Application Note

Cell Metabolism

Agilent Seahorse XF Plasma Membrane Permeabilizer and XF Electron Flow Assay

Data analysis and interpretation

Authors

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Abstract

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. Because changes in mitochondrial function may correlate with 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. Many mitochondrial substrates are unable to cross the plasma membrane freely, preventing experimental control over the substrates being oxidized by the mitochondria. Further, while the assay medium can be supplemented with various substrates, many cell types can store and oxidize endogenous pools of substrates (for example, glycogen and triglyceride), making it difficult to determine precisely which metabolic pathways are driving mitochondrial respiration.

The Agilent XF Plasma Membrane Permeabilizer (XF PMP) provides a solution to this challenge. It forms pores in the plasma membranes of cells, allowing experimental control over the specific substrates offered to mitochondria in situ. By exploiting the fact that substrates feed differentially into mitochondrial pathways, it is possible to isolate the pathways responsible for the altered oxygen consumption rate (OCR) originally observed in intact cells. This application note explains how changes in mitochondrial function, due to compound treatments or genetic interventions, can be investigated by permeabilizing cells using the Agilent XF PMP and Agilent XF Electron Flow assay. This combination of XF PMP, substrates, and inhibitors yields a powerful approach to understanding mitochondrial function when testing the effects of interventions (genetic manipulation/compound treatment) on mitochondria. It can facilitate therapeutic development for elucidation of target identification, mechanism of action, and for measuring efficacy and specificity.

Introduction

Relevance of mitochondria as a therapeutic target

It has been well established that mitochondrial dysfunction contributes to numerous pathologies and disease progression, including cancer, neurodegeneration, and metabolic disorders.[1] For example, mitochondria regulate many processes that are known to be altered in cancer cells, from metabolism to oxidative stress and apoptosis.[2] Mitochondrial function has also been implicated in the progression of neurodegenerative disorders, including Alzheimer's and Parkinson's disease.[3] More recently, mitochondrial function has been implicated as a key driver of immune cell differentiation, and understanding of immune cell metabolism and bioenergetics has been imperative for CAR T cell development.[4]

Mitochondria therefore represent an important target for areas of therapeutic development. Thus, detailed mechanisms of mitochondrial function, metabolism, and energy generation are central to understanding pathologies and successful development of therapeutic treatments. Critical components of therapeutic development include target identification, understanding the mechanism of action, and measuring the efficacy/specificity (including on/off target effects) of treatment. This information is not only imperative for favorable candidates, but it also allows continued refinement and improvement of development strategies and designs.

Assessment of mitochondrial function and the maximal respiration parameter

Mitochondrial function can be assessed by numerous methods, including Agilent Seahorse XF technology. Several XF assays, including the Agilent Seahorse XF Cell Mito Stress Test, Substrate Oxidation Stress Test, Mito Tox assay, and T and NK Cell Metabolic Profiling assays, provide a wealth of information regarding mitochondrial function, metabolism, and bioenergetics in real time, using live cells.[5-8] While these assays emphasize different parameters of mitochondrial function, all measure and report the same parameter of maximal respiration (Figure 1). The maximal respiration rate is predominantly set by substrate supply and oxidation, and changes (decreases) during interventions (compound treatment, genetic manipulation) often reflect changes in substrate transport, substrate oxidation (TCA), or the electron transport chain (ETC).[9]

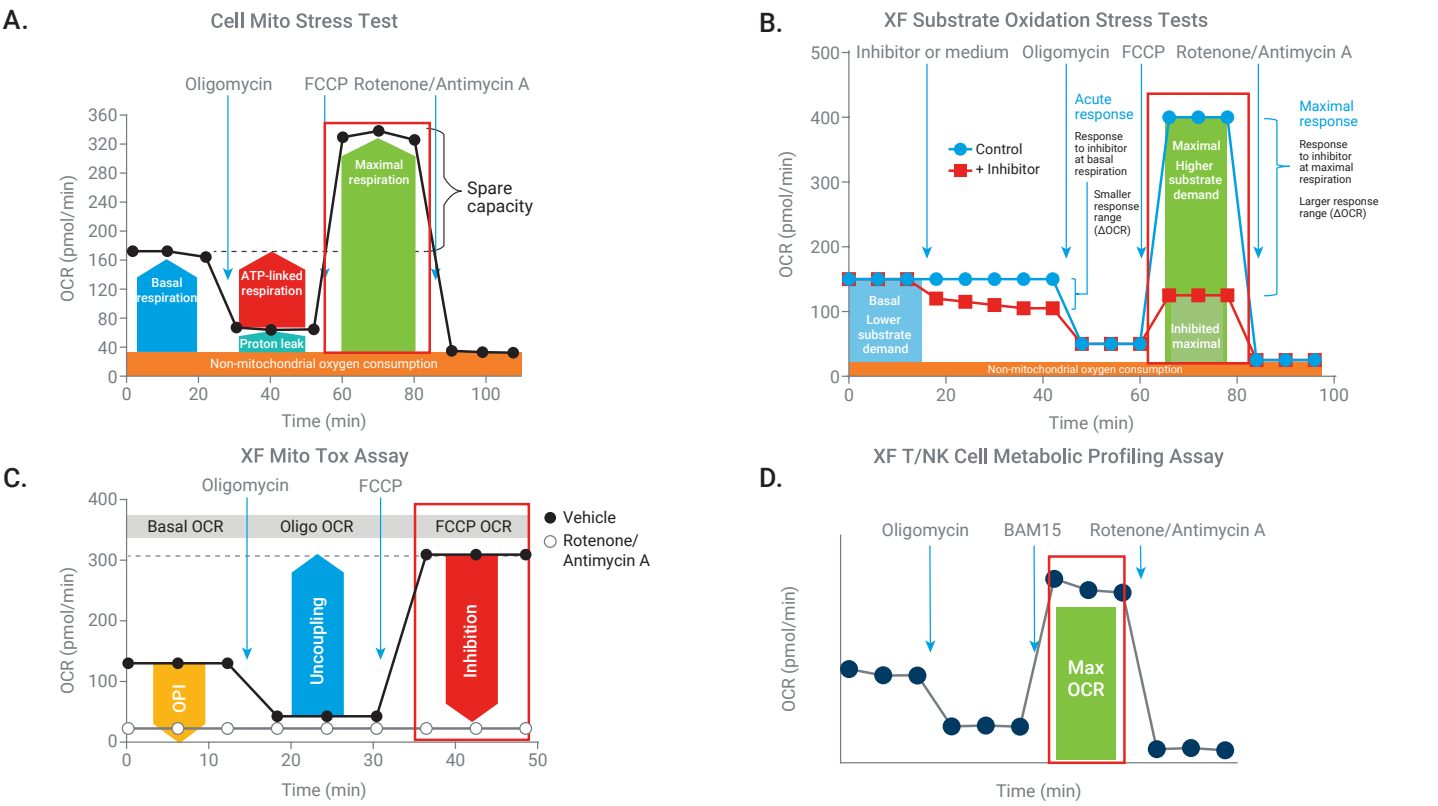


Figure 1. Agilent Seahorse XF assays that report changes in the mitochondrial maximal respiration parameter (also referred to as FCCP OCR or Max OCR), outlined in red. (A) Agilent Seahorse XF Cell Mito Stress Test. (B) Agilent Seahorse XF Substrate Oxidation Stress Tests. (C) Agilent Seahorse XF Mito Tox assay. (D) Agilent Seahorse XF T and NK Cell Metabolic Profiling assay. Decreases in maximal respiration (compared to the control) suggest that the intervention is affecting mitochondrial substrate supply/transport, substrate oxidation, and ETC activity.

These assays, however, may not provide direct information about the target identity, mechanism of action, or specificity (on/off target effects) of the treatment. For example, in Figure 2, HepG2 cells were treated with six compounds known to inhibit mitochondrial function via different mechanisms, and the XF Cell Mito Stress Test and Mito Tox assays were performed. The results indicate that each of the compounds has elicited a decrease in the maximal respiration parameter (MST) and negative MTI values (Mito Tox); however, neither of these assays are able to distinguish among the different mechanisms of inhibition elicited by the compounds. For example, UK5099 is a known inhibitor of the mitochondrial pyruvate carrier (MPC), blocking pyruvate transport into the mitochondria, while myxothiazol is a known inhibitor of complex III (CIII) in the ETC.

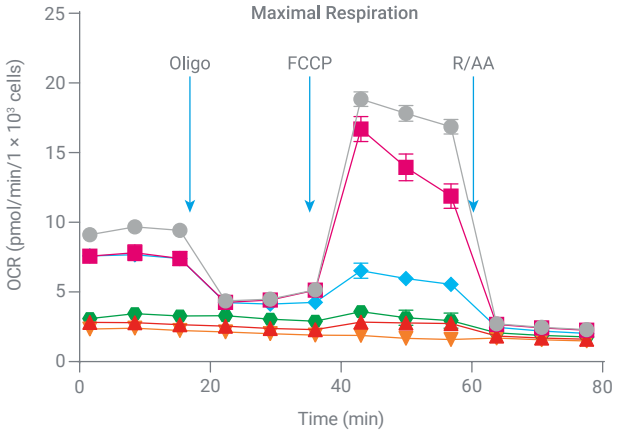
Application of XF PMP and the XF Electron Flow assay

Consequently, examining the precise enzyme or pathway driving observed changes in maximal respiration can provide additional insight and can further link specific alterations in metabolic enzymes with disease states. This application note explains how changes in mitochondrial function due to compound treatments or genetic interventions can be investigated by permeabilizing cells using XF PMP and the XF Electron Flow assay. First, the basis of the XF Electron Flow assay is described, followed by methods for analysis and interpretations of the assay data. Finally, key items for consideration in assay design and performance are discussed. These methods are ideal when testing the effects of interventions (genetic manipulation/compound treatment) on mitochondria, and can facilitate therapeutic development for elucidation of target identification, mechanism of action, and for measuring efficacy and specificity.

Notes:

- For information regarding the experimental details, reagent preparation, workflow, and performance of the XF Electron Flow assay with XF PMP, please see the Agilent XF PMP technical overview.[10]
- For information about differences in the use of intact cells, permeabilized cells, and isolated mitochondria, see Appendix I at the end of this document.

A.



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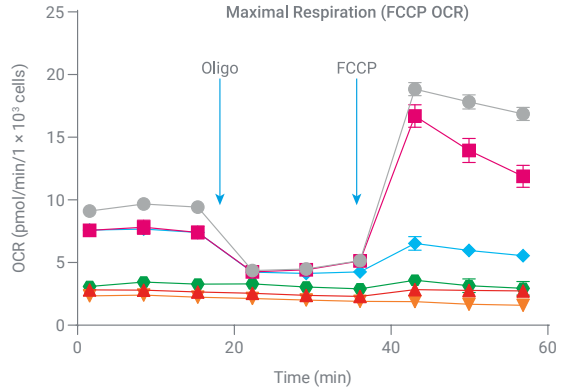

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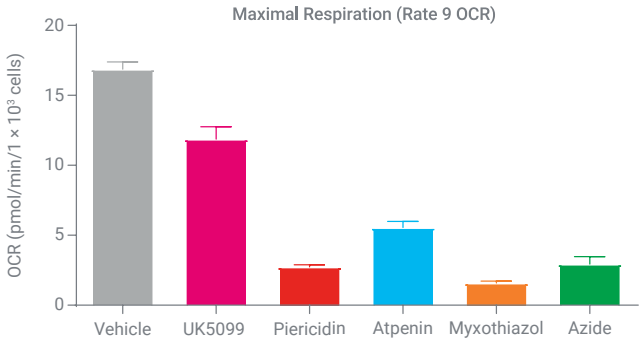
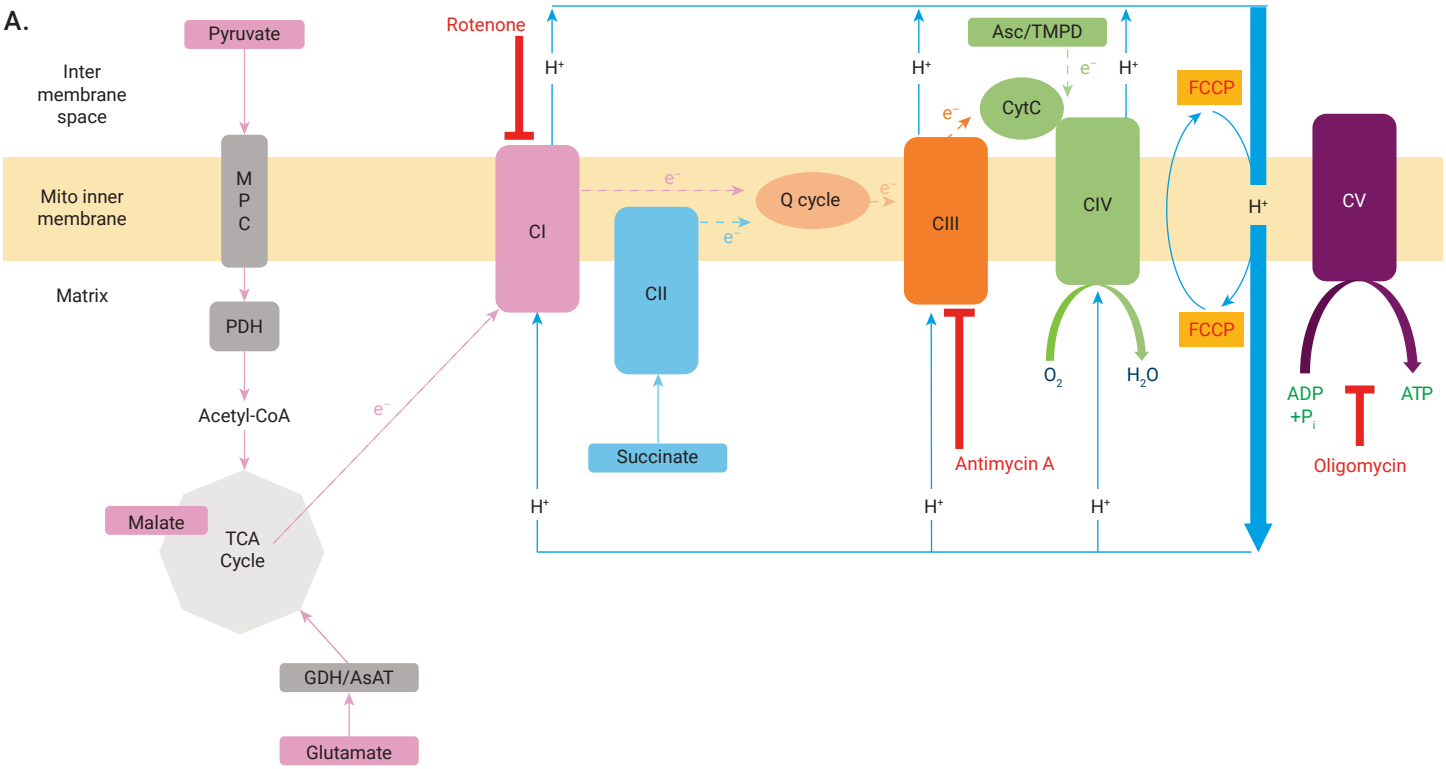


D.

Treatment	Concentration	Mode of Action	MTI
Vehicle	–	–	0.0
UK5099	2 µM	MPC inhibitor	-0.40
Piericidin	1 µM	CI inhibitor	-0.95
Atpenin	1 µM	CII inhibitor	-0.71
Myxothiazol	1 µM	CIII inhibitor	-1.0
Azide	20 mM	CIV inhibitor	-0.93

Figure 2. Agilent Seahorse XF Cell Mito Stress Test and (B) Agilent Seahorse XF Mito Tox assays were performed using HepG2 cells. Cells were acutely treated with compounds (or vehicle control) at the indicated concentration just before performance of the XF assay. All treatments elicit a decrease in the maximal respiration/FCCP OCR parameter compared to (C) the vehicle control, and display negative MTI values when tested using (D) the XF Mito Tox assay.

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 the 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

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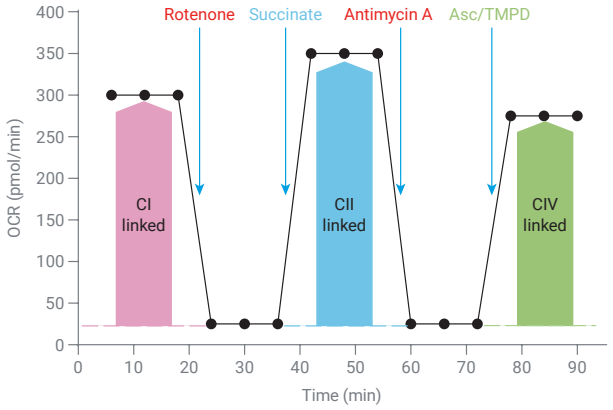


Figure 3. Agilent Seahorse XF Electron Flow assay design. As substrates enter the mitochondria using different pathways, controlling the specific substrate or group of substrates offered to permeabilized cells can correlate changes in molecular mechanisms of action with changes in the oxygen consumption rate (OCR). (A) Primary substrate oxidation pathways assessed by the XF Electron Flow assay. Complex I (CI), complex II (CII), and complex IV (CIV) linked substrates are labeled in light red, blue, and green, respectively. Inhibitors are shown in dark red. (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. Abbreviations: MPC = mitochondrial pyruvate carrier, PDH = pyruvate dehydrogenase; GDH = glutamate dehydrogenase; AsAT = aspartate aminotransferase; Asc = Ascorbate; TMPD = N,N,N',N' Tetramethyl-p-phenylenediamine; CytC = cytochrome C; Pi = inorganic phosphate. Adapted from Divakaruni, A. S. et al.[11]

Experimental

The XF Electron Flow assay: Theory and design

The XF Electron Flow assay can identify changes in mitochondrial substrate transport, substrate oxidation, or the electron transport chain

As noted, several XF assays provide multiparametric assessments of mitochondrial function using intact cells, each reporting the common parameter of maximal respiration (Figure 1). As the maximal respiration rate is governed by substrate supply and oxidation, changes (decreases) during an intervention can reflect changes in substrate transport, substrate oxidation, or the ETC. Therefore, examining the effects of interventions on specific substrate pathways is one approach to determining the mechanism underlying the observed differences. Using the XF Electron Flow assay with XF PMP treated cells allows assessment of specific mitochondrial substrate pathways and effects of interventions on mitochondrial substrate transport, oxidation, and ETC function.

The XF Electron Flow assay replicates the maximal respiration state of the respective intact cell XF assay (for example, the Cell Mito Stress Test or Mito Tox assay) while simultaneously allowing control of mitochondrial substrate provision via permeabilization with XF PMP (Figure 3). By exploiting the fact that different substrates feed differentially into mitochondrial pathways, it is possible to isolate the metabolic pathways that are responsible for decreases in the maximal respiration rate originally observed using intact cells (Figure 2).

The XF Electron Flow assay measures CI-, CII-, and CIV-linked 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, the intervention is applied (for example, compound treatment) to the XF PMP treated cells and the assay is started. As Figure 3C depicts, the first series of rate measurements assess the effect of the intervention on CI-linked respiration. Rotenone is then added to inhibit all CI-linked respiration and serve as a positive control for CI-linked inhibition. Next, succinate, a CII substrate, is injected to allow assessment of the effects of the intervention on CII-linked respiration. Antimycin A is then injected to inhibit CIII, blocking any CII, Q cycle, or CIII-linked respiration. Finally, a combination of ascorbate and TMPD, a CIV substrate, is injected to assess the effects of the intervention on CIV-linked respiration. It is important to note that the XF Electron Flow assay measures CI-, CII-, and CIV-linked respiration. For example, CI-linked respiration not only includes the activity of CI, but is also a sum of upstream events. This includes transport of CI substrates across the mitochondrial membrane (for example, pyruvate via the MPC), processing of the substrate via matrix enzymes (such as glutamate > α-ketoglutarate), and oxidation of the substrate via the TCA cycle (Figure 3A).The assay design is therefore ideal for investigating and assessing the effects of interventions on specific mitochondrial substrate oxidation pathways. It serves as an ideal next step in identifying the sources (targets) and causes (mechanisms of action) of decreases observed in maximal respiration using intact cells. For example, if the XF Electron Flow assay is performed using the same compounds as the XF Cell Mito Stress Test/Mito Tox assay (Figure 2), then the following results are obtained (Figure 4).

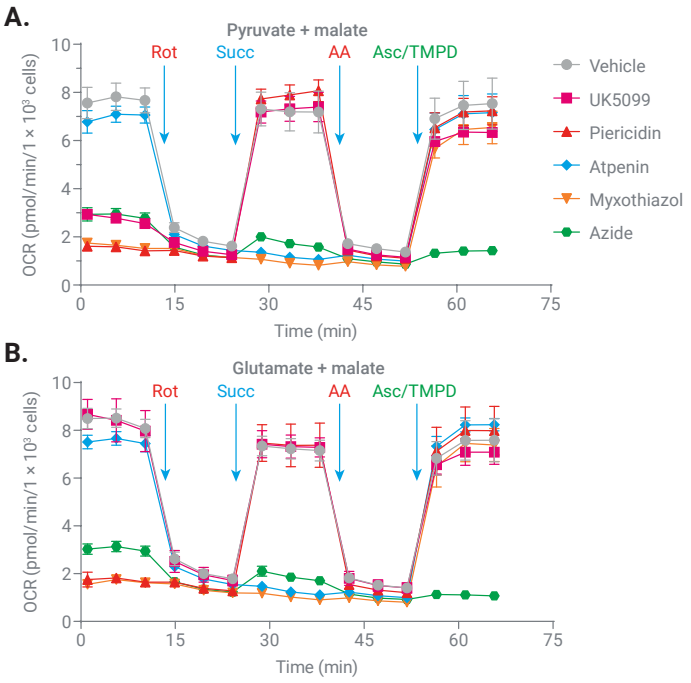


Figure 4. Agilent Seahorse XF Electron Flow assays. Cells were acutely treated with the same compounds as in Figure 2 (or vehicle control) just before performance of the XF assay. (A) Electron Flow assay using pyruvate + malate as the initial CI-linked substrate. (B) Electron Flow assay using glutamate + malate as the initial CI-linked substrate.

Data transformation, analysis, and interpretation

Qualitative interpretation using kinetic graphs: Patterns of inhibition

To begin interpreting XF Electron Flow assay data, it is recommended to review the kinetic graph, as each type of inhibitor will yield a unique pattern based on the site or mechanism of action. Note: before biological interpretation, the data should be inspected for quality or technical issues, as described in XF PMP technical overview, 5994-7548EN.[10]

CI-linked inhibition is revealed by a decrease in pyruvate or glutamate-driven respiration, measured in the first series of rate measurements (before the injection of rotenone), compared to the control group. As there are various substrates/points of entry to the TCA cycle and ultimately the CI (Figure 3A), it is recommended to perform the XF Electron Flow assay with both P/M and G/M as the primary initial CI-linked substrates to discriminate between different types of CI-linked inhibition.

Replotting the data in Figure 4 by compound (to show both P/M and G/M-driven CI respiration) illustrates key differences in the kinetic traces due to differences in the site or action of the inhibitor used (Figure 5). For example, UK5099, inhibits pyruvate-driven respiration but not glutamate-driven respiration, suggesting that the site of action is somewhere within the pyruvate oxidation pathway (for example, the MPC or PDH), upstream of CI (Figure 5A). In comparison, piericidin inhibits both pyruvate and glutamate-driven respiration (Figure 5B), suggesting that the site of action is common to both substrate pathways (such as the TCA cycle or CI).
Note that neither of these compounds elicit significant decreases in CII- (succinate-driven) or CIV-linked (Asc/TMPD-driven) respiration, indicating that the inhibition is specifically CI-linked.

CII-linked inhibition is revealed by decreases in succinate-driven respiration, measured in the third series of rate measurements (after the injection of succinate and before the injection of antimycin A), compared to the control group. For example, atpenin inhibits oxidation of succinate, but not CI- or CIV-linked respiration, suggesting that the site of action is specific to CII-linked respiration (Figure 5C).

Q cycle and CIII-linked inhibition are revealed by a decrease in both CI-driven (pyruvate and glutamate) and CII (succinate) driven respiration, as illustrated for the compound myxothiazol (Figure 5D). CIV-linked respiration does not decrease, indicating that the inhibition is specifically Q cycle/CIII-linked. Other explanations for this pattern may include interventions that affect regulation of Ca^{2+} concentration and flux within the mitochondria and among the cytoplasm/endoplasmic reticulum, respectively.

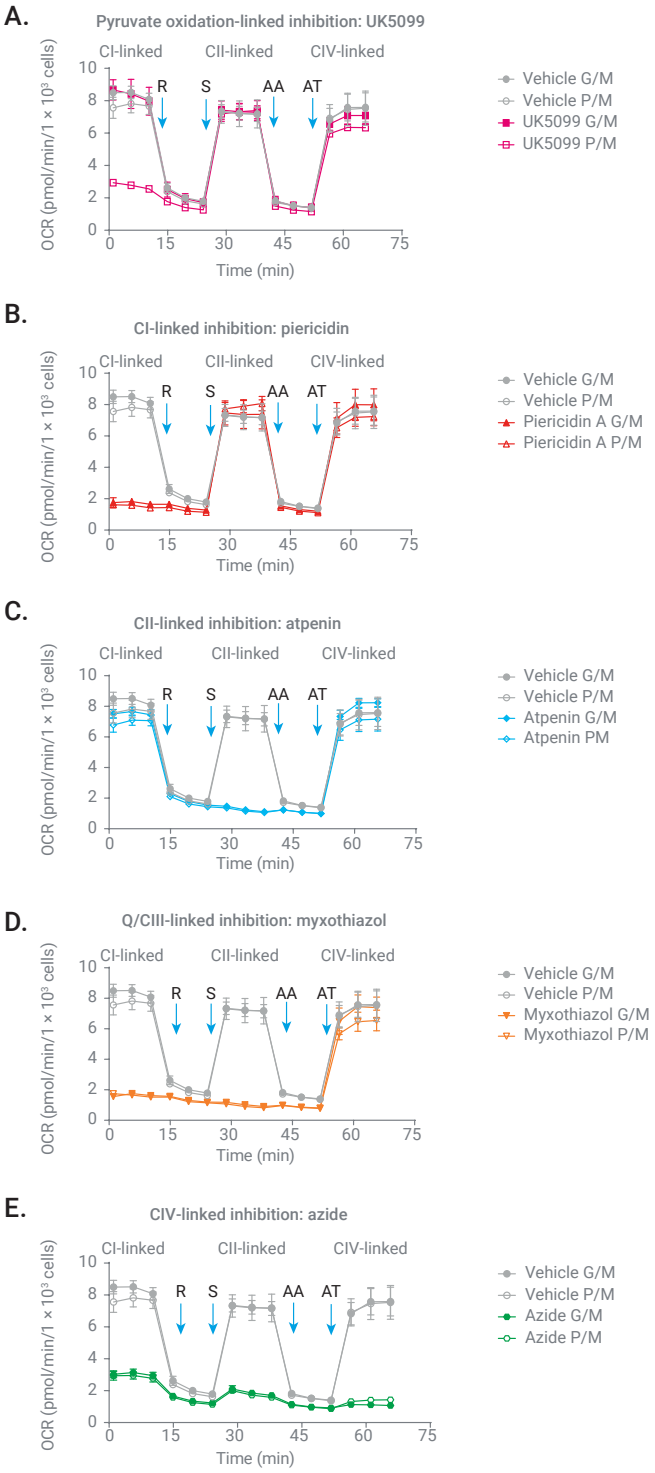


Figure 5. Agilent Seahorse XF Electron Flow assay examples of inhibition through different mechanisms. The data from Figure 4 were replotted for each individual compound to compare P/M (pyruvate + malate) and G/M (glutamate + malate) dependent CI-linked respiration. (A) UK5099, (B) piericidin A, (C) atpenin, (D) myxothiazol, (E) azide. The differences in kinetic patterns reveal different types of inhibition, summarized in Table 1 data.

CIV-linked inhibition is revealed by a decrease in all CI-, CII-, and CIV-linked respiration parameters compared to the control group, as observed with the CIV-inhibitor, azide (Figure 5E). These patterns for qualitative assessment of inhibition are summarized in Table 1.

Table 1. Qualitative assessment of inhibition type using the XF Electron Flow assay.

XF Electron Flow Assay: Qualitative Assessment of Inhibition Type				
Rates 1–3	Rates 7–9	Rates 13–15		Suggested inhibition type
Substrate provided				
P/M	G/M	Succinate	Asc/TMPD	
Nc	Nc	Nc	Nc	None
↓	Nc	Nc	Nc	Pyruvate oxidation/CI-linked
Nc	↓	Nc	Nc	Glutamate oxidation/CI-linked
↓	↓	Nc	Nc	CI-linked
Nc	Nc	↓	Nc	CII-linked
↓	↓	↓	Nc	Q/CIII-linked
↓	↓	↓	↓	CIV-linked
Nc defined as no significant change compared to control within experimental error ↓ Defined as significant change compared to control within experimental error				

Quantitative analysis of Electron Flow assay results: Calculation of CI-, CII-, and CIV-linked respiration

Quantitative analysis of CI-, CII-, and CIV-linked inhibition may also be performed using the equations below and summarized in Figure 6.

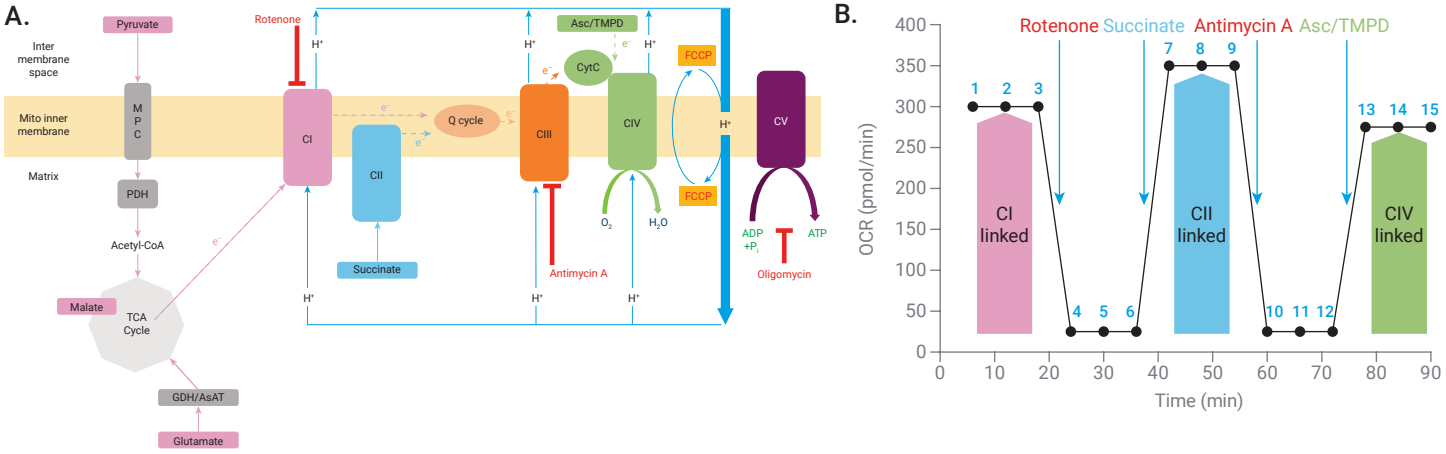
CI-linked OCR = Rate 3 OCR (rate before rotenone injection) – Rate 6 OCR (rate before succinate injection).

CII-linked OCR = Rate 9 OCR (before AA injection) – Rate 12 OCR (rate before Asc/TMPD injection).

CIV-linked OCR = Rate 13 OCR (rate post-Asc/TMPD injection) – Rate 12 OCR (rate before Asc/TMPD injection).

Quantitative analysis for Q/CIII-linked OCR cannot be performed using the XF Electron Flow assay. For further information on testing Q/CIII-linked respiration directly, see Appendix I.

The bar chart–delta calculator widget in Agilent Seahorse Analytics can be used to calculate delta OCRs between measurements for each well (Figures 7A to C), and the results can be exported to Microsoft Excel or GraphPad Prism for further analysis (Figure 8).



C.

			XF Electron Flow Assay Conditions					Measurement Points Used for Data Transformation		
ETC complexes tested			Injections					Calculations for vehicle control group and experimental groups		
CI	CII	CIV	Starting substrate	A	B	C	D	CI-linked OCR	CII-linked OCR	CIV-linked OCR
√	√	√	P/M or G/M	Rotenone	Succinate	Antimycin A	Ascorbate/ TMPD	(Rate 3 OCR) – (Rate 6 OCR)	(Rate 9 OCR) – (Rate 12 OCR)	(Rate 13 OCR) – (Rate 12 OCR)

Figure 6. Quantitative data transformation guidelines for XF Electron Flow assay results. (A) An overview of mitochondrial substrate transport, processing, oxidation, and ETC activity under the conditions of the XF Electron Flow assay. (B) A schematic of the XF Electron Flow assay outlining rate measurements and parameters of CI-, CII-, and CIV-linked respiration. (C) A summary of the XF Electron Flow assay and the rate measurements used for quantitative assessment of XF Electron Flow data.

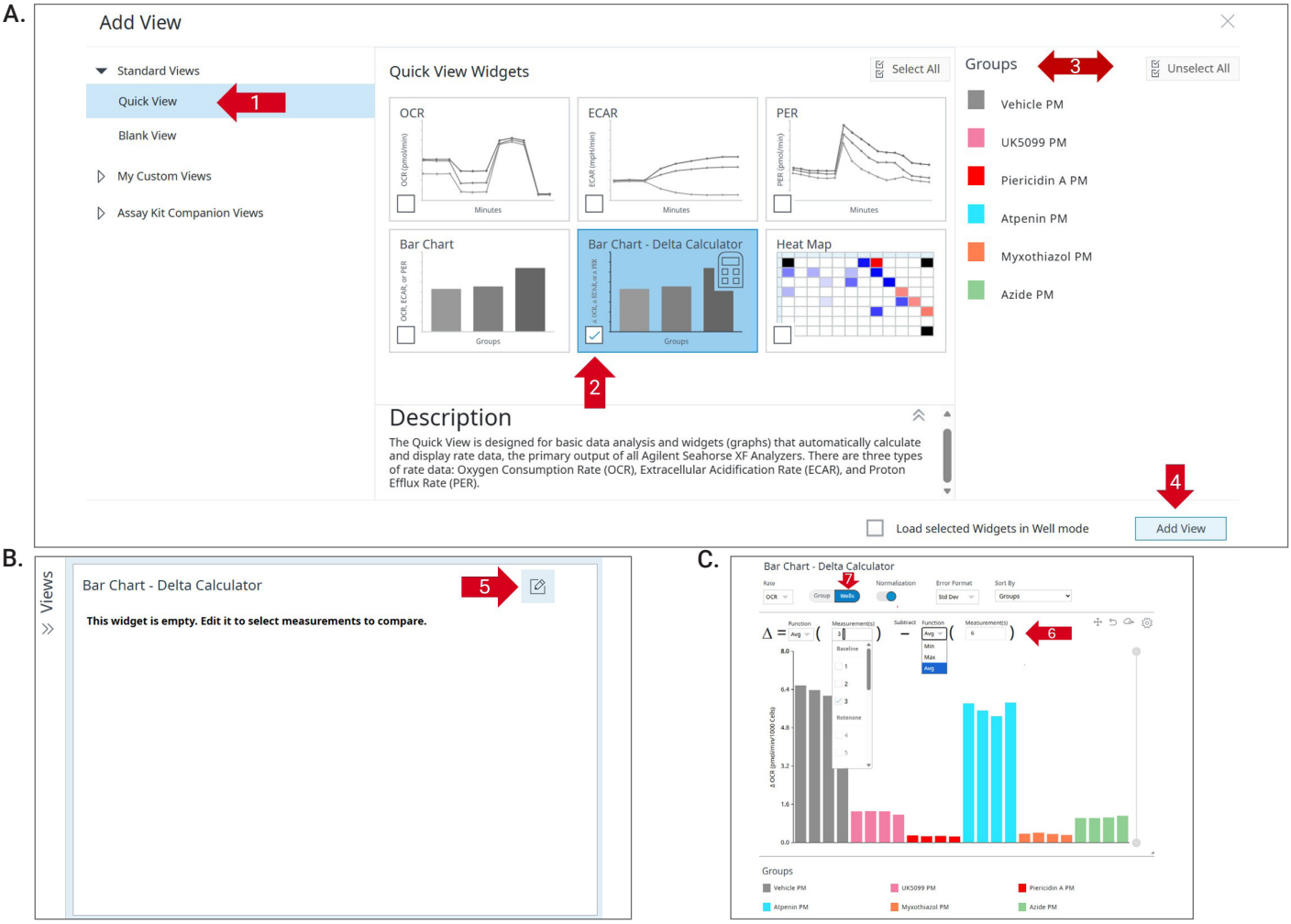
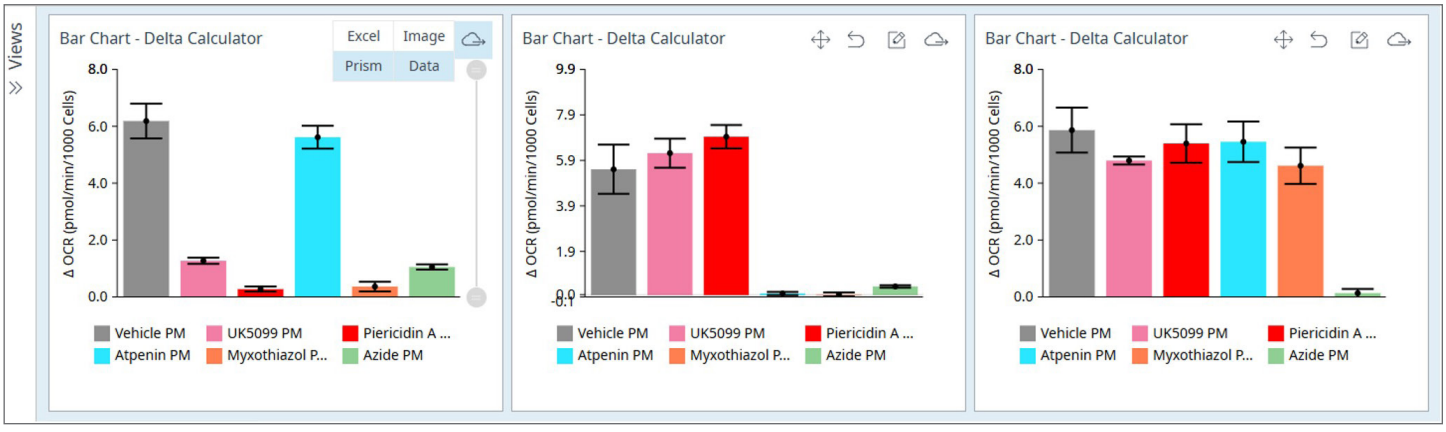


Figure 7. Using the Bar Chart–Delta Calculator to analyze XF Electron Flow data. (A) Steps to add the Bar Chart–Delta Calculator in Agilent Seahorse Analytics. (B) Empty Bar Chart–Delta Calculator widget. (C) Active Bar Chart–Delta Calculator window showing an example of Complex I–linked respiration calculation (Rate 3 minus Rate 6), displayed in well format.



CI-linked respiration (rate 3 minus rate 6) | CII-linked respiration (rate 9 minus rate 12) | CIV-linked respiration (rate 13 minus rate 12)

Figure 8. Pyruvate/malate dependent Complex I-, II-, and IV-linked respiration calculated using the Bar Chart–Delta Calculator and displayed in group format. Data and images can be exported by clicking the cloud icon.

The qualitative pattern of inhibition shown in Table 1 is still evident for each compound when comparing responses across CI-, CII-, and CIV-linked respiration (Figures 8). For example, piericidin shows inhibition only in CI-linked respiration, atpenin shows inhibition only in CII-linked respiration, while azide shows inhibition in CI-, CII-, and CIV-linked respiration.

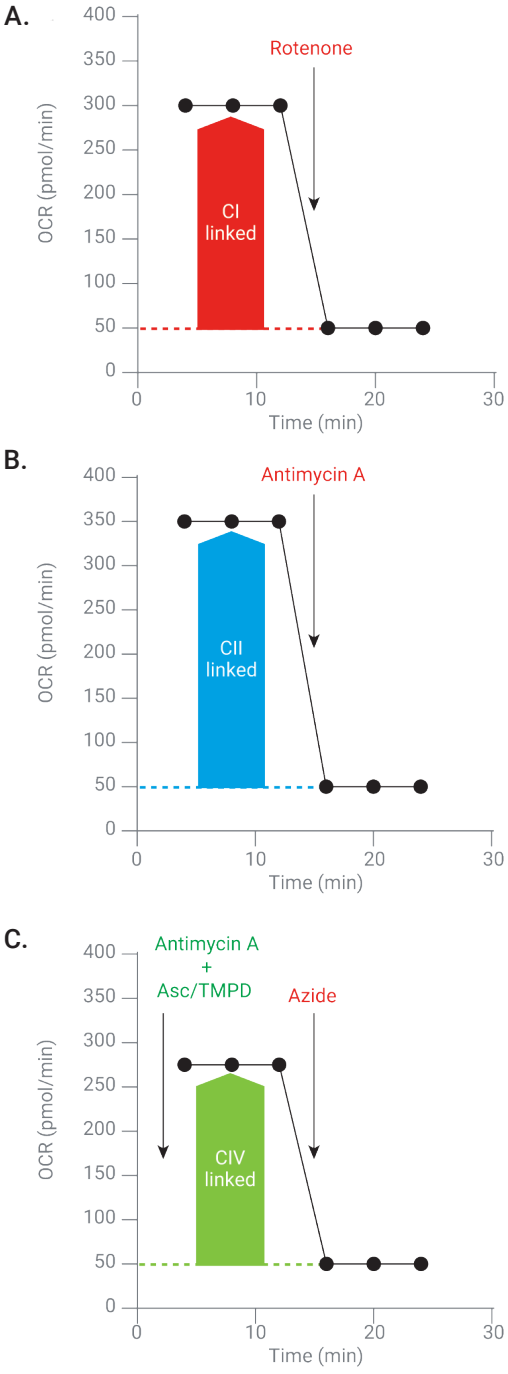


Figure 9. A schematic outline of single XF ETC complex assays for assessment of (A) CI-, (B) CII-, or (C) CIV-linked respiration.

Alternative assay workflows: Single and truncated XF ETC complex assays

Overview of single XF ETC complex assays

The XF Electron Flow assay may be divided into three separate assays, each measuring CI-, CII- and CIV-linked respiration. These assays are summarized in Figure 9 and their performance is described in 5994-7548EN.[10]

These assays are best applied after a standard XF assay has been performed. As noted above, treatments can exert both specific and nonspecific effects on mitochondrial function. These effects are often concentration-dependent, and 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, then single ETC complex assays are useful for further characterization or reducing assay time and materials required.

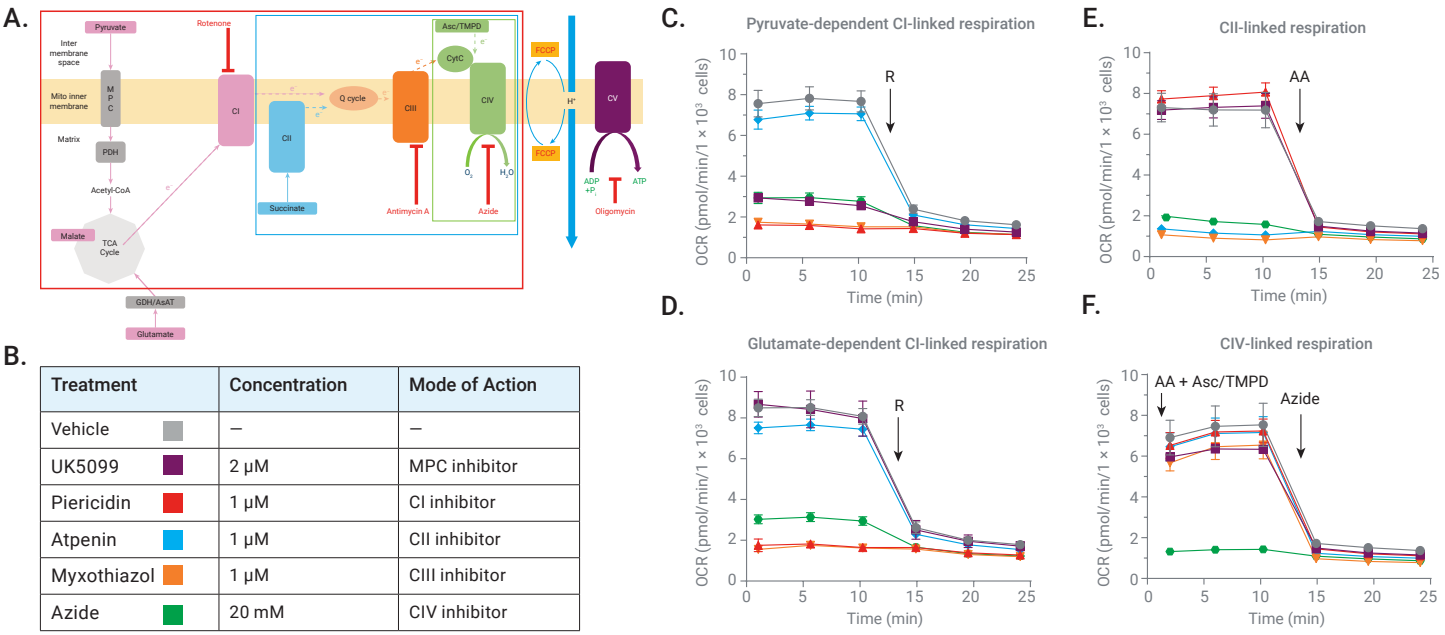
Patterns of inhibition: Qualitative interpretation using kinetic graphs

Data analysis, transformation, and interpretation for each assay is performed in a similar manner to the standard (four injection) XF Electron Flow assay, with minor differences outlined below.

CI-linked assay. This assay provides a CI-linked substrate to permeabilized cells and uses rotenone as a positive control for CI-linked inhibition. Similar to the standard Electron Flow assay, it is advised to perform the CI assay using more than one CI-linked substrate (such as pyruvate/malate and glutamate/malate) to identify specific substrate pathway inhibition versus direct inhibition of CI. For example, UK5099 inhibits pyruvate-dependent, but not glutamate-dependent, CI-linked respiration (Figure 10, C versus D). Other CI-linked substrates may be used as noted below for the standard Electron Flow assay (Figure A3).

It is imperative to realize that CIII-linked and CIV-linked inhibitors will also show inhibition of CI-linked respiration (for example, myxothiazol and azide, Figure 10, C and D). It is recommended that the standard Electron Flow assay be performed first to examine both specific and potential nonspecific effects of the treatment before using the individual CI assay.

CII-linked assay. This assay provides succinate + rotenone as the initial substrate (Figure 10E) and assays CII-linked respiration. Antimycin A is used as a positive control for inhibition. Compounds such as atpenin and malonate may be used if specific positive controls for inhibition of CII-linked respiration are desired.



B.

Treatment	Concentration	Mode of Action
Vehicle	–	–
UK5099	2 µM	MPC inhibitor
Piericidin	1 µM	CI inhibitor
Atpenin	1 µM	CII inhibitor
Myxothiazol	1 µM	CIII inhibitor
Azide	20 mM	CIV inhibitor

Figure 10. Single XF Electron Flow assay patterns of CI-, CII- or CIV-linked inhibition. (A) Primary substrate oxidation pathways assessed by the XF single ETC assays. CI-, CII-, and CIV-linked substrates are labeled in light red, blue, and green, respectively. Inhibitors are shown in dark red. The proton circuit (H+) has been omitted for clarity. (B) Treatment identity, concentration, and mode of inhibition. (C) XF CI assay using pyruvate/malate as the CI substrate. (D) XF CI assay using glutamate/malate as the CI substrate. (E) XF CII assay using succinate/rotenone as the CII substrate. (F) XF CIV assay using ascorbate/TMPD as the CIV substrate. In all cases, HepG2 cells were pretreated with inhibitors shown in (B) just before the assay.

Table 2. Single XF Electron Flow assay qualitative patterns of CI-, CII- or CIV-linked inhibition.

Qualitative Patterns of Inhibition Using Single ETC Complex Assays				
Complex tested	**Substrate**	**Rate 3**	**Inhibition type, compared to control**	**Considerations**
CI-linked	P/M	↓	Pyruvate-dependent, CI-linked	CIII- and CIV-linked inhibitors will also show decreases in OCR relative to control
	G/M	↓	Glutamate-dependent, CI-linked	
CII-linked	Succinate + rotenone	↓	CII-linked	CIII- and CIV-linked inhibitors will also show decreases in OCR relative to control
CIV-linked	AA + Asc + TMPD	↓	CIV-linked/other	An AA + Asc + TMPD injection is the first step in the assay, before rate measurement 1
↓ Defined as significant change compared to control within experimental error.				

Again, it is imperative to understand that CIII-linked and CIV-linked inhibitors will also show inhibition of CII-linked respiration (such as myxothiazol and azide, Figure 10E). It is recommended that the standard Electron Flow assay be performed first to examine both specific and potential nonspecific effects of the treatment before using the individual CII assay.

CIV-linked assay. This assay is slightly different in that it begins with succinate + rotenone as the initial substrate during preparation. A combination of AA + Asc + TMPD is injected before any rate measurements to inhibit CII/CIII-linked respiration and provide Asc/TMPD as a substrate for CIV respiration are carried out (Figure 10F).

In this example, only azide shows inhibition of CIV-linked respiration. These patterns for qualitative assessment of inhibition are summarized in Table 2.

Quantitative analysis of single complex assays

Data analysis, transformation, and quantitative analysis of single ETC complex XF Electron Flow assays are identical to that of the standard XF Electron Flow assay, except for the rate measurements used for calculations. In each case, the specific ETC complex-linked respiration is assessed using Rate 3 – Rate 6. The initial assay conditions, injections, and measurement points used in the data transformation for single ETC complex assays are outlined in Table 3.

Table 3. Single XF Electron Flow assays. Measurement points used for quantitative assessment of CI-, CII- or CIV-linked inhibition.

			XF Electron Flow Assay Conditions			Measurement Points Used for Data Transformation		
ETC complexes tested			Injections			Calculations for vehicle control group and experimental groups		
CI	CII	CIV	Starting substrate	A	B	CI-linked OCR	CII-linked OCR	CIV-linked OCR
√			Pyruvate + malate or glutamate + malate	Rotenone	-	(Rate 3 OCR) – (Rate 6 OCR)		
	√		Succinate + rotenone	Antimycin A	-		(Rate 3 OCR) – (Rate 6 OCR)	
		√	Succinate + rotenone	Antimycin A + Ascorbate + TMPD	Azide			(Rate 3 OCR) – (Rate 6 OCR)

Table 4. Truncated XF Electron Flow assays. Measurement points used for quantitative assessment of CI-, CII- or CIV-linked inhibition.

			XF Electron Flow Assay Conditions				Measurement Points Used for Data Transformation			
ETC complexes tested			Injections				Calculations for vehicle control group and experimental groups			Considerations
CI	CII	CIV	Starting substrate	A	B	C	CI-linked OCR	CII-linked OCR	CIV-linked OCR	
√	√		Pyruvate + malate or Glutamate + malate	Rotenone	Succinate	Antimycin A	(Rate 3 OCR) – (Rate 6 OCR)	(Rate 9 OCR) – (Rate 12 OCR)		CIII- and CIV-linked inhibitors will also show decreases in OCR relative to control.
√		√	Pyruvate + malate or Glutamate + malate	Antimycin A	Ascorbate + TMPD	Azide	(Rate 3 OCR) – (Rate 6 OCR		(Rate 9 OCR) – (Rate 12 OCR)	CIII inhibitors will also show decreases in OCR relative to control for CI-linked OCR.
	√	√	Succinate + rotenone	Antimycin A	Ascorbate + TMPD	Azide		(Rate 3 OCR) – (Rate 6 OCR	(Rate 9 OCR) – (Rate 12 OCR)	CIII inhibitors will also show decreases in OCR relative to control for CII-linked OCR.

Truncated XF Electron Flow assays

Truncated XF Electron Flow assays (for example, assessment of CI- and CII-linked respiration) can also be performed. The initial assay conditions, injections, and measurement points used for data transformation, as well as considerations for use, are outlined in Table 4.

XF Electron Flow assay workflow: Further considerations

Alternative CI-linked substrates

As shown in Figure A3 (Appendix), there are several other substrates that feed into CI via the TCA cycle, notably β-hydroxybutryate and acyl CoA, or acyl carnitine (palmitoyl CoA or palmitoyl carnitine), via ketone body and beta oxidation pathways, respectively.

These substrates can be employed to investigate the effects of interventions on these specific substrate oxidation pathways and can be used as alternatives to P/M or G/M as initial CI substrates in the Electron Flow assay.[11,12]

For long chain fatty acids, either palmitoyl carnitine (+malate), or palmitoyl CoA + carnitine (+malate) may be used as an initial CI-linked substrate in the XF Electron Flow assay. This is to isolate the effect of the intervention to the long chain fatty acid oxidation pathways. For more a detailed and mechanistic analysis of the long chain fatty acid oxidation pathways, alternative XF workflows using permeabilized cells (or isolated mitochondria) are recommended.[13]

Another CI-linked substrate, methyl pyruvate, which can permeate the mitochondrial membrane, can be used to bypass the active transport of pyruvate via the MPC. It was employed to illustrate that the target of UK5099 and TZDs is the MPC.[11]

CIII-linked substrates

As shown in Figure A3, Glycerol-3-phosphate (G3P) can serve as a specific Q/CIII-linked substrate.[11,12] However, the ability to use G3P depends on the expression levels of G3P dehydrogenase (G-3-PDH), which is cell type-dependent. As designed, the Electron Flow assay does provide a qualitative assessment of Q/CIII-linked inhibition.

Acute versus chronic interventions

Regarding compound treatments, it is recommended to provide the compound of interest acutely to the permeabilized cells, just before performance of the Electron Flow assay. This is to ensure that any effects observed are specific to mitochondrial function versus effects on cell viability, gene expression, or signaling events that can be influenced when cells are treated chronically (hours to days) with the compound of interest.

If an assessment of chronic treatments is desired, then it is recommended to design three sets of assays to access both acute (mitochondrial) and chronic effects (mitochondrial or cellular) of the intervention, outlined in Table 5.

Table 5. Chronic and acute compound treatment guidelines.

Treatment Type	Provide Compound in Cell Growth Media?	Provide Compound in XF Electron Flow Assay Media?
Chronic only	Yes	No
Acute only	No	Yes
Chronic + acute	Yes	Yes

Anytime a chronic intervention is performed, it is recommended to also perform a cell viability assay to assess if the compound induces long-term cytotoxic effects.

Compound treatments (drug permeability: Intact cells versus PMP treated cells)

Another consideration and potential source of difference when using intact versus permeabilized cells is the ability of the compound to cross the plasma membrane. As noted, many substrates and compounds are unable/kinetically slow to cross the plasma membrane. However, upon cell permeabilization, compounds that typically would not cross the membrane (thus exerting no effect on the mitochondria and showing no change in maximum respiration) are now accessible to, and, in turn, can potentially affect the mitochondria. A classic example of this is carboxyactractyloside, an inhibitor of the adenine nucleotide transferase (ANT), which shows no effect on respiration when provided to intact cells, but inhibits coupled (State 3) respiration when provided to permeabilized cells or isolated mitochondria.[14] These differences between intact and PMP-treated cells with respect to compound accessibility can also result in different apparent compound efficacy (for example, IC_{50} values).

Functional assessment of genetic manipulations

Similar to compound treatments, the XF Electron Flow assay may be used to test the specific effects of genetic manipulations on mitochondrial function. Effects may or may not persist depending on target location (nucleus, cytoplasm, mitochondria, and related signaling events). For example, this assay can be used to functionally confirm if a mitochondrial targeted genetic modification has resulted in changes in gene/protein expression levels. For example, functional proof of MPC1 and MPC 2 knockdown was illustrated by showing that KD cells can oxidize several CI-linked substrates (including methyl pyruvate), but are unable to utilize pyruvate.[11]

Permeabilized-cell mitochondrial function sequencing (PMF-seq), which has been recently introduced, combines the power of bioenergetics, where specific substrates and inhibitors can be provided to the mitochondria, with parallel genetic screening that employs modern technologies such as CRISPR.[15] For example, plasma membranes within a pool of CRISPR mutagenized cells are permeabilized, allowing detailed bioenergetic analysis. Cells with desired bioenergetic parameters can then be selected optically, using flow cytometry, and subjected to next-generation sequencing (NGS).

Cell growth and cytotoxic effects

As noted, chronic drug treatments and genetic manipulations may alter cell growth rates or cell viability. In these cases, it is recommended to perform both cell counting and cell viability assays to normalize/account for any changes in cell growth or cytotoxic effects of the intervention. The Agilent Cytation and NovoCyte instruments provide streamlined solutions for assessing the number of live cells when using adherent and suspension cells, respectively.[16,17]

Uncouplers and oxidative phosphorylation inhibitors

Due to the standard initial conditions of the XF Electron Flow assay (the presence of oligomycin and FCCP, to inhibit CV and uncouple oxidative phosphorylation from the ETC, respectively), it is not suitable for assessment of oxidative phosphorylation inhibitors (OPIs) and uncouplers. These types of modulation may be revealed when using XF assays that report basal respiration and proton leak (uncoupling) parameters, such as the XF Cell Mito Stress Test or Mito Tox assays. In such cases, both intact and permeabilized cells, as well as isolated mitochondria, are typically used to further characterize these types of effects, and different assay workflows using XF PMP are employed.[18,19]

ECAR and pH level data

It is important to note that under conditions of cell permeabilization, cytoplasmic function (including glycolysis) ceases due to the dilution of cytoplasmic contents and the presence of a nonionic buffer. ECAR is therefore not reflective of glycolytic activity, nor should any such interpretations be made using ECAR data. It is critical to review the pH-level data (described below) to ensure that the pH values remain within acceptable limits (pH 7.0 – 8.0) for the duration of the assay. Large changes in pH value due to compound treatments can significantly impact mitochondrial function, and can interfere with the accuracy of data interpretation and conclusions.

Materials and methods

HepG2 cells were seeded in Agilent Seahorse XF Pro M cell culture microplates at a density of 2.0×10^4 cells per well. They were cultured in low-glucose (5.5 mM) DMEM (Gibco 11885), supplemented with 2 mM GlutaMAX and 10% serum. Plates were kept in the thermostat cabinet for 1 hour after seeding, then incubated at 5% CO_2 at 37 °C in a humidified atmosphere for 24 hours.

The materials and methods used to perform the XF PMP Electron Flow assay are listed in Table 6.

Table 6. Agilent products required to perform the XF Electron Flow assay using XF PMP.

Item	Part Number
Agilent Seahorse XF Pro analyzer	-
Agilent Seahorse XF Pro M FluxPak	103775-100
Agilent Seahorse XF PMP	102504-100
Agilent Reservoir, 12 column, polypropylene	201280-100

Mitochondrial substrates, modulators, and reagents for the MAS buffer were prepared as described in the Agilent Seahorse XF Plasma Membrane Permeabilizer and XF Electron Flow Assay technical overview.[10] All XF assays were performed following the procedures described in the guide, including preparation of compounds, reagents, and sensor cartridges.[10]

Appendix I

Substrate provision and utilization—Intact cells vs permeabilize-cells and isolated mitochondria

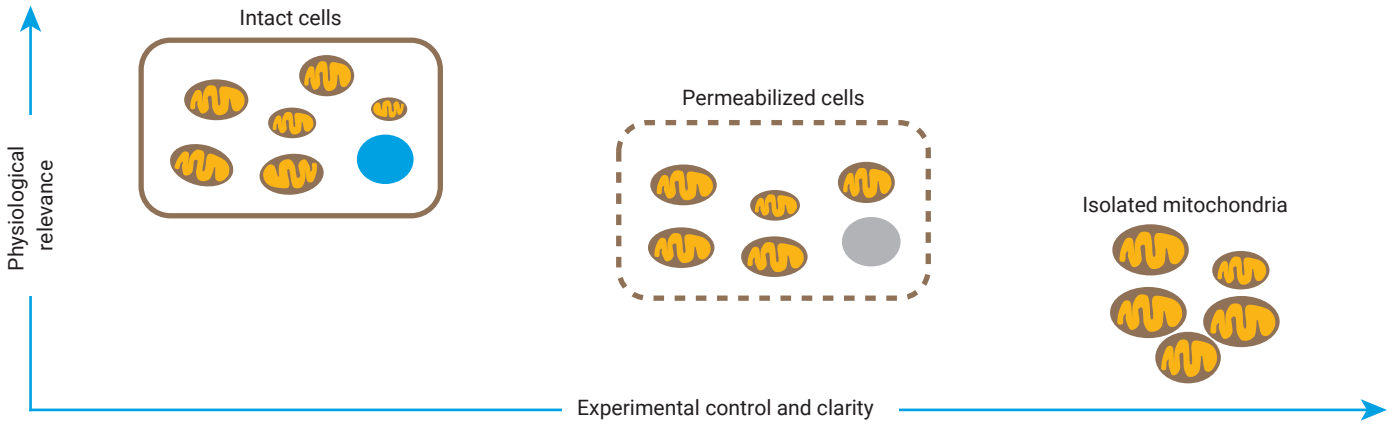
Use of intact cells versus permeabilized cells and isolated mitochondria in the XF analyzer: Application and advantages

It is important to define different types of sample materials that can be used to investigate mitochondrial function using XF technology, illustrated in Figure A1. The XF assay kits (Cell Mito Stress Test, for example) all use "intact cells" (cells that have not had the plasma membrane or internal contents altered in any physical way). The XF technology can also be used with permeabilized cells.[11,12] These are defined as cells that have been treated so that pores are formed in the plasma membrane, allowing experimental access to the internal contents of the cell and, for this purpose, the mitochondria. Isolated mitochondria, typically obtained from animal tissue, are defined as mitochondria that have been physically purified away from all other cellular contents and exist in an isolated state. They can also be assessed using XF technology.[20]

Each material has distinct advantages and disadvantages (biological and technical) when being used to investigate mitochondrial function, summarized in Figure A1. For example, while intact cells provide greater physiological relevance, there is much less control over the experimental conditions. On the other hand, using permeabilized cells or isolated mitochondria may offer less physiological relevance, but these sample types are often a better model for investigating details and mechanisms of mitochondrial function. This is due to greater control over variables and experimental conditions.

Permeabilized cells provide experimental control over substrate provision and utilization

The key advantage is that permeabilized (XF PMP-treated) cells and isolated mitochondria allow control over substrate provision and utilization that can be difficult, or even impossible to control using intact cells. When assaying intact cells, various cellular and mitochondrial substates are provided in the assay media, typically glucose, pyruvate, glutamine (and other amino acids). While these substrates



Intact Cells	XF PMP Treated Cells	Isolated Mitochondria
Use when hypothesis being tested requires intact cellular system for function (for example: change in signaling, gene expression, nutrient uptake)	Use when hypothesis being tested involves a change intrinsic to mitochondria	
Provides information on mitochondria, glycolysis, and ATP production rates	Mitochondrial mechanistic information (all nuclear and cytoplasmic function stops)	
Limited experimental control over substrate provision/utilization (The cell chooses which substrate(s) to use)	High degree of experimental control over substrate provision/utilization (well-established protocols and analysis of mechanism)	
No artifacts due to mitochondrial isolation		Mitochondria subject to damage during isolation
Best for studying interventions to primary cells and established cell lines		Best for studying tissue samples from adult animals (no cell model)
Physiological relevance		Mechanistic understanding

Figure A1. Intact versus permeabilized cells, versus isolated mitochondria. Use of XF PMP treated cells and isolated mitochondria allow experimental control over substrate provision and utilization.

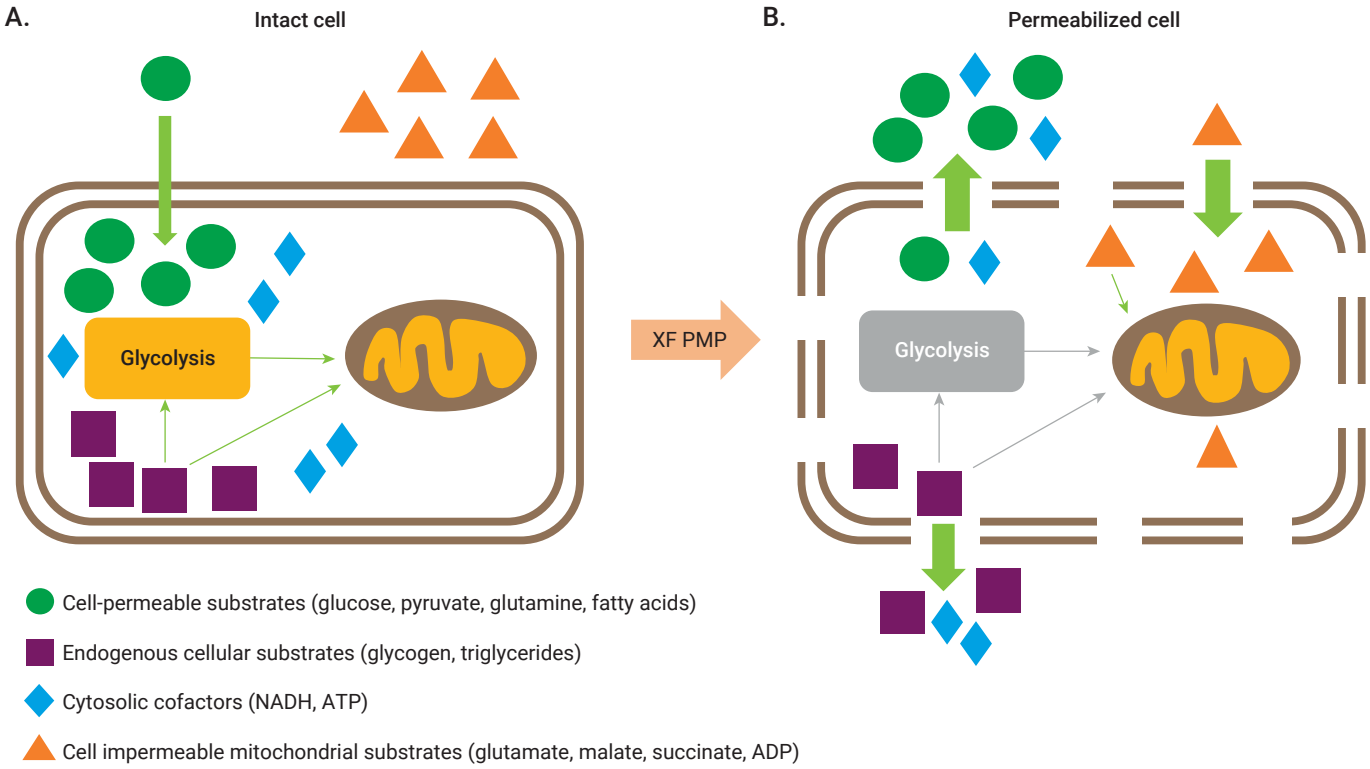


Figure A2. Permeabilization with XF PMP allows control over mitochondrial substrate provision and utilization. (A) Intact cells utilize a combination of provided and endogenous substrates to support glycolysis and mitochondrial function. Some mitochondrial substrates are impermeable to the plasma membrane. (B) Upon permeabilization of the plasma membrane using XF PMP, experimental control over the provision of mitochondrial substrates is achieved, allowing specific substrate oxidation pathways and downstream ETC complexes to be investigated. Further, upon permeabilization of the plasma membrane, cytosolic activity, including glycolysis, ceases due to the loss of substrates and cytosolic cofactors into the assay media.

may be provided, it is important to note that the cell may also have stored (endogenous) substrates at its disposal (including glycogen and triglycerides). Thus, despite having experimental options for substrate provision, there is little, if any, experimental control over actual substrate utilization when employing intact cells; an intact cell may (or may not) use any combination of provided or endogenous substrates. Another major limitation of intact cells is that many mitochondrial substrates (such as malate, glutamate, succinate, and ADP) are unable to cross the plasma membrane freely, also preventing experimental control over the substrates that the mitochondria are oxidizing (Figure A2, panel A).

The fundamental reason for using permeabilized cells or isolated mitochondria is having direct access to the mitochondria, allowing control over both substrate provision and utilization by the mitochondria (Figure A2, panel B). Different mitochondrial substrates use different pathways for transport, enzymatic modification, entrance to the TCA cycle and the electron transport chain (Figure A3).

Both pyruvate and glutamate, as examples, are CI-linked substrates, but pyruvate enters the TCA cycle through a different transport/dehydrogenase pathway compared to glutamate. In addition, there are substrates that can be linked to specific ETC complexes, such as succinate for CII. Because of this access and control, one can investigate the effects of treatments that result in changes to mitochondrial function, including target, mechanism of action, and assessment of treatment efficacy and specificity.

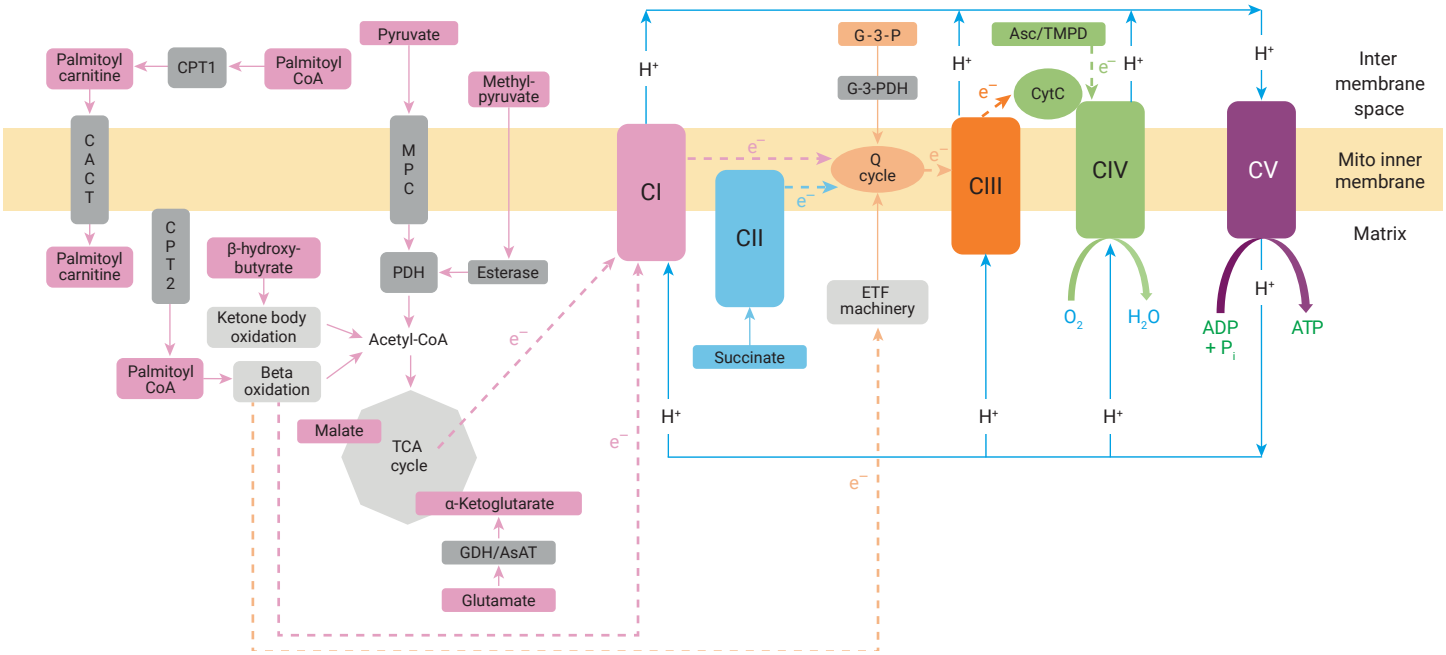


Figure A3. Oxidizable substrates feed into different parts of the respiratory chain. OCR is a measure of respiratory chain activity involving subsets of electron transport chain complexes. Complex I and Complex II-linked substrates are labeled in red and blue, respectively, and substrates linked directly to the ubiquinone (Q) pool are labeled in pale orange (note that fatty acid oxidation involves both). Electrons are delivered to cytochrome oxidase (Complex IV) with ascorbate and TMPD, labeled in green. MPC = mitochondrial pyruvate carrier, PDH = pyruvate dehydrogenase, GDH = glutamate dehydrogenase, AsAT = aspartate aminotransferase, CPT1 = carnitine palmitoyl transferase 1, CACT = carnitine acyl carnitine transferase, CPT2 = carnitine palmitoyl transferase 2, ETF = electron transfer flavoprotein related enzymes, G-3-PDH = Glycerol-3-phosphate dehydrogenase, TMPD = N,N,N',N' Tetramethyl-p-phenylenediamine, CytC = cytochrome C, Pi = inorganic phosphate. Adapted from 5994-7548.[11]

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Products used in this application

Agilent products

Agilent Seahorse XF plasma membrane permeabilizer

www.agilent.com/lifesciences/discoverXF

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