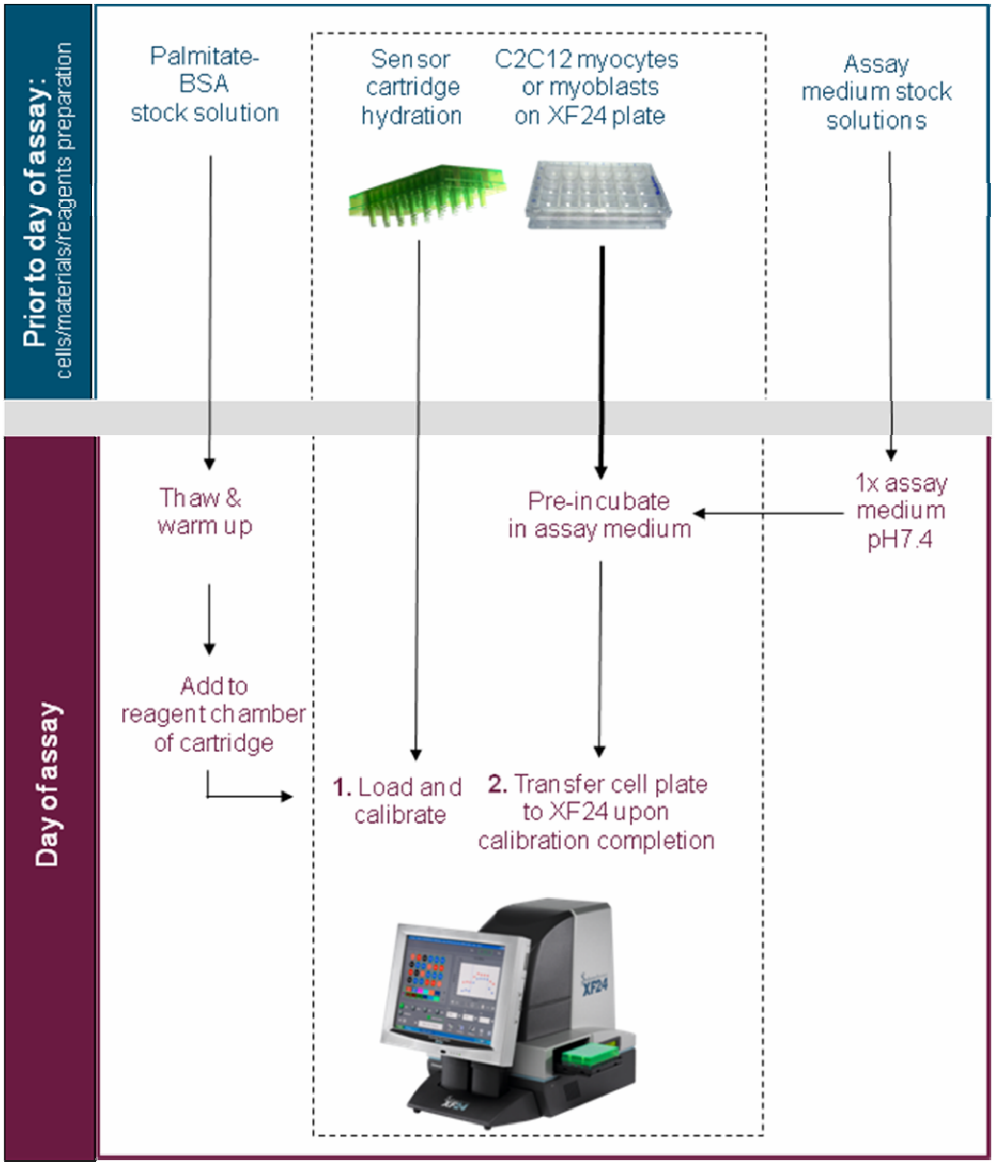
Fatty Acid Oxidation Assay on the XF24 Analyzer

Mitochondria oxidize a variety of fuels to generate ATP through oxidative phosphorylation. Cells can utilize fatty acid, glucose and amino acids as their fuels. Traditionally, fatty acid oxidation (FAO) assay is performed by quantifying radioactive byproducts, H_2O or CO_2 after incubating cells with radioactively-labeled fatty acids such as palmitate or oleate.

In this protocol, increased oxygen consumption rate (OCR) immediately following exposing cells to palmitate substrate is used as an indicator for FAO in XF24 Analyzer. This offers a rapid, easy and non-radioactive alternative assay for fatty acid oxidation. In addition, both kinetic and accumulative data can be obtained in a single assay.

$$C_{16}H_{32}O_2 + 23\ O_2 \longrightarrow 16\ CO_2 + 16\ H_2O$$



Assay Flow Chart

I. Reagents and Materials

I.1. Components of Krebs Henseleit Buffer (KHB)
NaCl (Sigma, S3014, dry, or Sigma S6316, 5M solution)
KCl (Sigma, P4504)
$MgSO_4$ (Sigma, M2643)
Na_2HPO_4 (Sigma, S3397)
Glucose (Sigma, G8270, dry, or Sigma G8769, 45% solution)
Carnitine (Sigma, C0158)

I.2. Palmitate-BSA conjugate and BSA vehicle control (see Palmitate preparation protocol)

I.3. XF24 plate of C2C12 myoblasts or myocytes (see C2C12 culture/seeding, and differentiation protocol)

I.4. XF24 Cartridge

I. 5. Calibration buffer (Seahorse Bioscience)

1.6. Multichannel pipettes and tips

II. Preparation of KHB buffer

II.1. Preparation of 5x KHB base from dry reagents

II.1.1. Prepare 50X or 100X stock of sodium phosphate (monobasic and dibasic) stock

II.1.2. To make 1 liter 5x KHB, add the following reagents separately in the listed order to 900 ml dH_2O in a 1 liter beaker containing a magnet stir bar. Allow each to dissolve before adding the next one:

Chemical	5X (mM)	5X (g/L)
NaCl	555	32.532
KCl	23.5	1.752
MgSO4	10	1.204
Na2HPO4	9	0.852

II.2.2. Adjust final volume to 1000 mL with dH_2O.

II.2.3. Filter-sterilize and store at 4ºC for later use.

II.2. Alternate preparation of 5x KHB base from stock solutions:

II.2.1. Use 5 M NaCl stock solution. Make 100 mL stock solutions of KCl (1 M), $MgSO_4$ (0.5 M), and Na_2HPO_4 (0.5 M) in dH_2O as follows:

Stock	Concentration (M)	Amount for 100 mL (g)
NaCl	5	--purchase--
KCl	1	7.456 g
$MgSO_4$	0.5	6.019 g
Na_2HPO_4	0.5	7.098 g

II.2.2. To make 1 liter 5x KHB, add the following to 833.5 mL dH_2O:

Component	Stock concentration (M)	5X (mM)	Amount for 1 L (mL)
NaCl	5	555	111 mL
KCl	1	23.5	23.5 mL
$MgSO_4$	0.5	10	20 mL
Na_2HPO_4	0.5	6	12 mL

II.2.3. Filter-sterilize and store at 4˚C for later use.

II.2.3. Preparation of 50 mM carnitine stock

II.2.3.1. Dissolve 81 mg carnitine in 10 mL dH_2O in a 25 ml conical tube.

II.2.3.3. Filter-sterilize.

II.2.3.3. Aliquot into 1 ml in 1.5 ml microfuge tubes.

II.2.3.4. Store at -20ºC. Frozen stock is good for one month and may be used within three days of thawing when kept at 4ºC.

II.2.4. Preparation of 1x KHB assay medium

II.2.4.1. Final composition of 1x KHB

Component	Concentration
NaCl	111 mM
KCl	4.7 mM
$MgSO_4$	2 mM
Na_2HPO_4	1.2 mM
glucose	2.5 mM
carnitine	0.5 mM

On day of assay, determine volume of medium needed from table below and prepare 1X KHB assay buffer as below.

II.2.4.2a. Add indicated volumes of 5X KHB, dH2O, 45% glucose solution and 50mM carnitine in a beaker containing a stir bar.

# of Plates	1x KHB Needed	dH_2O (mL)	5X KHB (mL)	45% glucose (mL)	50mM carnitine (mL)
1	50	40	10	0.05	0.5
2	100	80	20	0.10	1.0
3	150	120	30	0.15	1.5
4	200	160	40	0.20	2.0

II.2.4.2b. Alternatively, add dry glucose and carnitine to the 1x KHB as follows:

Name	g/L	g/100 mL
Glucose	0.45	0.045
Carnitine	0.081	0.008

II.2.4.3. Warm to 37ºC before adjusting pH.

II.2.4.4. Measure pH with pH meter and adjust pH to 7.4. For 100 mL 1x KHB, adjustment will typically require 10-15 µL 1 N HCl.

II.2.4.5. Store at 37ºC till ready to use.

III. Fatty Acid Oxidation Assay

III.1. Prepare an XF assay template (via the Assay Wizard) using the XF24 operation manual and incorporating proper experimental design. Upload the assay template to the XF24 Analyzer before starting the assay.

III.2. For one XF plate, remove one 4 mL aliquot 5x BSA and one 4 mL aliquot 5x Palmitate-BSA conjugate from the freezer and thaw in a 37˚C water bath.

III.2. Change the medium on XF cell plate to assay medium:

III.2.1. With a 200 µL multichannel pipette, remove and discard medium down to 50 µL remaining. (For example, remove 135 µL from 200 µL – evaporative loss is typically 5-15 µL.)

III.2.2. With a 1.2 ml multichannel pipette and a reservoir for KHB, add 950 µL assay medium to all wells.

III.2.3. With same pipette, remove 890 µL/well from all wells.

III.2.4. With same pipette, add 500 µL assay media/well to all wells.

III.3. Incubate the plate in 37°C incubator, non-CO_2 and non-humidified, to equilibrate cells in assay medium.

III.4. Palmitate and BSA should be completely thawed. Invert to mix.

III.5. Dilute in assay medium if necessary – **final** palmitate concentration should be 150 µM for myocytes or 200µM for myoblasts.

The palmitate preparation protocol yields a 1 mM stock which is used as-is for myoblasts (5x for 200 µM) or diluted for myocytes by mixing 3 mL 1 mM palmitate-BSA stock with 1 mL assay medium for 750 uM stock (5x for 150 µM). If palmitate-BSA is diluted, BSA control should be diluted in the same proportion. 4 mL 5x stock is needed for FAO assay on one XF24 plate.

Note: optimal palmitate concentration for different cell types can vary. A Palmitate concentration titration experiment is recommended for a specific cell type/line.

III.6. Transfer BSA-Palmitate and BSA into tissue culture reservoirs.

III.6. Load onto XF24 cartridge as indicated in assay template – load 75 µL into each of two ports per well, for example A and C using a 200 µl 8-channel pipette.

Large pore tip should be used for loading reagents into cartridge port..

III.7. BSA should be injected into at least three wells (not including empty wells for thermal control) for a vehicle control. An example layout for an FAO assay of a plate of myocytes might have BSA in column 1, ports A and C, and palmitate-BSA columns 2-6, ports A and C.

III.8. Open Assay template on XF24 instrument and begin calibration. Continue assay as guided by prompts (another phrase?).

III.9. Protocol Commands

Protocol Steps		For Tubes	For Blasts	
	Command	Time (min)	Time (min)	Port
	Calibrate			
	Mix	2	2	
	Wait	10	10	
	Mix	4	3	
	Wait	2	2	
	Measure	1.5	3	
	Mix	4	3	
	Wait	2	2	
	Measure	1.5	3	
	Mix	4	3	
	Wait	2	2	
	Measure	1.5	3	
	Inject			A
	Inject			C
	Mix	4	3	
	Wait	2	2	
	1.5	1.5	3	
	Mix	4	3	
	Wait	2	2	

	c	1.5	3	
	Mix	4	3	
	Wait	2	2	
	Measure	1.5	3	
	Mix	4	3	
	Wait	2	2	
	Measure	1.5	3	

Note:
1). The number of rate measurement should be determined according to specific goal of each experiment.
2). To assay the effect of FAO agonists such as metformin, cells can be treated for desired amount of time before fatty acid oxidation assay. Alternatively, rapidly permeating compounds can be injected and rate measured before palmitate-BSA injection.

References:

1. Harwood HJ Jr,et al.. J Biol Chem. 2003. 26;278(39):37099-111. Isozyme-nonselective N-substituted bipiperidylcarboxamide acetyl-CoA carboxylase inhibitors reduce tissue malonyl-CoA concentrations, inhibit fatty acid synthesis, and increase fatty acid oxidation in cultured cells and in experimental animals.