

Comprehensive Assessment of Mitochondria Complex Activity in Mature Adipocytes

Using the Agilent Seahorse XF plasma membrane permeabilizer

Authors

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Abstract

Mechanistic studies using isolated mitochondria in the Agilent Seahorse XF analyzer provide direct access to electron transport chain (ETC) complexes, precise substrate control, and often clearer interpretation of mitochondrial activity compared with intact cell studies. However, isolating mitochondria from cultured cells (particularly adipocytes) is technically challenging and frequently results in low yield or compromised quality. The Agilent Seahorse XF Plasma Membrane Permeabilizer (XF PMP), a recombinant cytolysin with selective affinity for cholesterol, selectively permeabilizes the plasma membrane while preserving mitochondrial integrity, enabling mechanistic studies without the need to isolate mitochondria.

This application note describes an optimized XF PMP-based workflow for comprehensive assessment of electron transport chain (ETC) activity in mature 3T3-L1 adipocytes. The assay enables simultaneous evaluation of multiple respiratory complexes in the same sample while preserving mitochondrial stability and maximal respiration during extended assay periods. A proof-of-concept study demonstrating the time- and dose-dependent inhibition of Complex I by metformin is also presented.

Introduction

The Agilent Seahorse XF Plasma Membrane Permeabilizer (PMP) is a recombinant cytolysin with selective affinity for cholesterol, enabling selective permeabilization of the plasma membrane while preserving mitochondrial membrane integrity.¹ In live-cell Seahorse XF assays like XF cell mito stress test, maximal respiration is influenced by many factors, and a decrease in maximal respiration can be caused by changes in substrate transport, substrate oxidation, or electron transport chain (ETC) activity. By utilizing the XF PMP, the Agilent Seahorse XF electron flow (EF) assay bypasses intact live cell limitations by reproducing the maximal respiration state observed in intact cell assays while allowing precise control of mitochondrial substrate availability. Because distinct substrates enter mitochondria through specific metabolic pathways, this targeted approach allows direct identification of the pathways responsible for impaired mitochondrial respiration.

Traditionally, revealing the mechanisms underlying metabolic dysfunction requires mitochondrial isolation. This process is technically demanding, time-consuming, and often yields mitochondria of variable quality, with a high risk of subselection, particularly when starting from cultured cells. Moreover, in situ interrogation of oxidative pathways has been limited by the lack of reagents that selectively permeabilize the plasma membrane without compromising mitochondrial integrity. Traditional detergents such as saponin and digitonin require careful titration and can adversely affect mitochondrial function, including loss of cytochrome c from the intermembrane space. Seahorse XF PMP addresses these limitations by enabling safe, reproducible plasma membrane permeabilization in situ, allowing direct control of mitochondrial substrates while preserving mitochondrial function. This approach eliminates the need for mitochondrial isolation and avoids the drawbacks associated with detergent-based permeabilization.

Experimental

Differentiation of 3T3-L1 preadipocytes

3T3-L1 fibroblasts were obtained from ATCC (part number CL-173) and cultured in DMEM (Gibco, part number 11995) supplemented with 10% calf bovine serum (ATCC, part number 30-2030). Cells were passaged at approximately 80% confluence. For differentiation, about 20,000 cells per well were seeded into gelatin-coated (0.2%, 100 μ L per well) 24-well Agilent Seahorse XF V28 plates (Part number 100882-004). One day post-confluence (designated day zero), adipogenic differentiation was induced using DMEM containing 2% fetal bovine serum (FBS)(HyClone, part number SH30070.03),

0.5 mM 3-isobutyl-1-methylxanthine (IBMX)(Sigma, part number I5879), 5 μ M rosiglitazone (Sigma, part number R2408), 1 μ M dexamethasone (Sigma, part number D4902), and 400 nM insulin (Sigma, part number I9278). After 48 hours (day two), the induction medium was replaced with DMEM containing 2% FBS and 400 nM insulin. From day four onward, cells were maintained in DMEM-based differentiation medium containing 2% FBS and 50 nM insulin. Medium exchange was performed every two days.

Seahorse XF assays

Day-six 3T3-L1 adipocytes were used for XF PMP and FCCP titration assays and day seven 3T3-L1 adipocytes were used for all other XF EF assays. All assays were performed using the Seahorse XF24 V28 cell culture microplates on the Agilent Seahorse XF Flex analyzer. Assay cycles consisted of 3 minute mixing, 0 minute waiting, and a 2 minute 10 second measurement period. Measurement duration can be adjusted as needed but should be $\geq$ 2 minutes to ensure data accuracy.

1) XF PMP titration in mature 3T3-L1 adipocytes

Succinate and ADP were used as substrates to titrate the optimal XF PMP concentration in mature 3T3-L1 adipocytes, as neither crosses the intact plasma membrane. Mitochondrial assay solution (MAS) buffer was prepared as in Table 1, and XF EF assay buffer in Table 2. Rotenone was added to prevent ETC backflow which can generate ROS through Complex I (CI). For this particular assay, XF PMP was prepared in MAS buffer supplemented with 0.2% BSA to reduce its binding to injection ports. In other XF EF assays, port injection reagents were prepared in basal MAS buffer to minimize bubble formation, and XF PMP was added directly to the cell wells as a component of the XF EF assay buffer. It is important to ensure that the final assay buffer pH is maintained between 7.2 and 7.4. Detailed compound catalog and preparation method can be found in Agilent Technologies Technical Overview 5994-7548EN (Determine Mitochondrial Complex Activities in Permeabilized Cells Using the Agilent Seahorse XF Electron Flow Assay with Agilent Seahorse XF PMP).²

Table 1. MAS buffer.

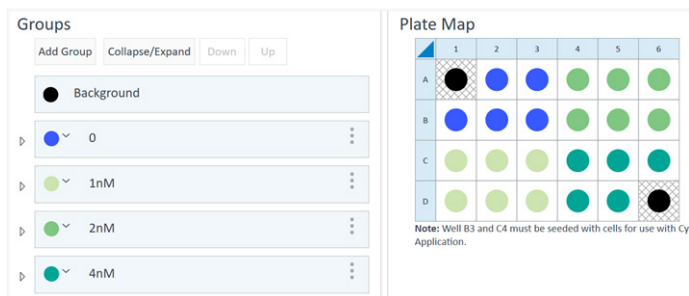
Compound	Final Concentration
Mannitol	220 mM
Sucrose	70 mM
KH ₂ PO ₄	10 mM
MgCl ₂	5 mM
HEPES	5 mM
EGTA	1 mM

Table 2. XF EF assay buffer for XF PMP titration (in MAS buffer).

Compound	Final Concentration
Succinate	10 mM
Rotenone	2 μ M
ADP	4 mM
Fatty acid free BSA	0.2%

The standard XF assay templates include a 12-minute equilibration which is disabled for XF PMP titration assays to minimize exposure of cells to hypertonic MAS buffer in the absence of permeabilization agent.

Following one basal measurement, XF PMP was injected through port A (0, 10, 20, or 40 nM), one concentration for of each quarter of the plate (Figure 1), yielding final concentrations of 0, 1, 2, or 4 nM. Oxygen consumption rate (OCR) was measured for 50 cycles, totaling 258 minutes after XF PMP injection.

**Figure 1.** Plate map for XF PMP titration experiment.

2) FCCP titration in XF PMP permeabilized mature 3T3-L1 adipocytes

The Complex II (CII) substrate, succinate, was used for FCCP titration because it provides cleaner and more stable measurements, whereas CI-driven respiration can be influenced by fluctuations in the NAD⁺/NADH ratio and upstream transport processes, such as the mitochondrial carrier protein (MCP) system.

An XF PMP concentration of 2 nM was determined to be optimal for mature adipocytes (Figure 3A) and was included in the XF EF assay buffer subsequently (Table 3). For this and subsequent assays, the 12-minute equilibration step in the XF assay protocol was enabled as OCR typically requires approximately 10 minutes to reach maximal levels following cell permeabilization (Figure 3A).

Table 3. XF EF assay buffer for FCCP titration (in MAS buffer).

Compound	Final Concentration
Succinate	10 mM
Rotenone	2 μ M
ADP	4 mM
Fatty acid free BSA	0.2%
XF PMP	2 nM

FCCP was prepared in MAS buffer and titrated at final concentrations of 0.5, 1, 2, and 4 μ M. The assay protocol is provided in Table 4. The extended measurement period following FCCP injection was designed to evaluate how long mitochondria can sustain maximal respiration, in this case, CII activity, under uncoupled conditions.

Table 4. FCCP titration assay protocol.

	Reagent	Final Concentration	Cycle
Basal	-	-	3
Port A	Oligomycin	2 μ M	4
Port B	FCCP	0.5 to 4 μ M	50

3) Assessment of respiratory control ratio

Respiratory control ratio (RCR) is a vital parameter in bioenergetics that measures the efficiency of oxidative phosphorylation in mitochondria. It is defined as the ratio of ADP-stimulated respiration (state 3) to resting respiration after ADP depletion (state 4). In XF EF assays, state 4o, obtained after oligomycin injection to inhibit ATP synthase, is commonly used in place of state 4. This oligomycin-induced state provides an estimate of nonphosphorylating respiration, which can help reduce the contribution of residual ATP turnover that may be present in traditional state 4 measurements.^{3,4}

For RCR determination, ADP, oligomycin, FCCP, and rotenone/antimycin A were injected sequentially as described in Table 5. Both CI- and CII-driven RCR were assessed using pyruvate/malate and succinate, respectively (Table 6).

Table 5. RCR assessment assay protocol.

	Reagent	Final Concentration	Cycle
Basal	-	-	3
Port A	ADP	4 mM	3
Port B	Oligomycin	2 μ M	4
Port C	FCCP	2 μ M	3
Port D	Rotenone/ Antimycin A	2 μ M/ 10 μ M	4

Table 6. XF EF assay buffer for RCR assessment (in MAS buffer).

Complex I		Complex II	
Compound	Final concentration	Compound	Final concentration
Pyruvate	10 mM	Succinate	10 mM
Malate	1 mM	Rotenone	2 μ M
XF PMP	2 nM	XF PMP	2 nM
Fatty acid free BSA	0.2%	Fatty acid free BSA	0.2%

4) Assessment of mitochondria respiratory complex activities

For sequential assessment of CI, CII and CIV activities in the same sample, day-seven 3T3-L1 adipocytes permeabilized with XF PMP were supplied with pyruvate/malate for CI-linked respiration. ADP, oligomycin and FCCP were added to collectively induce/mimic conditions of maximal respiration used for intact cell XF assays (Table 7). Rotenone was subsequently injected through port A to inhibit CI activity, followed by succinate injection through port B to assess CII-linked respiration.

Table 7. XF EF assay buffer for assessing mitochondria respiratory complex activity (in MAS buffer).

Compound	Final Concentration
Pyruvate	10 mM
Malate	1 mM
Oligomycin	2 μ M
ADP	4 mM
FCCP	2 μ M
Fatty acid free BSA	0.2%
PMP	2 nM

Antimycin A was then used to inhibit CIII activity, while *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD) reduced by ascorbate served as an artificial electron donor to enable measurement of CIV-linked respiration. Detailed reagent concentrations and XF assay protocol are provided in Table 8.

Table 8. XF EF assay protocol for sequential assessment of CI, II and IV activities.

	Reagent	Final Concentration	Cycle
Basal	-	-	3
Port A	Rotenone	2 μ M	3
Port B	Succinate	10 mM	4
Port C	Antimycin A	10 μ M	3
Port D	TMPD/Asc	0.1 mM/10 mM	4

Cells were pretreated immediately before the assay with DMSO (control), piericidin A (2 μ M; Cayman, part number 15379), atpenin A5 (1 μ M; Cayman, part number 11898), or sodium azide (20 mM; Sigma, part number S2002) to demonstrate representative inhibition of distinct respiratory chain complexes.

To distinguish between CI and upstream inhibition, day-seven 3T3-L1 adipocytes were divided into two substrate conditions following permeabilization. One group received pyruvate/malate as the initial substrate, as described in Table 7, while in the other group pyruvate/malate was replaced with glutamate/malate (10 mM each). Within each substrate condition, wells were further subdivided into vehicle control (DMSO) or 2 μ M UK5099 treatment for 45 minutes prior to initiation of the XF EF assay. All groups were then assayed using the same protocol outlined in Table 8.

To assess Complex I inhibition by metformin, day-seven 3T3-L1 adipocytes were permeabilized as described in Table 7 and assayed according to the protocol outlined in Table 9. Metformin stock solution (1 M prepared in Milli-Q H₂O; Sigma, part number D150959) was diluted in MAS buffer to 100 mM, 20 mM, and 400 μ M for port injection, yielding final concentrations of 10 mM, 2 mM, and 40 μ M, respectively. Control wells (0 metformin) received MAS buffer alone from the corresponding port.

Table 9. XF EF assay protocol for assessing complex I inhibition by metformin.

	Reagent	Final Concentration	Cycle
Basal	-	-	3
Port A	Metformin	0 to 10 mM	24
Port B	Rotenone	2 μ M	4

Results and discussion

Robust adipogenic differentiation of 3T3-L1 preadipocytes under reduced-serum conditions

To minimize oxygen depletion when maximal complex activity is measured under substrate-replete conditions, 3T3-L1 preadipocytes were differentiated using 2% FBS rather than the conventional 10% FBS protocol. Reducing serum concentration suppresses post-induction clonal expansion without compromising terminal differentiation, as confirmed by adipocyte morphology with evident lipid accumulation (Figure 2). This low-serum differentiation strategy has also been adopted in brown adipocyte XF assays to improve respiratory measurements by experienced users.⁵

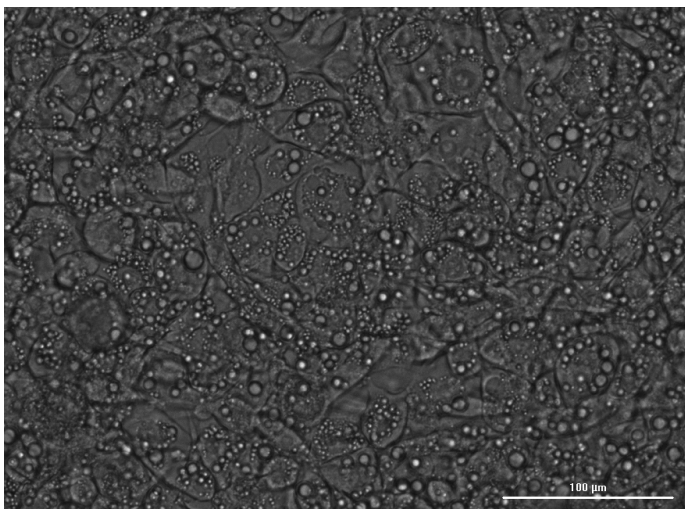


Figure 2. Representative 20x Agilent BioTek Cytation image of 3T3-L1 adipocytes at day seven of differentiation cultured in medium containing 2% FBS. Scale bar = 100μm

Optimization of XF PMP concentration for permeabilization studies in 3T3-L1 adipocytes

For most cell types, 1 nM XF PMP is recommended for assessing mitochondrial ETC complex activities, as described in the Agilent Technologies technical overview 5994-7548EN (Determine Mitochondrial Complex Activities in Permeabilized Cells Using the Agilent Seahorse XF Electron Flow Assay with Agilent Seahorse XF PMP) and previous publications.¹ However, due to the high culture density of adipocytes and their unusually high intracellular lipid content, we reoptimized the XF PMP concentration to ensure effective cell permeabilization.

As shown in Figure 3A, treatment with 1 nM XF PMP required approximately one hour to achieve maximal OCR and complete permeabilization, which is impractical for most XF assays. In contrast, full permeabilization was achieved within ~10 minutes when 2 or 4 nM XF PMP was used. At both concentrations, maximal OCR was sustained for at least four hours, and oxygen levels remained above the recommended threshold (50 mmHg) throughout the assay.

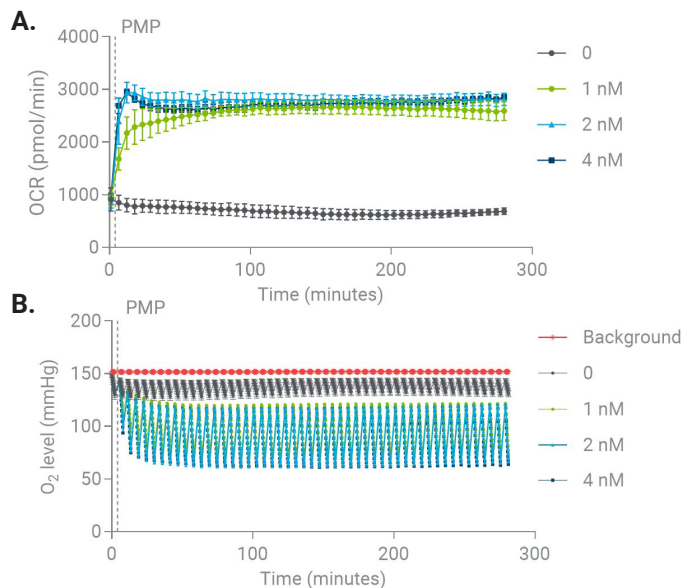


Figure 3. XF PMP titration in 3T3-L1 adipocytes. A) OCR kinetic traces, B) O₂ level traces.

Optimization of uncoupler concentration for permeabilized 3T3-L1 adipocytes XF assays

Typically, the optimal FCCP concentration established for intact cells is suitable for XF PMP electron flow assays. To determine whether this holds true for 3T3-L1 adipocytes, around 2 μM FCCP was titrated in XF PMP-permeabilized mature adipocytes, the concentration previously identified as optimal in intact live cells.

FCCP titration showed that all tested concentrations (0.5, 1, 2, and 4 μM) ultimately achieved comparable maximal respiration. However, 0.5 and 1 μM FCCP required a longer time to reach maximal OCR. Maximal CII-linked respiration was sustained throughout the entire 50 measurement cycles following FCCP injection (~258 minutes), indicating strong stability of maximal CII activity in XF PMP-permeabilized condition (Figure 4). Considering the fast and durable response, 2 μM FCCP was selected for subsequent experiments to assess maximal ETC complex activities.

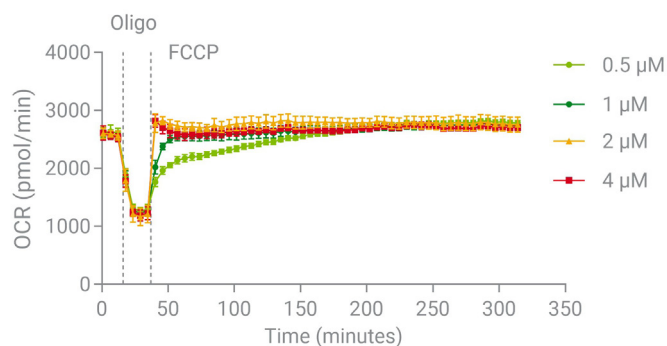


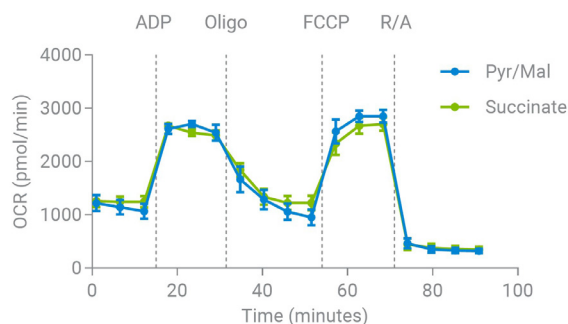
Figure 4. OCR kinetic traces of FCCP titration assay in XF PMP permeabilized 3T3-L1 adipocytes. Oligo: Oligomycin

XF PMP permeabilization results in healthy coupled mitochondria in 3T3-L1 adipocytes

Figure 5A shows the OCR kinetic traces under basal conditions and following sequential injections of ADP, oligomycin (Oligo), FCCP, and rotenone/antimycin A (R/A) in XF PMP-permeabilized day-seven 3T3-L1 adipocytes. The respiratory control ratio (RCR) was calculated using the following formula:

$$RCR = \frac{\text{State 3 respiration}}{\text{State 4o respiration}} = \frac{\max \text{ OCR(ADP)} - \min \text{ OCR(R/A)}}{\min \text{ OCR(Oligo)} - \min \text{ OCR(R/A)}}$$

A.



B.

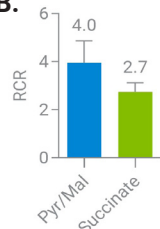


Figure 5. RCR assessment in XF PMP permeabilized 3T3-L1 adipocytes. A) OCR kinetic traces B) Calculated RCR using pyruvate/malate (Pyr/Mal) or succinate as substrate. Data are mean $\pm$ SD of seven to eight technical replicates. R/A: Rotenone

In XF PMP-permeabilized 3T3-L1 adipocytes, the RCR obtained is approximately four for CI-driven respiration and approximately 2.7 for CII-driven respiration (Figure 5B). These values are much higher than RCRs reported in the literature using digitonin-permeabilized white adipose tissue⁶ or isolated mitochondria from white adipocytes,⁷ indicating good mitochondrial integrity and coupling efficiency when using the XF PMP permeabilization procedure.

The Agilent Seahorse Analytics (SHA), a cloud-based data analysis tool, includes a **Bar Chart - Delta Calculator** widget capable of calculating delta OCR values, which can simplify RCR determination (Figure 6).

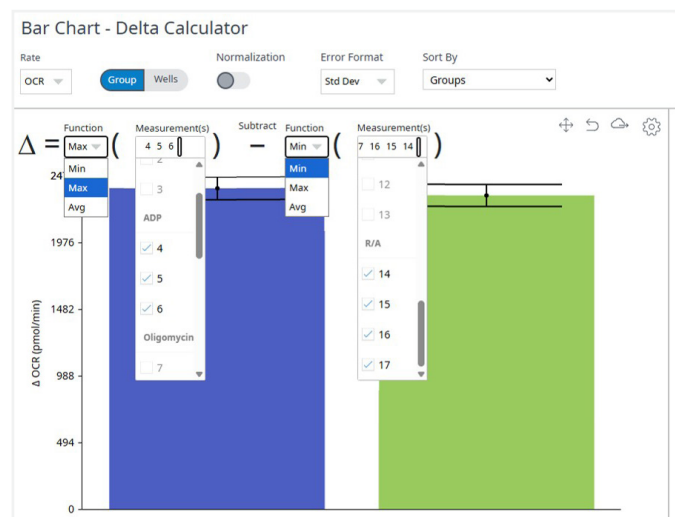


Figure 6. Bar Chart - Delta Calculator widget in Agilent Seahorse Analytics illustrating the calculation of RCR numerator, OCR max (ADP) - OCR min (R/A), across experimental groups; the resulting value represents state 3 respiration and can be exported to Microsoft Excel or Graph Prism.

Sequential assessment of CI, II, and IV activities in the same sample

Immediately before starting the assay, XF PMP-permeabilized 3T3-L1 adipocytes were treated with piericidin A (a CI inhibitor), atpenin A5 (a CII inhibitor), or sodium azide (a CIV inhibitor) as a proof-of-concept to demonstrate how XF electron flow assays can help identifying the site of mitochondrial inhibitor drug action. Control cells were treated with DMSO and assay was performed in MAS buffer containing pyruvate/malate as starting mitochondrial substrates.

Figure 7A shows the OCR kinetic traces for all four groups. As expected, piericidin A specifically impaired CI-linked respiration without affecting CII- or CIV-linked respiration. Atpenin A5 selectively inhibited CII-linked respiration while sparing CI- and IV-linked pathways. In contrast, sodium azide broadly suppressed respiration driven by CI, II, and IV (Figures 7A to D). The inhibition of CI- and CII-driven respiration by azide is indirect, resulting from a bottleneck at CIV that stalls electron flow. In comparison, piericidin A and atpenin A5 provide branch-specific inhibition without creating a downstream bottleneck, as electrons from the unaffected complex can still proceed through the remaining pathway: CI » Ubiquinone » CIII » CIV and CII » Ubiquinone » CIII » CIV, respectively.

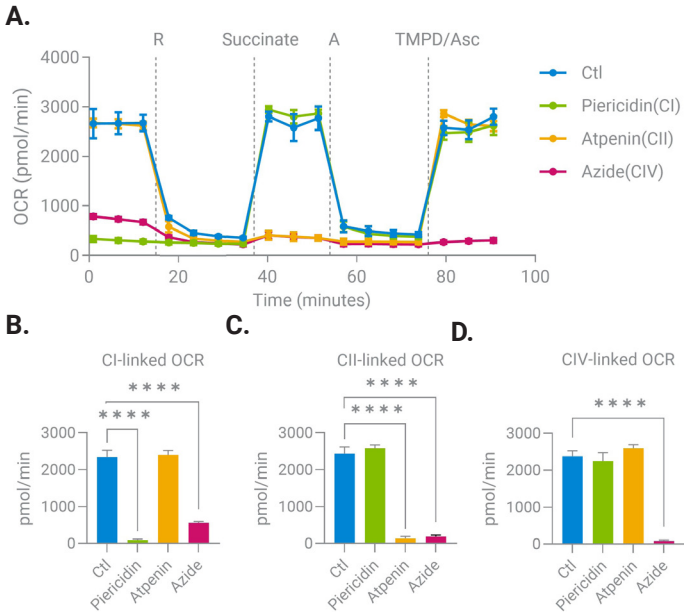


Figure 7. XF EF assay testing complex inhibitors in XF PMP permeabilized 3T3-L1 adipocytes. (A) OCR kinetic traces (B) Complex I-linked respiration (measurement 3 – minimum OCR after rotenone). (C) Complex II-linked respiration (maximum OCR after succinate – minimum OCR after antimycin A). (D) Complex IV-linked respiration (maximum OCR after TMPD/Asc – minimum OCR after antimycin A). R: Rotenone. A: Antimycin A. TPMD/Asc: *N,N,N',N'*-tetramethyl-*p*-phenylenediamine/Ascorbate. Data are mean $\pm$ SD of five to six technical replicates and representative of two independent experiments. *****P* < 0.0001.

The **Bar Chart - Delta Calculator** widget in SHA was used to calculate complex linked respirations shown in Figure 8.



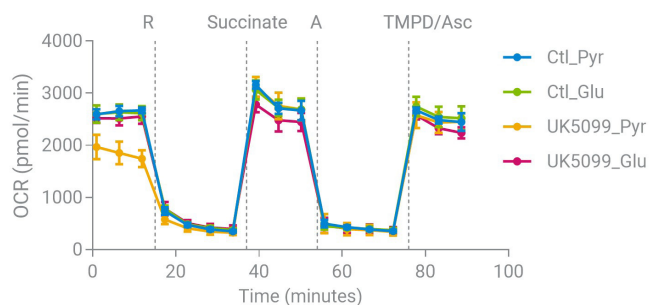
Figure 8. **Bar Chart - Delta Calculator** widget in Agilent Seahorse Analytics illustrating the well-wise calculation of OCR measurement 3 – OCR min (rotenone), the resulting value represents Complex I-linked respiration and can be exported to Microsoft Excel or Graph Prism.

Discriminating CI versus upstream inhibition

CI-linked respiration reflects not only CI enzymatic activity, but also the integrated contribution of upstream processes. These include transport of CI substrates across the mitochondrial membrane (such as pyruvate through the mitochondrial pyruvate carrier), substrate processing by matrix enzymes (such as conversion of glutamate to α -ketoglutarate), and subsequent oxidation through the tricarboxylic acid (TCA) cycle. To differentiate direct CI inhibition from impairment of upstream steps, it is recommended to perform the XF EF assay using both pyruvate/malate and glutamate/malate as initial CI-linked substrates.

UK5099, a mitochondrial pyruvate carrier (MPC) inhibitor, was used as a tool compound for this purpose. When pyruvate/malate is used as the initial substrate, the XF EF assay data suggests selective CI inhibition. In contrast, when glutamate/malate is provided, CI-linked respiration is largely preserved (Figure 9). This difference arises because UK5099 blocks pyruvate entry into mitochondria rather than directly inhibiting CI. As a result, only pyruvate-dependent CI-linked respiration is reduced, while glutamate-dependent CI-linked respiration remains intact.

A.



B.

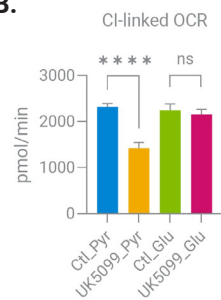


Figure 9. XF EF assay testing effect of UK5099 on CI-linked respiration using pyruvate/malate versus glutamate/malate as initial substrate in XF PMP permeabilized 3T3-L1 adipocytes. (A) OCR kinetic traces. (B) Complex I-linked respiration calculated as OCR measurement 3 minus the minimum OCR after rotenone injection. R: Rotenone. A: Antimycin A. TPMD/Asc: *N,N,N',N'*-tetramethyl-*p*-phenylenediamine/ Ascorbate. Data are mean $\pm$ SD of five to six technical replicates and representative of two independent experiments. **** $P < 0.0001$; ns, not significant.

Assessing Complex I inhibition by metformin

Metformin is an oral medication widely used to treat type 2 diabetes mellitus. It is a mild inhibitor of mitochondrial CI, although there is ongoing debate in the literature regarding whether therapeutic plasma concentrations are sufficient to induce meaningful CI inhibition. In addition to its mitochondrial effects, metformin exerts metabolic benefits through activation of AMP-activated protein kinase (AMPK). Notably, approximately 5 μ M metformin is sufficient to induce AMPK phosphorylation in hepatocytes,⁸ whereas substantially higher concentrations (2.5 to 10 mM) are required to induce AMPK phosphorylation in 3T3-L1 preadipocytes⁹ and adipocytes.¹⁰

Given that plasma metformin concentrations in patients typically range from ~ 10 to 40 μ M,¹¹ the time- and dose-dependent effects of metformin on CI-linked respiration at final concentrations of 40 μ M, 2 mM, and 10 mM were investigated in day-seven 3T3-L1 adipocytes permeabilized with XF PMP (Figure 10). At 10 mM, metformin significantly inhibited CI-linked respiration beginning approximately 25 minutes after injection (Figures 10A and B), and by the end of the two-hour monitoring period (measurement 27), CI activity was reduced by more than 50% (Figures 10C and D). In contrast, 2 mM metformin produced only a modest reduction in CI activity over two hours, while 40 μ M had no detectable effect. As expected, CII-linked respiration was unaffected by any concentration of metformin (Figures 10E and F).

Data normalization

In most cases, image-based cell number count or total protein content can be used to normalize XF EF assay results after the run. If normalizing to individual wells increases data variability, normalizing to the average protein content per treatment group is recommended.

When the experimental objective is to assess the treatment effect of a compound on maximal respiration, OCR measurements post treatment can be normalized to the baseline OCR, typically the last measurement before the first injection. In this case, all OCR measurements are expressed as a percentage of the baseline signal (such as Figures 10B, D, and F). This approach enables evaluation of the relative effects of test compounds without the need for additional sample processing or protein quantification.

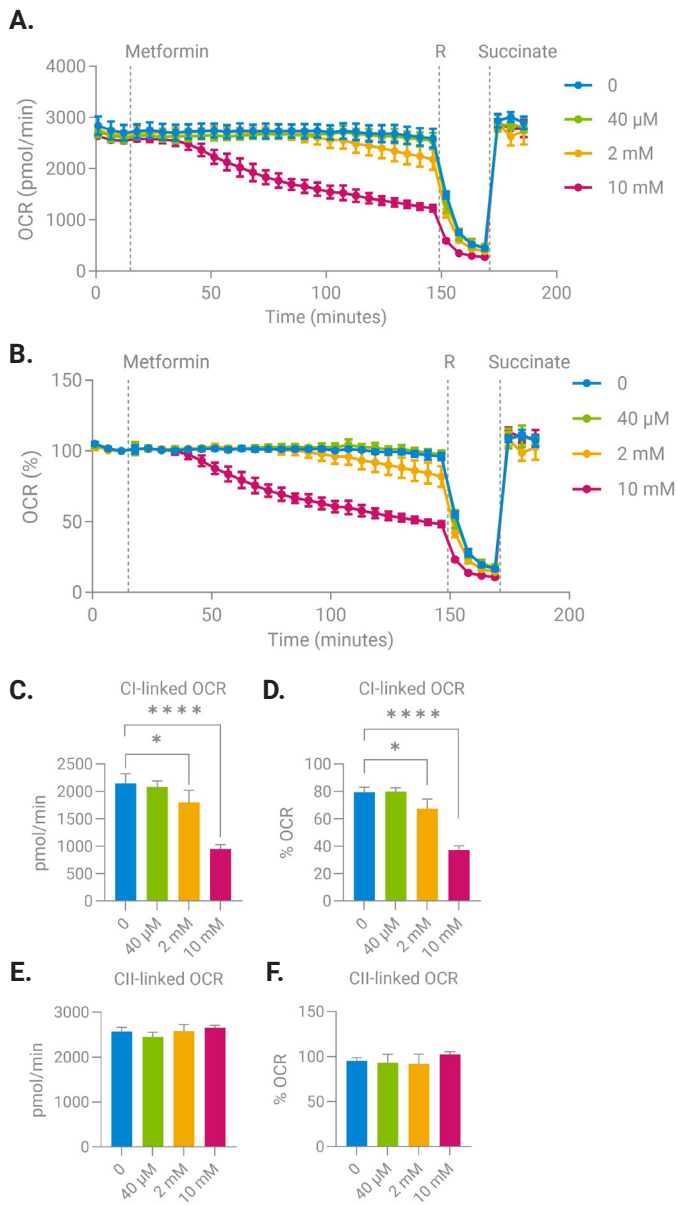


Figure 10. XF EF assay testing effect of metformin on CI-linked respiration in XF PMP permeabilized 3T3-L1 adipocytes. (A) and (B) are OCR kinetic traces before normalization and after normalized/baselined to measurement 3 (m3), respectively. (C) Raw and (D) m3 baselined CI-linked respiration two hours after metformin treatment, calculated as $OCR_{m27} - OCR_{min(R)}$. (E) Raw and (F) m3 baselined CII-linked respiration calculated as $OCR_{max(succinate)} - OCR_{min(R)}$. R: Rotenone. * $p < 0.05$, **** $p < 0.0001$, data = mean $\pm$ SD. n = four to six.

Conclusion

The Agilent Seahorse XF PMP provides a robust method for selectively permeabilizing mature adipocytes without compromising mitochondrial integrity, enabling direct measurement of ETC complex activities. This strategy supports screening for mitochondrial modulators, identification of gene targets, and characterization of drug mechanisms of action. Moreover, this approach can be extended to other applications that require selective plasma membrane permeabilization.

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Agilent products

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[Agilent BioTek Cytation portfolio](#) 

[Agilent Seahorse XF plasma membrane permeabilizer](#) 

[Agilent Seahorse XF24 V28 culture plate](#) 

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