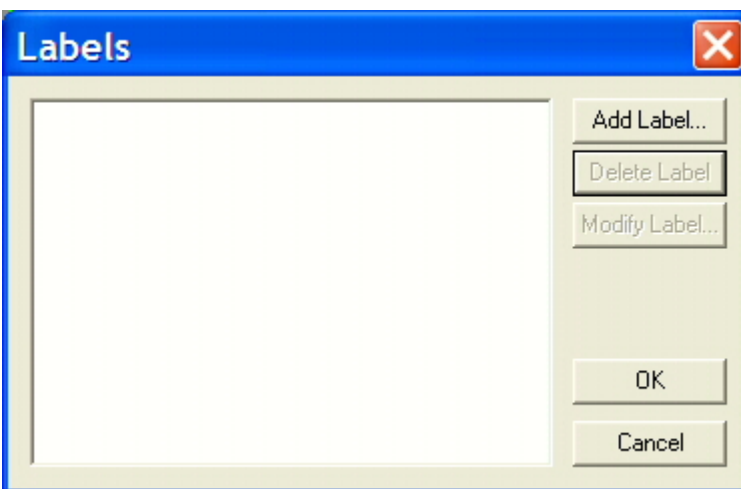
Labels dialog for the Graph

3. In the **Label Info** dialog, enter in the text for the label. Also, select a color and size for the text font. Click **OK** when finished. After all labels have been added, click **OK** on the **Labels** dialog
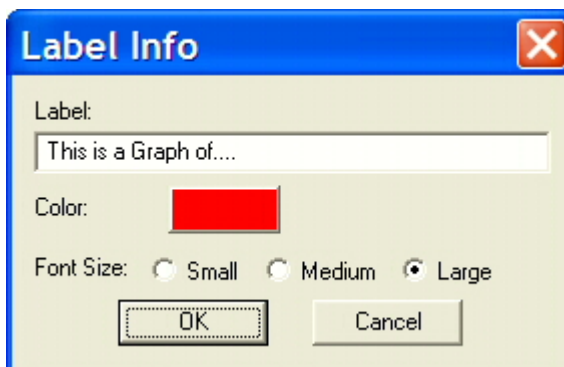
Label Info dialog

4. The labels are initially displayed in the upper left corner of the graph. Click and drag labels to the desired locations.

Note: When working with the data file in Excel, the labels are saved with the data file. But labels created in the XFReader96 software will not be saved to the data file.
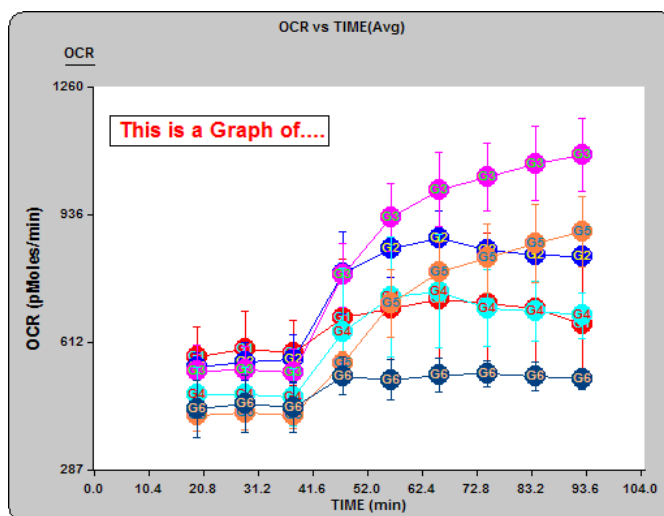
Label displayed on the Graph

Data Threshold Option

Overview

When viewing data in non-well group mode, a new thresholding option is now available.

A target value and range are specified and all data points are sorted based on this threshold. Data outside the threshold are colored blue and those points inside the threshold are colored red.

The figure to the right shows an example graph with a threshold applied.
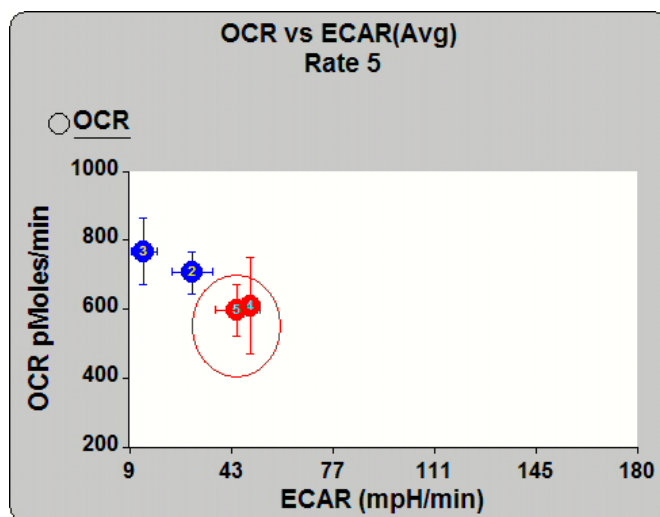
Threshold applied to scatter plot of OCR vs. ECAR.

![Seahorse Bioscience logo]

Setting the Threshold

To set the threshold,

1. Click on the [graph icon] button.

2. For each axis of interest, set the **Threshold** and **Threshold** range fields as shown in the figure to the right.

3. Close the graph settings dialog.

Note: a threshold cannot be defined for the x-axis when it is set to **TIME**.

	X	Y1
Minimum Value:	0	-20
Maximum Value:	90	220
Num Divisions:	9	12
Num Decimals:	0	0
Threshold:	40	100
Threshold range ±:	5	20
Show Error Bar:	Yes	Yes

Auto Scale X   Auto Scale Y1

Setting the Threshold

Displaying the Threshold

To view the thresholded data in the graph and well grid,

1. Click on the [graph icon] button.

2. Turn on the **Display threshold** option as shown to the right.

3. Close the graph settings dialog.

4. Click on the **Well Group Mode** button so it is not outlined in green.

Note: Well group mode is not compatible with the threshold feature.

Error Bar Format: **Standard Deviation**

BaseLine Options...   Time Zero Options...

Display threshold.   Threshold Options...

Link Y1 display variable.   Do Background Correction.

Advanced Graph Options...   Close

Enabling Display threshold.

Viewing the Threshold

Thresholded well grid.

When the threshold is displayed, the well grid will become colored blue and red. The red wells fall within the threshold and the blue wells fall outside the threshold. Scroll through the rates to see how the colors change over the course of the assay.

Threshold graphing an analyte vs. another analyte

When graphing an analyte versus another analyte, the threshold region is indicated by an ellipse. The ellipse is only an approximation. The actual threshold region is the rectangle that bounds the ellipse.

Threshold graphing an analyte vs. time

When graphing an analyte versus time, the threshold region is indicated by a solid red line bounded by two dotted red lines indicating the range.
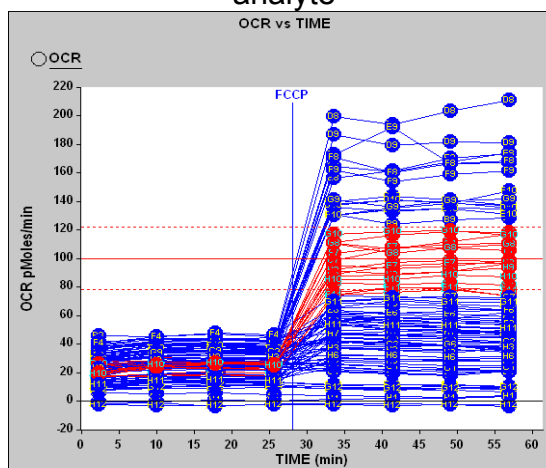
Working with Multiple Data Sets

The ability to view data from multiple data files in the same graph is a key new feature of the Data Viewer. The **Data Files** tab, as shown below, shows the contents of the Data Viewer. At minimum, the original assay saved to this file will be listed. To the right, an **Add** button is available to browse for and load the contents of other data files. The **Delete** button will remove data from the file. The **Clear List** button will remove all added data. The original data cannot be deleted or cleared.
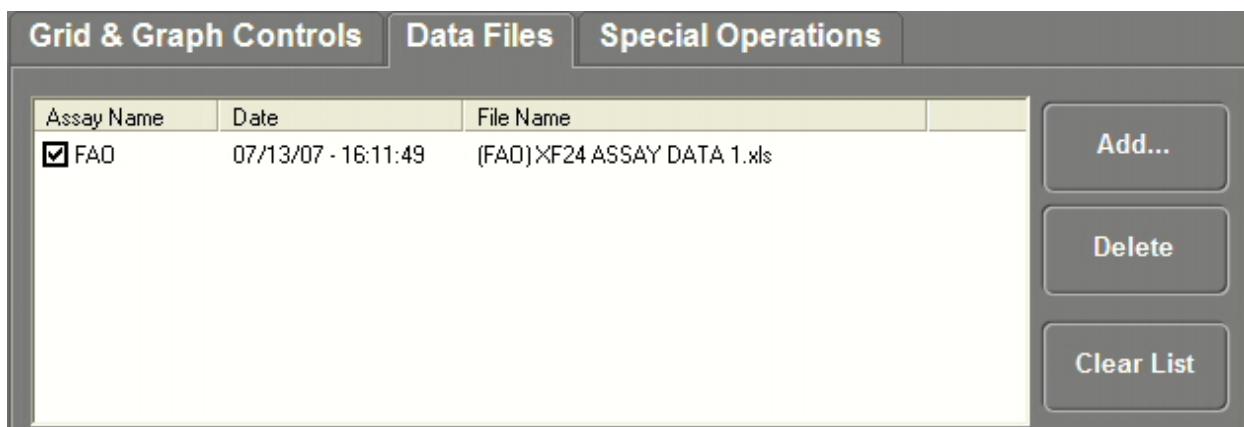
The Data Files tab

To add data from another file,

1. Click on the **Add** button.

2. Browse for and select either an XLS or XFD data file. Configure the browsing window to display only one file type by setting the **Files of type** option.

3. Once a file has been selected, click **Open**.
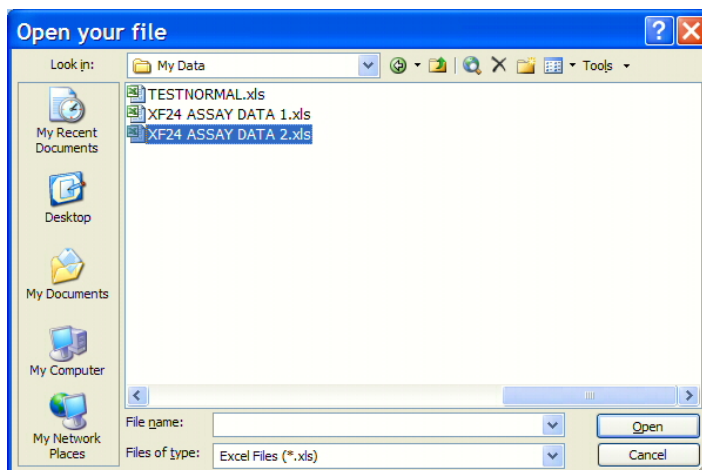
Open dialog

The list in the **Data Files** tab will update to indicate the contents from the selected file have been added.
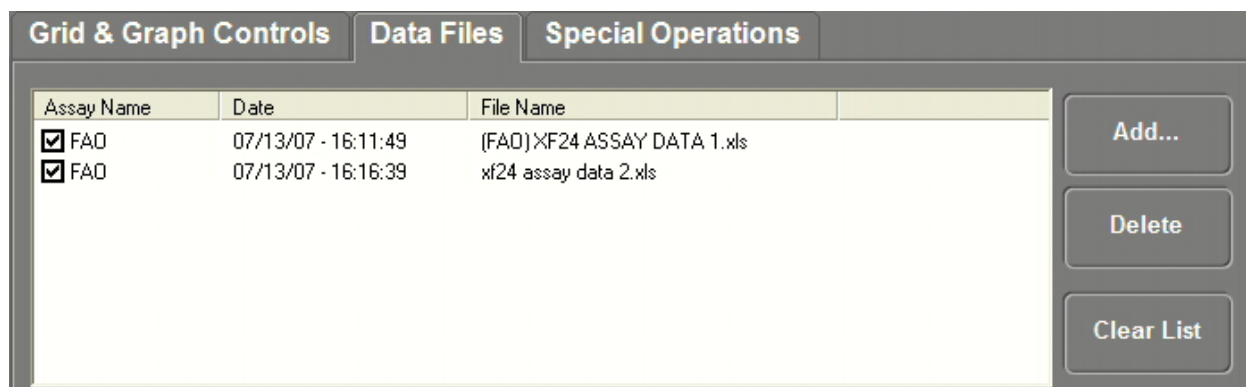
The Data Files tab showing two sets of data

Viewing Multiple Data Sets

After loading a second set, the graph will display the data from both files. In the figure below, the left graph shows the original data and the right graph includes the data added from another file.
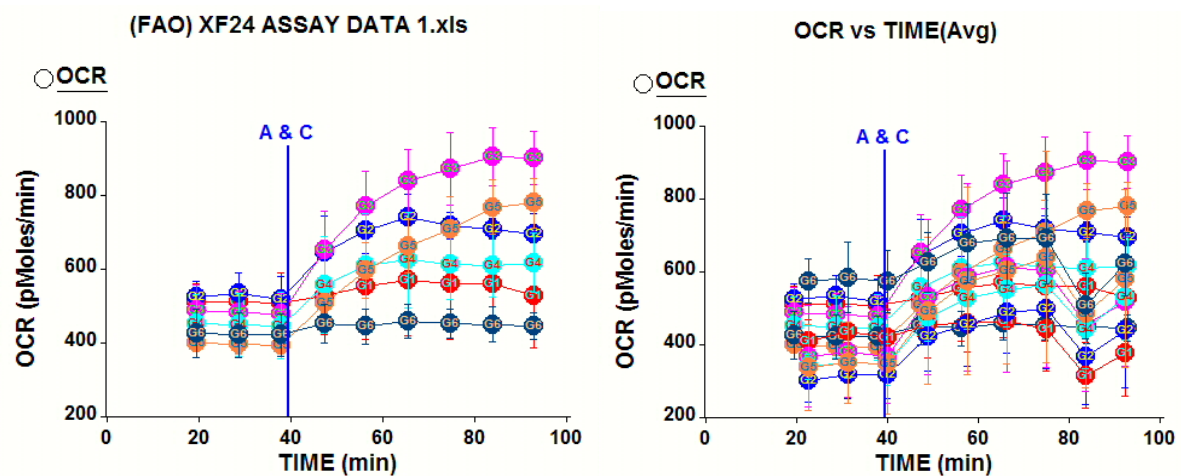
Graphs showing data from one file and from two files

To toggle a set of graphs one and off, use the checkboxes shown to the left of each file listing in the **Data Files** tab. If only one is selected, then the graph title will default to the name of the source file for that data. If multiple listings are selected, then the graph title will default to a generic indication of the graph content.

The well grid can only show data from one data set at a time. To switch between data sets, click and highlight the desired listing on the **Data Files** tab. Continue to use the controls on the **Grid & Graph Controls** tab to select which analyte to display in the grid.

The figure below shows the **Data Files** tab with the checkboxes and highlighted listings in use. In this example, the graph would show the data from the second data set and the well grid would show values from the first data set.
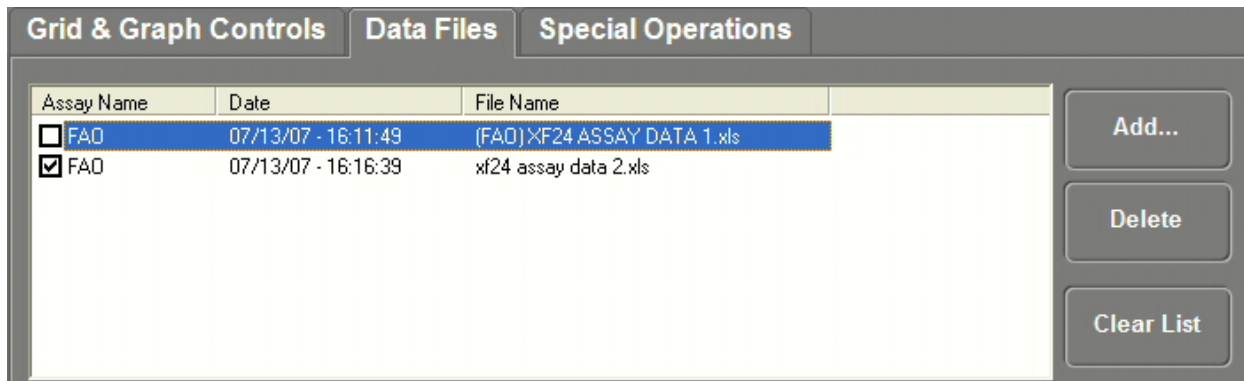
The Data Files tab showing use of checkboxes and highlighted listings

Averaging Multiple Data Sets

The **Average Profiles** button on the Grid **& Graph Controls** tab allows you to average data from multiple data sets. The averaging feature requires that the two data sets be from similarly run assays, with the same number of measurements and identically named groups. The figure below shows a group from two different data sets graphed separately on the left and averaged together on the right.
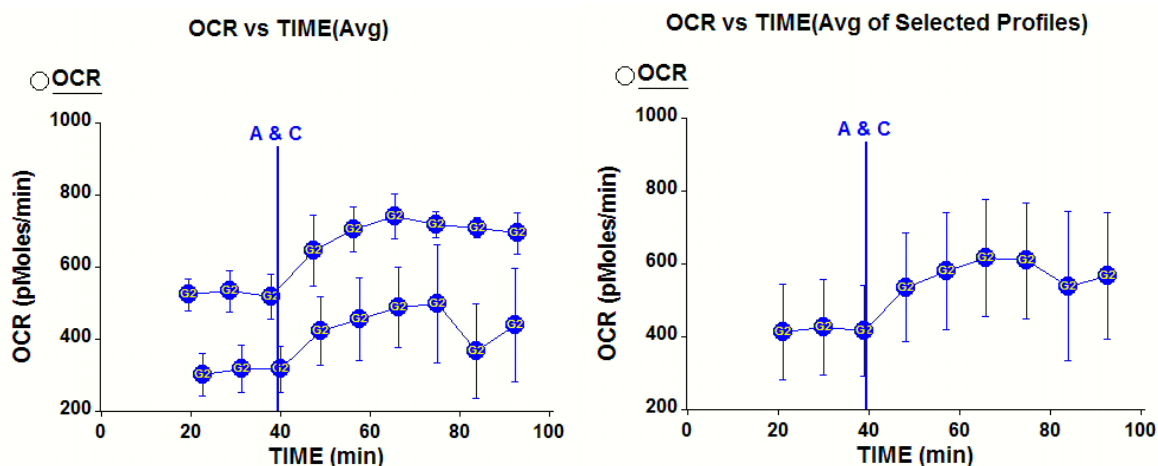
Averaging Data from Two Data Sets

Multi-View and Multi-Graph Display

Overview

To provide more flexibility in viewing data using the Data Viewer in Excel, up to four different views of the Data Viewer can be configured. Any combination of display options can be applied to each view, such as different data sets, different graphing options, or with different selected wells. Toggle between the view using tabs located at the upper right. Initially, each data file will start out with one tab representing the default view. More tabs can be added using the buttons at the lower right. This feature is useful for looking at several different graphs from the same assay at one time.
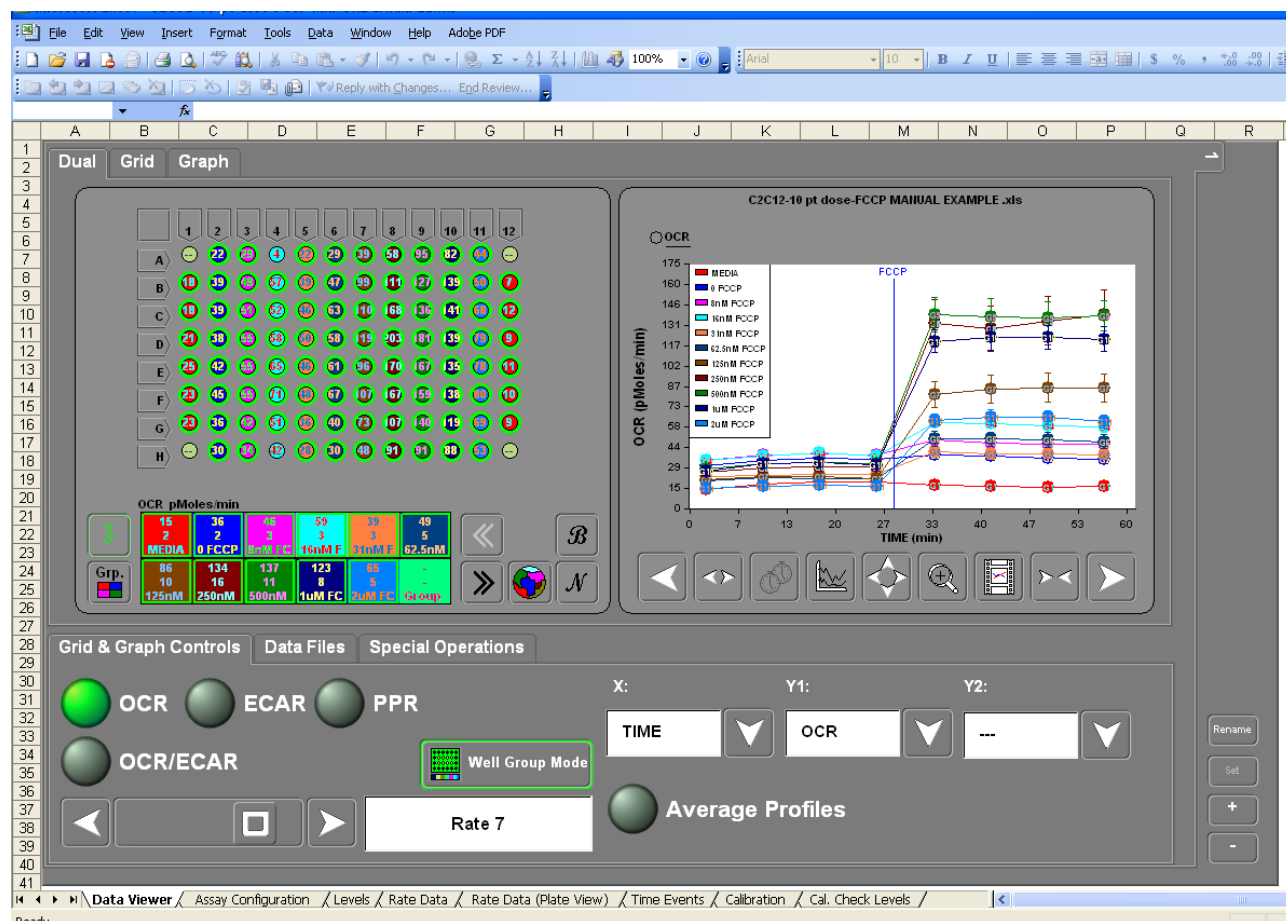
Data Viewer
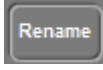
Adding Multiple Views

To add more views, click on the button. Up to four views can be added. By default, the views will be numerically labeled 1 to 4.

To remove a view, select the appropriate view tab and click on the button.
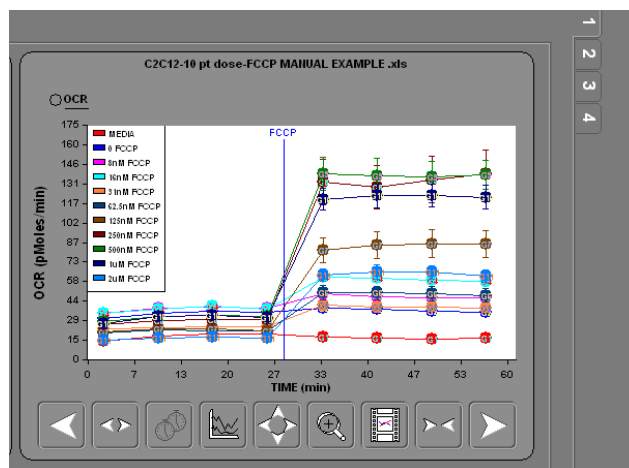
View Tabs with Default Labels

The labels for the views can be changed by clicking on the Rename button. Enter the new name in the dialog and click OK.
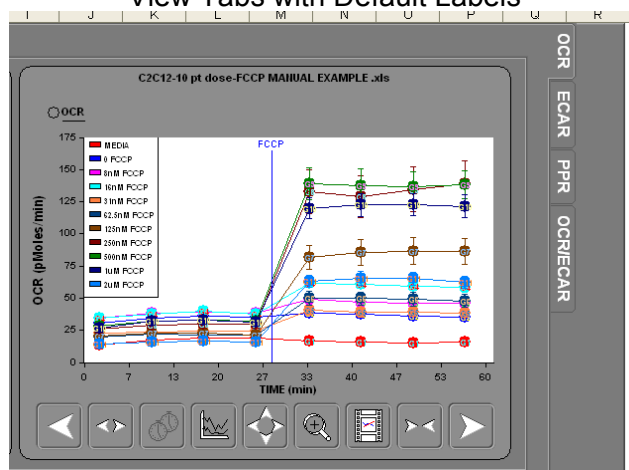
View Tabs with Custom Labels

Configuring and Graphing Multiple Views

After creating the views, click on each tab and customize each view by configuring the Data Viewer. In this example, the graph in each view has a different set of data and the views are labeled accordingly.

Click on the Graph tab to see a display of each graph from the four views. In this mode, the tabs include a button for showing and hiding the corresponding graph. So one, two, or three graphs can be displayed instead of all four.
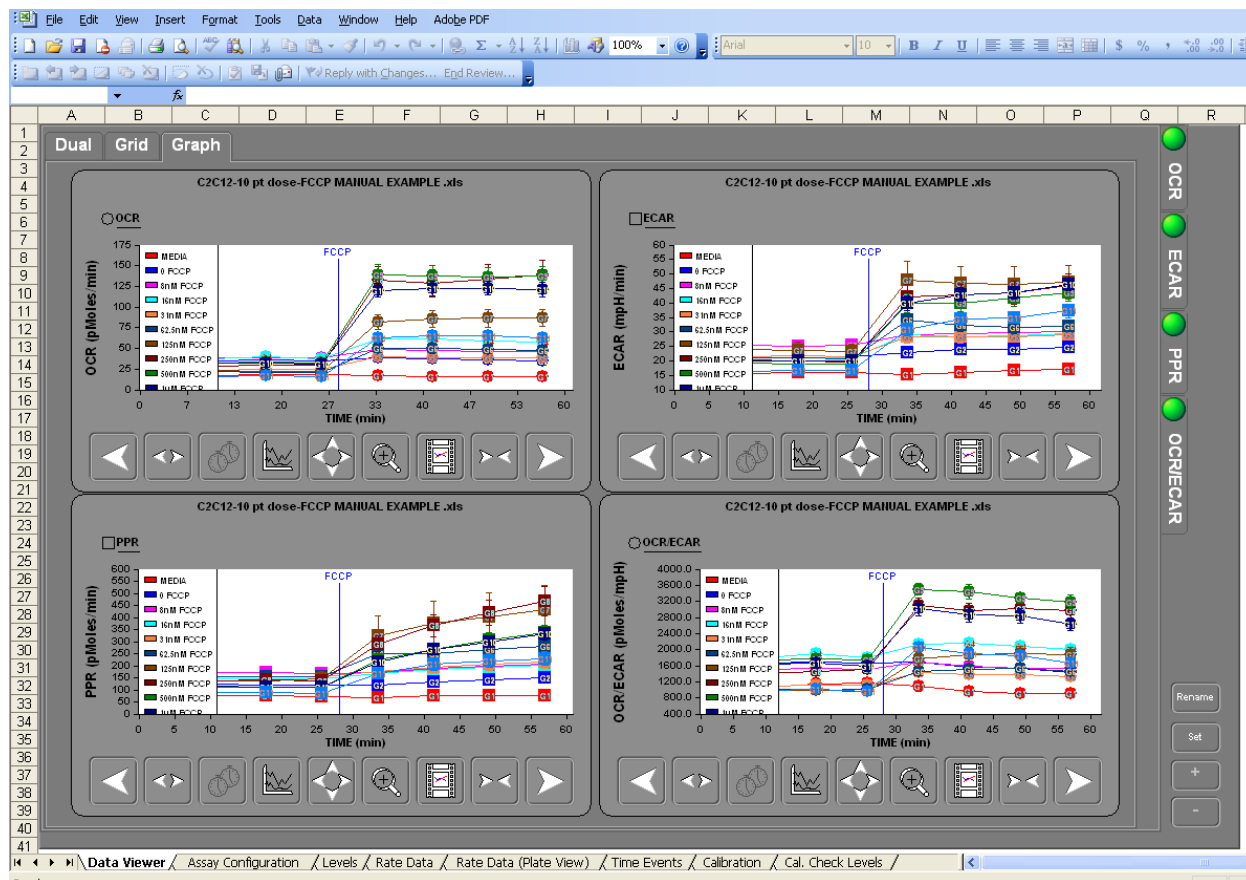
Graphs Displayed from each View

Saving and Exporting Graph Data

The graph's right-click context menu contains a new item called **Save Graph Data.** This features takes the data currently displayed in the graph and exports it to a new excel worksheet. By using the controls on the Data Viewer, the graph becomes a data filter and this feature provides an easy way to export that data for additional analysis.

When done from the Data Viewer in Excel, it adds a new worksheet to the existing data file. However, this can also be done from the application software. The user will be prompted to enter in a filename and a new excel file will be created with the generated worksheet.

As shown in the figure below, the worksheet will contain pictures of the well grid and graph. The data will be located below the pictures organized by group or well, depending on how the graph is configured.
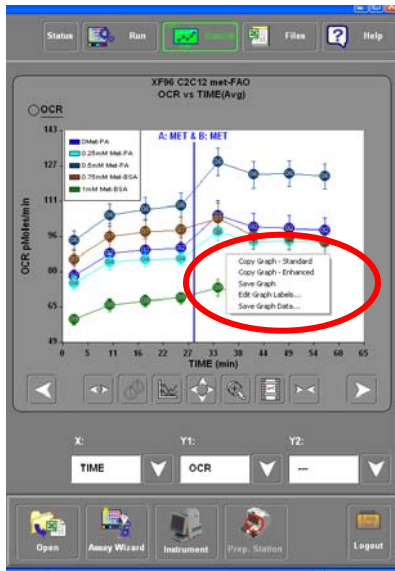
When performed using the Data Viewer in Excel, a new worksheet will be added to the existing data file. When performing from the XF reader Software, the user will be prompted to enter in a filename and a new excel file will be created containing the data from the graph.

Right click on Graph: choose "save graph data….."

The "save graph data" output will contain only the data shown in the graph:
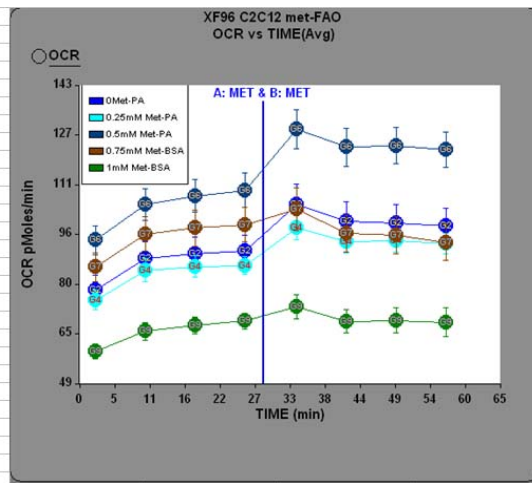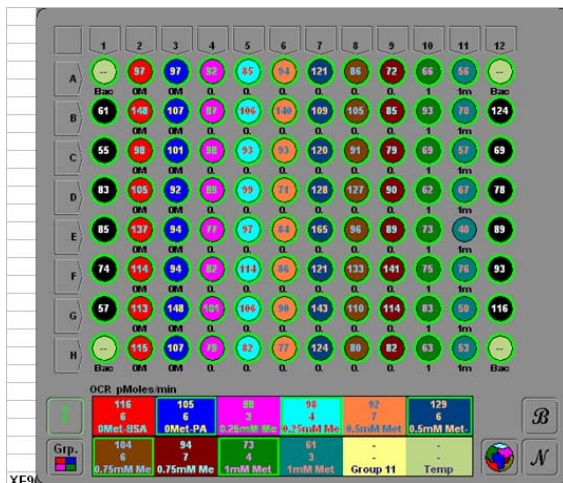
TIME (min)	0Met-PA		0.25mM Met-PA		0.5mM Met-PA		0.75mM Met-BSA		1mM Met-BSA
	Mean	Std Error	Mean	Std Error	Mean	Std Error	Mean	Std Error	Mean
2.516666889	78.35771942	4.697581768	75.16775513	3.116228819	93.95373535	4.535063267	85.53367615	4.556810856	58.84516144
10.23333359	88.23260498	5.283539295	84.46868896	3.628093243	105.2464981	4.981353283	95.98188019	5.400135517	65.30144501
17.93333435	89.72545624	5.136349678	85.73007202	3.286072016	107.9499207	5.154028416	97.74951935	5.650510788	67.22299957
25.65000153	90.57051849	5.110179901	86.01222229	2.708130121	109.6674652	5.438490868	98.84178162	5.442539215	68.48844147
33.71666718	105.1889191	6.463121414	97.83339691	3.806670189	128.8270264	6.148316383	103.7198868	6.718732357	72.95089722
41.41666794	100.0160751	5.962479591	93.40311432	3.318825006	123.2064896	5.975840092	96.1295929	5.751657963	68.46834564
49.13333511	99.28973389	5.80673027	93.74193573	3.338212013	123.6798782	5.664761543	95.5923996	5.862612247	68.7714386
56.85000229	98.37625885	5.738883495	92.84235382	3.239964008	122.5295258	5.554852962	93.26399994	5.69141674	68.19320679

Export from the Save Graph Data function: OCR

Copying, Saving and Exporting Graph and Well-Grid Images

New options have been added to the popup menus that appear when right-clicking on the well-grid and graph objects. Both menus have a **Save** option that supports saving a snapshot of the object to an image file.

To save an image of the well-grid,

1. Right-click on the well-grid and select **Save Grid** from the popup menu.

2. The Save As dialog will appear, as shown in the figure to the right. Enter in a filename and choose a file type. TIFF, JPEG, and BMP formats are supported.

3. If additional pixel resolution is required in the image, click on the checkbox to **Use enhanced mode for the save**. The saved image will be 3 times larger than what is displayed.
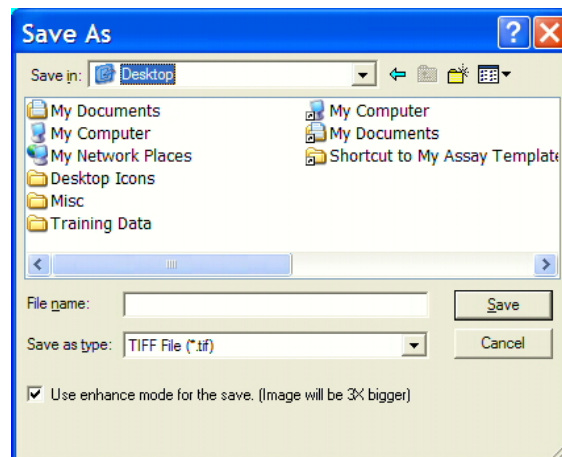
Save As dialog for the Well-grid

To save an image of the graph,

1. Right-click on the graph and select **Save Graph** from the popup menu.

2. The Save As dialog will appear, as shown in the figure to the right. Enter in a filename and choose a file type.

3. Select **Use enhanced mode for the save** for a larger image size.

4. Select **Image without border** to have a borderless image. This produces a result similar to **Copy Standard** when choosing to copy and paste the graph.
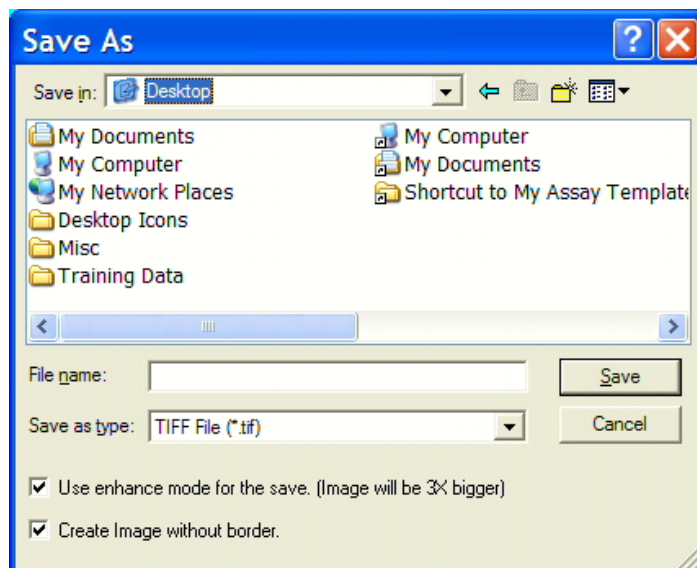
Save As dialog for the Graph

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Appendix V   -   The Media, Cell, & Compound Database
and Additional Features and Options

Option to Save Media, Cell, & Compound Definitions

Many users take advantage of the Assay Configuration worksheet template to define and store information regarding the cell types, media, and compounds used in assays. However, the process of creating these definitions and copying them from template to template can be time consuming. A **Database** feature has been added to the **Assay Wizard** to allow the separate storage of these definitions. Once stored, they can be recalled later and added to an existing assay configuration.

The **Assay Wizard** screens for defining growth media, cell type, running media, compound, and injection media all support this feature in the same way. To demonstrate, the instructions below will refer to running media but they apply similarly to the other assay materials that are part of the assay configuration.

Storing a Running Media Definition to the Database

1. In the **Media Info** screen of the **Media & PPR** tab, click on the **Add Media** button.

2. Select **New Media** in the popup screen.

3. **New Media Info** will appear highlighted in the Running Media List on the left. On the right, click on and edit the fields to define the media.

4. Click on the **Add to Database** button to store the definition.

Recalling a Running Media Definition from the Database
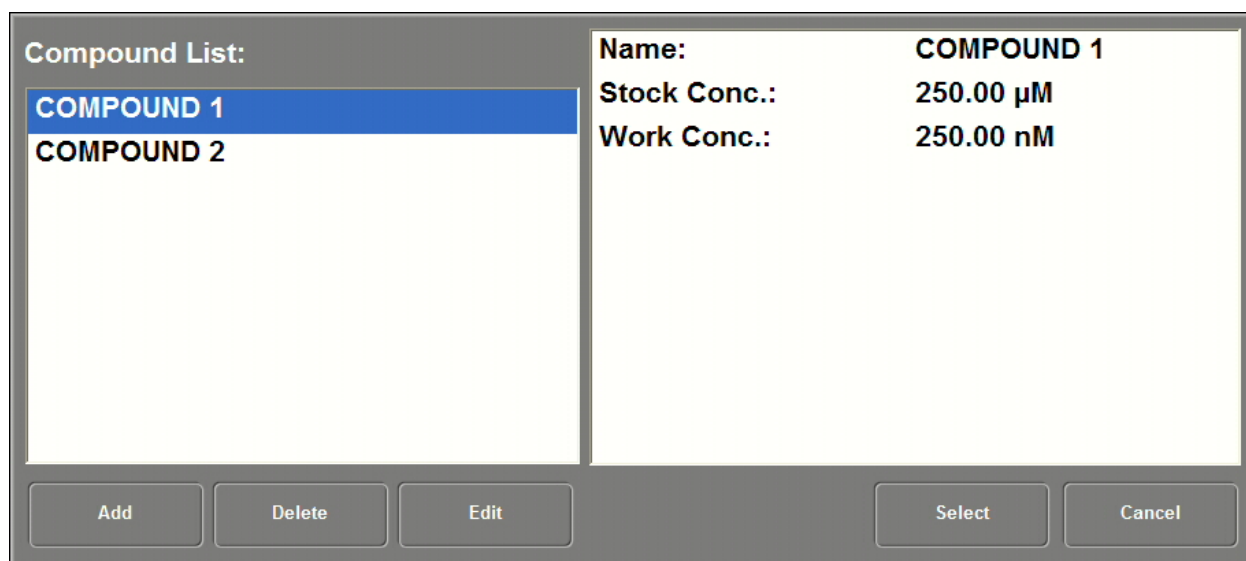
1. In the **Media Info** screen of the **Media & PPR** tab, click on the **Add Media** button.

2. Select **Load from Database** in the popup screen.

3. A window with a running media list will appear. Click on and highlight the desired media name from the list. The definition will be displayed on the right side window. Click **Select.**

4. The selected media should now appear in the **Running Media List** on the **Media Info** screen.

5. To complete the configuration, go to the **Media Layout** screen and assign the media to the appropriate locations of the wellplate.

Note: Unlike running media, a growth media is assigned to a cell type definition and not to a plate layout. Also, compounds and injection media are assigned on a port letter basis in addition to well location.

Database Functionality for Editing and Deleting Entries

In the **Assay Wizard**, the database feature is used to save the definitions of compounds and media used in the assay. These definitions can then be used later by loading them from the database. The figure below displays the selection dialog for browsing the database contents. **Edit** and **Delete** buttons have been added to support database maintenance. Existing entries can now be modified or completely removed.

Database Dialog

Where are the Definitions Stored?

When running this software for the first time, it creates three files in the program directory. The files are **CellDatabase.cif**, **MediaDatabase.mif**, and **CompoundDatabase.dif**. These files store the definitions of their respective content. The software can be configured to access these files from a different location. In addition, these database files can be pre-configured on a different computer and then copied to the instrument controller, or vice versa.

To configure the software to access these files from a different location

1. Click on the **Instrument** button to enter instrument setup.

2. Click on the **File Preferences** button in the right panel.

3. In the left panel, select **Database.**

4. For each database file, select the desired file location. When finished, click on **Apply Settings** and **End Instrument Setup Mode**.

Suggested Workflow

Simplify the creation of assay configurations by using the database feature to store definitions, or partial definitions, of frequently used assay materials. For example,

1. Load a partially completed assay configuration template into the **Assay Wizard** using the **Load from Template** button in the **General** tab.

2. Navigate to uncompleted sections of the assay configuration, load definitions, and perform layout assignments.

At the end of the wizard, use the **Save Template** button to save the completed assay configuration to an excel file that can be copied to the touch screen controller. If making these modifications on the touch screen controller, end the wizard and proceed directly to starting the assay.

User Login and Profile Preferences

Overview

User logins and passwords can now be used to access the XF software. The new Login screen, shown here in the figure below, is displayed when first starting the software. Various software settings will be associated with each login, including default assay configurations and folder locations. Each user login can also be configured with a default starting screen, such as the Results screen or Run screen. A Guest login, requiring no password, is provided. Editing or removing user logins can be done via the User Manager tool in the Instrument setup section.
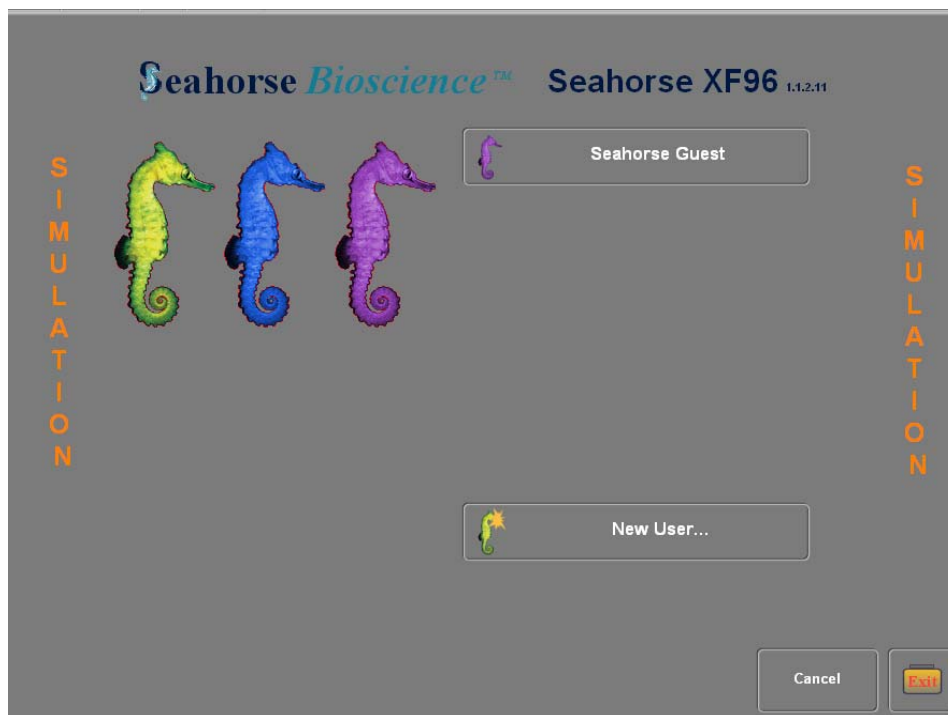
User Login Screen

Adding a New User

From the login screen, create a new user by clicking on the **New User…** button. In the User Info dialog, enter in the login and password. Though only three seahorse icons are available, there is no limitation on the total number of users that can be created.
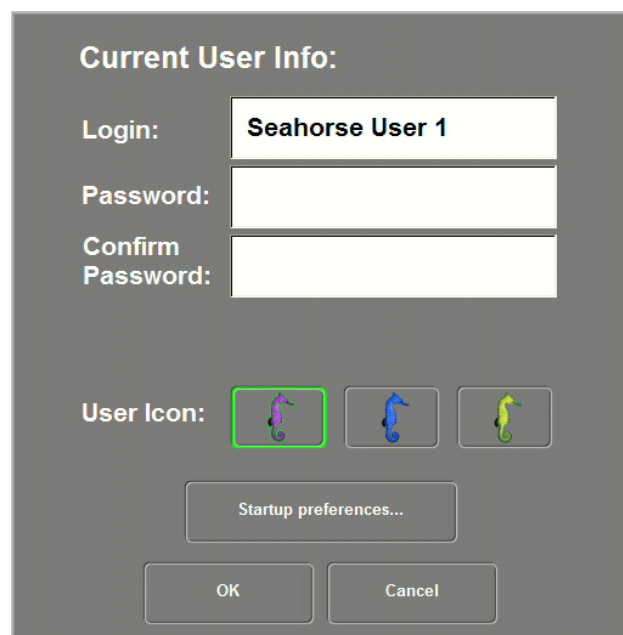
User Login Info

Click on the **Startup preferences…**
button to display this dialog. The Start
Screen setting determines the initial
screen the user sees after logging in.
Options include the Run Screen, Files,
Assay Wizard, Run Start, and Prep
Station.

The Assay Template Directory and the
Default Template File can also be
specified. These are the same settings as
found in the Assay section of File
Preferences.
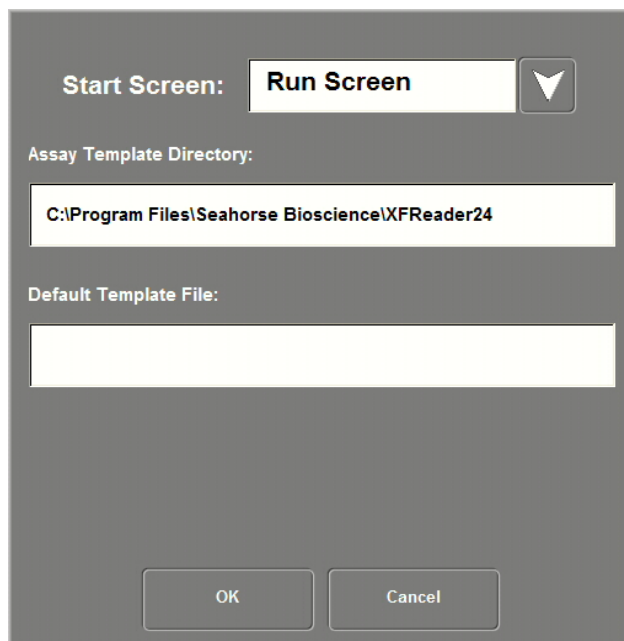
User Login Startup Preferences

Editing Users

After several user logins have been added,
it may be necessary to remove or edit
them. This administrative function can be
done from the User Manager section of
Instrument setup. Display this section by
selecting the **User Manager** button after
entering Instrument setup mode.

Accessing User Manager

Current User Info:

Login: Seahorse User 1

Edit User Info...

Edit User List...

Apply Settings   End Instrument Setup Mode
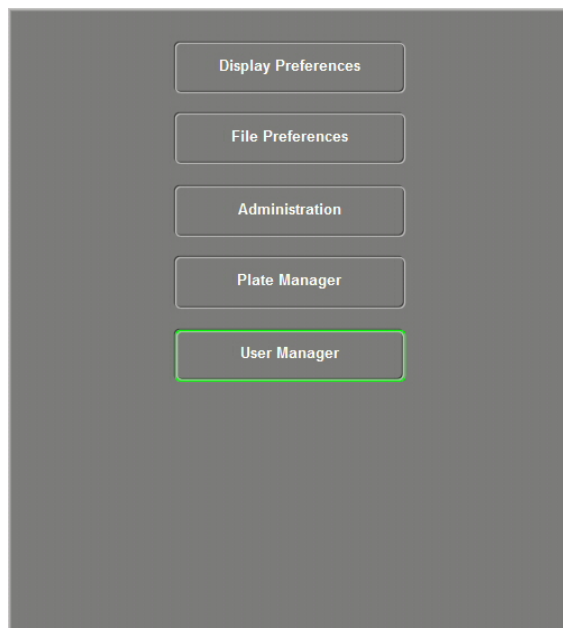
User Manager

The User Manager displays the current user. Click on **Edit User Info…** to edit the current user settings. To edit other users, or add new users, click on **Edit User List…**
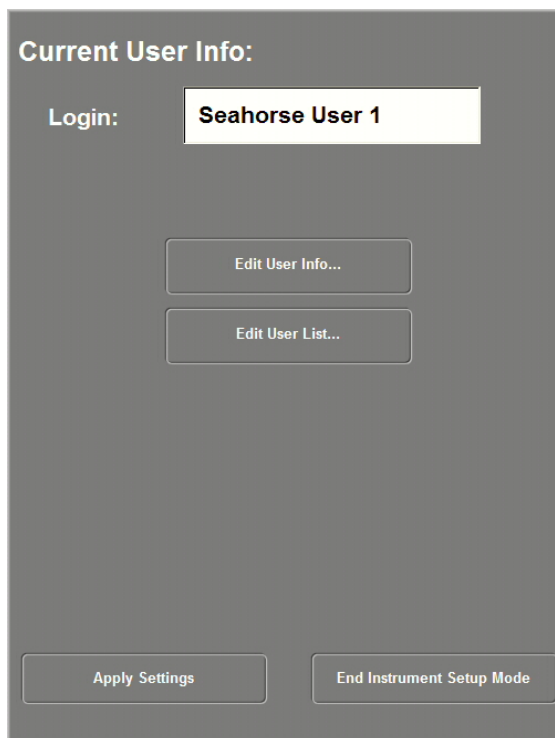
When accessing the Edit User List function for the first time, the Admin user password is required. The password initially defaults to **ADMIN.** This password, as well as any user password can be edited by selecting a user login and clicking on the **Modify** button. When modifying, any user can be elevated from a Normal User to an Administrator. Only Administrator level logins can access this section.

To remove unneeded users, select a user login and click on the **Delete** button.

New users can also be created here instead of at the main login screen.

Users:

Admin
Guest
Seahorse User 1

New user...   Modify   Delete

OK   Cancel

User Manager

Refresh, File Menu and File Filter

A **Refresh** button is located on the Files screen to update the list of available drives. In prior releases, the drive letter list was populated when starting the software and could only be updated by exiting and restarting.

After plugging in a USB flash drive to the instrument controller, or any other computer running the XF software, click on **Refresh** to expose the drive letter in the Files screen to allow immediate access to the device.

The File Menu Bar

Navigate to the parent folder of the current folder with the Up button. Create new folders with the new button.
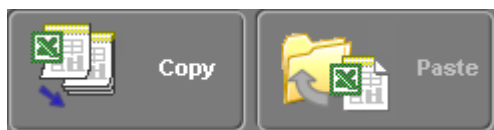
Copy and paste individual files to different folders or copy and paste entire folders and their contents to new locations.
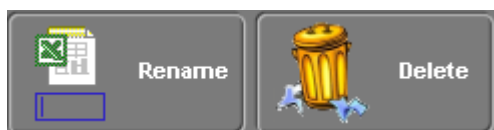
Update file or folder names using the Rename button and remove unneeded files or folders with the Delete button.

Save your data. The Save button replaces the Export button in previous versions. Use Save As to save to a new filename.

File Filter

At the bottom left of the Files screen, you have a few options for filtering which files are displayed. All files can be displayed, or just those files generated by the XF software. The data files come in two formats, *.xls and *.xfd.

Pop up Menus, Temperature and Auto Data Recovery

Popup Menu and Mouse Support

When interacting with the list of files, you can right-click on any filename using your mouse to display the pop-menu for quick access to some of the standard menu bar functions.

Click on the Cancel button to close the popup. Using the touch screen, you can also touch the file icon to the left of the filename to display the popup.
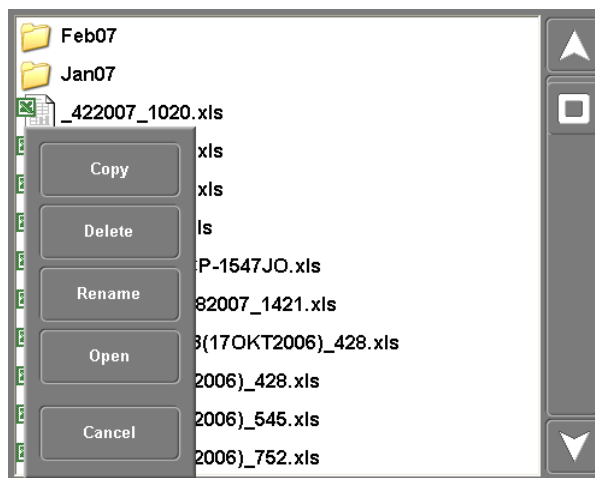
Similarly, right-clicking on a folder name, or touching the folder icon, will also display a popup menu. Again, close the popup by clicking or touching the Cancel button.

Double-clicking with the mouse is also supported on either files or folders. Double-clicking on a file opens that file and displays the data in the Results section. Double-clicking on a folder opens the folder and displays the contents on the right hand tree display in the Files section.
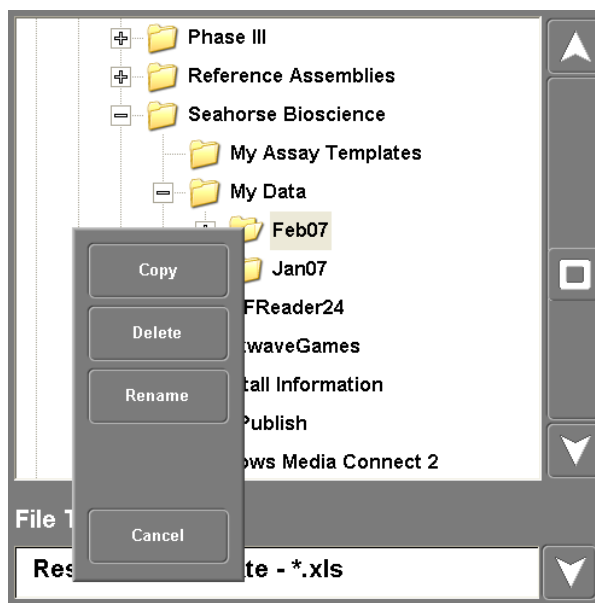
Option to Maintain Instrument Temperature when exiting XF Software

In prior releases, when exiting the software on the instrument's touch screen controller, the instrument heater was turned off, allowing the system to cool. In this release, when clicking the **Exit** button, the message prompt shown in Figure 3 will appear. The user can choose to have the instrument temperature maintained while the software is closed, or to turn off the heater.

If opting to turn the heater off, restarting the software will turn the heater back on. If the heater is left on, the instrument firmware will continue to safely regulate the system temperature. Safeguards are in place to prevent overheating.

If the **Exit** button is pressed by mistake, click on the **Cancel** button.

Improved Data Recovery when Auto-save Fails

At the end of an assay, if the software is unable to save the data to the specified target folder, the software will instead save the data files to the Recovered Data folder in the software installation directory. Figure 31 shows the error message that will be displayed if this occurs.

If the software has been installed to the default location, the Recovered Data folder can be found here: C:\Program Files\Seahorse Bioscience\XFReader96\Recovered Data.

Seahorse Bioscience
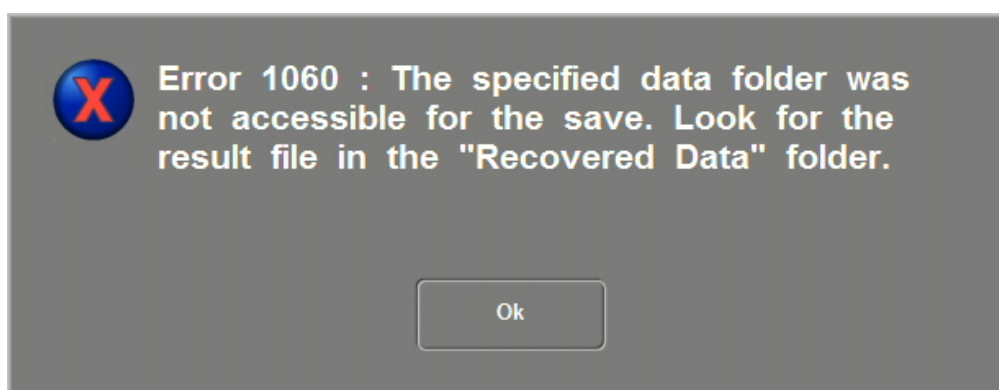
Appendix VI - Reagent and Solution Preparation

XF Assay Media, Specially Formulated DMEM (0 mM Glucose)
Part No. 100965

XF Assay Media is for use in conjunction with the Seahorse Bioscience XF Analyzer instruments, XF Biosensor cartridges and XF tissue culture plates. XF assays require a non-buffered media to accurately measure proton production rate and extra cellular acidification rate (PPR and ECAR) of cells growing in culture. The constituents are based on the formulation of Dulbecco's Modified Eagle's Medium, however no sodium bicarbonate (buffering agent), glucose, or L-glutamine is present.

Media Specifications

Volume	1 L
Osmolality	291 mOsm/Kg H_2O
Endotoxin	< 0.0052 EU/mL
Storage	2-8°C
Expiration	This liquid media has data supporting a shelf life of 18 months when properly stored

Media Constituents

Media Component	Final Concentration
Mg^{2+} (as $MgSO_4$)	0.8 mM
Ca^{2+} (as $CaCl_2$)	1.8 mM
NaCl	143 mM
KCl	5.4 mM
NaH_2PO4	0.91mM
Phenol Red	15 mg/ml

Preparation for use in XF Assay

XF Assay Media contains no added glucose, sodium pyruvate or Glutamax. To prepare media for an XF assay, do the following:
1. Warm media to 37°C in water bath.
2. Add 200 mM Glutamax (10mL per 1 L of media, 2 mM final concentration)
3. Add 100 mM Sodium Pyruvate. *Note this will be assay dependent* (10 mL per 1 L of media = 1 mM final concentration)
4. Add glucose for the desired final concentration based on the following table:

Final Glucose Concentration	Grams of Glucose per 1 L
0 mM	0.00g
2.5 mM	0.45g
5.0 mM	0.90g
10 mM	1.80g
25 mM	4.50g

5. Adjust pH of media to 7.4 using NaOH or HCl.
6. Filter sterilize if sterility was compromised.
7. Aliquot desired amount to use in XF assay. The reminder should be stored at 4°C. Re-adjustment of pH may be necessary if stored for longer than 7 days.

Buffer Capacity Based on Glucose Concentration

The buffer capacity of the XF Assay Media was determined for the following concentrations of glucose. Media was prepared as described in "Preparation for use in XF Assay."

Glucose Concentration	Buffer Capacity
0 mM	0.0009092
5.0 mM	0.0008779
10 mM	0.0008862
25 mM	0.0008557

Note that Seahorse Bioscience suggests that serum (e.g. FBS) be omitted from the final media formulation as it will affect the buffer capacity of the media. If FBS is required for cell viability or phenotypic maintenance, Seahorse Bioscience suggests using low amounts (~1% or less) and determining the resulting buffer capacity empirically for accurate PPR calculations.

Please follow the instructions below should you wish to make you own DMEM XF Assay media:

A. Reagents:
    a. Dulbecco's Modified Eagle's Medium Base 8.3g/L (Sigma D5030-1L)
    b. 100x (200mM) GlutaMax-1 (Gibco 35050-061)
    c. 100mM Sodium Pyruvate (Sigma S8636) (Assay Dependent)
    d. Glucose (Sigma G8270) (Assay Dependent)
    e. NaCl (Sigma S3014)
    f. Phenol Red (Sigma P-5530)

B. Dissolve DMEM Base in 500 mL dH$_2$O. Separately, dissolve 1.85g NaCl in 500 mL dH$_2$O. Combine NaCl solution with DMEM Base solution. Remove 20 mL from the combined solution. Add 10 mL 100x GlutaMax-1, 10mL 100 mM Sodium Pyruvate (assay dependent), and 15 mg Phenol Red. Add glucose powder for desired

concentration (4.5g = 25mM glucose). Warm media to 37°C, then pH to 7.4 using 5M NaOH (Sigma). Filter sterilize. Media can be stored at 4°C for later usage.

KHB XF Assay media

A. Reagents and Materials
      a. NaCl (Sigma, S3014, dry, or Sigma S6316, 5M solution)
      b. KCl (Sigma, P4504)
      c. MgSO$_4$ (Sigma, M2643)
      d. Na$_2$HPO$_4$ (Sigma, S3397)
      e. Glucose (Sigma, G8270, dry, or Sigma G8769, 45% solution)
      f. Carnitine (Sigma, C0158)

B. Preparation of 5x KHB base from dry reagents

To make 1 liter 5x KHB, add the following reagents separately in the listed order to 900 ml dH$_2$O in a 1 liter beaker containing a magnet stir bar. Allow each to dissolve before adding the next one:

Chemical	5X (mM)	5X (g/L)
NaCl	555	32.532
KCl	23.5	1.752
MgSO4	10	1.204
Na2HPO4	6	0.852

Adjust final volume to 1000 mL with dH$_2$O, then filter-sterilize and store at 4°C for later use.

C. Alternate preparation of 5x KHB base from stock solutions

Use 5M NaCl stock solution. Make 100 mL stock solutions of KCl (1 M), MgSO$_4$ (0.5 M), and Na$_2$HPO$_4$ (0.5 M) in dH$_2$O as follows:

Stock	Concentration (M)	Amount for 100 mL (g)
NaCl	5	--purchase--
KCl	1	7.456 g
MgSO$_4$	0.5	6.019 g
Na$_2$HPO$_4$	0.5	7.098 g

To make 1 liter 5x KHB, to 833.5 mL dH_2O add the following:

Component	Stock concentration (M)	5X (mM)	Amount for 1 L (mL)
NaCl	5	555	111 mL
KCl	1	23.5	23.5 mL
$MgSO_4$	0.5	10	20 mL
Na_2HPO_4	0.5	6	12 mL

Filter-sterilize and store at 4˚C for later use.

Preparation of 50 mM carnitine stock

Dissolve 81 mg carnitine in 10 mL dH_2O in a 25 ml conical tube.
Filter-sterilize and aliquot into 1 ml in 1.5 ml eppendorf tubes.
Store at -20ºC. Frozen stock is good for one month and may be used within three days of thawing when kept at 4ºC.

Preparation of 1x KHB assay medium

Final composition of 1x KHB

Component	Concentration
NaCl	111 mM
KCl	4.7 mM
$MgSO_4$	2 mM
Na_2HPO_4	1.2 mM
glucose	2.5 mM
carnitine	0.5 mM

On day of assay, determine volume of medium needed from table below and prepare 1X KHB assay buffer as below.

Add indicated volumes of 5X KHB, dH2O, 45% glucose solution and 50mM carnitine in a beaker containing a stir bar.

# of Plates	1x KHB Needed	dH_2O (mL)	5X KHB (mL)	45% glucose (mL)	50mM carnitine (mL)
1	50	40	10	0.05	0.5
2	100	80	20	0.10	1.0
3	150	120	30	0.15	1.5
4	200	160	40	0.20	2.0

Alternatively, add dry glucose and carnitine to the 1x KHB as follows:

Name	g/L	g/100 mL
Glucose	0.45	0.045
Carnitine	0.081	0.008

Warm to 37°C before adjusting pH.

Measure pH with pH meter and adjust pH to 7.4. For 100mL 1x KHB, adjustment will typically require 10-15 µL 1N HCl.

Store at 37°C until ready to use.

Stock compound preparation for injections:

Be sure to use *fresh* molecular biology grade DMSO for preparation of compounds.

Ensure that after the compound of interest is diluted in XF Assay media that the pH of the solution is 7.4.

10 mg/ml Oligomycin:

Resuspend 5 mg Oligomycin (Sigma, O4876-5mg) in 0.5 ml of *fresh* molecular biology grade DMSO.

Dilute in XF Assay media to achieve desired stock concentration for loading into injection ports. Typical working (final) concentrations are 0.1 – 1.0 µg/ml.

Aliquot and store at -20°C.

10 mM FCCP:

Dissolve 10 mg FCCP (Sigma, C2920-10mg) in 3.93 ml of *fresh* molecular biology grade DMSO.

Dilute in XF Assay media to achieve desired stock concentration for loading into injection ports. Typical working (final) concentrations are 0.1 – 5.0 µM.

Note that response to FCCP will be dependent in Assay media composition ([pyruvate], [glucose], presence of BSA, etc.)

Aliquot and store at -20°C.

100900-400_MANUAL XF96 W/ PREP STATION_RA

100 mM 2, 4-dinitrophenol (DNP):

Prepare a 100 mM solution by combining 18.4 mg 2, 4-DNP (Sigma D198501) with 1.0 ml of *fresh* molecular biology grade DMSO.

Dilute in XF Assay media to achieve desired stock concentration for loading into injection ports. Typical working (final) concentrations are 10 – 100 µM.
pH to 7.4 using 0.1 M NaOH.

Hint: DNP will change the pH of the media significantly. Use a pH meter or narrow band testing strip to check pH. Comparing the color of the DNP solution to the same volume of unbuffered DMEM media is not accurate for pH as the DNP is a bright yellow solution that alters the color of the media even at pH 7.4.

Aliquot and store at -20°C.

1.0 mM Rotenone:

Prepare a 50 mM solution by combining 19.7 mg Rotenone (Sigma R8875) with 1.0 ml of *fresh* molecular biology grade DMSO. Perform a 50X dilution in DMSO to achieve a concentration of 1.0 mM rotenone (e.g. 20 µl 50 mM Rotenone, 980 µl DMSO).

Dilute the 1.0 mM solution in XF Assay media to achieve desired stock concentration for loading into injection ports. Typical working (final) concentrations are 0.1 – 1.0 µM.

Aliquot and store at -20°C.

1.0 M 2-deoxyglucose (2-DG):

For a 1.0 M 2-DG solution combine 0.492 g 2-DG (Sigma D6134) in 2.7 ml DMEM or KHB XF Assay media. Check volume and bring to 3.0 ml final. pH to 7.4 using 4-8 µl of 0.1 N NaOH.   Check with a narrow band pH strip.

Hint: compare the color of 2-DG solution to the same volume of unbuffered DMEM media to accurately pH. Make sure both solutions are at the same temperature during comparison. You may also test with a narrow band pH strip.

Bulk Preparation of Palmitate-BSA Complex

I. Materials
Sodium palmitate (Sigma, P9767, mw 278.41)
Ultra fatty acid free BSA (Roche 03 117405 001, mw 68,000)
Tissue culture grade deionized water (Gibco 1523-0147)
5M NaCl solution (Sigma, S6316)
Glass beaker, 1 L, 2
Glass beakers, 250 mL, 2
Glass Erlenmeyer flask, 50 mL, 1
Small stir bars, 3
Heated stir plate, 2
Thermometer, 2
150 mL filter unit, 0.22 micron (Corning 430626)
Glass vials, 4 mL (National Scientific, B7999-2)
Ice

II. Preparation of 1 mM Sodium Palmitate /0.17 mM BSA Solution (6:1 FA:BSA)
Note: keeping each solution at the specified temperature is critical in this protocol
A. Setup prior to BSA and sodium palmitate solution preparation
1. Warm about 200 mL tap water in each of 2 1L beakers in 37°C water bath.
2. Warm 250 mL beaker with stir bar in 37°C incubator.
3. Make 300 mL 150 mM NaCl by adding 9 mL 5 M stock to 291 mL dH2O
B. BSA solution preparation
Note: ½ of the solution is intended for making aliquots for vehicle control in FAO assay. The remaining ½ is conjugated with sodium palmitate as in section C.
1. Weigh out 2.267 g ultra-fatty acid free (FAF) BSA,
2. Add to 100 mL 150 mM NaCl in 250 mL glass beaker while stirring in a stir plate.
2. Cover beaker with parafilm and place in one of the 1L water baths pre-warmed to 37°C on a heated stir plate; adjust heat as needed to maintain temperature near 37°C but never more than 40°C (keep a thermometer in water bath).
3. Stir till BSA is completely dissolved.
4. Transfer BSA solution to the upper chamber of 150 mL filter unit in laminar flow hood. Filter with vacuum.
5. Transfer 50 mL BSA to pre-warmed 250 mL beaker, cover with parafilm, return to 37°C water bath and resume stirring.
6. Dilute the remaining 50 mL BSA with 50 mL 150 mM NaCl for 0.17 mM BSA stock.
7. Aliquot 4 mL/glass vial and freeze at -20°C for use as BSA control.

C. Sodium palmitate solution preparation
1. Once BSA is stirring in water bath, weigh out 30.6 mg of sodium palmitate, and add to 44 ml of 150 mM NaCl solution in 50mL Erlenmeyer flask.
2. Cover flask with parafilm and weight with lead ring. Place in the other pre-warmed 1.0 L water bath on a heated stir plate; heat to 70°C while stirring (thermometer in water bath).

   3. Palmitate solution will appear increasingly cloudy as temperature reaches 50-60˚C but will become clear near 70˚C.

   D. Conjugating Palmitate and BSA
      1. Remove parafilm from both beaker and flask.
      2. Transfer 40 ml hot palmitate solution to the 50 ml of BSA solution stirring at 37˚C. Palmitate will precipitate if it is allowed to sit in a pipette – transfer 5 ml at a time, 8 times, in a 10 ml pipette, taking up and expelling quickly.
      3. Re-cover beaker with parafilm.
      4. Stir at 37˚C for 1 hour, monitoring temperature of water bath to keep between 35 and 40˚C. Add ice to water bath to lower temperature if it reaches 40˚C.
      5. Measure volume in 100 ml glass graduated cylinder and adjust final volume to 100 ml with 150 mM NaCl for 1 mM palmitate stock.
      6. Adjust pH to 7.4 with 5-10 µL 1 N NaOH.
      7. Aliquot 4 ml (or smaller volume if preferred) per glass vial and store at -20˚C.

III. Thawing Palmitate and BSA for FAO assay
   A. Thaw palmitate and BSA in a 37˚C water bath for ~10 minutes before loading into the XF cartridge. Suggestion: if palmitate is thawed immediately before pre-incubating cell plate, it will be ready to load by the time the medium change is accomplished.
   B. Palmitate and BSA are known to be stable at -20˚C for at least two weeks, and thought to be stable for up to one month.

The key aspect of successful preparation of the Palmitate-BSA complex is to monitor the time and temperature of each step carefully and accurately.

Notes: Specifications of Solutions
   Palmitate is 1 mM for a 5x injection to 200 µM final. It is complexed in a 6 to 1 molar ratio with BSA which is 0.17 mM for a 5x injection to 33.3 µM final.
   The conjugated palmitate can be diluted to a lower concentration with NaCl or KHB.
   This protocol can be adapted to make a 2 mM palmitate solution for 10x injection, or 8x, etc as desired.

Appendix VII – Coating of XF Cell Culture Plates

The XF cell culture plates are similar to any typical cell culture plate with respect to plasma treatment and cell adherence. If cell types are used that need a type of coating, the XF plates may be coated with any typical coating, including poly-D-lysine, collagen, fibronectin, and Cell-Tak®.

Please follow the respective manufacturer's instructions for coating procedures.
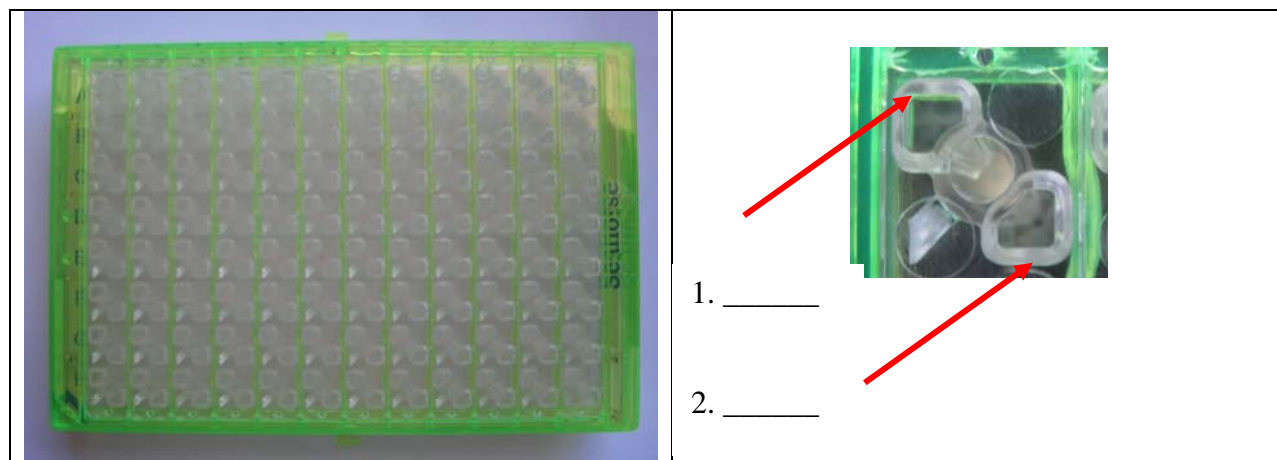
Appendix VIII - Troubleshooting

Please visit www.seahorsebio.com for FAQs.

For further information, please contact your local Seahorse Bioscience Field Application Scientist
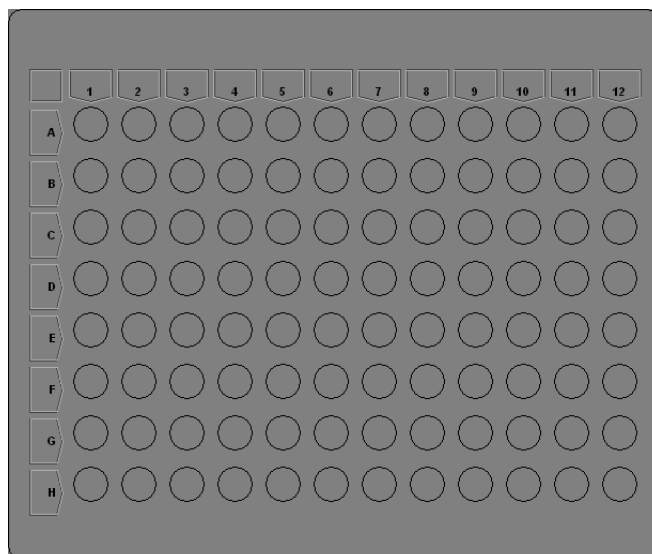
You may also contact Seahorse Bioscience Support via email or phone (see Appendix X below).

Appendix IX: XF96 Training Completion Check Questions

1.) Label the Injection Ports below:

1. _____

2. _____

2.) Identify the wells used for Temperature Correction below:

3.) True or False: A typical measurement cycle includes a mix and measurement period.

4.) What reagent is used for calibration?

_____

5.) What is the proper temperature for all plates being run in the XF24?_____

6.) Sensor Cartridges need to be hydrated for _____ before being run in the XF96.
   A. 30 minutes
   B. Overnight
   C. 1 hour

7.) True or False: Compounds are loaded into the sensor cartridge before calibration.

8.) What is typically the shortest measurement period?_____

9.) True or False: A 1-Step seeding procedure is used for seeding cells in the XF96 cell culture plates.

10.) When seeding a new cell line in XF96 cell culture plates, how long should you wait before adding the final amount of growth media?_____

11.) What are the minimum and maximum volumes for each well when starting an XF96 assay?_____
__

12.) How many times can an XF96 sensor cartridge be run on its matching cell plate?_____
__

13.) What is the recommended volume of compound to load in each port?_____

14.) After which number measurement is the baseline typically stable?_____

15.) True or False: The Bar Code on the XF96 sensor cartridge should be facing towards the user.

16.) True or False: The seeding surface of the XF96 cell culture plate is the same size as a typical 96-well plate.

17.) After removing the growth media from a cell plate and replacing it with unbuffered running media, where should the plate be stored?-

_____

18.) What is the Seahorse Bioscience Customer Support email address?_____

19.) When the XF96 is set up in YOUR lab, perform a baseline cell run with an acute drug response (2,4-DNP, CCCP, etc.) following the guidelines detailed in this protocol. Sample templates may be seen in Sections 12-13. Send the data file to Seahorse Bioscience to ensure proper function and set-up of your new instrument.

Seahorse *Bioscience*

Appendix X – Contact, Support and Ordering Information

**For questions about XF technology, the XF96 instrument,
Prep Station, XF experimental design, data analysis,
troubleshooting and other information:**

Email: support@seahorsebio.com

Phone (USA): (800) 671-0633, option #3
Phone (EU): +1 (978) 671-1600, option # 3

**For ordering XF96 cell culture plates,
XF96 sensor cartridges and calibrant solution:**

Email: orders@seahorsebio.com
Phone: (978) 671-1600 option #2
Fax Purchase Orders to: (978) 671-1611