

Assessing Mitochondrial Respiratory Complexes Using Cells Permeabilized with XF Plasma Membrane Permeabilizer (PMP)

Technical Overview

Introduction

The XF Plasma Membrane Permeabilizer (PMP) reagent forms pores in the cellular plasma membrane without causing any damage to the mitochondrial outer membrane. This protocol describes how to interrogate mitochondrial respiratory complexes using intact cells, permeabilized with XF PMP and run on an Agilent Seahorse XF or XFe Analyzer. While this protocol describes how to use the XF PMP reagent, please refer to the XF PMP Technical Overviews *Using XF PMP to measure mitochondrial respiratory complexes in limited biomaterial without isolating mitochondria* and *Using XF PMP to measure substrate oxidation in mitochondrial respiratory complexes without isolating mitochondria* and Agilent Technical Support when designing an XF PMP assay.



Materials and Methods

The assay workflow (Figure 1) describes the procedure used to prepare the cells and the XF PMP reagent.

Note: When planning an XF PMP assay, Seahorse recommends characterizing the cell seeding density and optimal reagent concentrations prior to starting the assay. Please contact Agilent Technical Support with any questions.

Reagents

- Adherent cells of interest and appropriate growth medium
- XF Calibrant (p/n 100840-000)
- 3x Mitochondrial Assay Solution (MAS) (For solution recipe, refer to Table 1). MAS is a nonionic mannitol and sucrose-based buffer used in permeabilization assays.
- Ultrapure or Tissue-culture grade water
- 10 % (w/v) fatty acid-free BSA or powdered BSA
- Oxidizable substrates and inhibitors (Refer to Table 2).
- XF PMP (p/n 102504-100).

Table 1. MAS recipe.

Reagent	3x MAS	Amount for 1.0 L of 3x MAS
Mannitol	660 mM	120.23 g
Sucrose	210 mM	71.88 g
KH ₂ PO ₄	30 mM	4.08 g
MgCl ₂	15 mM	15 mL of 1.0 M solution
HEPES	6 mM	6 mL of 1.0 M solution
EGTA	3 mM	12 mL of 0.25 mM solution
Fatty acid free (FAF) BSA	0.6 % (w/v)	6.0 g

Table 2. Substrates and inhibitors.

Substrate	Final conc.	Add with ...	Relevant inhibitors
Pyruvate	10 mM	1 mM Malate; 2 mM DCA*	2 μM Rotenone; 2 μM UK5099
Glutamate	10 mM	10 mM Malate	2 μM Rotenone; titrated aminooxyacetate
β-Hydroxybutyrate	10 mM	1 mM Malate	2 μM Rotenone
Palmitoylcarnitine/ Octanoylcarnitine	40 μM	1 mM Malate	2 μM Antimycin A
α-Ketoglutarate	10 mM	No additions necessary	2 μM Rotenone
Succinate	10 mM	2 μM Rotenone	2 μM Antimycin A; 2 μM Myxothiazol; 20 mM Malonate
Glycerol-3-phosphate	5–10 mM	2 μM Rotenone	2 μM Antimycin A; 2 μM Myxothiazol
Ascorbate	10 mM	100 μM TMPD, 2 μM Antimycin A	20 mM Azide

NADH-linked (Complex I) substrates
Q-linked (Complex II or III) substrates
Cytochrome oxidase-linked (Complex IV) substrates

Table 2 is adapted from¹.

*Optional (DCA will relieve potential kinase inhibition of pyruvate dehydrogenase)

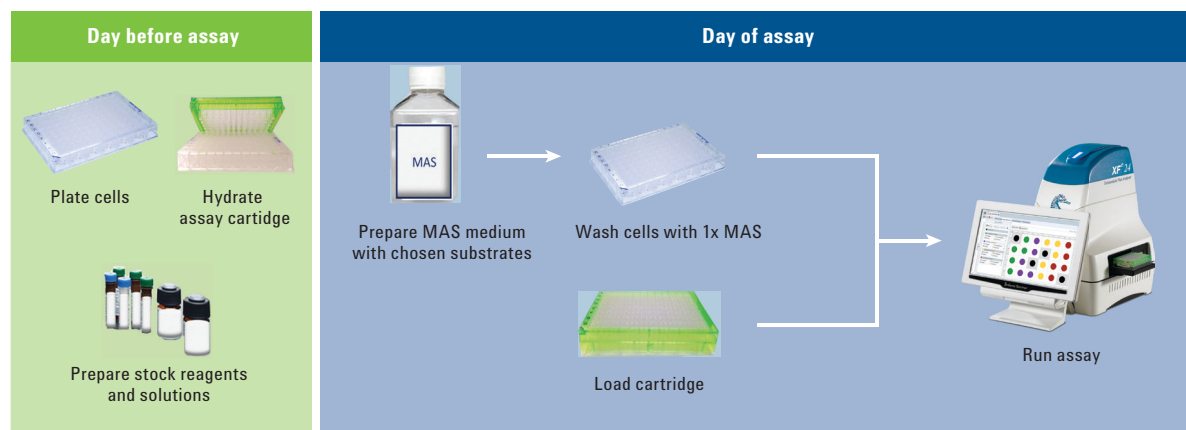


Figure 1. XF PMP assay workflow.

Other materials

- 37 °C water bath (required)
- Multichannel pipettes (not required, but highly recommended)
Note: Volumes provided for the Agilent Seahorse XF24 and XF96 Analyzers apply to the corresponding Agilent Seahorse XFe Analyzers.

Day before the assay

1. Seed cells in an XF Cell Culture Microplate at previously optimized cell density.
2. Hydrate the XF Sensor Cartridge using XF Calibrant then incubate overnight at 37 °C.

Day of the assay

1. Place the desired mitochondrial effectors and inhibitors on ice. If necessary, thaw frozen stocks.
2. Prepare 1x MAS buffer using Ultrapure or Tissue-grade water. Make 80 mL for either 24 or 96-well format.
Optional: Add fatty acid-free BSA at a final concentration of 0.2 % (w/v).
Note: If adding BSA, reduce the volume of water accordingly.

Prepare reagents

1. Set aside 30 mL of 1x MAS per cell culture microplate to be used as assay medium. Warm the remaining medium at 37 °C, which is for plate washing (See *Review and analyze data* section).
2. Add the specific substrates to the assay medium at the desired concentration base on assay design (refer to Table 2). To compensate for the MAS dilution following substrate addition, add another 0.5 mL of 3x MAS Buffer for every 1 mL of substrate. (Example: for every 30 mL of assay medium, add 600 µL of 0.5 M pyruvate (10 mM final

concentration), 60 µL of 0.5 M malate (1 mM final concentration), and 330 µL of 3x MAS Buffer).

3. Set aside 3 mL of substrate-containing assay medium per injection port to be used in the assay.
4. Based on the assay design, prepare the desired concentrations for each injection, and load into the appropriate ports on the cartridge.
5. To the remaining substrate-containing medium (Step 2), add a final concentration of 4 mM of ADP and 1 nM XF PMP Reagent.
Note: Further titration of XF PMP may be required depending on the cell type used. Contact Agilent Technical Support for more details.
6. Mix thoroughly and gently, then warm to 37 °C.

Design assay

Create an assay template, and calibrate the instrument, per instructions. Include the following modifications:

1. Use mix/wait/measure times of 0.5 min/0.5 min/2 min.
2. Do not include an equilibration step in the command protocol.
3. Take three measurements per step.

Run assay

1. Remove the cell plate from the incubator (See *Day of the assay* section) and wash twice quickly with either 0.15 mL or 0.5 mL (24 or 96-well format, respectively) using 1x MAS Buffer (*Day of the assay* section, step 2).
2. Following the last wash, replace the medium with warm MAS buffer supplemented with substrate(s), ADP, and XF PMP (*Prepare reagents* section, step 5) to a final volume of 0.5 or 0.18 mL for either 24 or 96-well format, respectively.

3. Insert the plate into the XF Analyzer, and run the assay.

Review and analyze data

Transfer the appropriate data file to a desktop or laptop computer, and use Wave Desktop for data review and analysis.

Assay optimization hints

1. Cells may have substrate preferences based on the tissue origin and culture conditions. Refer to Divakaruni; *et al.* 2014¹ for additional substrate information.
2. The assay duration should be as short as possible to avoid cell lifting from the microplate.
3. Perform the wash steps as quickly (but gently) as possible to minimize cell-exposure time to the MAS buffer.
4. When pipetting BSA-supplemented assay medium, bubbles can form in the assay plate. Therefore, do not push past the stopper when either washing or loading the microplate.

Reference

1. Divakaruni; *et al.* Measuring mitochondrial function in permeabilized cells using the Seahorse XF Analyzer or Clark-type oxygen electrode. *Curr. Protoc. Toxicol.* **2014**, 60, 25.2.1-25.2.16.

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