Simultaneously Measuring Oxidation of Exogenous and Endogenous Fatty Acids using the XF Palmitate-BSA FAO Substrate with the XF Cell Mito Stress Test

The regulation of metabolic pathways and energy expenditure, and how differential substrate utilization contributes to metabolically-regulated signaling mechanisms is an important topic for researchers in fields such as immunology and cancer. Of particular interest is fatty acid oxidation (FAO) by the cell.

The XF FAO Assay described in this Technical Brief measures FAO in cells under both basal and stressed energy demands. This assay strategy is most relevant for determining the intrinsic rate and capacity of a cell to oxidize fatty acids (FAs) in the absence or limitation of other exogenous substrates. Moreover, a change in respiration can be caused by utilization of exogenous FAs, endogenous FAs, and/or uncoupling by FAs. Unlike other assays, this XF FAO Assay can measure FAO respiration from these three sources simultaneously. The immortalized cell lines tested displayed minimal changes in basal respiration (low relative energetic demand) and large changes in maximal respiration (high relative energetic demand) when exogenous palmitate was added.

Measuring exogenous FAs in respiration assays requires a consistent and reliable FAO substrate. Conjugating palmitate to BSA is tedious, and resulting free fatty acid (FFA) concentration can vary significantly with each preparation. Using XF Palmitate-BSA FAO Substrate ensures controlled ratio of palmitate to BSA and a known concentration of bioavailable substrate.

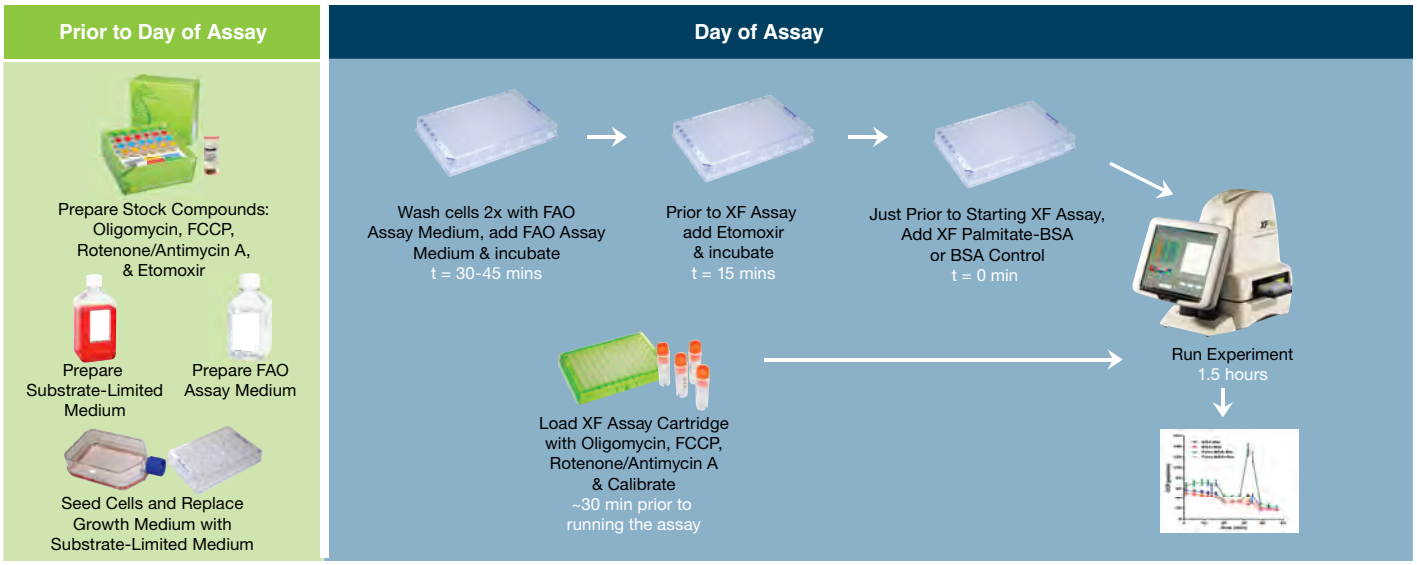
The XF FAO assay described uses the XF96 Extracellular Flux Analyzer*, XF Palmitate-BSA FAO Substrate, the XF Cell Mito Stress Test Kit, and Etomoxir (Eto), a CPT-1 (carnitine palmitoyl transferase-1) inhibitor. The XF Analyzer allows for the simultaneous measurement of the two major energy pathways of the cell: mitochondrial respiration and glycolysis. The XF Cell Mito Stress Test measures the key parameters of mitochondrial function: basal respiration, ATP production, proton leak, and spare respiratory capacity; it may also reveal the substrate choices and preferences of cells under basal and maximal energy demands.

The combination of XF Palmitate-BSA FAO Substrate, Eto, and the XF Cell Mito Stress Test enables the design of an assay to determine the relative utilization of exogenous and endogenous FAs.

This Technical Brief describes the following methods:

1. A method for measuring oxidation of exogenous fatty acids.
2. A method for determining oxidation of endogenous fatty acids in the same experiment.
3. Other aspects of the FAO assay, and items to be aware of when conducting these experiments.

Figure 1 | **FAO Assay Workflow**



*This Technical Brief is also applicable to the XF24 Analyzer and XF®24/96 Analyzers

Assay Design Overview

The FAO Assay Workflow (Fig. 1) describes the steps used to prepare the cells and conduct the XF assay for oxidation of exogenous and endogenous FAs.

Cells were seeded in XF Cell Culture Microplates and allowed to grow overnight in typical growth medium. The growth medium was then replaced with medium containing reduced concentrations of glucose, GlutaMAX™, and serum to deplete endogenous substrates within the cell (glycogen, triglycerides, amino acids), thus priming the cells to oxidize exogenous FAs.

Four different conditions were used to inform the analysis:

1. BSA control without Eto (BSA-Eto)
2. BSA control with Eto (BSA+Eto)
3. Palmitate-BSA without Eto (Palm:BSA-Eto)
4. Palmitate-BSA with Eto (Palm:BSA+Eto)

The assay workflow included two pre-treatments:

1. Eto or vehicle was added to appropriate wells on the microplate 15 minutes before the XF assay was initiated (t = -15 min).
2. Palmitate-BSA or BSA was added to appropriate wells on the microplate just prior to initiating the XF assay (t = 0).

The groups were then assayed using the XF Cell Mito Stress Test Kit.

Materials and Methods

The experiments described in this Technical Brief were performed on an XF96 Analyzer, and part numbers are provided for that platform; however, this assay may also be adapted for all XF/XF^e Analyzer platforms.

Reagents

1. Growth Media
   - C2C12 Medium: DMEM, 10% fetal bovine serum (FBS), 25 mM glucose, 2 mM GlutaMAX , 1% penicillin/streptomycin.
   - HepG2 Medium: DMEM, 10% fetal bovine serum (FBS), 5.5 mM glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX, 1% penicillin/streptomycin.
2. Substrate-Limited Medium
   - DMEM: 0.5 mM glucose, 1 mM GlutaMAX, 0.5 mM carnitine, and 1% FBS. Carnitine was added fresh the day of the media change.
3. FAO Assay Medium
   - KHB: (111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 2 mM $MgSO_4$, 1.2 mM NaH_2PO_4) supplemented with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES on the day of the assay, adjusted to pH 7.4 at 37°C.
4. XF Palmitate-BSA FAO Substrate (Seahorse Bioscience, Part # 102720-100).
5. Respiration Reagent Stocks
   - XF Cell Mito Stress Test Kit Reagents (Seahorse Bioscience, Part # 101706-100) (Oligomycin, FCCP, rotenone and antimycin A), prepared as recommended.
   - 0.5 mM carnitine and 10 mM Eto were made in H_2O and adjusted to pH 7.4.
   - XF Palmitate-BSA FAO Substrate and respiration reagents were stored at -20°C.
   - All other chemicals were purchased from Sigma-Aldrich.

Cell Culture

C2C12 myoblasts and HepG2 cells were obtained from American Type Culture Collection and cultured as suggested by the supplier. Cells were seeded in XF96 Cell Culture Microplates (C2C12 at 15,000 cells/well, HepG2 at 20,000 cells/well) and cultured overnight in their respective growth medium.

The following day, growth medium was replaced with substrate-limited medium and the cells incubated for an additional 24 hours.

XF Assay

Respiration was measured using the Seahorse XF96 Extracellular Flux Analyzer with XF96 FluxPaks (Seahorse Bioscience, Part # 102310-001). Volumes provided for the XF96 Analyzer also apply to XF^{e}96 Analyzers. Volumes and protocol commands are also provided for running the assays in an XF/XF^{e}24 Analyzer. Unless specified otherwise, cells were assayed in the FAO assay medium described. Oxidizable substrates and compounds that affect mitochondrial function were provided as indicated.

Assay Workflow

- Seed cells in an XF96 Cell Culture Microplate.
- 24 hours prior to the assay, replace growth medium with substrate-limited medium.
- 45 minutes prior to the assay (t = -45 min), wash the cells one to two times with FAO Assay Medium, depending on the relative adherence of the cells being used.
- Add 135 µL/well FAO assay medium to the cells (375 µL/well in XF/XF^{e}24 microplates) and incubate in a non-CO_2 incubator for 30-45 minutes at 37°C.
- Load the assay cartridge with XF Cell Mito Stress Test compounds (final concentrations: 2.5 µg/mL oligomycin, 0.8 or 1.6 µM FCCP, 2 µM rotenone/4 µM antimycin A.). Note: oligomycin and FCCP concentrations must be optimized for specific cell type and seeding density.
- 15 minutes prior to starting the assay (t = -15 min), dilute 10 mM stock solution of Eto to 400 µM in FAO Assay Medium. Add 15 µL Eto or vehicle to each well (37.5 µL/well to XF/XF^{e}24 microplates). The final concentration in the wells will be 40 µM. Add Eto at this point to allow time for it to be taken up by the cells and bind to CPT-1.
- Incubate for 15 minutes at 37°C in a non-CO_2 incubator.
- Just prior to starting the assay (t = 0 min), add 30 µL XF Palmitate-BSA FAO Substrate or BSA to the appropriate wells (87.5 µL/well to XF/XF^{e}24 microplate).
- Immediately insert the XF Cell Culture Microplate into the XF96 Analyzer and run the XF Cell Mito Stress Test with the command protocol in Table 1.

Table 1 | Instrument Run Protocol

Command	# of loops/ cycles	XF/XF^{e}24 times (min)	XF/XF^{e}96 times (min)
Calibrate	-	-	-
Equilibrate	auto	auto	auto
Mix, Wait, Measure	4	3, 2, 3	3, 0, 3
Inject A	-	-	-
Mix, Wait, Measure	3	3, 2, 3	3, 0, 3
Inject B	-	-	-
Mix, Wait, Measure	3	3, 2, 3	3, 0, 3
Inject C	-	-	-
Mix, Wait, Measure	3	3, 2, 3	3, 0, 3
The number of loops may be adjusted based on the kinetics of the particular experiment.			

Interpretation of Results

All data were analyzed using XF software and displayed as 'middle-point' (average) oxygen consumption rates (pmol O_2/min/well). Data is the average of replicate wells +/- S.E.M.

Utilization of Fatty Acids

The ability to oxidize exogenously added FAs is influenced by ATP demand, as well as the presence of other substrates (such as glucose) in the assay medium, and stores of endogenous substrates (such as glycogen or triglycerides). Moreover, FFAs are weak lipophilic acids and can mildly uncouple mitochondria and cause an increase in oxygen consumption not due to FAO[1].

Differentiating Oxidation of Exogenous Fatty Acids from Uncoupling

The assay for exogenous FAO by HepG2 cells is illustrated in Fig. 2 and demonstrates how the XF FAO Assay Parameters (Table 2) are used to determine the change in respiration (OCR) due to oxidation of exogenous FAs or uncoupling. All calculated results may be found in Table 3.

In this experiment, the HepG2 cells provided with Palm:BSA-Eto (green) had a basal respiration rate 15 pmol O_2 /min greater than the BSA-Eto (blue) group. By calculating the difference in proton leak between the two groups, one can determine that uncoupling contributed 6 pmol O_2/min to the increase in basal respiration rate, while oxidation of exogenous FA contributed 9 pmol O_2 /min.

A striking result in this example is that the BSA-Eto (blue) group has little increase in maximal respiration when treated with FCCP, while the Palm:BSA-Eto (green) group shows greatly increased maximal respiration. This indicates that exogenous FA (as opposed to endogenous FA) is primarily being oxidized when a bioenergetic stress is placed on the system. This experiment shows a slight change in the basal respiration when exogenous palmitate is added, with a much larger change in the maximal respiration.

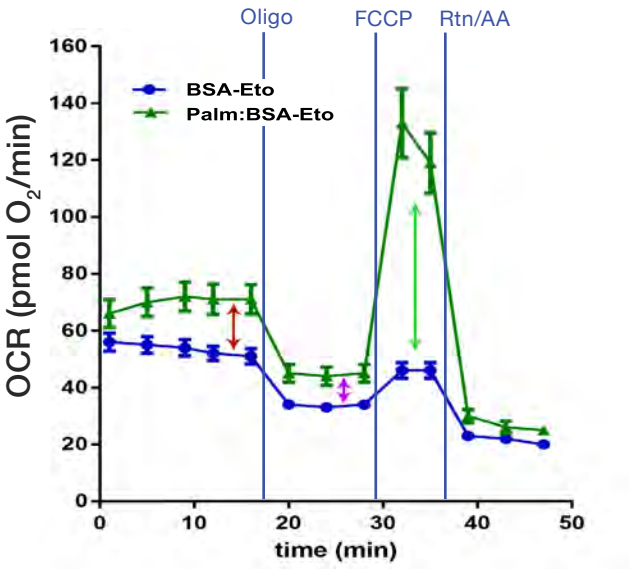


Figure 2 | Determination of Exogenous Fatty Acid Utilization and Uncoupling by Free Fatty Acids in HepG2 cells

Basal respiration of HepG2 cells provided Palm:BSA-Eto (green) is 15 pmol O_2/min greater than that of the BSA-Eto group (blue) due to oxidation of exogenous FFAs plus uncoupling by FFAs (red arrow). The proton leak respiration of HepG2 cells provided Palm:BSA-Eto (green) is 6 pmol O_2/min greater than that of the BSA-Eto group (blue) due to uncoupling by FFAs (purple arrow). Maximal respiration of HepG2 cells provided Palm:BSA-Eto (green) is 87 pmol O_2/min greater than that of the BSA-Eto (blue) group demonstrating a significant reliance on utilization of exogenous FAs to respond to a stress energy demand (green arrow).

Table 2 | XF FAO Assay Parameters

Basal Respiration
Rate to prior to oligomycin injection minus non-mitochondrial respiration.

Proton Leak
Rate prior to FCCP injection minus non-mitochondrial respiration.

ATP Production
Basal respiration minus proton leak.

Maximal Respiration
Maximal FCCP rate minus non-mitochondrial respiration.

Spare Capacity
Maximal respiration minus basal respiration.

Non-mitochondrial Respiration
Final rate after rotenone/antimycin A injection.

Oxygen Consumption due to uncoupling by FFA
Oligomycin Palm:BSA-Eto rate minus Oligomycin BSA-Eto rate.

Basal Respiration due to utilization of exogenous FAs
Basal Palm:BSA-Eto rate minus Basal BSA-Eto rate minus OCR due to uncoupling by FFA.

Maximal Respiration due to utilization of exogenous FAs
Maximal Palm:BSA-Eto rate minus Maximal BSA-Eto rate minus OCR due to uncoupling by FFA.

Basal Respiration due to utilization of endogenous FAs
Basal BSA-Eto rate minus basal BSA+Eto rate.

Maximal Respiration due to utilization of endogenous FAs
Maximal BSA-ETO rate minus maximal BSA+Eto rate.

Measuring Oxidation of Endogenous Fatty Acids

In Fig. 3, the use of Eto in cells exposed only to BSA (pink) is used to probe for utilization of endogenous FA. The fraction of respiration (OCR) attributable to endogenous FAs can be easily calculated as shown in Table 3.

The Palm:BSA+Eto group (orange) confirms that the significant increase in maximal respiration in the Palm:BSA-Eto group (green) was driven by oxidation of exogenous FAs, as OCR fell below the BSA-Eto group (blue). Basal respiration (OCR) declined 9 pmol O_2/min in the BSA+Eto group (pink) (purple arrow). This indicates that under these conditions, a small fraction of the basal respiration was due to oxidation of endogenous FAs and/or minor amounts of residual FAs in the BSA preparation.

Similarly, for the maximal respiration, OCR declined 17 pmol O_2/min in the BSA+Eto group (pink), again indicating that under these conditions a fraction of the maximal respiration was due to oxidation of endogenous FAs, and/or minor amounts of residual FAs in the BSA preparation. See Appendix for additional examples of this assay.

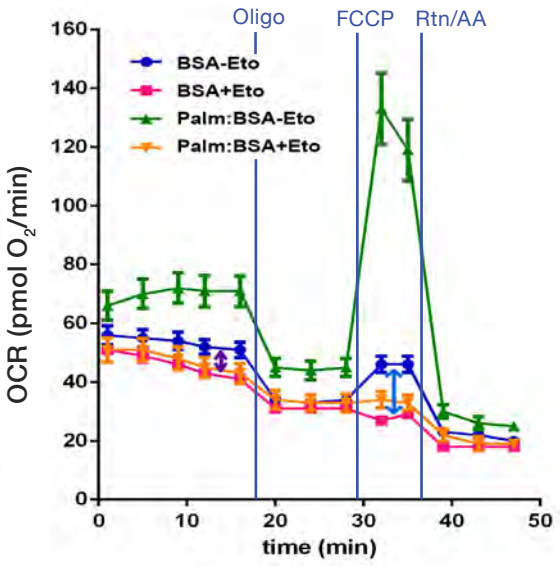


Figure 3 | Determination of Endogenous Fatty Acid Utilization

Basal respiration of HepG2 cells provided BSA-Eto (blue) is 9 pmol O_2/min greater than that of the BSA+Eto group (pink) due to the oxidation of endogenous FAs and/or residual FAs in the BSA preparation (blue) (purple arrow). Maximal respiration of HepG2 cells provided BSA-Eto (blue) is 17 pmol O_2/min greater than that of the BSA+Eto group (pink) indicating that a portion of maximal respiration was due to oxidation of endogenous FAs and/or minor amounts of residual FAs in the BSA preparation (blue arrow).

Assay Optimization Hints

1. The optimal medium conditions for substrate limitation should be investigated for each cell type. This Technical Brief offers a starting point for substrate limitation conditions for cultured cells. However, it is encouraged that substrate limitation conditions for the particular cell type of interest are examined (e.g. lower or higher concentrations of glucose, GlutaMAX, FBS, etc.). For example, cells that do not store large amounts of endogenous substrates may require less or no substrate limitation to observe oxidation of exogenous FAs.
2. Certain cell types may be depleted of substrates for 2 days (~48 hrs), which will further reduce endogenous substrates and can provide a larger difference in FCCP stimulated OCR between BSA control and Palmitate-BSA groups.
3. FCCP and oligomycin should be titrated in the presence of BSA (same assay medium composition and BSA concentration as will be used for the FAO assay) to obtain the optimal concentration for maximal respiration. Note that the FCCP concentration-response will shift to the right due to the presence of BSA in the assay medium, as BSA can bind FCCP.
4. Etomoxir concentrations should not exceed 40 µM final, due to potential off-target effects. Note that etomoxir is provided 15 minutes before the BSA control or Palm:BSA is given to the cells to ensure inhibition of CPT-1.
5. If desired, the Palm:BSA can be diluted with BSA to reduce the effective FFA concentration in the assay. (see page 6)
6. Note that most preparations of FA free BSA (used for conjugation to palmitate) do contain trace amounts of residual FAs which may contribute slightly to changes in O_2 consumption rates when the BSA reagent is added to the cells (Figs. 2 and 3).
7. Please ensure that you are using the correct XF^e/XF FluxPaks and volumes for your particular XF^e/XF Analyzer, as they are not interchangeable.

Table 3 | XF FAO Assay Results with HepG2 Cells

	BSA -Eto	BSA +Eto	Palm:BSA -ETO	Palm:BSA +Eto
Basal Respiration	30	23	45	24
Proton Leak	14	13	20	15
ATP Production	16	9	26	9
Maximal Respiration	26	9	107	16
Spare Capacity	-5	-14	62	-8
Non-mitochondrial Respiration	20	18	25	19
Change in Basal OCR due to oxidation of exogenous FAs and/or uncoupling by FFAs	0	-8	15	-7
OCR due to uncoupling by free FAs	-	-	6	6
Change in maximal OCR due to utilization of exogenous fatty acids	-	-	87	-
Basal OCR due to oxidation of endogenous FAs	0	-1	9	1
Change in maximal OCR due to utilization of endogenous fatty acids	-	-	17	-

FFA concentration = 125 nM.
All data were analyzed using XF software and displayed as average oxygen consumption rates (pmol O_2/min/well). Data is presented as an average of replicate wells +/- S.E.M.

Summary

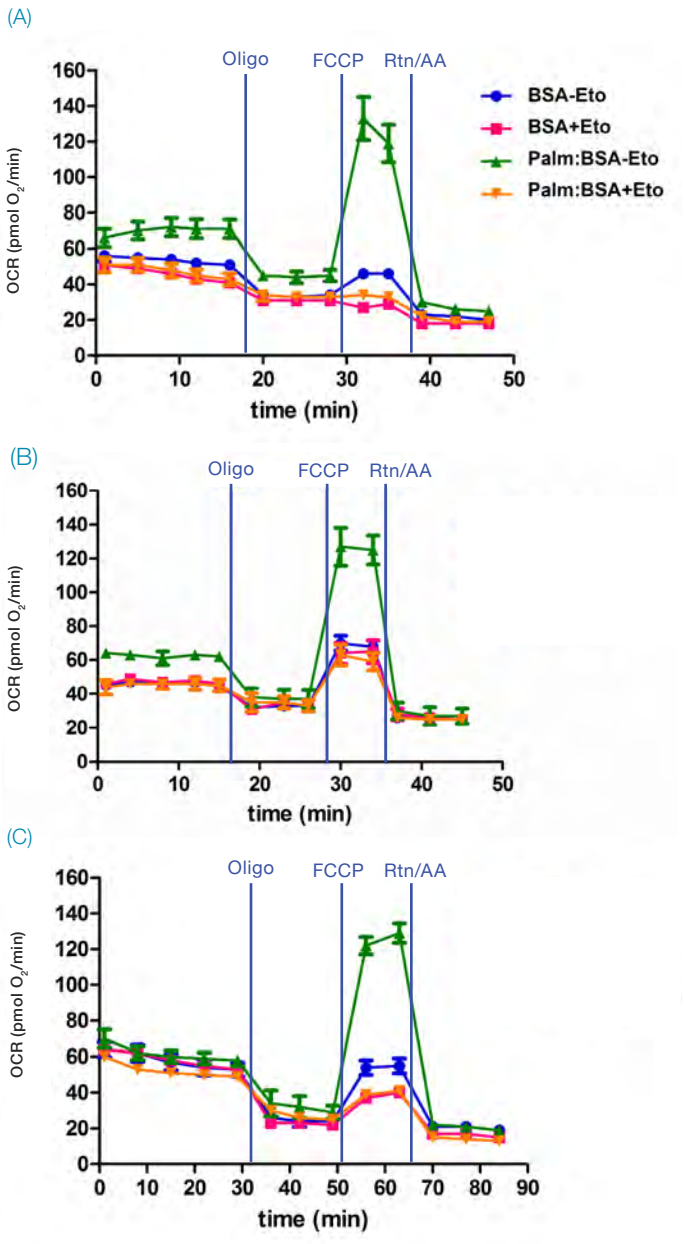
The value of combining the XF Cell Mito Stress Test with the XF Palmitate-BSA FAO Substrate and Eto is that one may drive cells to oxidize exogenous FAs and determine its contribution to basal and maximal respiration.

This was illustrated with the immortalized cell lines used in this Technical Brief. Their responses to exogenous FAs were most pronounced under conditions of high energetic demand, while basal conditions revealed only low to moderate responses.

In summary, this Technical Brief describes a method and assay design for determining the intrinsic and inducible ability of a cell to specifically oxidize exogenously added FAs. By using a combination of the XF Palmitate-BSA FAO Substrate, the XF Cell Mito Stress Test and Eto, one may also reveal FAO due to endogenous FAs and distinguish it from FA mediated uncoupling.

Appendix

While the basic pattern of the FAO is similar in these examples, there are subtle, but significant differences related to both differences in FA utilization and differences in mild uncoupling of the cells by the exogenously added FFA.



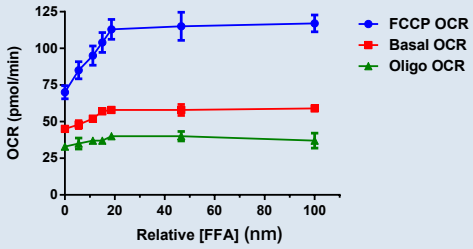
Fatty Acid Utilization Profiles
HepG2 (A), C2C12 myotubes (B), and C2C12 myoblast (C) cells grown in substrate -limited medium overnight to prime the cells for utilization of exogenous FAs and tested with XF Palmitate-BSA FAO Substrate and the XF Cell Mito Stress Test. Eto was used to inhibit FAO and confirm specificity of respiration (OCR) due to FAO.

XF BSA-Palmitate FAO Substrate Provides Lot-Specific, Saturating Concentration of Bioavailable Free Fatty Acid

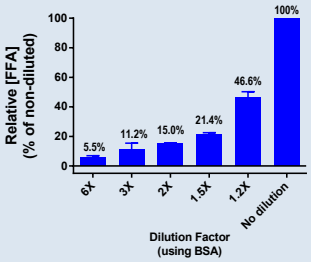
It is the Free Fatty Acid that is the bioavailable substrate for FAO, and therefore the concentration of FFA will determine the degree of utilization of exogenous FAs[2, 3]. The concentration of FFA in the XF Palmitate-BSA FAO Substrate is reported for each lot and provides a saturating concentration of FA for most cells. XF Palmitate-BSA FAO Substrate may also be diluted with BSA to reduce the concentration of free FA.

The dose dependence of respiration of HepG2 cells on the concentration of FFA is illustrated in Panel A. Basal and maximal (FCCP OCR) respiration increase with increasing concentration of FFA, plateauing at ~ 20 nM, indicating that 20 nM FFA is saturating under these assay conditions. Increases in the oligomycin insensitive rate indicate mild uncoupling from the FFAs, and can be determined using the XF Cell Mito Stress Test as described in this Technical Brief.

If a lower concentration of FFA is desired, XF Palmitate-BSA FAO Substrate should be diluted with BSA. It is also important to note that dilution with BSA yields an exponential dilution of the FFA (Panel B). The final concentration of FFA may be quantified with an ADIFAB (acrylodan labeled intestinal fatty acid binding protein) assay[2, 3]. The lot-specific FFA concentration for each lot of XF Palmitate-BSA FAO Substrate provided on the product data sheet allows for precise control of the bioavailable substrate in your XF FAO Assay.



A) Dose response dependence of basal, oligomycin and maximal OCR (FCCP rates) on the concentration of FFA. Note that maximal OCR is achieved at ~ 18.7 nm FFA (1.5x fold-dilution).



B) Quantitation of relative FFA concentration when Palmitate-BSA is diluted with BSA using an ADIFAB assay. The concentration of FFA decreases exponentially relative to the dilution factor.

References

1. The regulation and physiology of mitochondrial proton leak. (2011) Divakaruni AS, Brand MD. Physiology (Bethesda), 26 (3), pp 192-205.
2. Interactions of long-chain fatty acids and albumin: Determination of free fatty acid levels using the fluorescent probe ADIFAB (1993) Richieri GV, Anel A, Kleinfeld AM. Biochemistry, 32 (29), pp 7574–7580.
3. The measurement of free fatty acid concentration with the fluorescent probe ADIFAB: a practical guide for the use of the ADIFAB probe. (1999) Richieri GV, Ogata RT, Kleinfeld AM. Mol Cell Biochem. 192 (1-2), pp 87-94.

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