Agilent

Agilent Mito-rOCR Assay Kit

User Guide

Notices

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Contents

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1 Introduction

Assay background

The Agilent Mito-rOCR assay is an innovative fluorescence plate reader-based solution designed to measure mitochondrial respiration by determination of relative oxygen consumption rates (rOCR) in adherent cells in a 96-well microplate format. The Mito-rOCR assay has been designed for use with time-resolved fluorescence plate readers and is suitable for both monochromator- and filter-based detection modes. It is based on the Agilent Mito-rOCR reagent, a chemically stable, inert, water-soluble, and cell impermeable scalable mix-and-measure reagent for use in adherent cell culture conditions.

In this assay, the Mito-rOCR reagent is added to extracellular media in a cell culture microplate. The fluorescence signal of the Mito-rOCR reagent depends on oxygen concentration in the culture media since it is quenched by O_2 through molecular collision. As the extracellular O_2 is consumed by mitochondrial respiration, the fluorescence signal increases. The signal detected is inversely proportional to the amount of extracellular O_2 in the media.

The Mito-rOCR assay has been designed for use with Agilent cell culture imaging microplates, which have been shown to have low autofluorescence and deliver optical clarity comparable to glass. The Mito-rOCR assay kit also contains an innovative 96-well, custom plastic lid, the Mito-rOCR Seal Lid, which is inserted into the microplate wells immediately before starting the assay measurements. The seal lid generates a semiclosed microchamber that limits oxygen diffusion from the atmosphere, removing the need to add sealant reagents such as oil and facilitating the assay workflow. The small chamber created by the seal lid contributes to the high increase in sensitivity observed when compared to oil-based solutions for the measurement of changes in oxygen concentration. It delivers reliable rOCR measurements within minutes, even when working with low respiratory cell types. The seal lid is compatible with bottom and top fluorescence reads. In addition, it can be easily removed at the end of the assay, allowing easy access to the cells to perform other cellular assays.

The assay measurements are performed by placing the Mito-rOCR microplate and seal lid into the Mito-rOCR magnetic holder. It keeps the Mito-rOCR seal lid position at a fixed distance from the bottom of the well and guarantees uniform microchamber volume and measurements across the entire 96-well plate, with a significant increase in well-to-well assay consistency and sensitivity compared to oil-based assays.

From the raw time-resolved fluorescence measurements, relative rates of oxygen consumption (rOCR) in the sample can be easily obtained using the Mito-rOCR Analysis Module. The module is found within Agilent Seahorse Analytics, a cloud-based platform that enables plate reader data import, signal linearization, autoslope calculation, and generates data tables and bar charts for easy data interpretation.

In addition, the assay includes positive and negative control reagents to help define the assay's signal window and dynamic range, avoid out-of-range signals, and support data analysis and interpretation.

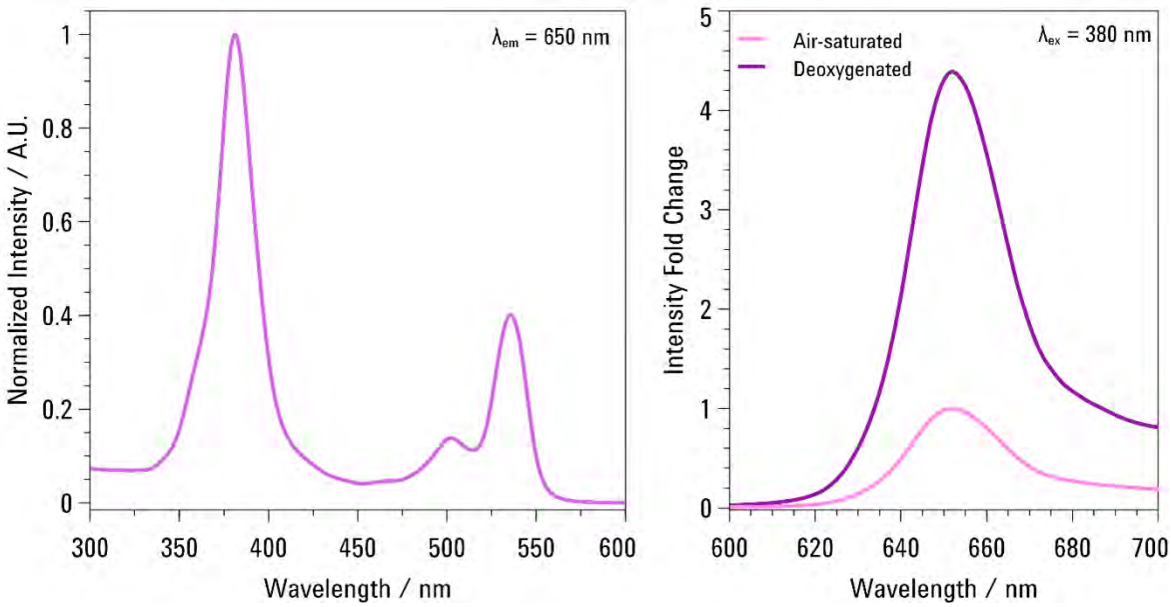


Figure 1. Excitation and Emission spectra of the Mito-rOCR reagent. The left panel shows a normalized excitation (excitation 360 to 400 nm; peak 380 nm). The right panel shows emission (emission 630 to 680 nm; peak 650 nm) in oxygenated and deoxygenated conditions.

Glossary

OCR

Oxygen consumption rate

rOCR

Relative oxygen consumption rate

RFU intensity

The raw, time-resolved intensity signal the rOCR reagent measured in the fluorescence plate reader expressed as relative fluorescence unit (RFU)

Linearized intensity

Intensity data normalized relative to the background signal and linearized using a natural logarithm equation (see section 4), expressed in arbitrary units (AU)

Mito-rOCR reagent

Soluble phosphorescence sensor reagent detecting extracellular oxygen level

Assay media

Cell culture media used during Mito-rOCR assay. It can be either Agilent XF assay media supplemented with glucose, pyruvate, and glutamine or the cell culture media used during cell culture growth and maintenance

Agilent XF assay media

DMEM- or RPMI-based assay media free of phenol red, bicarbonate, glucose, glutamine/GlutaMAX, or sodium pyruvate, allowing specific customization. The pH is preadjusted to 7.4 for easy assay media preparation. Recommended in Agilent Seahorse XF analysis

Mito-rOCR assay media

Assay media containing Mito-rOCR reagent

Blank well

Control group containing assay media only (without Mito-rOCR reagent or cells). Used for the calculation of assay signal-to-blank ratio in Mito-rOCR data QC

Background well

Control group containing Mito-rOCR assay media only (with the Mito-rOCR reagent but without cells). Use for background well signal detection. This measurement is used during data analysis in Seahorse Analytics for signal linearization and rate calculations

GOx

Glucose oxidase enzyme. Catalyzes the oxidation of glucose to d-glucono-δ-lactone, consuming oxygen that is converted in hydrogen peroxide (H_2O_2)

GOx well

Positive control group containing Mito-rOCR assay media and GOx reagent. Glucose needs to be supplemented if the assay media doesn't include it. Does not contain cells. Used as a positive control of the assay, ensuring that the assay is functioning correctly by producing a strong signal. Used to adjust instrument gain for fluorescence measurements

Rot/AA well

Negative control group well, containing cells in culture media, Mito-rOCR reagent, and mitochondrial complex I and III inhibitor mix (1 µM rotenone and antimycin A, each). Helps to identify nonspecific signals, due to nonmitochondria-dependent oxygen consumption

Vehicle well

Well(s) with cells treated with solvent or medium used to dissolve compounds tested in other experimental wells. Contain cells in culture media and Mito-rOCR reagent and the same volume of vehicle solvent added to experimental wells. Only included in the assay as a control group when comparing the effect of test compounds in mitochondrial respiration

Mito-rOCR seal lid (seal lid)

Microplate lid with pillars specifically designed to form a microchamber in each well when assembled to the cell culture microplate. Minimize oxygen exchange with the environment and increase assay sensitivity

Condensation ring lid

Conventional plate lid for Agilent 96-well microplate.

2 Kit Information

Kit contents

Table 1 Mito-rOCR Assay Kit and Accessories

Part Number	Product Name	Contents	Quantity
MO-400-4	Mito-rOCR Assay Starter Kit	Mito-rOCR Magnetic Holder	1
		Mito-rOCR Analysis Module	3 users
		Mito-rOCR Microplate Pack, lids/plates	4
		Mito-rOCR Assay Pack, reagents	4
MO-300-4	Mito-rOCR Assay Kit	Mito-rOCR Microplate Pack, lids/plates	4
		Mito-rOCR Assay Pack, reagents	4
MO-200-4	Mito-rOCR Microplate Pack	Seal lid and 96-well microplate	4
MO-100	Mito-rOCR Magnetic Holder	Metal top plate and magnetic base plate	1
MO-500	Micro-rOCR Analysis Module	Software license key	3 users

Additional required items

The assay requires the following items that are not included in the kit:

- Fluorescence plate reader with time-resolved fluorescence (TRF) function and equipped with temperature control (see the Instrument and Software Compatibility section)
- Cell culture media
- dd H_2O
- XF Assay media and supplements (optional, part number 103680-100 or 103681-100)
- Microplate heating block for plate preparation (e.g. Agilent RTCA E-Plate Temperature Control Accessory, part number 6488447001)
- Multichannel pipette and tips
- Pipetting reservoir

Note: Mito-rOCR can be measured in the presence of culture media and serum. The XF DMEM or RPMI, pH 7.4 media supplemented with fuel substrates (glucose, glutamine, pyruvate) can provide control assay conditions free from phenol red and serum, facilitating rOCR measurements and live-cell fluorescence imaging.

Kit storage and handling

- The Agilent Mito-rOCR Assay Kit is shipped at room temperature. Upon arrival, the Mito-rOCR reagent vials and Agilent Glucose Oxidase vials are stored between +2 to 8 °C before being reconstituted. If reconstituted in water, the stock is stored at –20 °C for use within one month (avoid freeze-thaw cycles).
- Agilent Rotenone + Antimycin A (Rot/AA) reagent vials are stored at room temperature and once reconstituted, must be used immediately.
- The Mito-rOCR Seal Lid and 96-well microplate is stored at room temperature.
- The Mito-rOCR magnetic holder is stored at room temperature. Do not leave the metal top plate unsupported between the magnets. If unsupported, the metal top plate can bend over time, requiring a replacement of the magnetic holder. Keep the top plate either supported by a microplate or place it beneath the magnetic holder metal base plate (Figure 2).
- The reagents are stable for one year from the manufacturing date. The actual expiration date is printed on the label of the assay kit box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 3 and 12 months.

Table 2 Storage condition of kit contents and accessories

Item	Storage
Mito-rOCR reagent	+2 to 8 °C
Glucose Oxidase	+2 to 8 °C
Rotenone/Antimycin A	Room temperature
96-well cell culture microplate/seal lids	Room temperature
Magnetic holder (metal top lid and base plate)	Room temperature

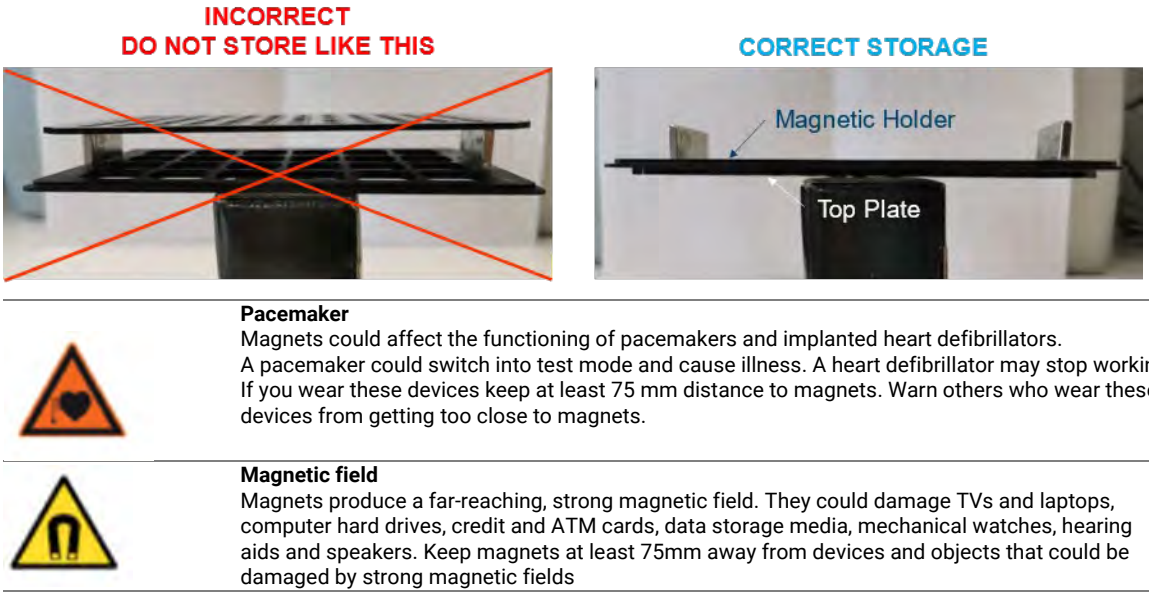


Figure 2. Magnetic holder recommended storage condition,

Instrument and software compatibility

The Agilent Mito-rOCR assay can be run using Agilent BioTek fluorescence plate readers equipped with temperature control and with monochromator- or filter-based time-resolved fluorescence capabilities using Gen5 software version 3.16 or higher. Additional instrument compatibility information is available in section 5. Plate Reader Setup Guide.

3 Assay Workflow

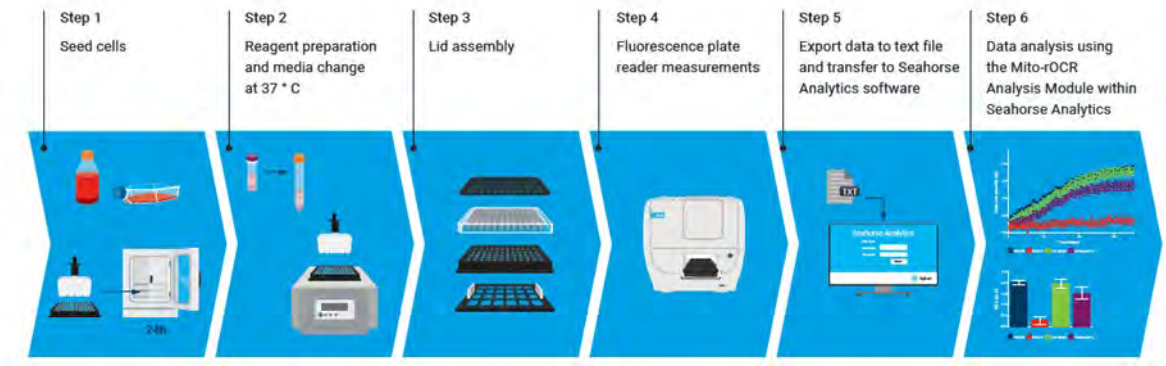


Figure 3. Summary workflow for Mito-rOCR.

Note: To ensure accurate assay setup, calculations, and troubleshooting, it is crucial to follow the critical steps:

- Blank and background wells must be assigned for a signal-to-blank assessment and background correction, respectively.
- GOx wells must be assigned to set the gain (sensitivity) appropriately.
- The plate temperature must be maintained at 37 °C until loaded into a prewarmed plate reader. It is highly recommended that the plate be prepared and handled on a plate heating block or warmer.

Pre-assay preparation

Plate reader setup (Agilent Gen5 protocol preparation)

- Create or download a Mito-rOCR Gen5 protocol file, choosing the Agilent Mito-rOCR plate definition and recommended instrument settings. Do not use default or standard plate definitions.
- Optimal wavelengths are 380 nm for excitation and 650 nm for emission with TRF options. The key parameters required for the instrument setup are listed in Table 3.
- Gen5 protocols and the Mito-rOCR plate definition file are available to download in the Support section of the Mito-rOCR website: www.agilent.com/lifesciences/mito-rocr.

Table 3 Gen5 protocol recommended set up parameters for Mito-rOCR assay

Required Parameters	Monochromator	Filter Cube
Temperature setpoint	37 °C	37 °C
Kinetic/run time (min)	45	45
Kinetic/interval (min)	1	1
Excitation (nm)	380/20	380/20
Emission (nm)	650/20	645/15
Dichroic mirror (nm)	NA	400
Top (nm)	NA	100
Optics Position	Bottom (preferred)/top	Bottom (preferred)/top
Measurement option/Delay after plate movement (msec)	30	30
Measurement option/Measurements per data point	10	10
Measurement option/Dynamic range	Standard	Standard
Time-resolved option/Delay (µsec)	30	30
Time-resolved option/Data collection time (µsec)	30	30

Note: Plate layout and additional information added in Gen5 experiment files will not be imported to Mito-rOCR Analysis Module of Seahorse Analytics. Plate layout information should be added to the Mito-rOCR Analysis Module during data analysis.

Plate and magnetic holder preparation

- Before starting the assay, it is important to prewarm all the assay components to ensure proper temperature equilibration. It will minimize temperature drift during plate reader measurements.
- Place the Mito-rOCR Magnetic Holder (including base plate and metal top plate) and the packaged Mito-rOCR Seal Lid into a 37 °C incubator for at least one hour.
- Overnight incubation while the cells are adhering to the microplate is recommended. Refer to the storage and handling guidelines in the previous section (Figure 2).
- Prewarm and maintain assay media at 37 °C until plate preparation to ensure temperature consistency.

Note: The Agilent Mito-rOCR assay can measure the rOCR of cells stably adhered to Agilent 96-well cell culture microplates. The cell culture duration prior to the assay varies depending on the cell type, although an overnight culture is commonly used for most cell lines. Optimal cell seeding number varies by cell type but is typically between 1.5 and 6 x 10^4 cells per well. Generally, densities resulting in 50 to 90% confluency generate rOCR in the recommended dynamic range of the assay. A cell density titration experiment is recommended to determine the optimal cell number per well.

Mito-rOCR assay setup

Step 1: Cell culture plate preparation

1 Harvest and resuspend the cells to the desired final concentration to seed in 100 µL of growth medium.

2 Seed 100 µL of cell suspension per well on the Agilent 96-well cell culture microplate provided in the kit using standard cell culture procedures. Do not seed cells in control wells assigned for Background, Blank and GOx. The recommended template uses wells H1 to H6 for no cell reference group as shown in Figure 4 and Table 4. Be sure to add medium only (no cells) in these wells.

3 IMPORTANT: Allow the plate to rest at room temperature in the tissue culture hood for one hour to facilitate even cell distribution and reduce edge effects for some cell types. Monitor adherence using a microscope.

4 Allow the cells to grow overnight in a cell culture incubator. Evaluate the growth and health of cells using a microscope.

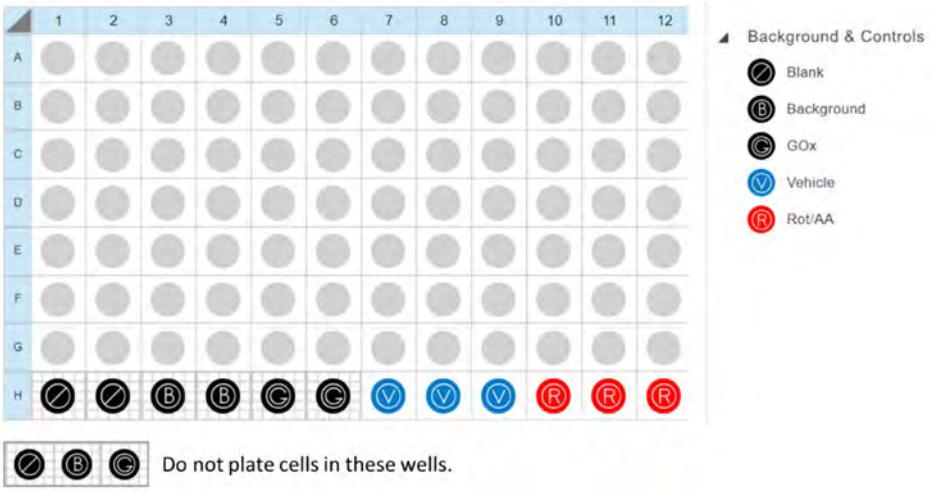


Figure 4. Recommended cell seeding layout using wells H1 to H6 as the cell-free control groups Background, Blank, and GOx.

Table 4 Control group well assignment

Control Groups	Cell	Mito-rOCR Reagent	GOx	Rot/AA	No. of wells[2]	Location[2]
Blank	No	No	No	No	2	H1, H2
Background	No	Yes	No	No	2	H3, H4
Gox[1]	No	Yes	Yes	No	2	H5, H6
Vehicle	Yes	Yes	No	No	3	H7, H8, H9
Rot/AA	Yes	Yes	No	Yes	3	H10, H11, H12

[1]Add glucose if the assay media does not contain any GOx substrate

[2]Recommended but can be modified

Note: Ensure Blank, Background, and GOx wells do not contain any cultured cells. The location and number of the control group wells (Blank, Background, GOx, Vehicle, and Rot/AA) can be modified depending on the user preference.

Step 2. Reagent preparation and media change

Step 2.1. Reagent preparation

1 Place assay media (cell culture media or supplemented XF assay media) in a 37 °C water bath.

2 Resuspend the Mito-rOCR reagent in 1 mL of assay media by pipetting up and down several times.

3 Resuspend the Agilent GOx in 100 µL of ddH_2O.

4 Resuspend the Agilent Rot/AA in 106 µL of assay media to prepare a 51x stock solution (51 µM).

5 Glucose is essential for the GOx controls. If you are unsure whether your media contains glucose, add 1 µL of 1 M glucose to only the GOx wells.

6 (Optional) Prepare stocks of test compounds/treatments and vehicle (51x final concentration for 1 µL additions or 11x final concentration for 5 µL additions). See sstep 5.

Note: Make sure that all the reagents are fully resuspended before starting the assay.

Step 2.2. Media preparation

1 Add 1 mL of Mito-rOCR reagent (from step 1) to 5.5 mL of assay media to make 6.5 mL of rOCR assay media and keep at 37°C.

2 Keep an aliquot of assay media without Mito-rOCR reagent at 37°C.

Table 5 Reagent and media preparation

Compound	Resuspend in:
Mito-rOCR reagent	1 mL assay media
GOx	100 µL ddH_2O
Rotenone/Antimycin A (51x)	106 µL assay media
Test compound (11x)	11x stock in vehicle or assay media
Media	**Preparation**
Assay media	Growth media or XF assay media with supplements
Mito-rOCR assay media	1 mL Mito-rOCR reagent + 5.5 mL assay media

Step 2.3. Media exchange

1 Carefully aspirate the culture media in all the wells of the cell culture microplate using an aspirator or pipette, ensuring minimal residual media.

2 Add 50 µL of Mito-rOCR assay media to all the microplate wells, *excluding the blank wells.*

3 Add 50 µL of assay media to blank wells - *no Mito-rOCR reagent.*

Note: We recommend that all assay media and stock solutions to be used in the assay are prewarmed at 37 °C prior to use. Use a plate block heater for plate preparation and prewarm the fluorescence plate reader to measurement temperature.

Note: Background wells are critical for rOCR calculation during analysis on Seahorse Analytics. It is imperative that the assay has at least one background well in the plate, otherwise, rate calculation cannot be performed.

Step 2.4. (Optional) Acute test compound treatment

1 Test compounds can be added to assigned wells when cotreatment is required during Mito-rOCR reading.

2 Add compounds and vehicle control to the test wells. The recommended maximum final volume is 55 µL and the stock concentration is from 51x to 11x to add 1 to 5 µL, respectively.

3 Vehicle well: Add vehicle used for test compound preparation to wells H7 to H9 (1 to 5 µL depending on the volume used for compound treatment).

Step 2.5. Control well setup

1 Incubate the plate in the microplate reader at 37 °C for 10 minutes with the condensation ring lid to prevent evaporation.

2 Remove the cell culture microplate (containing Mito-rOCR reagent) from the microplate reader and close the plate reader to maintain temperature. Add the following assay control reagents:

- Rot/AA well: Add 1 µL of Agilent Rot/AA to wells H10 to H12 (final concentration 1 µM) as negative controls for mitochondrial respiration
- GOx wells: Add 5 µL of Agilent GOx to GOx wells (H5-H6).

Note: GOx addition should be performed immediately before adding the rOCR seal lid and starting the run. GOx wells are critical for proper instrument gain adjustment for fluorescence measurements.

Step 3. Lid and magnetic holder assembly

Note: GOx addition should be performed immediately before adding the rOCR Seal Lid and starting the run. GOx wells are critical for proper instrument gain adjustment for fluorescence measurements.

1 Remove Mito-rOCR Magnetic Holder (plate base and metal top lid) and rOCR Seal Lid from the incubator.

2 On a plate warmer, place the microplate onto the prewarmed magnetic holder base plate.

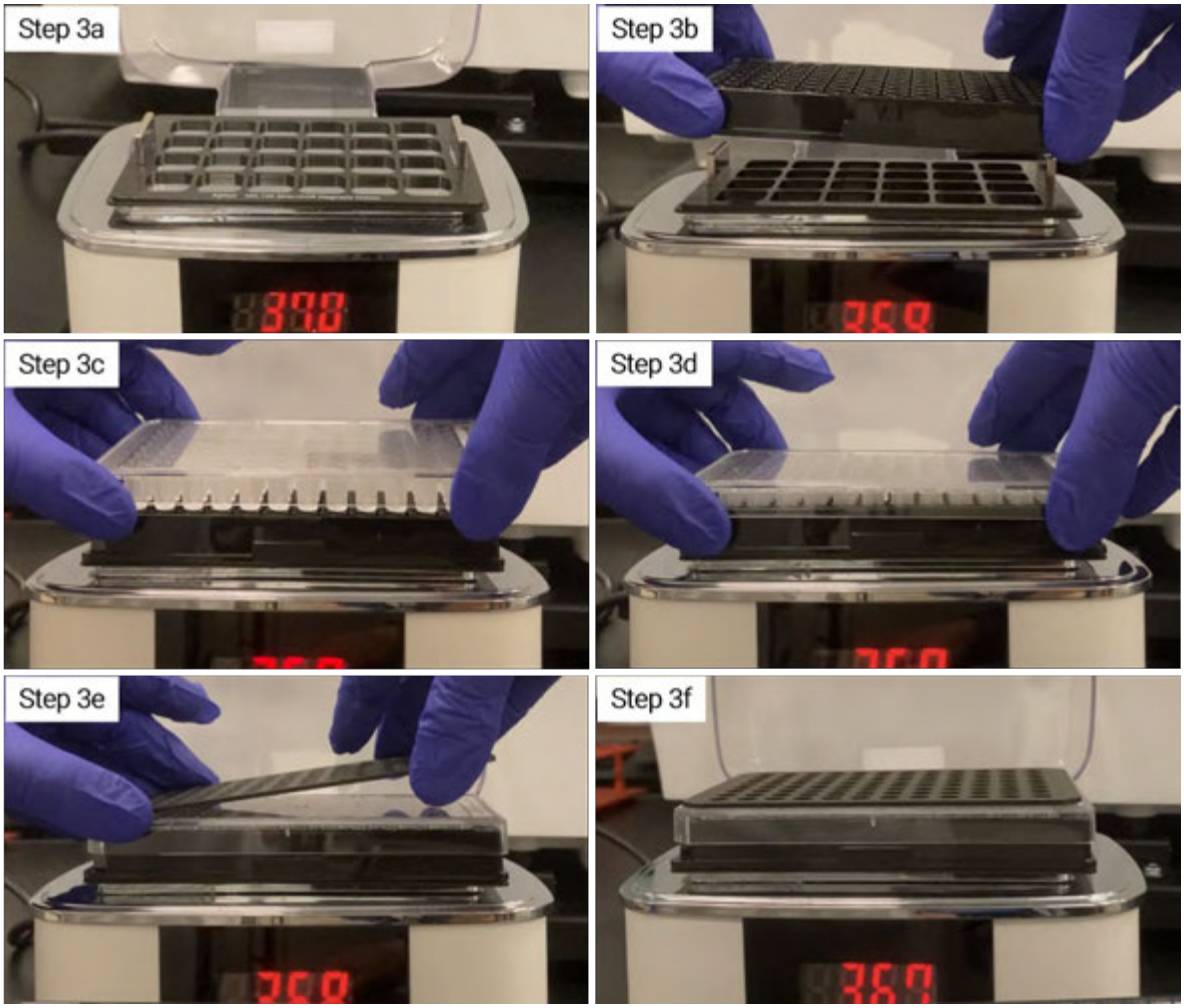
3 Remove the condensation ring lid from the cell culture microplate.

4 Align the rOCR Seal Lid with the plate.

5 Carefully insert the rOCR Seal Lid vertically down into the microplate (See Figure Steps 3a-c).

6 Carefully add the metal top plate on top of the rOCR Seal Lid (See Figure Step 3d).

7 Make sure that the cell plate sits flat on the magnetic base plate (Figure Step 3e).



Step 4. Fluorescence plate reader measurements

Step 4.1. Mito-rOCR measurements

1 Read the plate immediately in the temperature-controlled fluorescence microplate reader. The plate should be read kinetically for at least 45 mins.

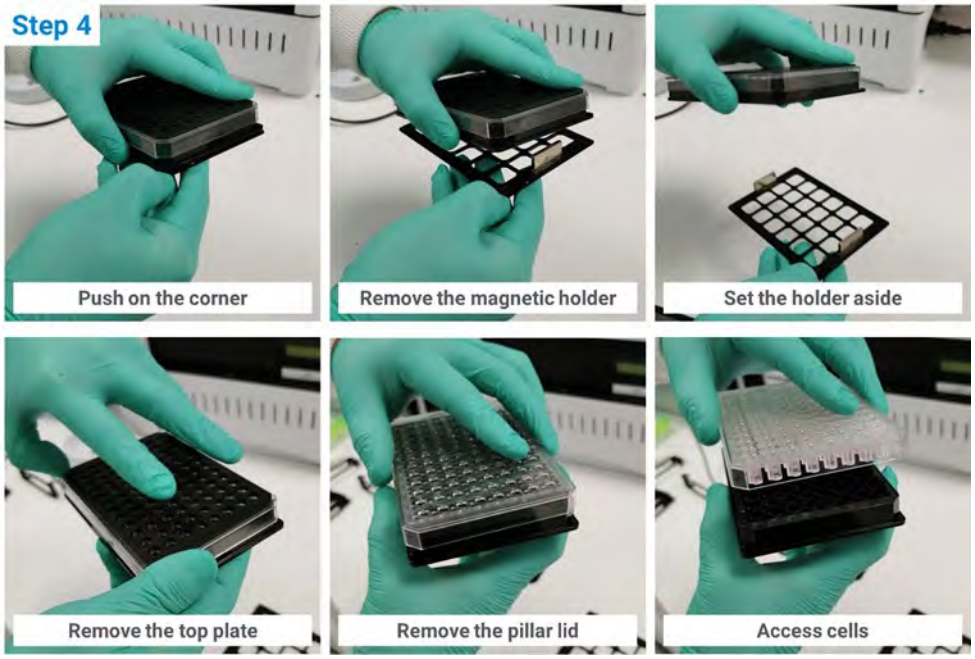
2 When prompted, select the wells containing Agilent GOx, (default H5 and H6) for gain adjustment.

3 Upon completion, remove the Mito-rOCR Magnetic Holder containing the cell culture microplate and save the experiment file.

Step 4.2. Plate disassembly

1 Once the run has been completed, disassemble the Mito-rOCR magnetic holder plate base, and top metal plate to remove the cell culture microplate. It is easier to remove the magnetic holder base plate first by pushing down on a corner or grabbing the bottom of the holder using the grid for grip (see image). Once the base plate with the magnets have been removed, remove the top metal plate.

2 Remove the rOCR Seal Lid from the microplate.



Step 4.3. (Optional) Post-assay image assessment

1 After completing the Mito-rOCR assay, cells can be used for multiplexing analysis. Microscopic imaging or any multimodal plate-reader-based analysis may be feasible even without removal of the rOCR seal lid if probes were added previously and there is no spectral interference between mito-rOCR and additional fluorescent probes. If desired, rOCR assay media can be replaced with fresh assay media for sequential multiplexing analysis.

2 If the rOCR Seal Lid interferes with the post-assay workflow, remove the rOCR Seal Lid as described above and replace it with the cell culture microplate lid. In addition, prolonged incubation in the presence of the seal lid can cause extended hypoxic stress on cells because the microchamber formed by the seal lid highly limits oxygen exchange.

Step 5. Data export to text file and transfer to Seahorse Analytics software

1 Export data as a text file (.txt) to perform data analysis using the Mito-rOCR Analysis Module within Seahorse Analytics. Exporting data in the incorrect format will result in error messages when data is uploaded to Seahorse Analytics. See FAQ section for the parameters required for data export set up.

2 The Mito-rOCR Gen5 protocol files contain a predefined exporting function which is ready to use.

Note: The exported data do not include any layout information regarding the cell seeding or treatments. The layout information must be recorded separately and applied through the template design function in the Seahorse Analytics software.

4 Data Analysis

Note: A Mito-rOCR Analysis Module software license key is required and can be used to support three users. One software license is included in the Mito-rOCR Assay Starter Kit (part number MO-400-4). Please ensure that users have a Seahorse Analytics account before assigning a Mito-rOCR license seat. Additional licenses can be purchased separately (part number MO-500). For more details, see the FAQ section.

Data import to Mito-rOCR Analysis Module within Seahorse Analytics

- Login to Seahorse Analytics: https://seahorseanalytics.agilent.com/. Make sure the Mito-rOCR software license key is activated, and your User ID is assigned to one of the three license seats. See the FAQ section for instructions about registering your license key.
- Upload the exported .txt file from your experiment.

Template design

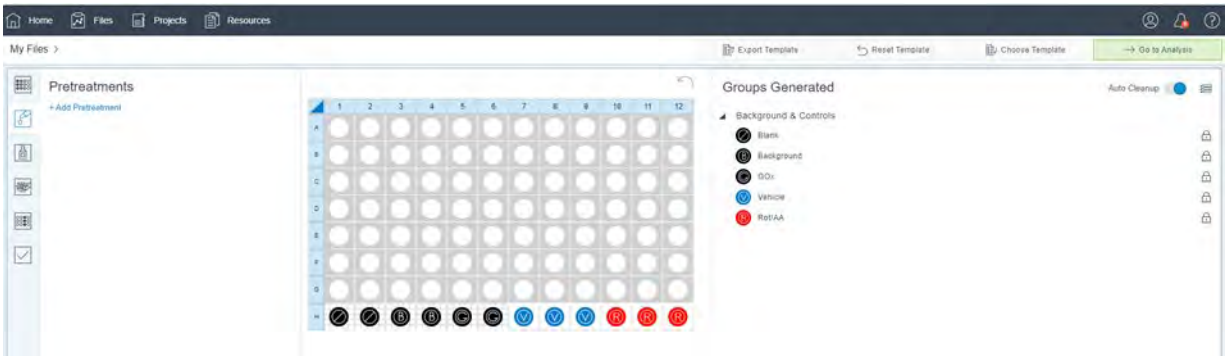
Note: The exported data do not include any layout information regarding the cell seeding or treatments. The layout information must be recorded separately and applied through the template design function in the Seahorse Analytics software.

1 Define plate layout ensuring that Control wells are assigned correctly. Control wells assignment is required for data analysis.

2 Create Experimental Groups and assign to the Plate Map. The setup menus for pretreatment and compounds titrations are available. Additional information about assay conditions can be included in the Plate Layout definition.

3 When Plate Layout is completed, click on the Go to analysis button (→ Go to Analysis), on the top/right corner of the screen.

4 (Optional) Use the Catalog menu in User Information tab ( ) at the top/right to create or edit frequently used group definitions such as pretreatments, media, and cells.



Mito-rOCR analysis view

1 Select in the Mito-rOCR View the Widgets that you want to display in the final report (Kinetic Chart, Bar Chart/Heat Map, Titration, Data Table) as well as what groups to display.

- Kinetic Chart

  The kinetic graph displays the linearized intensity or the non-linearized intensity versus time for each assay group. This kinetic data is used to calculate the slope (rOCR) using the linear portion of the signal curve.

- Bar Chart/Heat Map

  The automatically calculated rOCR of each well and group are displayed either as a bar chart or a heat map.

- Titration

  The rOCR versus a point-to-point trace of compound concentration is available when a titration template setup is applied.

- Data Table

  A summary of assay results is reported as in a table format.

2 A data analysis report can be exported to Microsoft Excel and GraphPad Prism data format.

3 A data QC function (yellow triangle icon on the top right bar) is provided to review any warnings that require attention.

Data analysis

Relative oxygen consumption rates are seamlessly calculated using the Mito-rOCR Analysis View within Seahorse Analytics. The rOCR is obtained by applying the four steps described below and rates are expressed as AU/h.

1 RFU Intensity Plotting

The raw, time-resolved intensity signal data obtained from a plate reader is uploaded and plotted as RFU.

2 Data Linearization

The raw time-resolved intensity is normalized relative to the background signal, using a natural logarithm equation:

$$\text{Linearized Intensity (AU)} = ln\left(\frac{[\text{RFU}]_{Sample}}{[\text{RFU}]_{Background}}\right)$$

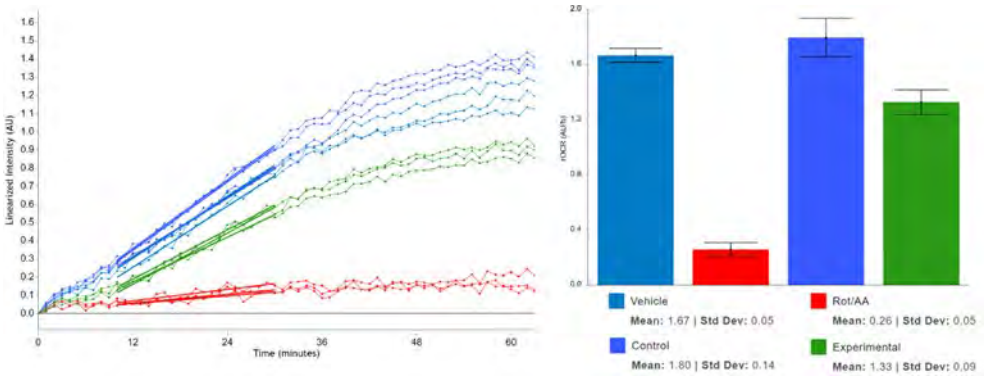
where $[\text{RFU}]_{Sample}$ and $[\text{RFU}]_{Background}$ represent time-resolved signals at a given time obtained from a sample well and the background control group, respectively. This transformation accounts for the nonlinear RFU probe signal response respective to oxygen concentration. Baseline of the linearized kinetic curve is available, by using the linearized intensity at t = 0 as reference.

3 Data Smoothing

A Savinsky-Golay filter is used to apply smoothing to the linearized data. A more aggressive secondary smoothing option becomes available when the software detects a high noise level in the kinetic data. The secondary smoothing applies a wider smoothing window than the default setting.

4 rOCR calculation

The rOCR is obtained by identifying the linear portion of the linearized intensity kinetic data and calculating the slope. A linear regression fit is applied, and the fitted slope value in AU/h unit is used for rOCR. The linear region of the kinetic graph is automatically assigned by the software depending on the signal increase rate. For wells where the kinetic curve doesn't reach a signal plateau during the assay, the slope is calculated from the linear fit of the data between 10 and 30 minutes. For the kinetic curves where the signal has a fast increase and reaches a plateau, the linear interval and regression are automatically determined by iterative linear regression calculation of the data between 0 min and the time around the beginning of the plateau.



5 Agilent Plate Reader Setup Guide

Monochromator-based reader setup

Models

- Synergy H1
- Synergy Neo2
- Cytation 1
- Cytation 5
- Cytation 7
- Cytation C10

There is no filter setup needed for model configurations including monochromator-based fluorescence capability. Use predefined Mito-rOCR Gen5 protocols or define the parameters in the Gen5 protocol as described above (Table 3).

Filter-based reader setup

Models

- Synergy H1
- Synergy Neo2
- Cytation 1
- Cytation 5

To run the Agilent Mito-rOCR assay on a filter-based fluorescence plate reader configuration, specific filter cubes are required (Table 7).

Table 7 Fluorescence filter cube selection for Agilent F-model instruments

Instrument	Part number
Synergy H1	8040587
Synergy Neo2 Hybrid	1035123 (plus single emission top cube 1035003 for dual PMT configurations)
Cytation 1	8040587
Cytation 5	8040587

Plate type setup

Plate type setup is required for both filter and monochromator plate readers.

Option 1: Importing plate type

In Gen5, system control setup

✓ Select System > Plate Types > select Import > select the Mito-rOCR Plate Type.xml file

Option 2: Adding plate type

✓ Select the Add Plate button and then enter the values in Figure 5 and name the plate Mito-rOCR Plate.

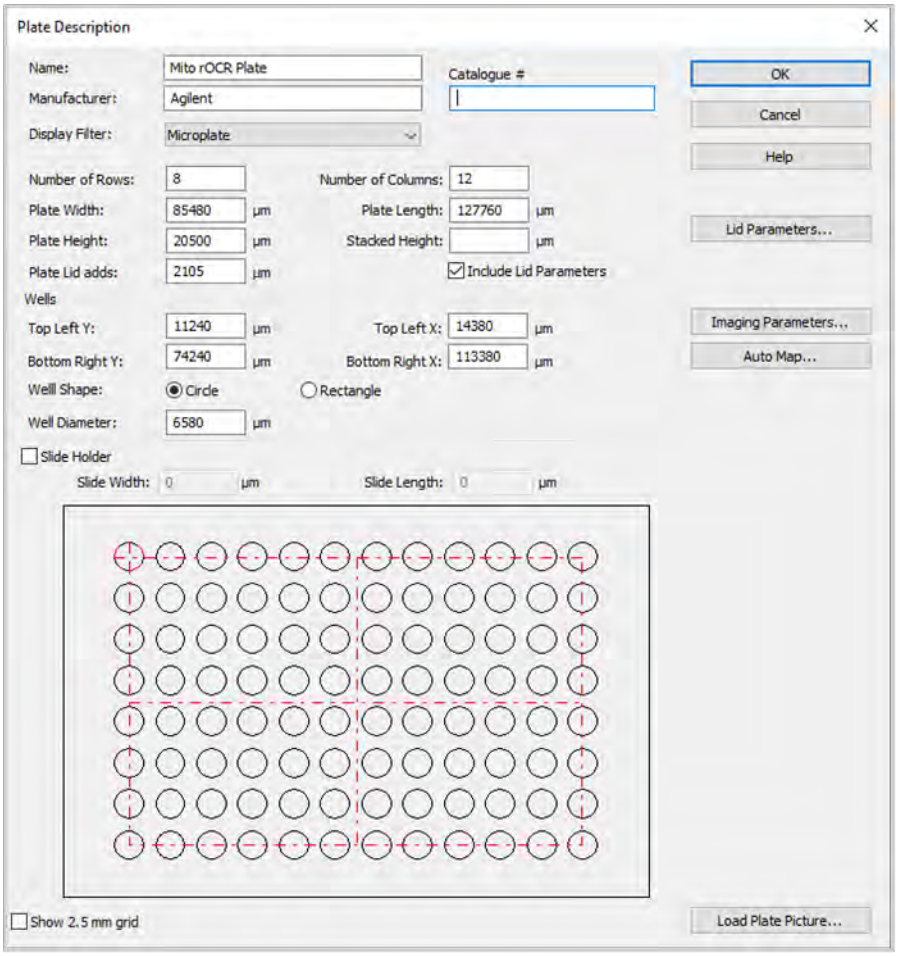


Figure 5. Mito-rOCR plate type parameters.

6 Frequently Asked Questions

- How do I register the Mito-rOCR Analysis Module license key?

  The license key is provided on a card included in the Mito-rOCR Assay Starter Kit (MO-400-4) or Mito-rOCR Analysis Module (MO-500). One Mito-rOCR Analysis Module license key is valid to be used by three users at a time and can be transferred between users up to a maximum of three times.

  Keep a safe record of this license key for registrations and license seat transfers. If lost, a sales order number will be required to retrieve the license key.

  Registration Instructions:

  - Create a Seahorse Analytics account or log on to an existing account: https://seahorseanalytics.agilent.com/
  - Go to User Information (upper right) and click Settings & User Data
  - Select Mito-rOCR License Keys and add license key

- Is modification of the position or the number of control group wells allowed?

  The position of control wells in the plate map can be modified. If a modified plate layout is used or there is a change in the control well location or number due to pipetting errors or by misplacing them, then they should be correspondingly assigned in Seahorse Analysis software by editing the template.

- What if all the wells were seeded with cells and no control groups were assigned?

  If you plated cells into background wells, you cannot process the results using Seahorse Analytics.

- Can the Mito-rOCR reagent be stored after reconstitution?

  Once reconstituted, Mito r-OCR reagent and GOx should be stored at –20 °C. Use the reconstituted material within one month, avoiding additional freezing-thawing. Rot/AA reagent should be reconstituted immediately before use.

- What is the maximum volume of assay media per well that can be utilized?

  The Mito-rOCR Assay media volume per well should not exceed 55 µL. The addition of bigger volumes of assay media can cause liquid overflow when the seal lid is assembled, and the pillars are inserted in wells.

- Why did I receive the message "An error occurred performing operation" when I tried to assign a user seat in the Mito-rOCR software license?

  When assigning a license seat, each email address first needs to be registered for an SHA account. Please ensure the User ID is correct and has already been registered for a Seahorse Analytics account before assigning to the Mito-rOCR software module users.

- Can I modify the data exporting setting?

  The setting can be newly generated or modified. However, the key parameters shown below need to be kept to analyze the data with Seahorse Analytics because data in the incorrect format can cause uploading failure.

Parameters	Setup
Content/Data/include	Raw data
Content/data/general format	Row-wise table Regroup data in one matrix/table when possible
Content/data/kinetic/spectrum/scanning data format	Row-wise table
Workflow	Auto-execute on completion of the procedure
Workflow/export mode	Each plate in a separate file
Format/include	Headings Statistic column labels
Format/separator	TAB
File/extension	txt

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