Technical Overview

Cell Metabolism

Agilent

Trusted Answers

Determine Mitochondrial Complex Activities in Permeabilized Cells

Using the Agilent Seahorse XF Electron Flow Assay with Agilent Seahorse XF PMP

Authors

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Abstract

The study of mitochondrial function is central to both clinical and basic scientific research. Mitochondrial respiration measurements using intact cells can help define metabolism and its dysregulation in fields such as cancer, immunology, and mitochondrial toxicology. Using intact cells with Agilent Seahorse XF assays can provide an overall cellular and mitochondrial bioenergetic profile. However, because changes in mitochondrial function may correlate to altered mitochondrial substrate oxidation, examining the oxidation of specific substrates is one approach to determining the mechanism underlying differences observed in mitochondrial activity using intact cells.

The Agilent Seahorse XF Plasma Membrane Permeabilizer (PMP) allows for experimental control over specific substrate provision to the mitochondria. It does this by providing safe and consistent permeabilization of the plasma membrane in situ and eliminating the need for isolation of mitochondria from cultured cells or use of detergents (digitonin) for cell permeabilization. This enables assessment of multiple mitochondrial substrate oxidation pathways in a single experiment, providing direct functional measurement of Complex I (CI)-, Complex II (CII)-, and Complex IV (CIV)-linked respiration. Thus, in cases where a relevant cell model is available, including established cell lines and primary cells, the permeabilization of the plasma membrane in situ via XF PMP is highly advantageous compared to isolation of mitochondria from cultured cells.

This technical overview describes how to combine XF PMP, substrates, and inhibitors with the XF electron flow assay to create a powerful approach for understanding mitochondrial function when testing the effects of interventions (genetic manipulations/compound treatments) on mitochondria. Best practices of ensuring a successful XF Seahorse electron flow assay are also included in the frequently asked questions section at the end of the document.

Introduction

Product description

The Agilent Seahorse XF Plasma Membrane Permeabilizer (PMP) is a proprietary reagent that permeabilizes intact cells in culture. It selectively targets the cellular plasma membrane while leaving the mitochondrial membrane intact. This selectivity enables deep characterization of key components in mitochondrial function, including substrate transporters, enzymes, and electron transport chain (ETC) complexes, without the need to isolate mitochondria. The exclusive reagent is a recombinant protein capable of forming pores in the cytoplasmic membrane through oligomerization, leading to permeabilization of cultured cells.

Since the action of XF PMP is consistent, a fixed concentration sufficiently permeabilizes a broad range of cell types, including cell lines, primary cells, and immune cell types. This reduces the time spent optimizing assay conditions. In experiments with XF PMP, permeabilized cells are provided with specific mitochondrial substrates. By comparing the profiles obtained with different substrates, users can identify the specific components that are responsible for altered mitochondrial function.

Standard XF electron flow assay overview

The standard Seahorse XF electron flow assay sequentially assesses CI-, CII-, and CIV-linked respiration in a single well. It can also provide information about CIII-linked inhibition. It is a comprehensive and thorough method for measuring multiple discreet mitochondrial substrate pathways. This allows a rapid and sensitive way of assessing specific and potential nonspecific effects of an intervention (for example, genetic manipulation, compound treatment) on several substrate pathways in succession.

Several XF assays, including the Cell Mito Stress Test, Substrate Oxidation Stress Tests, XF Mito Tox assay, and XF T cell and NK Cell Metabolic Profiling assays, provide multiparametric assessments of mitochondrial function using intact cells. Each of these assays reports the common parameter of maximal respiration.[1-4]

As the maximal respiration rate is governed by substrate supply and oxidation, changes (decreases) during an intervention can reflect changes in substate transport, substrate oxidation, or the ETC.[5] Examining the effect of interventions on specific substrate pathways is therefore one approach to determining the mechanism underlying the observed decreases in maximal respiration. Using the XF electron flow assay with XF PMP treated cells allows assessment of specific mitochondrial substrate pathways and the effects of interventions on mitochondrial substrate transport, oxidation, and ETC function.

The XF electron flow assay replicates the maximal respiration state of intact cell XF assays (for example, XF Cell Mito Stress Test) while simultaneously allowing control of mitochondrial substrate provision via permeabilization with XF PMP or use of isolated mitochondria. By exploiting the fact that different substrates feed differentially into mitochondrial pathways (Figure 1), it is possible to isolate which metabolic pathways are responsible for decreased maximal respiration.

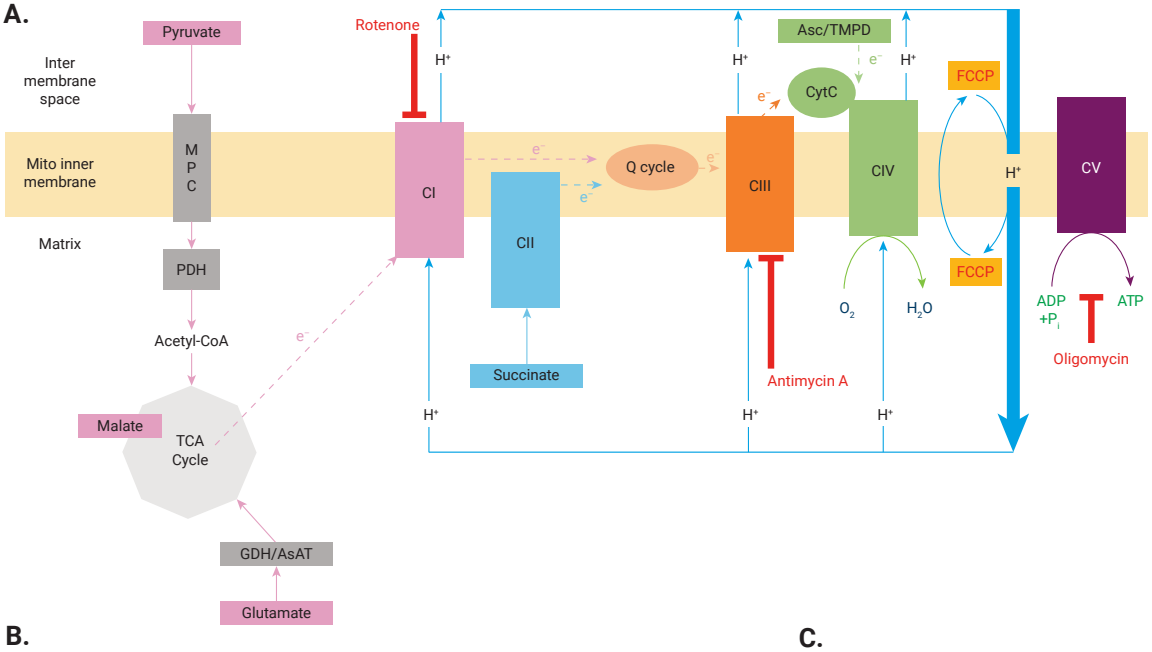
Intact cells are permeabilized with XF PMP and simultaneously provided with oligomycin, FCCP, ADP, and a complex I-linked substrate, typically a combination of pyruvate and malate or glutamate and malate. At this point, an intervention may be applied (for example, compound treatment) to the XF PMP-treated cells, and the assay is initiated.

The first series of rate measurements assess complex I-linked respiration. Rotenone is then added to inhibit all CI-linked respiration and serve a positive control for CI-linked inhibition. Next, succinate, a CII substrate is injected to CII-linked respiration. Antimycin A is then injected to inhibit CIII, blocking any CII, Q, or CIII-linked respiration. Finally, a combination of ascorbate and TMPD, a CIV substrate, is injected to assess CIV-linked respiration.

This assay design is therefore ideal for testing the effects of interventions (genetic manipulation/compound treatment) on mitochondrial function. It can offer information on elucidation of target identification, mechanism of action, and measuring efficacy and specificity.

Note: For detailed information on XF electron flow assay theory, including data analysis and interpretation, please see the companion document: Agilent Seahorse XF Plasma Membrane Permeabilizer and XF Electron Flow Assay: Data Analysis and Interpretation.[6]

A.



B.

Component	Function	Purpose
Initial assay conditions		
XF PMP	Permeabilization of plasma membrane	In situ experimental control of mitochondrial substrate provision
Oligomycin	CV inhibitor	Collectively induces/mimics conditions of maximal respiration used for intact cell XF assays
FCCP	Uncoupler	
ADP	CV/ANT substrate	
Pyruvate + malate	CI-linked substrate	Assessment of pyruvate-driven CI-linked respiration
Glutamate + malate	CI-linked substrate	Assessment of glutamate-driven CI-linked respiration
Injections		
Rotenone	CI inhibitor	+ Control for CI-linked inhibition
Succinate	CII-linked substrate	Assessment of CII-linked respiration
Antimycin A	CIII inhibitor	+ Control for CII- and CIII-linked inhibition
Ascorbate/TMPD	CIV-linked substrate	Assessment of CIV-linked respiration

C.

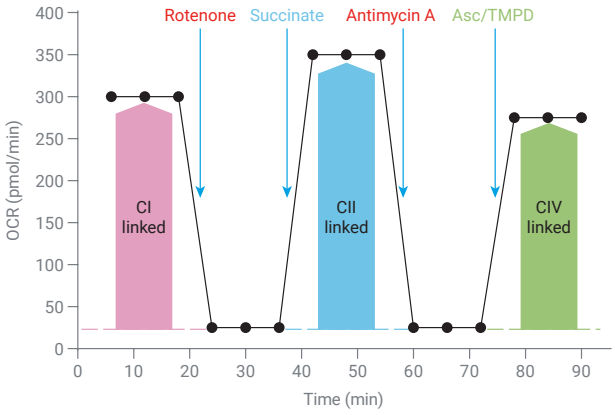


Figure 1. Agilent Seahorse XF electron flow assay design. (A) Primary substrate oxidation pathways assessed by the XF electron flow assay. Complex I-, Complex II-, and Complex IV-linked substrates are labeled in red, blue, and green, respectively. Inhibitors are shown in red. MPC = Mitochondrial pyruvate carrier, PDH = pyruvate dehydrogenase, GDH = glutamate dehydrogenase, AsAT = aspartate aminotransferase, TMPD = *N,N,N′,N′* tetramethyl-p-phenylenediamine, CytC = cytochrome C, Pi = inorganic phosphate. (B) Components, function, and purpose of the initial XF electron flow assay conditions and injection compounds. (C) Graphic depiction of XF electron flow assay kinetic trace, outlining parameters of CI-, CII-, and CIV-linked respiration.

Advantages of XF PMP and when to apply

Traditionally, pinpointing the precise mechanism underlying metabolic changes has typically involved isolating mitochondria. Respiration measurements using isolated mitochondria enable control over which substrates are oxidized. However, the isolation process can be complex, time consuming, and the yield and quality of the end product is often poor and prone to subselection during isolation.[7,8] This is especially true when attempting to isolate mitochondria from cultured cells.

Further, the ability to interrogate specific oxidative pathways in situ has been limited by not having a reagent that permeabilizes the plasma membranes of cells while also not damaging mitochondrial membranes. Historically, amphipathic glycosides (saponin and digitonin) have been used for cell permeabilization. But they typically require precise and repeated titration of the agent to minimize detrimental effects on mitochondrial function, including loss of cytochrome C (CytC) from the inter membrane space (Figure 2B).[9,10]

The XF PMP provides a solution to both challenges, safely and consistently permeabilizing the plasma membrane in situ.[10, 11] It also allows for experimental control over specific substrates and eliminates the need for isolation of mitochondria or use of detergents for permeabilization. In cases where a relevant cell model is available (cell line, primary cell), the permeabilization of the plasma membrane in situ via XF PMP is therefore highly preferable to isolation of mitochondria from cultured cells.

It should be noted, however, that in certain cases, the use of isolated mitochondria may be the only alternative to using intact cells or permeabilized cells. Experimental designs using whole organisms or mammals (for example, mice) require isolation of mitochondria. In these instances, isolated mitochondria can be seeded onto Seahorse XF microplates and the XF electron flow assay can be performed using Seahorse XF analyzers.[12-14]

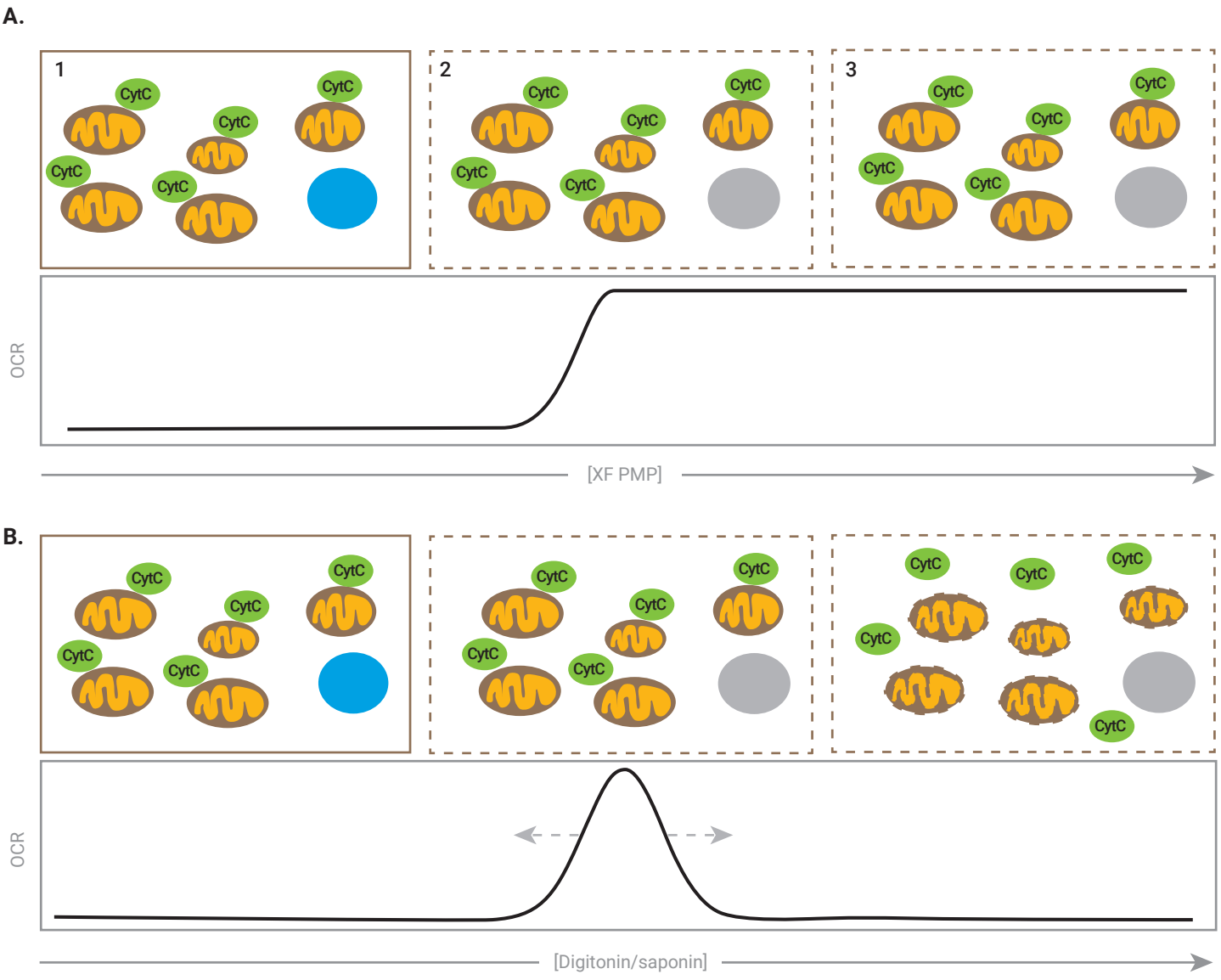


Figure 2. Agilent Seahorse XF PMP provides consistent and safe permeabilization of cells across a broad concentration range. (A) A schematic illustrating the effects of XF PMP concentration on mitochondrial integrity and OCR. Higher than saturating concentrations of XF PMP (3) do not cause decreases in the respiration rate (OCR) or result in loss of CytC from the mitochondrial membrane. (B) A schematic illustrating the effects of amphipathic glycosides (saponin or digitonin) concentration on mitochondrial integrity and OCR. In contrast, higher than optimal concentrations of amphipathic glycosides (3) cause decreases in the respiration rate (OCR) and result in loss of CytC from the mitochondrial membrane. Note the narrow optimal concentration of amphipathic glycosides, and that this optimal concentration range can shift depending on cell number and assay conditions (broken gray arrows).

Sample types compatible with XF PMP

The XF PMP reagent is compatible with numerous types of cells, including cell lines, primary cells and both adherent and suspension/immune cell types.[10, 15] Ensure the proper XF cell culture plate type is used for the cell type being investigated.

Prerequisites and considerations

The XF electron flow assay is considered an advanced assay. It is most often performed following intact cell XF assays to investigate decreases in the maximal respiration parameter and pinpoint mitochondrial targets and sites of action.

Before performing an XF electron flow assay, the following points should be carried out or considered:

- Performance of an XF assay using intact cells, typically an XF Cell Mito Stress Test or XF T Cell Metabolic Profiling assay with the cell types of interest to evaluate optimal cell density and FCCP concentration.[1, 4]
- Performance of an XF electron flow assay requires basic knowledge of running an XF assay, including cell seeding,[16] sensor cartridge handling, and injection port loading.
- XF PMP is a reagent only. It is not compatible with DMEM- or RPMI-based XF assay media designed for the assessment of intact cells. It must be prepared in mitochondrial assay solution (MAS). Other reagents and solutions must also be prepared in MAS buffer.
- The XF electron flow assay is designed for assessment of mitochondrial function only (via OCR). While ECAR is measured during the assay, it is important to note that ECAR and PER are not reflective of glycolytic activity and should not be interpreted as such.

This technical overview will show users how to perform an XF electron flow assay. Please consult the companion document for assay background and data interpretation.[6]

Nomenclature and glossary

- Permeabilized cells: Cells that have been treated so that pores are formed in the plasma membrane of the cell, allowing experimental access to the internal contents of the cell.
- Intact cells: Cells that have not had the plasma membrane permeabilized.
- Isolated mitochondria: Mitochondria that that have been separated or purified from all other cellular material.
- XF PMP: XF Plasma Membrane Permeabilizer (PMP) is a proprietary reagent that permeabilizes intact cells in culture. It selectively targets the cellular plasma membrane while leaving the mitochondrial membrane intact.
- Maximal respiration: Maximal respiration, typically induced by an optimized concentration of uncoupler (for example, FCCP, Bam15), is governed by the rate of substrate transport, rate of substrate oxidation, and ETC activity.
- Intervention: Anything done to the cell before performing the XF electron flow assay. It is also known as a pretreatment. Examples of interventions include genetic manipulation, as well as short (acute) and long (chronic) term compound exposure.
- XF electron flow assay: A four-injection XF assay that sequentially measures CI-, CII-, and CIV-linked respiration in a single well, using XF PMP-treated (permeabilized) cells or isolated mitochondria.
- Mitochondrial assay solution (MAS): Mannitol and sucrose + HEPES + $MgCl_2$ + KH_2PO_4 + EGTA
- Base XF electron flow assay medium: MAS + oligomycin + FCCP + ADP + PMP
- Complete XF electron flow assay medium: Base XF electron flow assay medium + oxidizable substrate (for example, CI-linked substrate)
- CI-linked respiration: Mitochondrial respiration linked to substrates that provide electrons (via NADH) to ETC complex I (for example, pyruvate, glutamate). This includes CI activity, as well as upstream processes, such as substrate transport, enzymatic reactions, and the TCA cycle.
- Pyruvate-dependent CI-linked respiration: Mitochondrial respiration linked specifically to oxidation of pyruvate, including upstream processes such as substrate transport and enzymatic reactions.
- Glutamate-dependent CI-linked respiration: Mitochondrial respiration linked specifically to oxidation of glutamate, including upstream processes such as substrate transport and enzymatic reactions.
- CII-linked respiration: Respiration linked to ETC complex II activity, meaning the oxidation of succinate, which generates electrons in the form of $FADH_2$.
- Coenzyme Q/CIII-linked respiration: Respiration linked to the Q cycle and ETC CIII activities that shuttle electrons from CI and CII through coenzyme Q and complex III to CytC.

– CIV-linked respiration: Respiration linked to CytC/CIV activity that results in the consumption of oxygen (O_2). Artificial substrates, such as ascorbate + TMPD, may be used to drive and measure CytC/CIV activity.

XF PMP product information

Product content

One microfuge tube containing 25 µL of 10 µM PMP solution in HNG buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol [v/v]). It contains enough material for six 24- or 96-well microplates.

Table 1. Agilent Seahorse XF Plasma Membrane Permeabilizer product content.

Compound	Function	Concentration	Quantity Per Vial
XF PMP	Selective Plasma Membrane Permeabilization agent	10 µM	25 µL

Product storage

Upon arrival, make 5 µL aliquots into fresh tubes (user supplied) and store at –20 °C. Alternatively, store at –20 °C upon arrival and make aliquots after the initial thaw/use. The reagent box contains five slots to store the 5 µL aliquots. Multiple freeze-thaw cycles are not recommended. Storage of diluted PMP reagent is also not recommended.

Product use

For most cell types, including cell lines and primary cells, a final concentration of 1 nM XF PMP is recommended. .

Additional required Agilent products

The Agilent products in Table 2 are also required for performing Seahorse XF electron flow assay in 96-well format. For information to perform the assay in 24- or 8-well formats, please refer to Appendices I and II, respectively.

Additional required reagents

Table 2. Additional required items.

Item	Part Number
Agilent Seahorse XF Pro analyzer	-
Agilent Seahorse XFe96/XF Pro FluxPak[1,2]	103792-100
Agilent Seahorse XF Pro M FluxPak[1,2]	103775-100
Agilent Seahorse XFe96/XF Pro PDL FluxPak[1,3]	103798-100
Agilent reservoir, 12 column, polypropylene	201280-100

1. Part numbers 103792-100, 1037775-100, and 103798-100 contain Agilent Seahorse XFe96 cartridges and respective cell culture plates to perform XF assays.
2. Recommended for use with adherent cell types.
3. Recommended for use with suspension cell types.

To perform the XF electron flow assay in combination with XF PMP, stock solutions of MAS, mitochondrial substrates and modulators are required. Preparation of stock solutions are outlined in Tables 3 and 4.

Preparation of MAS

Compared to intact cells, XF PMP and isolated mitochondria assays use a nonionic mannitol and sucrose-based buffer, referred to as MAS buffer (Table 3).
Note: It is not recommended to use intact cell assay media (for example, XF DMEM, RPMI) due to the high sodium/ionic content of these solutions.

Table 3. 1x MAS buffer preparation.

1x MAS Buffer			
Compound	**Final Concentration (mM)**	**Amount for 1.0 L**	**CAS No.**
Mannitol	220	40.08 g	69-65-8
Sucrose	70	23.96 g	57-50-1
KH_2PO_4	10	1.36 g	7778-77-0
$MgCl_2$	5	5 mL of 1.0 M solution	7786-30-3
HEPES	5	5 mL of 1.0 M solution	7365-45-9
EGTA	1	0.38 g	67-42-5

Add mannitol then sucrose to 700 mL of distilled/tissue culture grade water, allowing each to fully dissolve before adding the remaining components. Adjust the pH to ~ 6.5 to 7.0 by adding one to two pellets of KOH at a time. Adjust the pH to 7.3 to 7.4 with 10 and 1 M KOH solutions. Adjust the final volume to 1.0 L using distilled/tissue culture grade water. Sterilize the final solution using a 0.2 µm filter and store at 4 °C.

Preparation of mitochondrial substrates and modulators

XF electron flow assays require several mitochondrial substrates and modulators. Prepare stock solutions as described Table 4. Prepare aliquots and store at –20°C for up to two months.

Table 4. Mitochondrial substrates, modulators, and stock solutions.

Compound	Relevance	[Stock]	Amount	Final Volume (mL)	Solvent or Buffer	CAS No.
Pyruvate[1] (pyruvic acid)	CI-linked substrate	1 M	695 µL[2]	10	1x MAS	127-17-3
Glutamate (glutamic acid)	CI-linked substrate	1 M	1.47 g	10	1x MAS	56-86-0
Malate (malic acid)	Required for CI-linked respiration	1 M	1.34 g	10	1x MAS	6915-15-7
Succinate (succinic acid)	CII-linked substrate	1 M	1.18 g	10	1x MAS	110-15-6
Ascorbate (ascorbic acid)	CIV-linked substrate	1 M	1.76 g	10	1x MAS	50-81-7
TMPD	CIV-linked substrate	10 mM	23.72 mg	10	1x MAS	637-01-4
ADP (potassium salt)	ANT and CV substrate	500 mM	1 g	4	1x MAS	72696-48-1
Oligomycin A	CV inhibitor	10 mM	5 mg	0.632	DMSO or 95% ETOH	579-13-5
FCCP	Uncoupler	10 mM	10 mg	3.934	DMSO or 95% ETOH	370-86-5
Rotenone	CI inhibitor	100 mM[3]	39.4 mg	1.0	DMSO or 95% ETOH	83-79-4
Antimycin A	CIII inhibitor	100 mM[3]	25 mg	4.557	DMSO or 95% ETOH	1397-94-0
Azide	CIV inhibitor	2 M	130 mg	1.0	1x MAS	26628-22-8

1. Pyruvate solution should be made fresh every 2 weeks.
2. Amount based on a density of 1.267 g/mL.
3. 100 mM rotenone and antimycin A stock solutions should be further diluted to 10 mM (in DMSO or 95% ETOH) for a convenient working stock concentration.

1 M pyruvate, glutamate, malate, and ascorbate stock solution preparation:

1. Resuspend pyruvic, glutamic, malic, or ascorbic acid in 7 mL 1x MAS buffer.
2. Monitoring the pH and stirring constantly, add KOH pellets, one to two at a time, allowing them to dissolve completely in between additions until the pH reaches ~ 6 to 7.
3. Adjust to pH 7.3 to 7.4 using 10.0 M, then 1.0 M of KOH solutions, dropwise.
4. Add 1x MAS buffer to the final volume (10 mL).
   Note: Ascorbic acid will not dissolve at low pH. It is necessary to add the KOH pellets one to two at a time and mix until the solution clarifies (~ pH 7).

10 mM TMPD stock solution preparation:

1. Combine 23.72 mg of TMPD with 10 mL 1x MAS buffer. The solution will be blue (oxidized state).
2. Add 100 to 200 µL of the 1 M ascorbate solution (pH 7.4) prepared previously. The solution should become light brown/tan (reduced state).
3. If the solution becomes blue in storage, prepare a fresh solution.

500 mM ADP stock solution preparation:

1. Add 2.8 mL 1x MAS buffer to 1.0 g potassium (K^+) ADP
2. Adjust the pH to 7.4 using 10.0 M, then 1.0 M of KOH solutions, dropwise.
3. Add 1x MAS buffer to the final volume (4.0 mL).
   Note: ADP will not dissolve at low pH. It is necessary to add KOH and mix until the solution clarifies (~ pH 7).

Oligomycin, FCCP, rotenone, antimycin A stock solution preparation:

These compounds may be resuspended in 100% DMSO or 95% ethanol and the resulting solutions do not require adjustment of pH.

Take note of the recommended amounts and resuspension volumes to ensure the proper concentration is achieved. Rotenone and antimycin A can be prepared as 100 mM solutions, and a subsequent 1:10 dilution may be used to prepare 10 mM stocks.

2 M azide stock solution preparation:

Azide is only required if the single CIV complex assay is being performed. It is not required for the XF electron flow assay, unless a specific control for CIV-linked inhibition is desired.

1. Combine 130 mg of azide with 0.9 mL 1x MAS buffer.
2. Adjust the volume to 1.0 mL with 1x MAS buffer.

Standard XF electron flow assay workflows

XF electron flow assays may be performed using either adherent or suspension cell types. The basic workflow for each cell type is outlined in Figures 3 and 4. Note that other than differences in cell seeding timing and methods, the workflows are identical.

Adherent cell workflow

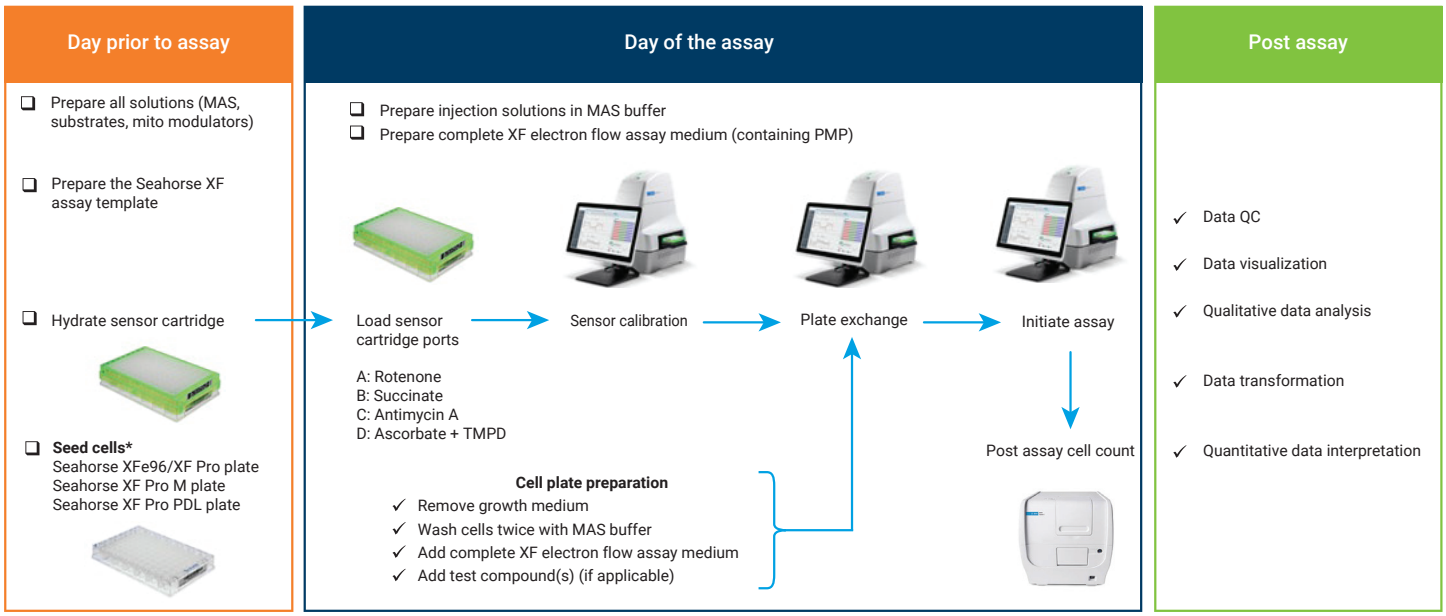


Figure 3. The Agilent Seahorse XF electron flow assay workflow using Agilent Seahorse XF PMP for adherent cell types. *Cells may be seeded more than one day before the assay if necessary, for example, for primary cell types.

Suspension cell workflow

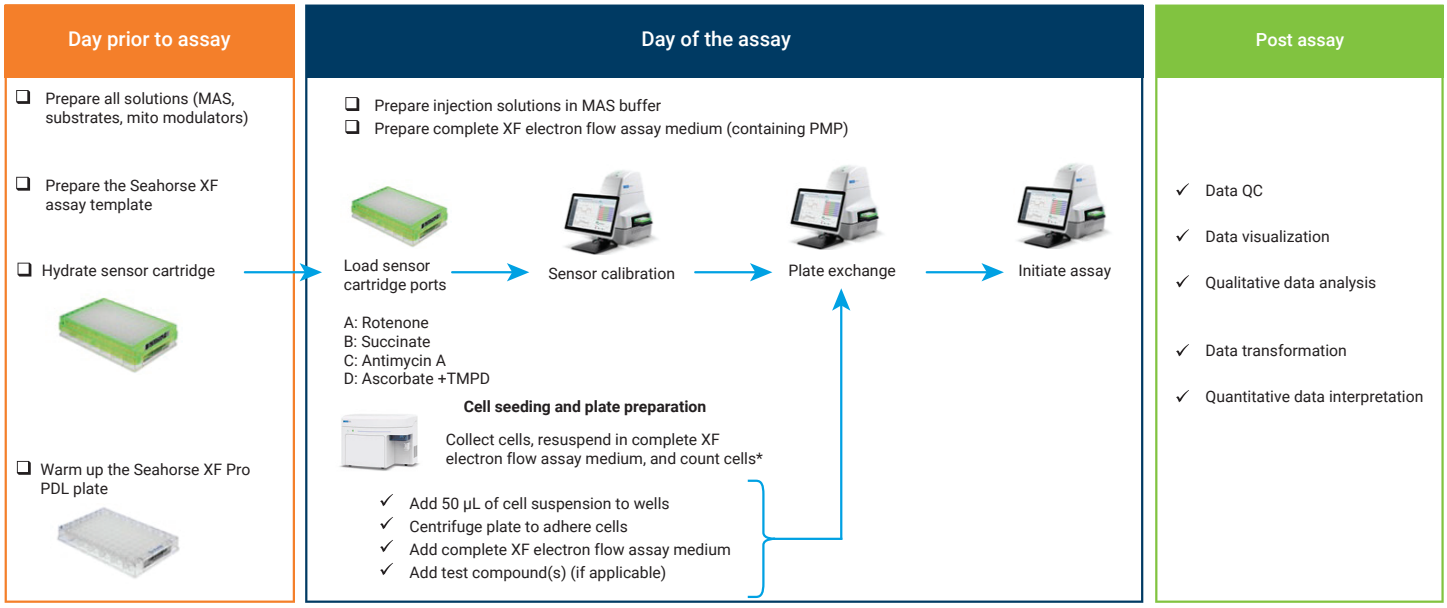


Figure 4. The Agilent Seahorse XF electron flow assay workflow using Agilent Seahorse XF PMP for suspension cell types. *It is recommended to count cells using the method outlined in Agilent publication 5994-6245EN.[21]

Day before assay

Step 1.

Electron flow assay reagent preparation:
To perform the XF electron flow assay, the following reagents and solutions are required.

- MAS buffer, Table 3
- Mitochondrial substrate stock solutions, Table 4
- Mitochondrial modulator stock solutions, Table 4

Step 2.

Prepare the XF electron flow assay template:
Prepare an XF electron flow assay template using the Agilent Seahorse Wave Pro controller software and the following guidelines for completing the injection strategy, pretreatments, assay media, and cell fields. Begin using a "blank" template and complete the fields as follows:

Group definitions

XF electron flow assay injection strategy, Table 5:

Rotenone (20 µM port, 2 µM final)

Succinate (100 mM port, 10 mM final)

Antimycin A (20 µM port, 2 µM final)

Ascorbate/TMPD (100 mM/1 mM port, 10 mM/100 µM final)

XF electron flow assay pretreatments: Pretreatments are user-defined. Typically, a compound is added just before initiating the assay. Groups may be designed in combinations of compound identity, compound dose, and number of replicates per condition, as well as starting CI-linked substrate (pyruvate/malate or glutamate/malate).

Note that genetic manipulations may also be considered pretreatments and may be combined with acute interventions (compound treatments).

XF electron flow assay medium (Tables 6, 7, 8):

Complete XF electron flow assay medium (solution to be prepared the day of the assay)

- MAS + 10 mM pyruvate/1 mM malate + 4 mM ADP + 2 µM oligomycin + optimal [FCCP] + 1 nM PMP

And/or

- MAS + 10 mM glutamate/10 mM malate + 4 mM ADP + 2 µM oligomycin + optimal [FCCP] + 1 nM PMP

Cell type: User-defined. Employ the same seeding density as optimized for XF assays using intact cells (for example, the Seahorse XF Cell Mito Stress Test or XF T Cell Metabolic Profiling assay).

Adherent cells are seeded in XFe96/XF Pro (or XF Pro M, or XF Pro PDL) plates the day (or days) before the assay, depending on the cell type and experimental design.

Note that suspension cells must be seeded in XF Pro PDL plates on the day of the assay.

Plate map: The plate map will be defined by the experimental design, number of groups/conditions tested, and the number of replicate wells per group. Figure 5 shows an example plate map for testing five experimental conditions (pretreatments) using both pyruvate/malate and glutamate/malate as initial CI-linked substrates (10 groups in total).

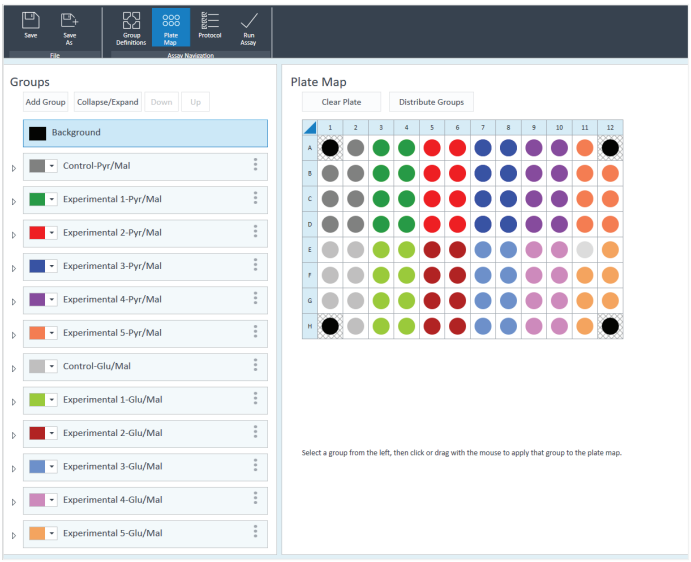


Figure 5. An example plate map for testing five experimental (pretreatment) conditions with seven to eight replicates per condition using both pyruvate/malate and glutamate/malate as initial CI-linked substrates.

Dose–response and compound screening assay designs:
The XF electron flow assay is applicable to both compound screening and dose–response assay designs (Figure 6).

- Compound screening: The CSV import feature in the Seahorse Wave Pro software may be used to upload compound libraries for screening assays. Up to 80 individual compounds (at a single concentration) may be screened in a single assay.
- Dose response: Use the Titration Set Up feature in the Agilent Seahorse Wave Pro software to create dose-response assays.

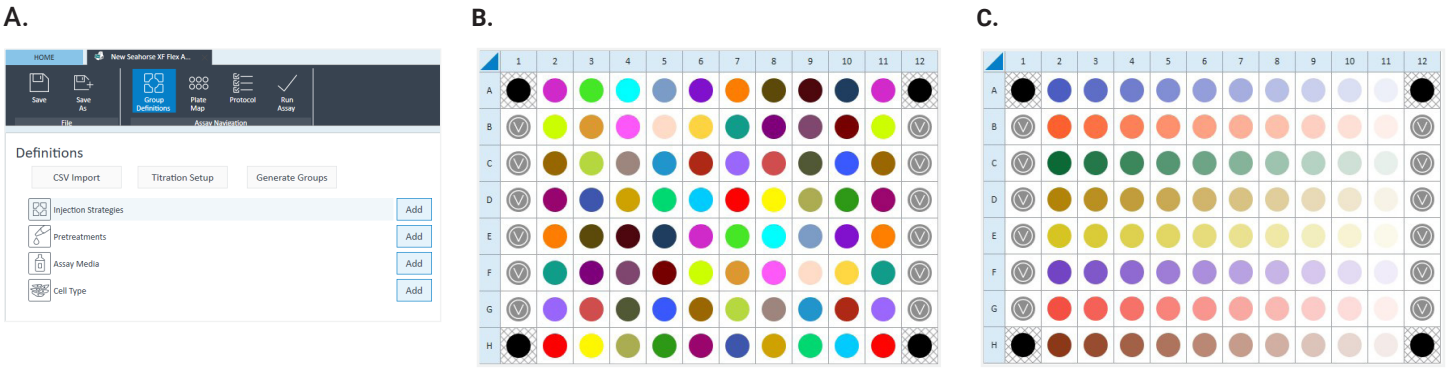


Figure 6. Agilent Seahorse XF Electron Flow assay screening and dose–response assay design. Examples of an 80-compound screening assay and 10-point dose response using eight compounds.

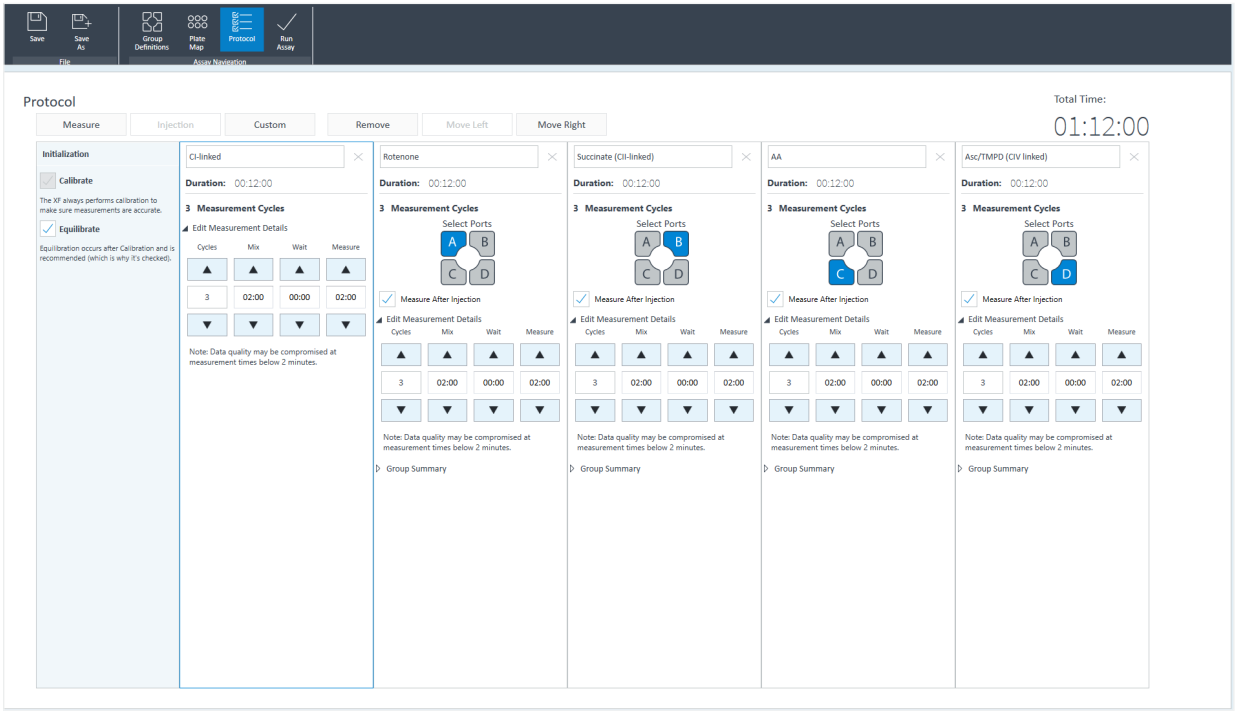


Figure 7. An Agilent Seahorse XF electron flow assay protocol. Note that the **Mix** and **Measure** times are two minutes.

Assay protocol: Create the assay protocol as shown in Figure 7, using three measurement cycles with 2 minute **Mix**, 0 minute **Wait**, and 2 minute **Measure** timings for each measurement period.

Step 3.

Cell seeding (adherent): Seed adherent cells in the appropriate XF cell culture microplate at the optimal density in 80 µL of cell growth/maintenance media (cell type-specific) (Figure 8). Do not add cells to background correction wells A1, A12, H1, and H12.

Cartridge hydration: Begin sensor cartridge hydration as described in Agilent publication 5994-4361EN.[17]

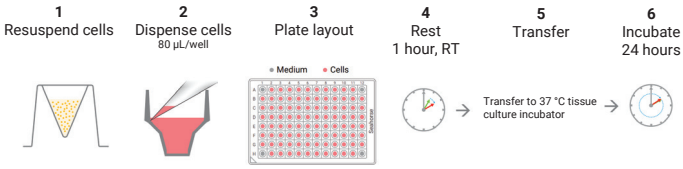


Figure 8. Seeding adherent cells in Agilent 96-well Seahorse XF cell culture microplates.

Day of assay

1. **Cartridge hydration:** Complete the sensor cartridge hydration, as described in Agilent publication 5994-4361EN, from 2023.[17]
2. **Prepare injection solutions:** Prepare injection solutions by combing the reagents in Table 5.

Table 5. Agilent Seahorse XF electron flow assay injection solution preparation.

To Make 4 mL of Each Injection Solution							
Port	**Vol**	**Reagent**	**[Stock]**	**Volume Reagent**	**Volume MAS**	**[Port]**	**[Well] (Final)**
A	20 µL	Rotenone	10 mM	8 µL	4 mL	20 µM	2 µM
B	22 µL	Succinate	1 M	400 µL	3.6 mL	100 mM	10 mM
C	25 µL	Antimycin A	10 mM	8 µL	4 mL	20 µM	2 µM
D	28 µL	Ascorbate	1 M	400 µL	3.2 mL	100 mM	10 mM
		TMPD	10 mM	400 µL		1 mM	0.1 mM

Table 6. Standard Agilent Seahorse XF electron flow assay medium preparation.

To Make 20 mL of Base XF Electron Flow Assay Medium		
Solution	**Volume Per 20 mL Assay Medium**	**[Final Concentration]**
MAS buffer	19.5 mL	1x
500 mM ADP	160 µL	4 mM
10 mM oligomycin	4.0 µL	2 µM
10 mM FCCP Final concentration is cell type-dependent	1.0 µL	0.5 µM
	2.0 µL	1.0 µM
	4.0 µL	2.0 µM
	8.0 µL	4.0 µM
10 µM XF PMP	2.0 µL	1.0 nM*
* For most cell types, a final concentration of 1 nM XF PMP is recommended.		

3. **Load injection solutions:** Load the appropriate volume of each injection solution into the respective port listed. See Agilent publication 5994-0239EN r2 for further instructions on loading XF sensor cartridge injection ports.[18]
4. **Calibrate sensors:** After ports are loaded, calibrate the loaded sensor cartridge as described in Agilent publication 5994-4582EN.[19]
5. **Prepare base XF electron flow assay medium:** Prepare the base XF electron flow assay medium by combining the solutions (20 mL per XF microplate) shown in Table 6. Next, using Table 7 or 8, combine the appropriate volume of CI-linked substrate with the appropriate volume of XF electron flow assay medium. It is recommended to test both pyruvate/malate and glutamate/malate-dependent CI-linked respiration to discriminate between specific CI inhibitors and those that inhibit substrate pathway-dependent processes upstream of CI.

Table 7. Complete Agilent Seahorse XF electron flow assay medium preparation for use with pyruvate/malate and glutamate/malate as the initial CI-linked substrate.

Pyruvate/Malate and Glutamate/Malate	**[Stock]**	**Vol**	**[Final]**	**Volume Base XF EF Assay Medium**
Initial CI-linked substrate	1 M pyruvate	75 µL	10 mM	7.5 mL
	1 M malate	7.5 µL	1 mM	
	And			
	1 M glutamate	75 µL	10 mM	7.5 mL
	1 M malate	7.5 µL	10 mM	

Table 8. Complete Agilent Seahorse XF electron flow assay medium preparation for use with pyruvate/malate or glutamate/malate as the initial CI-linked substrate.

Pyruvate/Malate or Glutamate/Malate	**[Stock]**	**Vol**	**[Final]**	**Volume Base XF EF Assay Medium**
Initial CI-linked substrate	1 M pyruvate	150 µL	10 mM	15 mL
	1 M malate	15 µL	1 mM	
	Or			
	1 M glutamate	150 µL	10 mM	15 mL
	1 M malate	150 µL	10 mM	

6. **Prepare pretreatments (if applicable):** Pretreatments are typically compounds of interest, often first identified by the XF Cell Mito Stress Test, XF Substrate Oxidation Stress Test, XF Mito Tox Assay, XF T Cell Metabolic Profiling assay, or orthogonal methods. Prepare 20 µL per well of a 10x compound solution in 1x MAS buffer of each compound/dose to be tested in the assay.
   Example:
   Eight replicates at a single dose = 160 µL of 10x compound
   Single replicates at 10 doses = 20 µL of 10x compound at each dose

7. **Prepare the cell plate and perform the assay:**

   Adherent cells

   a. Remove all but 20 µL of the cell growth medium from each well.

   b. Wash cells twice with 200 µL of 1x MAS buffer (Agilent publication 5994-0240EN r3).[20]

   c. Add 142 µL of Complete XF electron flow assay medium to each well (162 µL total).

   d. Add 18 µL of 10x compound solution or vehicle control in complete XF electron flow assay medium to the appropriate wells (180 µL final volume). Note: there is no need to incubate the plate at 37 °C, no CO_2.

   e. After the sensor cartridge calibration is complete, exchange the XF utility plate with the cell plate and start the assay.

   Suspension cells

   a. Count and seed suspension cells as described in Figure 9. See Agilent publications 5994-6245EN and 5994-3493EN Rev. A0 for detailed instructions.[21-22]

   b. Dilute an appropriate volume of quantitated cell suspension in 5.0 mL of Complete XF electron flow assay medium to achieve the desired seeding concentration, using 50 µL of cell suspension per well.

   c. Add 50 µL of cell suspension to each well, omitting the background correction wells A1, A12, H1, and H12.

   d. Centrifuge the cell plate for 1 to 2 minutes at 200 g.

   e. Add 112 µL of Complete XF electron flow assay medium to each well (for a total volume of 162 µL).

   f. Incubate at 37 °C, no CO_2, for 20 minutes. It takes about 30 minutes for suspension cells to become fully permeabilized: 20 minutes of incubation plus approximately 12 minutes of equilibration.

   g. Add 18 µL of 10x compound or vehicle control in complete XF electron flow assay medium to the appropriate wells (180 µL final volume).

   h. After the sensor cartridge calibration is complete, exchange the XF utility plate with the cell plate and begin the assay.

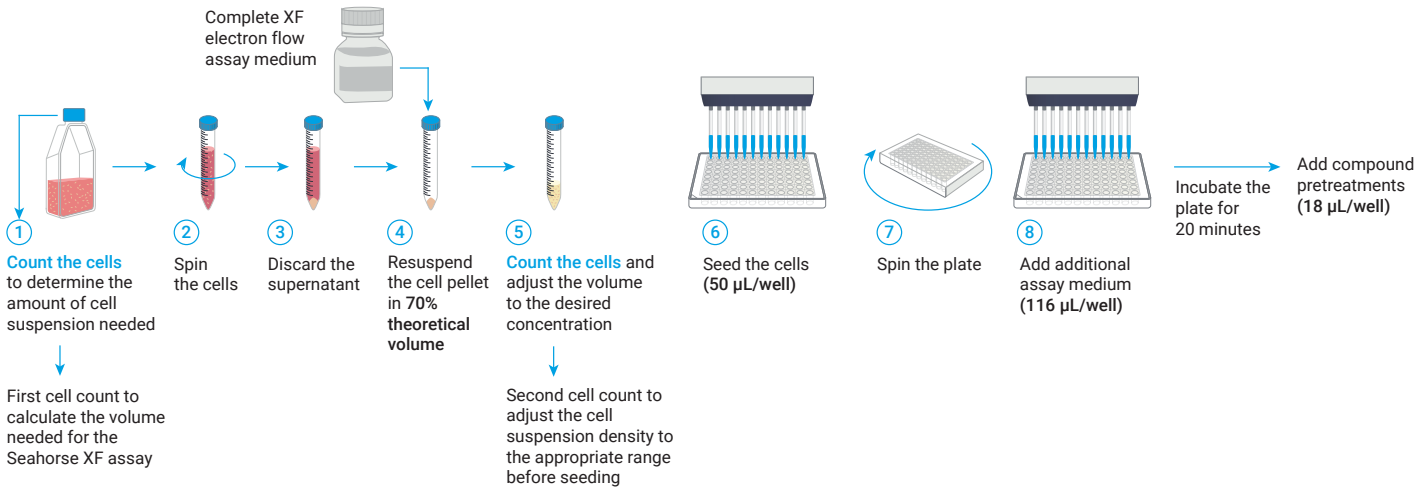


Figure 9. Seeding suspension cells in Agilent 96-well Seahorse XF cell culture PDL microplates.

Post assay operation/procedures

Data normalization

Adherent cells: It is recommended to perform a cell count after the assay using an Agilent Cytation instrument, as described in Agilent publication 5991-9385EN.[23]

Suspension cells: Performing a cell count before the assay is recommended using an Agilent NovoCyte instrument, as described in Agilent publication 5994-6245EN.[21]

Cell counts may then be applied to normalize OCR values to a per cell basis in the Wave Pro software or in Seahorse Analytics.

Data QC: OCR and O_2, ECAR, and pH

Before data analysis and interpretation, it is recommended to first examine the data quality to ensure technical accuracy.

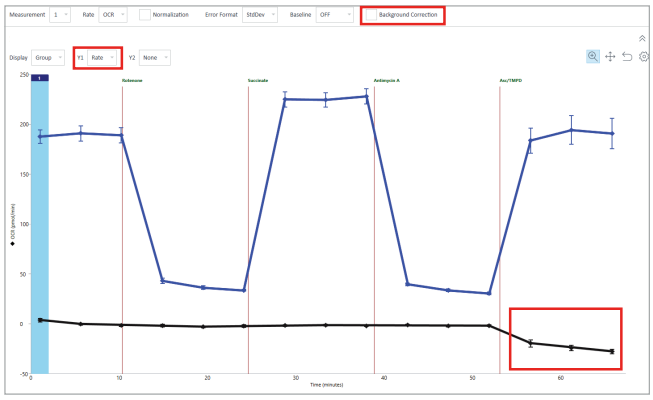
OCR: The XF electron flow assay is performed under conditions of maximal respiration. Under optimal conditions, CI-, CII-, and CIV-linked respiration rates can easily reach ~ 300 to 350 pmol/min, providing a large dynamic range to access changes (decreases) due to an intervention. In most cases, the optimal cell number per well and FCCP concentrations used in intact/live cell assays can be applied to the XF electron flow assay. However, if hypoxia occurs (O_2 levels drops to below 45mmHg) or if assay dynamic range is too small, further optimization with cell density or FCCP may be considered.

O_2 level data: Injection of ascorbate/TMPD causes a decrease in the background OCR values as well as decreases in the level O_2 data. This is normal and due to auto-oxidation of the ascorbate/TMPD (Figure 10).

Note: Because of this decrease in background OCR levels, the Data QC feature in Seahorse Analytics/Wave Pro software is not compatible with the XF electron flow assay and should not applied in these circumstances.

O_2 level data should always be inspected to ensure changes in respiration rate are mitochondrial-based and not due to assay artifacts, such as sensor interference. This is particularly true with colored compounds or compounds with strong auto-oxidative properties.

A.



B.

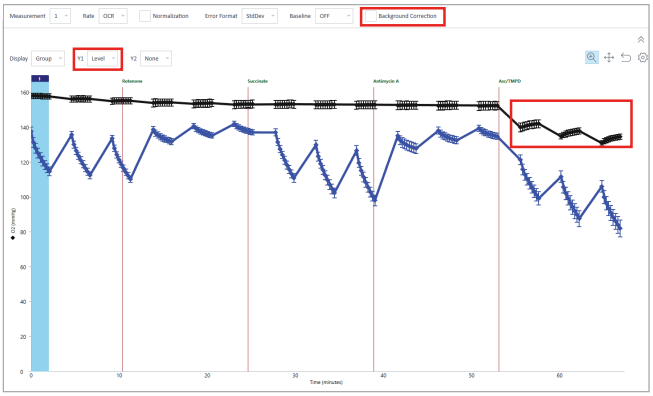
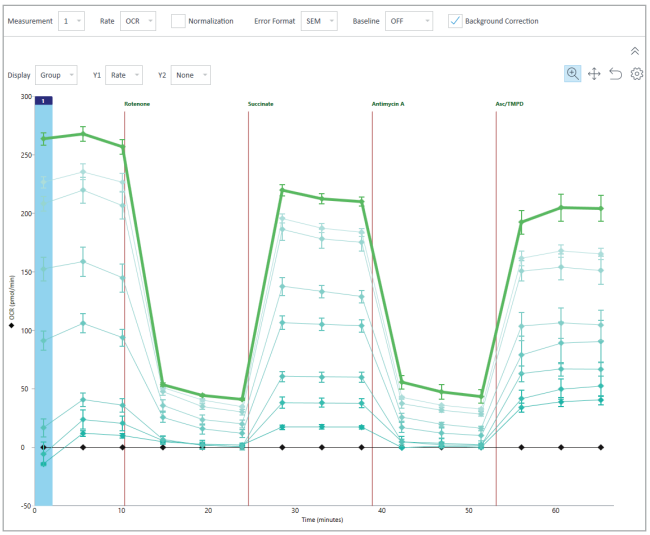


Figure 10. Agilent Seahorse XF Electron Flow assay background OCR (A) and level O_2 data (B). Decreases in the background OCR values and O_2 data upon injection of ascorbate/TMPD are normal.

For example, what appears to be a dose–dependent decrease in OCR for CI-, CII-, and CIV-linked respiration (Figure 11A) is actually an artifact caused by interference of the O_2 sensor with the compound being tested, in this case, pyrvinium, which is dark red. In this instance, there is an apparent dose-dependent decrease in the initial O_2 level data, rendering the assay inappropriate for further biological interpretation (Figure 11B).

ECAR: As noted earlier, ECAR is not relevant in the XF electron flow assay. Therefore, ECAR data should not be included in biological interpretation.

A.



B.

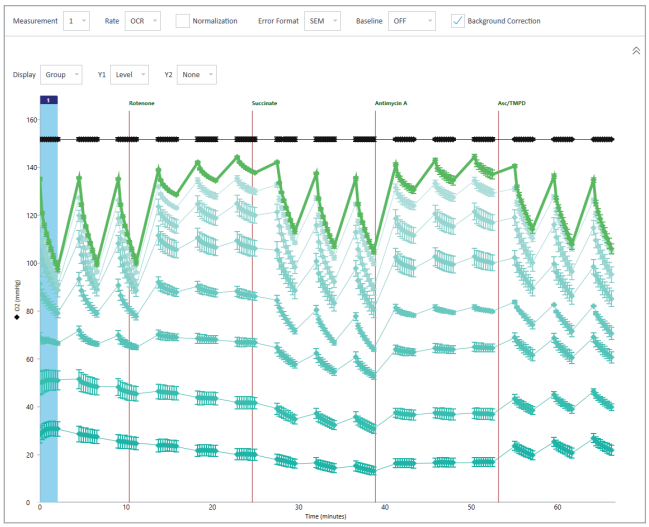


Figure 11. Example of dose–dependent O_2 sensor interference. Agilent Seahorse XF electron flow assay OCR (A) and level O_2 (B) data for control (dark green) and increasing concentrations of pyrvinium.

The optimal pH for the XF electron flow assays is 7.4 and the pH level data should therefore be reviewed carefully to ensure that an intervention has not shifted the pH below 7.2 or above 7.8. It is recommended to check and adjust the pH of the compound solutions, if necessary, to avoid large changes in pH during application to the cells, especially for treatments where the final concentration exceeds 5 mM.

Data visualization, transformation, and interpretation

Please see the companion document: Agilent Seahorse XF Plasma Membrane Permeabilizer and XF Electron Flow Assay: Data Analysis and Interpretation.[6]

Single ETC complex assay workflows

The XF electron flow assay can be divided into three separate assays, each measuring CI-, CII-, and CIV-linked respiration, respectively. These assays are summarized in Figure 12.

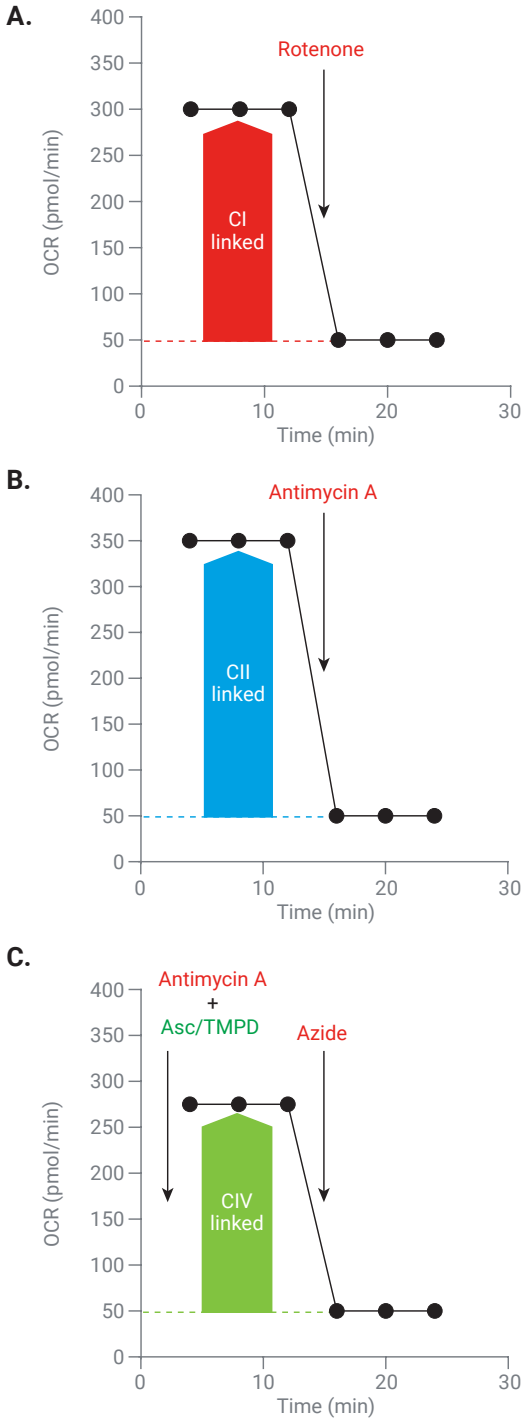


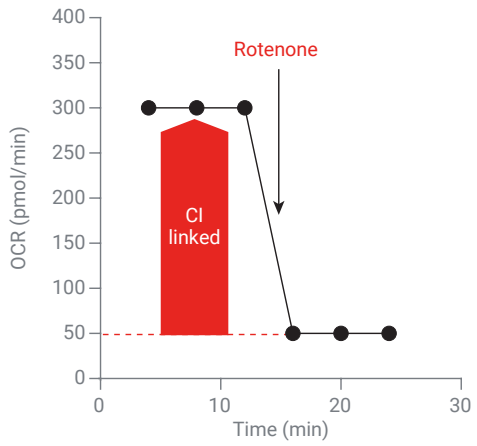
Figure 12. A schematic outline of single Agilent Seahorse XF ETC complex assays for assessment of CI-, CII-, or CIV-linked respiration.

Single XF ETC complex assays are best applied after the standard (four injection) XF electron flow assays have been performed, including dose-response analysis. Treatments can exert both specific and nonspecific effects on mitochondrial function. These effects are often concentration-dependent. Use of the standard XF electron flow workflow for performing dose-response assays is therefore an ideal method for assessing specific and nonspecific effects of interventions. Once the effects of the treatment are characterized and known to be specific for a particular substrate/ETC complex pathway, single ETC complex assays can be used for further characterization or reducing assay time and materials required. Prepare the base XF electron flow assay medium according to Table 6.

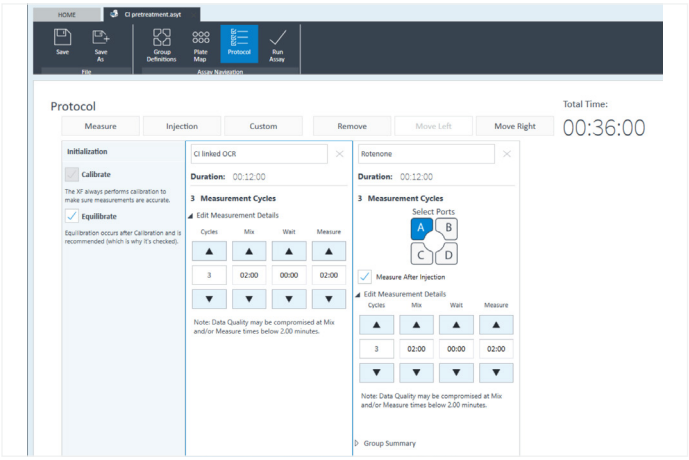
Next, using Figures 13, 14, or 15, combine the appropriate volume of the desired substrate with the appropriate volume of the base XF electron flow assay medium to make the complete XF electron flow assay medium for the ETC complex activity of interest. Also, prepare the XF assay template and injection solutions required for the specific single ETC complex assay chosen.

CI-linked Seahorse XF assay

A.



B.



C.

To Make 4 mL of Injection Solution							
Port	Volume	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Rotenone	10 mM	8 µL	4 mL	20 µM	2 µM

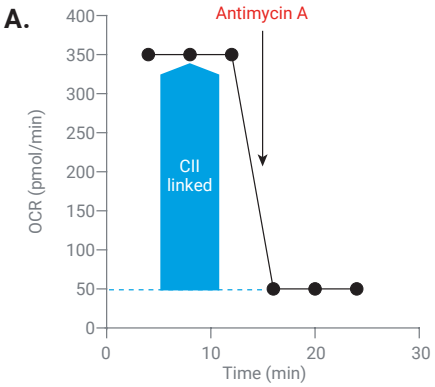
D.

Pyruvate/Malate or Glutamate/Malate	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CI-linked substrate	1 M pyruvate	200 µL	10 mM	20 mL
	1 M malate	20 µL	1 mM	
	And/or			
	1 M glutamate	200 µL	10 mM	20 mL
	1 M malate	200 µL	10 mM	

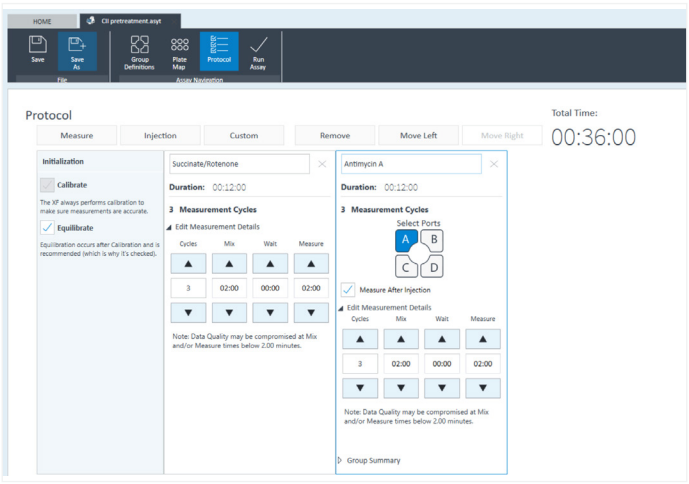
Figure 13. The ETC CI-linked Agilent Seahorse XF assay. (A) A schematic of the ETC CI-linked assay. (B) The CI-linked assay template instrument protocol. (C) CI-linked assay injection solution preparation. (D) CI-linked assay complete medium preparation using pyruvate/malate and/or glutamate/malate. (E) CI-linked assay complete medium preparation using pyruvate/malate or glutamate/malate.

CII-linked assay

A.



B.



C.

To Make 4 mL of Injection Solution							
Port	Volume	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Antimycin A	10 mM	8 µL	4 mL	20 µM	2 µM

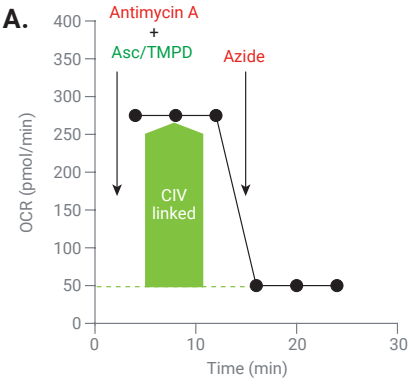
D.

Succinate/ Rotenone	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CII-linked substrate	1 M succinate	200 µL	10 mM	20 mL
	10 mM rotenone	4 µL	2 µM	

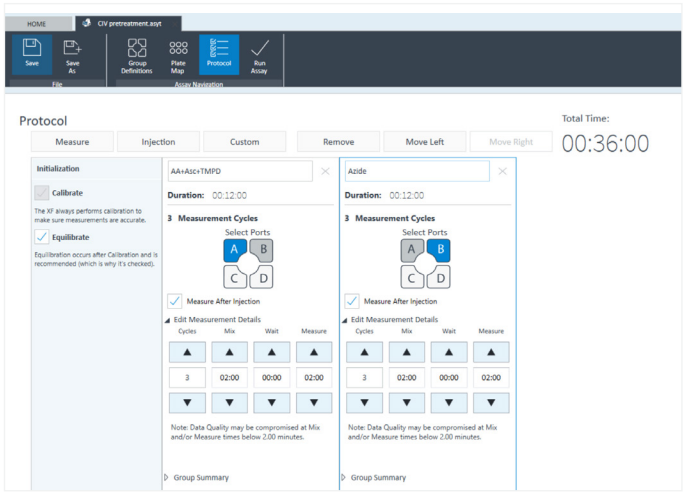
Figure 14. The ETC CII-linked Agilent Seahorse XF assay. (A) A schematic of the ETC CII-linked assay. (B) The CII-linked assay template instrument protocol. (C) CII-linked assay injection solution preparation. (D) CII-linked assay complete medium preparation using succinate/rotenone. Atpenin (2 µM final) or malonate (20 mM final) may be used in place of antimycin A if a specific CII-inhibitor is desired.

CIV-linked assay

A.



B.



C.

To Make 4 mL of Each Injection Solution							
Port	Volume	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Antimycin A	10 mM	8 µL	3.2 mL	20 µM	2 µM
		Ascorbate	1 M	400 µL		100 mM	10 mM
		TMPD	10 mM	400 µL		1 mM	0.1 mM
B	22 µL	Azide	2 M	400 µL	3.6 mL	200 mM	20 mM

D.

Succinate/ Rotenone*	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CII-linked substrate*	1 M succinate	200 µL	10 mM	20 mL
	10 mM rotenone	4 µL	2 µM	

* CIV-linked substrates ascorbate and TMPD are provided in the first injection, before any rate measurements.

Figure 15. CIV-linked Agilent Seahorse XF electron flow assay injection solution preparation. (A) A schematic of an ETC CIV-linked assay. The assay is prepared using a CII-linked substrate (succinate/rotenone) and the CIV-linked substrate, ascorbate/TMPD (+antimycin A), is injected before the first measurement period. (B) The CIV-linked assay template instrument protocol. (C) CIV-linked assay injection solution preparation. (D) CIV-linked assay complete medium preparation using succinate/rotenone.

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Frequently asked questions

Q: Where can the detailed information about how to interpret the XF electron flow assay results be found?

A: See Agilent Seahorse XF Plasma Membrane Permeabilizer and XF Electron Flow Assay: Data Analysis and Interpretation. Agilent Technologies, Application Note, 2026, publication number 5994-7549EN.[6]

Q: What variables are required to be optimized before performing an XF electron flow assay?

A: Cell seeding density and FCCP concentration must be optimized before performing an XF assay. Typically, the optimal values for cell seeding density and FCCP concentration used for intact cells in XF assays (such as the XF Cell Mito Stress Test, XF Substrate Oxidation Stress Tests, XF Mito Tox assay, and the XF T (or NK) Cell Metabolic Profiling assay) will be appropriate for XF PMP Electron Flow assays.

Q. Can Bam15 be used instead of FCCP?

A: Yes. Optimization of Bam15 concentration is performed the same way as for optimization of FCCP concentration using intact cells.

Q: What cell types can be used in the XF electron flow PMP assay?

A: The XF PMP reagent is compatible with numerous types of cells, including cell lines and primary cells. This includes both adherent and suspension/immune cell types.[10, 15] Ensure that the proper XF cell culture plate type is used for the cell type being investigated.

Q: Does the concentration of XF PMP in the XF electron flow assay need to be optjmized?

A: For most cell types, including (adherent or suspension) established cell lines and primary cells, a final concentration of 1 nM XF PMP is recommended. In particular cases, this concentration may need to be further optimized. Performing an XF electron flow assay with increasing concentrations of XF PMP (for example, 0, 0.125, 0.25, 0.5, 1.0 and 2.0 nM) is suggested. In these instances, the concentration of XF PMP that yields the highest OCR values (within the dynamic range of the instrument), with minimal error, is typically the optimal concentration of XF PMP to use in the assay.

Q: Do any other parameters or reagent concentrations (substrates, inhibitors) need to be optimized before an XF electron flow assay is performed?

A: No. All other components, including substrate and inhibitor concentrations have been optimized, and agree with the concentration values conventionally used when working with permeabilized cells or isolated mitochondria.[11-14]

Q: Can long chain fatty acids (for example, palmitoyl carnitine, palmitoyl CoA + carnitine) be used as a substrate in the XF electron flow assay?

A: Yes. Either palmitoyl carnitine (+malate), or palmitoyl CoA + carnitine (+malate) may be used as an initial CI-linked substrate to isolate the effect of the intervention to just the long chain fatty acid oxidation pathways. For more detailed and mechanistic analysis of the long chain fatty acid oxidation pathways, alternative XF workflows using permeabilized cells (or isolated mitochondria) are recommended.[13]

Q: Is there a Q/CIII-linked substrate to test Q/CIII-linked respiration directly?

A: Glycerol-3-phosphate (G3P) can sometimes be used to drive Q/CIII-linked respiration. This will depend on the expression level of glycerol-3-phosphate dehydrogenase (G3PDH) in the cell type of interest. The XF electron flow assay, as designed, can provide qualitative information regarding the effects of interventions on Q/CIII-linked respiration.[15]

Q: Why does the CIV assay start with a CII substrate?

A: Due to the rapid oxidation of ascorbate/TMPD by CIV, ascorbate/TMPD (+ antimycin A to block CI-, CII- and CIII-linked respiration) is injected just before measuring CIV activity to achieve the highest OCR signal. The CII-linked substrate, succinate (+ rotenone), is provided as an initial substrate to keep the mitochondria in an energized state upon cell permeabilization.

Q: Does ECAR or PER correlate with glycolysis in the XF electron flow assay?

A: ECAR and PER does not correlate with glycolysis in XF PMP assays. After plasma membrane permeabilization, most cytoplasmic functions, including glycolysis, are lost. This is due to the soluble contents of the cytoplasm (including redox cofactors) being diluted into the assay medium, as well as the interruption of ionic-driven processes, due to the assay medium being nonionic (based in mannitol and sucrose).

Q: Can I perform the XF electron flow assay using isolated mitochondria?

A: Yes. The assay could be performed exactly as described in this technical overview, using isolated mitochondria as the sample material instead of cells and XF PMP. Isolated mitochondria may be adhered to XF plates via centrifugation. Typically, 0.5 to 5 µg of mitochondrial protein is used per well.[12-14]

Q: Can the XF electron flow assay be performed using 24- or 8-well XF analyzers?

A: Yes. The XF electron flow assay using XF PMP may be adapted for 24- and 8-well XF analyzers. All instructions are the same; however, different amounts are required for the well and compound injection volumes. See Appendices I and II in this document for translating the XF electron flow assay to 24- and 8-well XF analyzers, respectively.

Q: Can the XF electron flow assay be performed under conditions of coupled respiration (such as State 3), by omitting oligomycin and FCCP from the XF electron flow assay medium?

A: While possible, this is not recommended. As designed, the XF electron flow assay is performed under conditions wher complex V (ATP synthase) is both inhibited (via oligomycin) and uncoupled from the electron transport (via FCCP). This design enables detailed characterization of interventions (genetic manipulation, acute compound treatment) on the function of specific mitochondrial components, including substrate transporters, TCA enzymes, and electron transport chain (ETC) complexes. By running the assay under conditions of coupled (State 3), interventions affecting the OXPHOS machinery (complex V, adenine dinucleotide, and phosphate transporters) will be independent of the substrate used, and may obscure accurate data interpretation. If an investigation requires using permeabilized cells or isolated mitochondria under coupled respiration conditions, an alternative workflow is suggested in which a desired substrate is provided, and the sample is sequentially measured under conditions of State 3, State 4o, State 3u (uncoupled), and inhibited respiration.[12, 24]

Q: Can the XF electron flow assay be used to measure respiratory control ratio (RCR)?

A: As designed, the XF electron flow assay cannot be used to measure RCR, as the assay is performed under conditions of uncoupled respiration (in the presence of oligomycin and FCCP) to elicit maximal respiration rates. The RCR (using permeabilized cell or isolated mitochondria) can be measured using an alternative workflow where a desired substrate is provided, and the sample is sequentially measured under conditions of State 3, State 4o, State 3u (uncoupled), and inhibited respiration.[12, 24]

Appendix I

Performing the XF electron flow assay using Seahorse XF 24-well instruments

The XF electron flow assay and single ETC complex assays may be performed using XF 24-well analyzers. The assay is performed in the same way as for XF 96-well analyzers, except for changes in injection solution volumes, assay media, and substrate solutions.

For information about the required materials, assay template creation, cartridge hydration, cell seeding, and more when using XF 24-well instruments, please visit the Seahorse XF Learning Center.

Prepare 1x MAS buffer, mitochondrial substrates, and modulators as described in Tables 3 and 4.

Use the following tables and figures to adapt the methods previously outlined to XF 24-well analyzers. The tables and figures have been numbered in analogous sequences to the figures and tables for the XF 96-well analyzers. For example, Table 5 is listed as Table 5-24.

Standard XF electron flow assay: 24-well XF analyzers

Table 5-24. Agilent Seahorse XF electron flow assay injection solution preparation.

To Make 4 mL of Each Injection Solution							
Port	**Vol**	**Reagent**	**[Stock]**	**Volume Reagent**	**Volume MAS**	**[Port]**	**[Well] (Final)**
A	56 µL	Rotenone	10 mM	80 µL	4 mL	20 µM	2 µM
B	62 µL	Succinate	1 M	400 µL	3.6 mL	100 mM	10 mM
C	69 µL	Antimycin A	10 mM	8 µL	4 mL	20 µM	2 µM
D	75 µL	Ascorbate	1 M	400 µL	3.2 mL	100 mM	10 mM
		TMPD	10 mM	400 µL		1 mM	0.1 mM

Table 6-24. Standard Agilent Seahorse XF electron flow assay medium preparation.

To Make 15 mL of Base XF Electron Flow Assay Medium		
Solution	**Volume Per 15 mL Assay Medium**	**[Final Concentration]**
MAS buffer	14.6mL	1x
500 mM ADP	120 µL	4 mM
10 mM oligomycin	1.5 µL	2.0 µM
10 mM FCCP Final concentration is cell type-dependent	0.75 µL	0.5 µM
	1.5 µL	1.0 µM
	3.0 µL	2.0 µM
	6.0 µL	4.0 µM
10 µM XF PMP	1.5 µL	1.0 nM*
* For most cell types, a final concentration of 1 nM XF PMP is recommended.		

Table 7-24. Complete Agilent Seahorse XF electron flow assay medium preparation for use with pyruvate/malate and glutamate/malate as the initial CI-linked substrate.

Pyruvate/Malate and Glutamate/Malate	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
Initial CI-linked substrate	1 M pyruvate	75 µL	10 mM	7.5 mL
	1 M malate	7.5 µL	1 mM	
	And			
	1 M glutamate	75 µL	10 mM	7.5 mL
	1 M malate	7.5 µL	10 mM	

Table 8-24. Complete Agilent Seahorse XF electron flow assay medium preparation for use with pyruvate/malate or glutamate/malate as the initial CI-linked substrate.

Pyruvate/Malate or Glutamate/Malate	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
Initial CI-linked substrate	1 M pyruvate	150 µL	10 mM	15 mL
	1 M malate	15 µL	1 mM	
	Or			
	1 M glutamate	150 µL	10 mM	15 mL
	1 M malate	150 µL	10 mM	

Single complex-linked assays: 24-well XF analyzers

ETC CI-linked XF assay

A.

To Make 4 mL of Injection Solution							
Port	**Vol**	**Reagent**	**[Stock]**	**Volume Reagent**	**Volume MAS**	**[Port]**	**[Well] (Final)**
A	56 µL	Rotenone	10 mM	8 µL	4 mL	20 µM	2 µM

B.

Pyruvate/Malate or Glutamate/Malate	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CI-linked substrate	1 M pyruvate	150 µL	10 mM	15 mL
	1 M malate	15 µL	1 mM	
	And/or			
	1 M glutamate	150 µL	10 mM	15 mL
	1 M malate	150 µL	10 mM	

Figure 13-24. The ETC CI-linked Agilent Seahorse XF assay. (A) CI-linked assay injection solution preparation. (B) CI-linked assay complete medium preparation using pyruvate/malate and glutamate/malate. C) CI-linked assay complete medium preparation using pyruvate/malate or glutamate/malate.

ETC CII-linked XF assay

A.

To Make 4 mL of Each Injection Solution							
Port	**Vol**	**Reagent**	**[Stock]**	**Volume Reagent**	**Volume MAS**	**[Port]**	**[Well] (Final)**
A	56 µL	Antimycin A	10 mM	8 µL	4 mL	20 µM	2 µM

B.

Succinate/ Rotenone	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CI-linked substrate	1 M succinate	150 µL	10 mM	15 mL
	10 mM rotenone	3 µL	2 µM	

Figure 14-24. The ETC CII-linked Agilent Seahorse XF assay. (A) CII-linked assay injection solution preparation. (B) CII-linked assay complete medium preparation using succinate/rotenone. Atpenin (2 µM final) or malonate (20 mM final) may be used in place of antimycin A if a specific CII-inhibitor is desired.

ETC CIV-linked XF assay

A.

To Make 4 mL of Each Injection Solution							
Port	**Vol.**	**Reagent**	**[Stock]**	**Volume Reagent**	**Volume MAS**	**[Port]**	**[Well] (Final)**
A	56 µL	Antimycin A	10 mM	8 µL	3.2 mL	20 µM	2 µM
		Ascorbate	1 M	400 µL		100 mM	10 mM
		TMPD	10 mM	400 µL		1 mM	0.1 mM
B	62 µL	Azide	2M	400 µL	3.6 mL	200 mM	20 mM

B.

Succinate/ Rotenone*	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CII-linked substrate*	1 M succinate	150 µL	10 mM	15 mL
	10 mM rotenone	3 µL	2 µM	
* CIV-linked substrates, ascorbate and TMPD, are provided in the first injection, before any rate measurements				

Figure 15-24. CIV-linked Agilent Seahorse XF electron flow assay injection solution preparation. The assay is prepared using a CII-linked substrate (succinate/rotenone) and the CIV-linked substrate, ascorbate/TMPD (+antimycin A), is injected before the first measurement period. (A) CIV-linked assay injection solution preparation. (B) CIV-linked assay complete medium preparation using succinate/rotenone.

Appendix II

Performing the Seahorse XF electron flow assay using Seahorse XF 8-well analyzers

The XF electron flow assay and single ETC complex assays may performed using XF 8-well analyzers. The assay is performed in the same way as for XF 96-well analyzers, except for changes in injection solution volumes, assay media, and substrate solutions.

For information about the required materials, assay template creation, cartridge hydration, cell seeding, and more. when using XF 8-well instruments, please visit the Seahorse XF Learning Center.

Prepare 1x MAS buffer and mitochondrial substrates and modulators as described in Tables 3 and 4.

For pipetting accuracy, make further dilutions of the following reagents:

Table 10. Reagent dilutions.

Stock Solution (from Table 4)	Further Dilute to	Using
10 mM oligomycin	1 mM	95% ETOH or 100% DMSO
10 mM FCCP	1 mM	95% ETOH or 100% DMSO
10 mM rotenone	1 mM	95% ETOH or 100% DMSO
10 mM antimycin A	1 mM	95% ETOH or 100% DMSO
10 µM XF PMP	1 µM	MAS buffer, at the time of the assay

Use the following tables and figures to adapt the methods previously outlined to XF 8-well analyzers. The tables and figures have been numbered in analogous sequences to the figures and tables for the XF 96-well analyzers. For example, Table 5 is listed as Table 5-8 here.

Standard XF electron flow assay: 8-well XF analyzers

Table 5-8. Agilent Seahorse XF electron flow assay injection solution preparation.

To Make 0.5 mL of Each Injection Solution							
Port	Vol.	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Rotenone	1 mM	10 µL	0.49 mL	20 µM	2 µM
B	22 µL	Succinate	1 M	50 µL	0.45 mL	100 mM	10 mM
C	25 µL	Antimycin A	1 mM	10 µL	0.49 mL	20 µM	2 µM
D	28 µL	Ascorbate	1 M	50 µL	0.4 mL	100 mM	10 mM
		TMPD	10 mM	50 µL		1 mM	0.1 mM

Table 6-8. Standard Agilent Seahorse XF electron flow assay medium preparation.

To Make 2 mL of Base XF Electron Flow Assay Medium		
Solution	Volume Per 2 mL Assay Medium	[Final Concentration]
MAS buffer	1.95 mL	1x
500 mM ADP	16 µL	4 mM
1 mM oligomycin	4.0 µL	2.0 µM
1 mM FCCP Final concentration is cell type-dependent	1.0 µL	0.5 µM
	2.0 µL	1.0 µM
	4.0 µL	2.0 µM
	8.0 µL	4.0 µM
1 µM XF PMP	2.0 µL	1.0 nM*
* For most cell types, a final concentration of 1 nM XF PMP is recommended.		

Table 8-8. Complete Agilent Seahorse XF electron flow assay medium preparation for use with pyruvate/malate or glutamate/malate as the initial CI-linked substrate.

Pyruvate/Malate or Glutamate/Malate	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
Initial CI-linked substrate	1 M pyruvate	20 µL	10 mM	2.0 mL
	1 M malate	2 µL	1 mM	
	Or			
	1 M glutamate	20 µL	10 mM	2.0 mL
	1 M malate	20 µL	10 mM	

Single complex-linked assays; 8-well XF analyzers

ETC CI-linked XF assay

A.

To Make 0.5 mL of Injection Solution							
Port	Vol.	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Rotenone	1 mM	10 µL	0.49 mL	20 µM	2 µM

B.

Pyruvate/Malate or Glutamate/Malate	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CI-linked substrate	1 M pyruvate	20 µL	10 mM	2.0 mL
	1 M malate	2 µL	1 mM	
	Or			
	1 M glutamate	20 µL	10 mM	2.0 mL
	1 M malate	20 µL	10 mM	

Figure 13-8. The ETC CI-linked Agilent Seahorse XF assay. (A) CI-linked assay injection solution preparation. (B) CI-linked assay complete medium preparation using pyruvate/malate and glutamate/malate. C) CI-linked assay complete medium preparation using pyruvate/malate or glutamate/malate.

ETC CII-linked XF assay

A.

To Make 0.5 mL of Each Injection Solution							
Port	Vol.	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Antimycin A	1 mM	10 µL	0.49 mL	20 µM	2 µM

B.

Succinate/ Rotenone	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CII-linked substrate	1 M succinate	20 µL	10 mM	2.0 mL
	1 mM rotenone	4 µL	2 µM	

Figure 14-8. The ETC CII-linked Agilent Seahorse XF assay. (A) CII-linked assay injection solution preparation. (B) CII-linked assay complete medium preparation using succinate/rotenone. Atpenin (2 µM final) or malonate (20 mM final) may be used in place of antimycin A if a specific CII-inhibitor is desired.

ETC CIV-linked XF assay

A.

To Make 0.5 mL of Each Injection Solution							
Port	Vol.	Reagent	[Stock]	Volume Reagent	Volume MAS	[Port]	[Well] (Final)
A	20 µL	Antimycin A	1 mM	10 µL	04 mL	20 µM	2 µM
		Ascorbate	1 M	50 µL		100 mM	10 mM
		TMPD	10 mM	50 µL		1 mM	0.1 mM
B	22 µL	Azide	2 M	50 µL	0.45 mL	200 mM	20 mM

B.

Succinate/ Rotenone*	[Stock]	Volume	[Final]	Volume Base XF EF Assay Medium
CII-linked substrate*	1 M succinate	20 µL	10 mM	2.0 mL
	1 mM rotenone	4 µL	2 µM	
* CIV-linked substrates, ascorbate and TMPD, are provided in the first injection, before any rate measurements				

Figure 15-8. CIV-linked Agilent Seahorse XF electron flow assay injection solution preparation. The assay is prepared using a CII-linked substrate (succinate/rotenone) and the CIV-linked substrate, ascorbate/TMPD (+antimycin A), is injected before the first measurement period. (A) CIV-linked assay injection solution preparation. (B) CIV-linked assay complete medium preparation using succinate/rotenone.

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