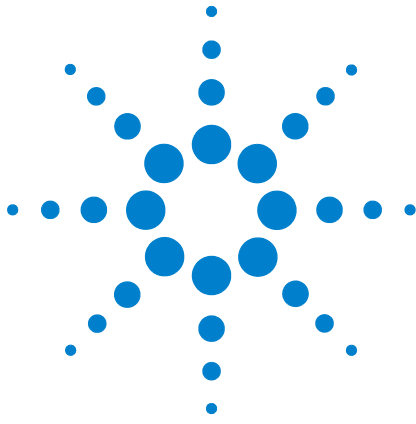




Agilent Seahorse XF Palmitate-BSA FAO Substrate Quickstart Guide

For use with Agilent Seahorse XFe, XF, and XFp Analyzers

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Kit part number: 102720-100



Agilent Technologies

Product Description

The Agilent Seahorse XF Palmitate-BSA fatty acid oxidation (FAO) Substrate is a bio-available palmitate reagent for researchers studying substrate utilization who require a reliable source of conjugated fatty acids. The XF Palmitate-BSA FAO Substrate is manufactured at a 6:1 Palmitate:BSA ratio. Each lot is tested for activity and free fatty acid (FFA) concentration.

Contents

- 1 mM palmitate conjugated to 0.17 mM BSA in 150 mM NaCl, pH 7.2, 3 × 2 mL vials
- 0.17 mM BSA control in 150 mM NaCl, pH 7.2, 3 × 2 mL vials. Lot-specific FFA is listed on each box

Storage conditions

Upon arrival, store at -20 °C, aliquot and refreeze if desired. Glass vials are recommended for storage. The stability of the product is still under evaluation, but has a minimum 6-month shelf life when properly stored. Do not store in a frost-free freezer.

Reagent preparation

- Thaw solution at room temperature.
- Prior to beginning the assay, add undiluted substrate or control directly to the well. We recommend adding the substrate directly to the microplate instead of to the injection ports to avoid inconsistent injection performance due to viscosity.

Table 1 Recommended volumes: Palmitate-BSA or BSA control

Component	Volume
XFe24/XF24	87.5 µL
XFe96/XF96	30 µL

Introduction

The ability to oxidize exogenously added fatty acids (FAs), as indicated by an increase in O₂ consumption rate (OCR), will be influenced by ATP demand, other substrates present in the assay medium (such as glucose), and stores of endogenous substrates (such as glycogen or triglycerides). Exogenously added FAs can also uncouple mitochondria, and cause an increase in the OCR not due to fatty acid oxidation (FAO).

Palmitate-BSA has been used extensively to examine FA oxidation. The addition of etomoxir to a cell, and the subsequent return to the control rate of respiration, demonstrate utilization of exogenous fatty acids^{1,2}. A drop in OCR in both the palmitate and BSA control indicates that endogenous FAs were also being oxidized³. Finally, if the OCR signal is insensitive to etomoxir or oligomycin, this indicates that the FAs are uncoupling the mitochondria⁴.

Because a change in OCR can be caused by utilization of exogenous FAs, endogenous FAs, or uncoupling by FAs, Agilent Technologies developed the XF FAO Assay that can measure all three sources. With the combined use of the XF Palmitate-BSA FAO Substrate, etomoxir, and the XF Cell Mito Stress Test, one can drive cells to oxidize exogenous fatty acids while revealing the portion of the OCR signal generated by endogenous and exogenous fatty acid oxidation, as well as uncoupling.

The assay requires components that are not included with the this product, which is referenced in [Table 2](#). A guideline on how to perform FAO assay is provided in this document. This assay was developed using immortalized cell lines. Use of primary cultures may require optimization of reduced nutrient conditions.

Table 2 Items required but not provided

Component	Details
XFe24/96 or XF24/96 Analyzer	www.agilent.com/en-us/products/cell-analysis-(seahorse)
XFe/XF FluxPak or FluxPak Mini	www.agilent.com/en-us/products/cell-analysis-(seahorse)
Substrate-Limited Medium	See formulation below
FAO Assay Medium	See formulation below
Etomoxir (10 mM Stock Solution in H ₂ O)	Sigma-Aldrich, catalog #E1905, (+)-etomoxir sodium salt hydrate
XF/XFp Cell Mito Stress Test Kit	Agilent Technologies, catalog #103015-100, #103010-100

Medium and Buffer Preparation

Table 3 Substrate-limited medium

Component	Details
DMEM	Corning #17-207-CV or Life Technologies catalog #A14430-01 are DMEM formulations without glucose, glutamine, sodium pyruvate, or HEPES
0.5 mM Glucose	D-(+)-Glucose, catalog #G7021, Sigma-Aldrich
1.0 mM GlutaMAX	GlutaMAX Supplement, catalog #35050061, Life Technologies
0.5 mM Carnitine	L-Carnitine hydrochloride, Sigma-Aldrich, catalog #C0283
1 % FBS	Fetal Bovine Serum

Table 4 FAO Assay buffer

Component	1x Concentration (mM)	5x Concentration (mM)
NaCl	111	555
KCl	4.7	23.5
CaCl ₂ *	1.25	6.25
MgSO ₄	2.0	10
NaH ₂ PO ₄	1.2	6
Supplements	On the day of the assay, supplement 1x KHB with 2.5 mM glucose, 0.5 mM carnitine and 5 mM HEPES (final concentrations). Warm to 37 °C, and adjust pH to 7.4 with NaOH.	
Carnitine may be purchased as L-carnitine hydrochloride from Sigma. Make as a 0.5 M stock solution in H ₂ O and adjust pH to 7.2 with NaOH.		

* This document was updated to include CaCl₂ in the FAO Assay Medium

Running Fao Assay

Note: The XF assay work flow deviates from standard XF assays, and includes two pretreatment steps:

- Selected groups are treated with etomoxir 15 minutes prior to the XF assay being initiated ($t = -15$ minutes).
- Selected groups are provided BSA control or Palmitate:BSA prior to the XF assay being initiated ($t = 0$).

FAO Assay Workflow

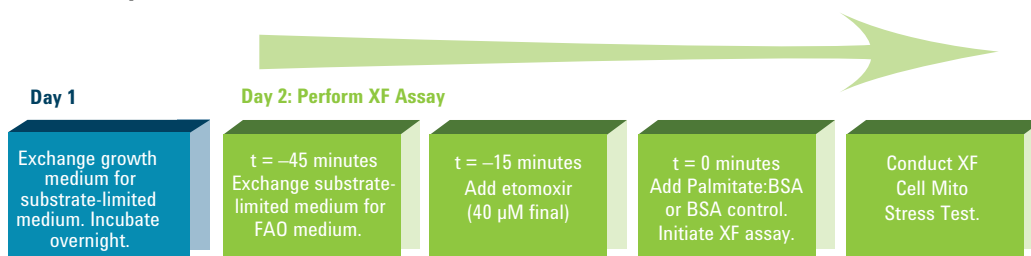


Figure 1 FAO Assay workflow.

Table 5 Recommended volumes

Recommended volumes	XFe/XF24 (μL)	XFe/XF96 (μL)
Starting volume	375	135
400 μM Etomoxir (40 μM final)	37.5	15
BSA control or Palmitate:BSA	87.5	30
XF Cell Mito Stress Test Injections (all 10X) - final concentration		
Port A: 25 $\mu\text{g}/\text{mL}$ Oligomycin (2.5 $\mu\text{g}/\text{mL}$ final)	50	20
Port B: FCCP (TBD by user [*])	55	22
Port C: 20 μM Rotenone/40 μM Antimycin A (2 and 4 μM final, respectively)	60	24

^{*} Determination of maximal FCCP stimulation (maximal exogenous FA oxidation) should be conducted by performing an FCCP titration in the context of the FAO assay workflow (that is, cells should be grown in substrate-limited medium, assayed in FAO assay media, and be FCCP titrated in the presence of BSA Control and Palmitate-BSA).

Determination of Fatty Acid Utilization in the XFe/XF Analyzer

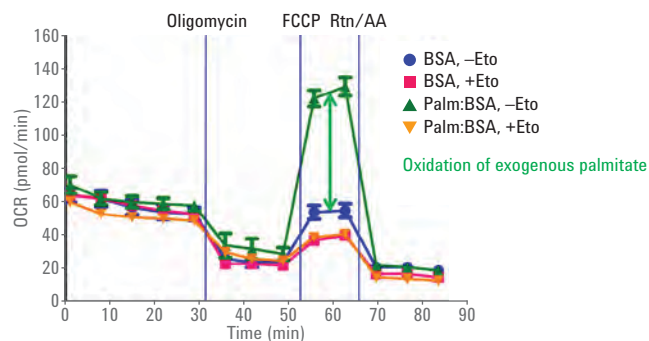


Figure 2 Kinetic graph of the FAO/XF Cell Mito Stress Test assay. The green arrow indicates utilization of exogenous fatty acids. **Note:** in this case, utilization of exogenous fatty acids was dependent on placing energetic stress (via FCCP) on the cells. This experiment illustrates that under conditions of substrate limitation, and the combined use of the XF Palmitate-BSA FAO Substrate, etomoxir, and the XF Cell Mito Stress Test, one can determine the proportion of respiration that is supported by exogenous fatty acids.

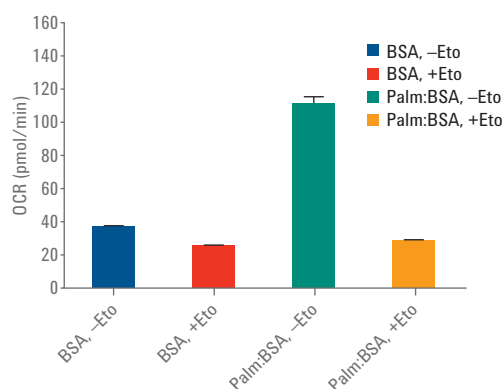


Figure 3 Bar chart highlighting the differences in maximal respiration. Data taken from the rate 10 measurement in Figure 2. The use of the XF Cell Mito Stress Test can elucidate different metabolic parameters allowing the determination if the cells are oxidizing the exogenous palmitate, as well as oxidizing endogenous fatty acids or causing uncoupling.

References

- 1 Ferrick, D. A.; *et al.* Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov. Today* **2008**, 13(5-6), 268-74.
- 2 Rowe, G. C.; *et al.* Disconnecting mitochondrial content from respiratory chain capacity in PGC-1 deficient skeletal muscle. *Cell Rep.* **2013**, 3(5), 1449-1456.
- 3 Canto, C.; *et al.* AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature* **2009**, 458(23), 1056-1062.
- 4 Divakaruni, A. S.; Brand, M. D. The regulation and physiology of mitochondrial proton leak. *Physiology* **2011**, 26, 192-205.

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