CELLULAR BIOENERGETICS
FOR THE 21st CENTURY

海馬生物能量測定儀
XF24實驗手冊

Seahorse Bioscience

海馬生物能量測定儀
中文操作手冊

本實驗手冊為基於
Seahorse Bioscience XF24 Extracellular Flux Analyzer Installation and Operation Manual
節錄並經翻譯增補而成
英文版作者：SHB & GWR
本中文手冊：David Li
本手冊著作權屬於
尚博生物科技公司
Seahorse Bioscience Inc

有關於本產品進一步的資訊請洽

WWW.CELL-BIO.COM.TW
WWW.SEAHORSEBIO.COM

相關意見以及其他服務，歡迎洽詢

尚博生物科技有限公司

Tel：02-2785-5860 Fax：02-2785-7237
www.cell-bio.com.tw
cell.bio@msa.hinet.net
台北市南港區園區街三號十七樓ER17

Contents

來吧！我們開始吧！

前言

由於生物體的代謝運作狀態與其生理狀態息息相關，因此儀器設定與實驗細胞品質與材料對於結果穩定性及再現性有關鍵的影響。在本手冊之中，我們以貼附性細胞為範例，為各位使用者說明典型的海馬代謝實驗的流程，以及與實驗過程之中所需要注意的一些小細節。在文末則有數個衍生性的實驗流程，包含有懸浮性細胞、粒線體與組織樣本的分析方法。

為協助各位快速上手，我們將典型的海馬實驗流程依據時間將其分為兩天，包含有第一天「準備日」與第二天「分析日」，各位可以透過每個實驗系統的流程表了解各步驟的執行順序為何。

預祝各位研究工作順利

David Li

海馬生物能量測定儀實驗流程

貼附型細胞

Day 1

Cell

訂定細胞數

背景校正設定

兩段式細胞培養

更換培養基

37°C無CO_2
培養一小時

分析培養基

製備培養基

調整pH值

XF Analyzer

開機

暖機

實驗流程設計

探針校正

樣本分析

結果輸出

探針組

探針浸潤

加入藥物

Day 2

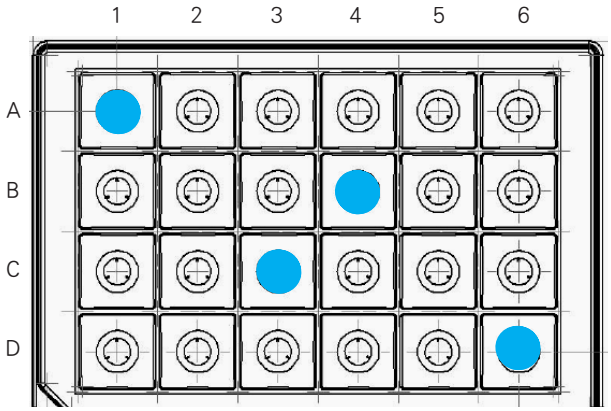
實驗第一天

準備實驗分析所需要的細胞

XF生物能量測定儀專用培養盤
XF測定儀所使用的細胞培養盤與一般所使用的二十四孔培養盤略有不同，原則上同樣具有4乘6的格式，但是每個孔都具有類似漏斗的構造，底部平坦的部分才是實際用於細胞培養的區域，其面積與一般常用的九十六孔盤底面積相當。

背景校正

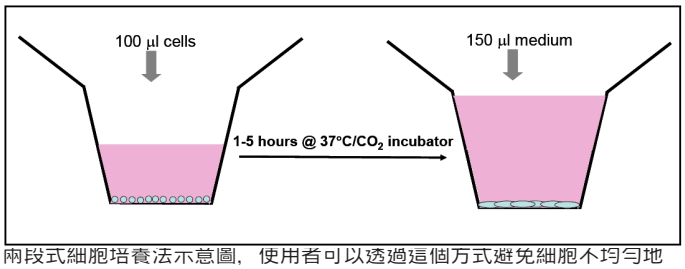
考量氧氣消耗速率對於溫度的變化相當敏感，在準備實驗用的細胞時，我們建議在每個24孔微孔盤中保留A1、B4、C3、D6四個位置，置入等體積的培養基做為背景校正之用。在實驗分析完成之後軟體將會自動將背景值扣除。



XF專用之細胞培養盤，擺放時缺角(左下角處)為前端，兩端均有用於辨識之條碼，圖中藍色位置為背景校正用，僅需要置入等量之培養基，不需種入細胞。

實驗所需的細胞數量

原則而言，海馬生物能量測定儀分析的是貼附性的細胞樣本(懸浮性細胞、粒線體或其他類型的樣本均有專用的實驗程序)，當這些細胞在專用培養盤底部形成一均勻的單層細胞的時候可以得到最佳的實驗結果，因此建議研究者可以依據所使用的細胞類型，評估其於96孔微孔盤之中形成單層細胞所需的數量作為起始的細胞數，而有些細胞類型在密度不足或過高的情況之下可能會喪失某些生理特徵，同時細胞的數量亦會影響實驗使用的藥劑濃度，因此建議針對各實驗需進行實驗最佳化。



兩段式細胞培養法示意圖，使用者可以透過這個方式避免細胞不均勻地分布於培養盤的底部，提供更穩定的訊號。

兩段式細胞培養法

利用兩段式的細胞培養法，研究者可以有效的製造出平整的單層細胞，因此強烈建議採用此方法準備研究所需要使用的樣本。

1. 將每個孔所需的細胞懸浮於100µ l培養基之中，一般而言每個well所需要使用的細胞約略介於兩萬至六萬個細胞之中。
2. 將製備好的細胞懸浮液加入well之中，加入培養基時建議將pipetor的頂端抵於底部小孔的孔壁上，徐徐將培養基加入孔中以達到最佳的效果，注意的是於「背景校正Well」中只可以加入培養基，不需要加入任何的細胞。
3. 將細胞培養盤置於二氧化碳培養箱中培養，直到細胞已經貼附於培養盤的底部。一般而言較容易貼附的細胞大約需要一到兩個小時，一些較不容易貼附的細胞可能需要六個小時左右。
4. 待細胞貼附之後，再於每個孔中加入適量的培養基，一般而言以隔夜培養來說建議加入約150µ l左右的培養基即可，注意加入培養基的時候請貼於容器壁緩緩的加入以避免擾動已經貼附於底部的細胞。
5. 將加入培養基之後的細胞培養盤置於二氧化碳培養箱隔夜培養

實驗所需使用的培養基

在實驗的過程之中，研究人員會需要準備兩種不同類型的培養基，第一種是準備細胞的時候讓細胞生長的培養基，第二種則是實際上機的時候所使用的分析培養基。
生長用培養基與一般用於細胞培養的培養基相同，而分析用的培養基則會除去一般培養基之中具有pH緩衝能力的分子，以期可以觀察到pH值的變化。除了pH緩衝之外，亦需要注意培養基之中所含有的養分分子是否切合實驗的需求。因此原則上需要除去包含有HEPES、Sodium bicarbonate、以及各類的血清、BSA等分子，加入所需的養分之後，將pH值確實的調整至pH 7.4。另外需注意的是實驗中所添加的各類養分本身也具有緩衝pH值的能力，如果需要計算其精確的氫離子產量(PPR, proton production rate)，則需要計算其緩衝力。

準備實驗所需的探針組

為了達到最佳實驗結果，建議將實驗所需使用的探針組於校正液中充分浸潤隔夜之後再進行實驗。

1. 於每個探針組所附的24孔盤的每個孔之中加入1ml的專用校正液
2. 將探針組置於加入校正液的24孔盤上之後置於無二氧化碳培養箱之中隔夜放置之後使用
3. 注意，探針組一旦接觸校正液之後須於72小時之內使用完畢，如果需要於培養箱中放置較長的時間，建議以封膜或保鮮膜將探針組與校正盤密封，以避免校正液過度蒸散。

設定海馬生物能量測定儀

由於細胞本身的呼吸作用對於溫度的變化非常的敏感，因此建議在實驗前先將海馬生物能量測定儀開啟並啟動溫控裝置，放置隔夜使之溫度確實達到37℃

如果希望將控制電腦關閉，使用者可以先將分析儀主機啟動後啟動控制電腦以及XF軟體，隨後關閉軟體，在關閉軟體的同時軟體會詢問是否繼續啟動溫度控制系統，選擇啟動溫控之後即可關閉控制電腦。

設計實驗進行流程

海馬生物能量測定儀的最大特色在於其非接觸性的偵測技術，進行偵測時，探針系統貼近細胞，於培養盤的底部形成一個暫時性的封閉空間，並且透過觀察這個微小空間中氧氣消耗的狀況以及pH變化的情況來了解細胞其代謝系統的變化。在每次偵測完成之後，儀器會重新將探針頭回復到偵測前的位置，以便細胞周圍氧氣已被消耗的培養基可以充分的跟其餘的培養基混合並且回復到偵測前的狀態。

OCR (pmol/min)	Mix Time	Wait	Measure Time
25–50	2 min.	2 min.	5 min.
50–100	2 min.	2 min.	4 min.
100–200	3 min.	2 min.	3 min.
200–400	4 min.	2 min.	2.5 min.
400–800	5 min.	2 min.	2 min.
800+	5 min.	2 min.	2 min.

海馬生物能量測定儀測定模式時間表，一般而言面對未知的樣本，研究者可以先以表格中淺藍色的3–2–3模式進行測定，隨後再依據樣本的呼吸率進行最佳化。

測定模式

考量到這樣的需求，我們將訊號偵測的流程分為三部分：混合、等待與偵測。原則上而言，為避免每次偵測過程中細胞週邊培養基的氧氣被耗盡，耗氧量越高的細胞的偵測時間越短，對應偵測後需要的回復時間(混合+等待)越長。一般而言如果今天處理的樣本條件不明，則建議使用者從標準「混合–等待–偵測=3–2–3」進行實驗，隨後依據實測結果，參照右表決定最佳的偵測模式。

流程設計

決定測定模式後，下一步就是設計實驗的流程，一般而言海馬系統的實驗流程如下：實驗開始的時候，操作人員需要將含有校正液的校正盤與探針組一起置入儀器之中，校正指令用意在針對每組探針組進行校正，以確保資料的穩定性。隨後儀器會將校正盤退出，並要求使用者將要偵測的細胞培養盤置入，並於置入後等候約12分鐘以平衡整個儀器的溫度，待溫度穩定之後，偵測細胞本身未加入任何藥物下的基礎代謝值，須注意的是我們建議在偵測基礎值的時候，細胞的代謝率在三次偵測之後會較為穩定，因此建議至少需要進行三次偵測，並以第三次偵測的結果為準作為基礎代謝值。

偵測完基礎代謝值之後，後續研究人員可依據其需求注入藥物，隨後重複偵測的動作以偵測藥物所產生的反應，此部分可依據實驗需求延長或調整。

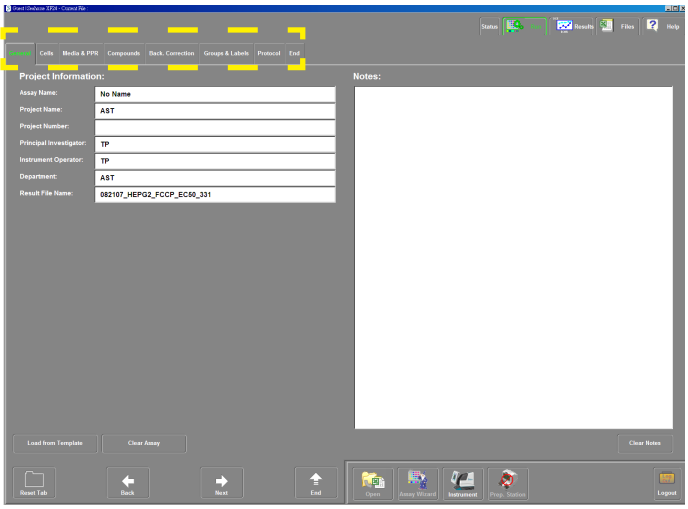
目的	指令	時間	重複次數
校正	Calibration	25	1
平衡	Equilibrate	12	1
偵測基礎值	Mix	3	N, N>3
	Wait	2	
	Measure	3	
注射藥物	Injection		
偵測藥物反應	Mix	3	K, K≒ 2 or 3
	Wait	2	
	Measure	3	

海馬生物能量測定儀典型的實驗流程。，依據測定模式時間表決定測定時間後，隨後即可依據實驗的需要設計實驗流程。原則而言，研究人員可以依據需求重複偵測的次數，觀察樣本的變化。

撰寫流程

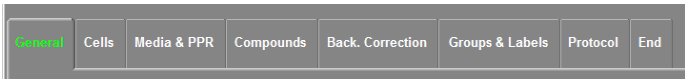
設計完實驗流程之後，下一步是設定這些指令以便儀器執行

首先啟動海馬控制軟體，並且啟動實驗精靈(Assay Wizard)，啟動實驗精靈之後會看到下面的畫面，這是實驗精靈的主要設定畫面。



實驗精靈設定畫面，黃色框內為各設定子頁面

可以注意到，設定畫面中分為數個獨立的子頁面，每個頁面分別用於紀錄實驗中各類參數的細節，這些資訊用意在協助研究人員追蹤實驗的流程，除了protocol此項之外其餘的內容均不會直接影響實驗的結果，不過強烈建議使用者確實的紀錄實驗的細節，以便後續的追蹤。

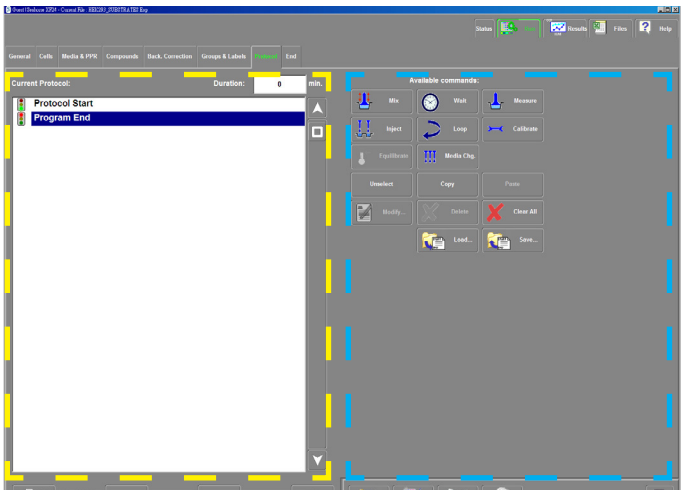


子頁面的切換工具，允許使用者完整的紀錄從細胞、培養基、實驗流程等相關完整背景資訊

子頁面的導航工具，可以快速的在各頁面間跳躍

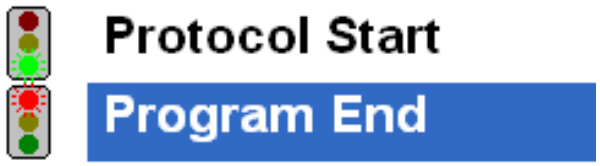
另外於設定畫面的下方還有一組導航鈕，可以讓使用者快速的在各頁面之間跳躍，以便進行設定。

在各子頁面之中，最重要的就屬於實驗流程(protocol)這一項，其頁面外觀如下：



流程設計頁面，左側黃色框為流程一覽，右側藍框為指令集

頁面左側是已編寫的實驗流程，上方顯示的是整個流程所需要花的時間。右側的按鈕則是各類不同類型的指令。每個新增的指令會自動增加在左側指令列藍色光條的上方，當編寫完後，儀器就會依據所撰寫Protocol，自start至End執行每一個指令。



已編寫的流程一覽，執行指令時會由上向下執行，每當使用者新增指令的時候，指令會新增在圖中藍色光棒的上方。

在每個實驗流程開始時，第一步請先加入校正以及平衡兩個步驟。在校正的步驟中，儀器先利用溶於校正液之中的氧氣，校正每組獨立的探針組的訊號，以維持結果的一制性，隨後再完成校正步驟之後，儀器會將探針組留在儀器內部，僅將校正用的空盤退出，之後請使用者將細胞盤置入之後開始進行平衡指令。平衡指令原則上則是由一系列的混合與等待的步驟所構成的，用意在使細胞確實的到達一個穩定的狀態以便進行分析。

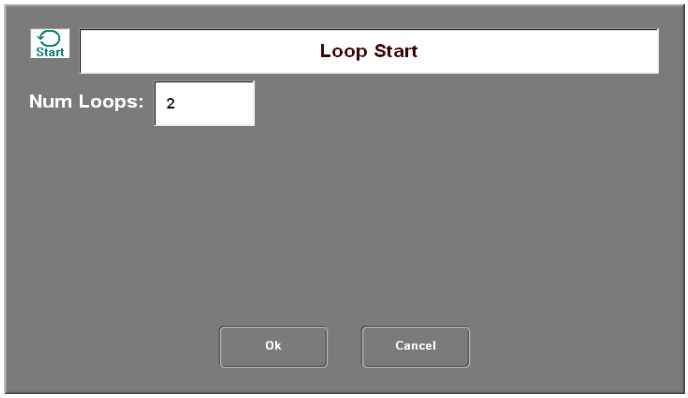
校正與平衡指令，原則上這是每個實驗必要的步驟，如果使用者並未加入這兩個指令的話，程式會主動發出警告訊息

完成校正與平衡之後，接下來要偵測樣本的基礎代謝值，為得到較穩定的結果，原則上會重複至少三次偵測。當我們要進行重複偵測動作的時候，可以透過迴圈(loop)這個指定來設定，減少撰寫時的複雜度。

迴圈指令的按鈕，可以讓使用者新增迴圈的指令

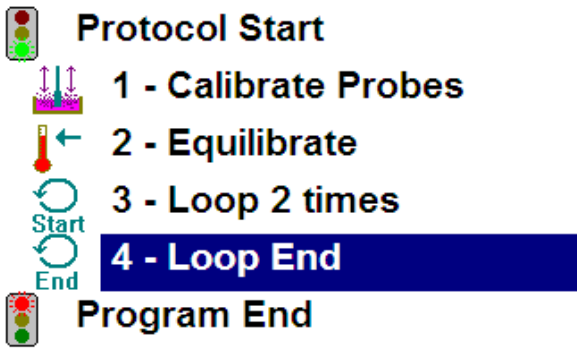
迴圈指令的結構包含有一個起始與一個結束，起始指令上所標示的數字表示迴圈指令重複的次數，介於起始與結束間即為重複的指令

迴圈的結構基本上包含一個迴圈起始與迴圈結束，迴圈上的數字說明起始與結束間的指令重複的次數，原則上無法重複的指令，例如注射的指令是無法加入到迴圈之中的。

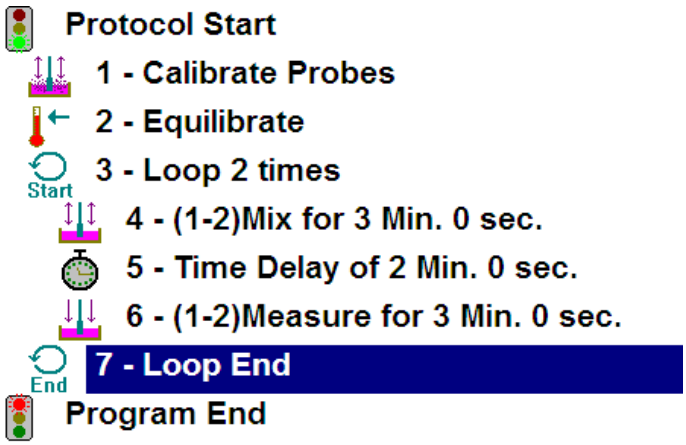


按下迴圈指令之後，首先程式會出現一個蹦現式的對話框，詢問所需要的迴圈數，按下ok之後流程列表中就會出現迴圈的指令

加入迴圈指令後，接下來則要在迴圈中輸出所需重覆的指令。在開始這麼做之前，請先確認藍色的光棒目前位於迴圈結束的位置。如下圖所示：

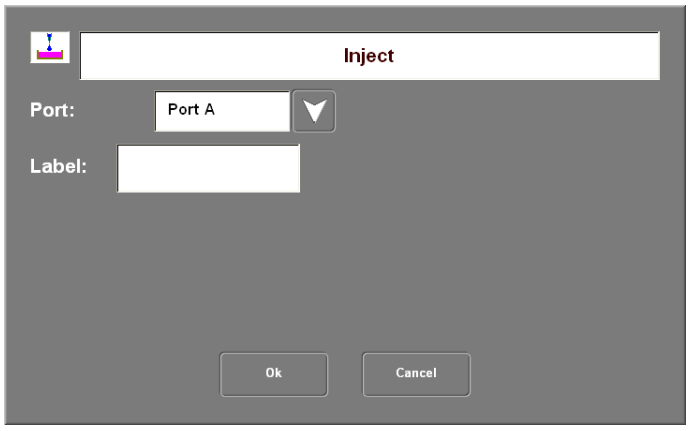


以一個典型的偵測模式為例，我們在迴圈指令之中新增一組「混合-等待-偵測=3-2-3」，新增指令後就會出現如下的結構，這樣在實際執行時迴圈內的指令就會依據使用者的需求重複



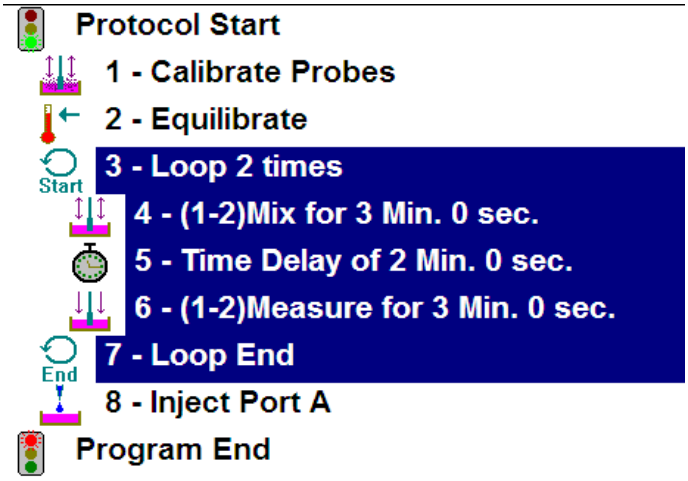
典型偵測指令與迴圈的結合，可以省去重覆設定偵測的困擾

接下來我們插入一組藥物注射的指令，要插入注射指令時，須注意藍色光棒不可以在迴圈之內，否則無法加入注射的指令，按下注射指令之後會出現如右的對話框詢問所需要注入的藥物注射槽為何，以及所需要注入藥物的名稱。確定之後按下ok，指令集之中就會新增一個注入的指令。



注射指令的設定頁面可以設定注入藥物的名稱以及要注入的藥物槽編號，需注意注射指令並沒有辦法重複

完成注入的指令之後，接下來我們要在注射後重複偵測的動作，想要節省指令的撰寫時間，我們可以重覆已經撰寫好的指令，透過複製功能複製所需指令之後，隨後運用貼上即可在需要的時候重複已有的動作。首先先將要複製的動作反白之後，按下右方工具列之中的複製(copy)，之後再將光棒移到所需新增指令的位置之後按下貼上(paste)如此一來就可以快速的重複指令了。



需要複製指令的時候，只需要先將所要複製的指令反白，隨後再將光棒移至所需新增的位置按下貼上即可

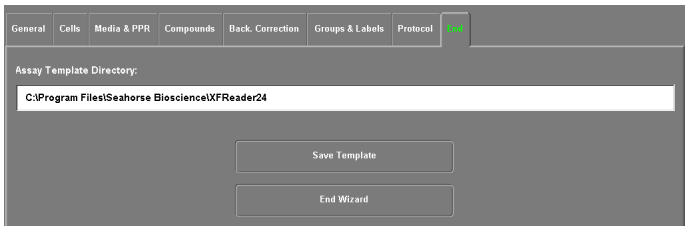
最後，當你完成整個實驗流程的設定之後，不要忘記檢查整個流程的結構，包含有：

1. 是否有校正與平衡的指令？
2. 指令重複的次數是否正確？
3. 混合、等待、偵測的時間長短？
4. 藥物注射的次序？
5. 注射後的偵測頻率與時間？
6. 整個實驗所需要的時間？

如此一來就完成了一個實驗流程的設計。

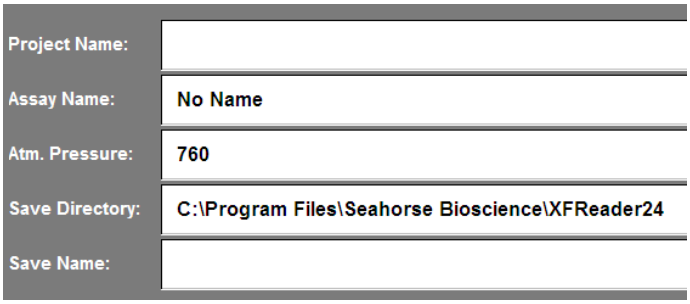
儲存與進行實驗

最後，在設定完成所有實驗流程之後，請切換到結束頁面，設定完成檔案儲存的路徑之後，按下儲存，並且按下結束設定精靈，即完成設定的工作。隨後軟體會自動跳轉至實驗執行的頁面，按下啟動鈕之後就可以開始實驗。



結束頁面，包含有檔案路徑的設定以及儲存功能

在按下啟動鈕之後，操作者會看到起始畫面，左側顯示所有預計要執行的指令，右側則讓使用者可以變更檔案儲存的路徑以及名稱，在最後按下下方的執行指令前，別忘了要檢查實驗的流程是否符合設計，以及檔案儲存的路徑以及名稱是否正確。



開始實驗前畫面右測的檔案儲存路徑設定，於真正開始實驗前須特別注意檔案儲存路徑與名稱的設定

實驗第二天

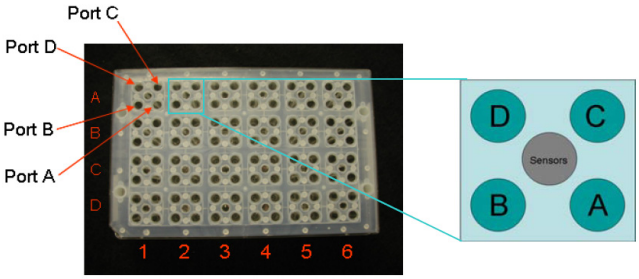
準備分析所需要使用的細胞

1. 確實將分析用培養基(無緩衝能力的DMEM、RPMI、KHB或其他的培養基)回溫至37℃
2. 使用你最好的朋友：顯微鏡觀察實驗所需要使用的樣本，確認細胞確實且平均地貼附於培養盤底部
3. 使用微量分注器或是任何你覺得適當的工具從每個well中吸取除去大約150-200μ l的培養基，使得每個well中都殘留有大約50-100μ l的培養基。注意在這個過程之中請不要讓你使用的工具接觸到培養盤的底部，以免擾動到細胞，也不要傾斜培養盤或作出任何會讓細胞細胞暴露於空氣中的舉動。
4. 於每個Well中加入1ml的分析用培養基，隨後利用同於3.的技巧移除這些培養基，使得每個well中都殘留有大約50-100μ l的培養基。
5. 於每個well中加入適量培養基直到他們達到適當的體積(500-1000μ l)
6. 將已經添加培養基之後的細胞置於無二氧化碳的培養箱之中至少放置一個小時之後再進行實驗，以確保細胞已經適應於新的培養基環境。

注意事項：3.與4.中所提到的實驗技巧用意僅在於防止細胞暴露於空氣之中後影響其生理狀態，每個well中所留下的培養基多寡其實並沒有一定的標準，但需要注意的是請確定每個well中所殘留的量一致，如此一來可以得到穩定性較高的結果。

把所需研究的目標藥物注入探針組中

當由上往下觀察一個XF24的探針組你可以注意到他是一個四乘六的格式，在其中一個角落有截角，請將這個截角向著自己(如下圖)，可以注意到每個Well的對應位址之中包含有五個獨立的凹孔，中央的孔是當這個探針組進入儀器中的時候光纖頭所使用的位置，請不要將任何的試劑加入中間的位置之中，剩下四個孔由右下到左上分為ABCD四個藥物注射槽，研究者可以在這四個槽之中加入感興趣的藥物並且要求海馬系統於指定的時間將這些藥物注射到細胞所處的培養基中。



藥物注射槽的結構：當我們將海馬探針組面對自己橫放，截角朝左下角時，注射槽由右下往左上分別為A、B、C、D。

在將探針置入儀器中進行校正之前，將所需要注入的藥物取75μ l確實回溫至37℃之後以分注器加入對應的藥物注射槽之中。一般而言建議將要注入的藥物溶於測試用培養基之中再注入，體積以75μ l為最佳，25μ l至100μ l均可接受。需特別注意的是如果要注入A槽的藥物，則該探針組上所有的A槽(包含背景控制組)均需要加入藥物(或對應培養基)，且體積需要完全相同(即所有A槽體積均要相同、所有B槽體積均要相同，依此類推)。另外於加入藥物的時候請使用微量分注器貼管壁傾斜加入，切忌直接對注射槽底端直射。

校正探針組與進行實驗

如果已經確實於前一天將XF生物能量測定儀啟動並且開啟溫控裝置，此時儀器應已經確實達到37℃，那麼使用者只要直接啟動控制電腦以及軟體，就可以開始準備進行探針校正。若無，請同樣開啟控制電腦與軟體，等待儀器本身的溫度穩定下來之後再進行校正的動作。

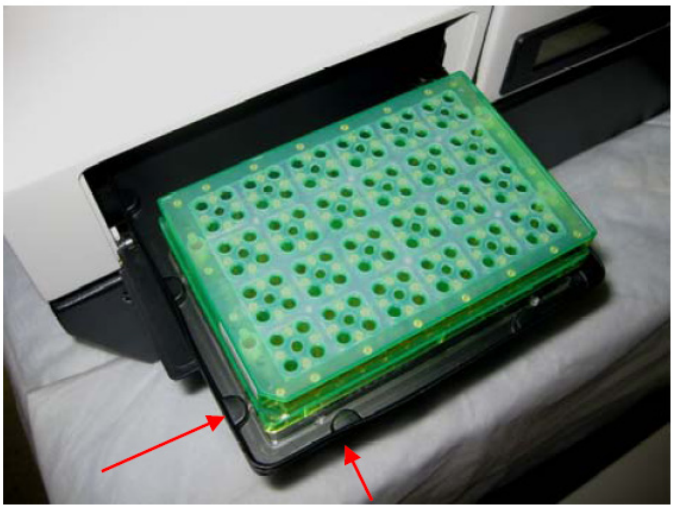
在啟動軟體之後請開啟所要進行的實驗樣板，有關於建立實驗樣本的詳細說明請參閱相關的說明文件。

開啟實驗樣板之後，請先確定所使用的探針組是否已經確實的加入了所需的藥物，接下來請按下螢幕上的綠色啟動按鈕(start)，按下之後會出現類似右圖的確認畫面，確定實驗的流程無誤之後，請確實的鍵入分析結果儲存的路徑以及檔案的名稱，隨後即可按下開始鈕進行實驗。

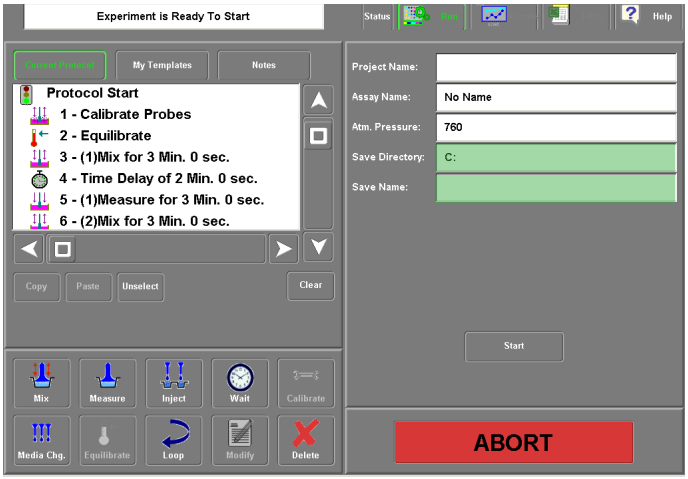
啟動鈕，用於啟動實驗流程。

按下啟動鈕之後，儀器內部的托盤就會移動到機器外，此時將已經加入了藥物以及包含有校正液的探針組放入儀器之中，注意請將探針組有截角的一側向著儀器的前方，並確實將他平放於托盤上，確認探針組已經卡入托盤上紅色箭頭所指的卡榫內之後就可按下螢幕上的確認，此時儀器就會自動開始進行校正的動作。

整個校正的程序大概需要使用大約三十分鐘的時間當校正完成之後，儀器會要求研究者將所要分析細胞盤置入儀器之中，此時只需要將托盤上的校正盤取下，將細胞以相同的方向(缺角向前)放入後確認即可。當分析完成之後資料會直接儲存在所指定的路徑以及名稱之下。



探針組置入時的方向，需注意其條碼向後，截角朝向儀器的前方，且探針組需確實的卡入噴濺盤的卡榫間，圖中紅色箭頭所指即為卡榫。



實驗開始後之啟動畫面，需注意確實填入圖中綠色兩個欄位中的資訊，包含檔案儲存路徑及名稱。

範例Protocol

Bioenergetic profile of A549:

Day 1:

細胞製備：

1. 依據一般細胞培養技巧，將A549細胞懸浮於Growth medium之中，調整濃度至3x10^5/ml。
2. 於XF專用細胞培養盤中除Temp. Ctrl.的所有Well之中，以傾斜角度加入100μl細胞懸浮液，置於37℃ 5%CO_2培養箱中培養1-5小時，待細胞貼附之後添加額外培養基隔夜培養備用

探針浸潤

1. 取一組XF assay kit，開封後與24孔盤中每個Well中加入500μl XF Calibrant solution，將探針至於盤上後置於37℃無CO_2培養箱之中備用。
2. 探針應於初次浸潤於Calibrant之後72小時使用。如需於培養箱中置放超過24小時，需以Parafilm密封邊緣以防水分散失。

XF細胞能量測定儀暖機：

1. 啟動測定儀主機電源與電腦電源，開機完成之後啟動XF分析軟體，儀器會自行加溫至37℃，靜置隔夜備用。
2. 如需將電腦電源關閉，可先完成1.之程序後結束XF分析軟體，並於軟體結束時選擇：啟動溫控系統之選項，隨後即可將電腦電源關閉。

Day 2:

準備分析用培養基：

1. 分析用培養基應以不含血清、Bicarbonate細胞培養用培養基為基準，依據所需進行實驗添加適當養分來源，使用前須確實回溫至37℃後調整pH值至7.4後置於37℃無CO2培養箱一個小時。
2. 脂肪酸氧化或其他特殊＂養份缺乏＂相關實驗須使用KHB緩衝液。
3. 如需進行含血清之實驗請與本公司連繫。

更換培養基：

1. 待分析用培養基回溫完成之後，取Day 1製備之細胞，以微量分注器吸取培養基至well中僅殘留50μl培養用培養基。
2. 加入1ml分析用培養基，再以pipetor吸取培養基至well中僅殘留50μl培養基。
3. 加入625μl分析用培養基，使每個well最終體積為675μl。

添加代謝調節藥物

1. 於 Temp. Ctrl.對應位置之藥物注射槽中加入分析用培養基。
2. 將所需研究之藥物加入探針的藥物注射槽之中，每種體積建議為75μl (25-100μl內皆可，但須注意保持每個well所注入之藥物體積相同)

探針校正與分析：

1. 將電腦軟體啟動之後載入預先設計之實驗protocol
2. 按下啟動鈕之後依據軟體所指示，將含有校正液的探針組放入儀器之中，按下確認後等待約三十分鐘待儀器完成校正
3. 儀器會將含有校正液的校正盤退出，取下校正盤之後放上所製備之細胞，按下確認。
4. 待儀器分析完成。

附錄一：非貼附性細胞實驗流程

懸浮性細胞

懸浮式細胞分析的流程，原則上是以貼附式細胞的流程為基礎修改而成。利用Cell-Tak先行處裡培養盤後，在加入細胞，透過Cell-Tak，研究者得以將分析的懸浮細胞樣本貼附於配養盤的底部，比照貼附性細胞處理

材料

1. BD Cell-Tak Cell and Tissue Adhesive (BD Biosciences, CN 354240)
2. NaHCO3 (Sigma, S5761)
3. Tissue Culture Grade Sterile Water (Invitrogen, CN 15230)
4. XF plates
5. Multi-channel pipette

培養盤處理

1. 取420mg $NaHCO_3$溶於50mL TC水中，調整pH值至 8.0，以0.22 um濾網過濾後存放於4°C備用，此為0.1M之NaHCO3溶液.
2. 以Cell-Tak處理細胞培養盤。
   計算使用量：Cell-Tak的使用量是每平方公分3.5ug，XF專用細胞培養盤每個孔槽的底面積為0.32平方公分，處理體積為50uL以XF24細胞培養盤而言，每個培養盤需準備1.5ml的Cell-Tak溶液。
   舉例而言：Cell-Tak原液為2mg/ml，取17ul加入1.5ml的0.1M NaHCO3溶液中，隨後於每個well中加入50ul進行Coating。
3. Coating中的培養盤需置於無菌操作台乾燥20分鐘，隨後吸去多餘的溶液，加入200ul的無菌水後再吸去以洗去多餘的bicarbonate，之後在於無菌操作台中乾燥20分鐘
4. 乾燥後的培養盤需以保鮮膜或封膜包裹，保存於4°C冷藏，一週內用畢。 refrigerator with rim wrapped in parafilm to avoid condensation.
5. 使用前先將處理過的培養盤置於市溫下回溫20分鐘，注意不可回溫至37°C，否則Cell Tak 會失去活性。

把細胞黏上去

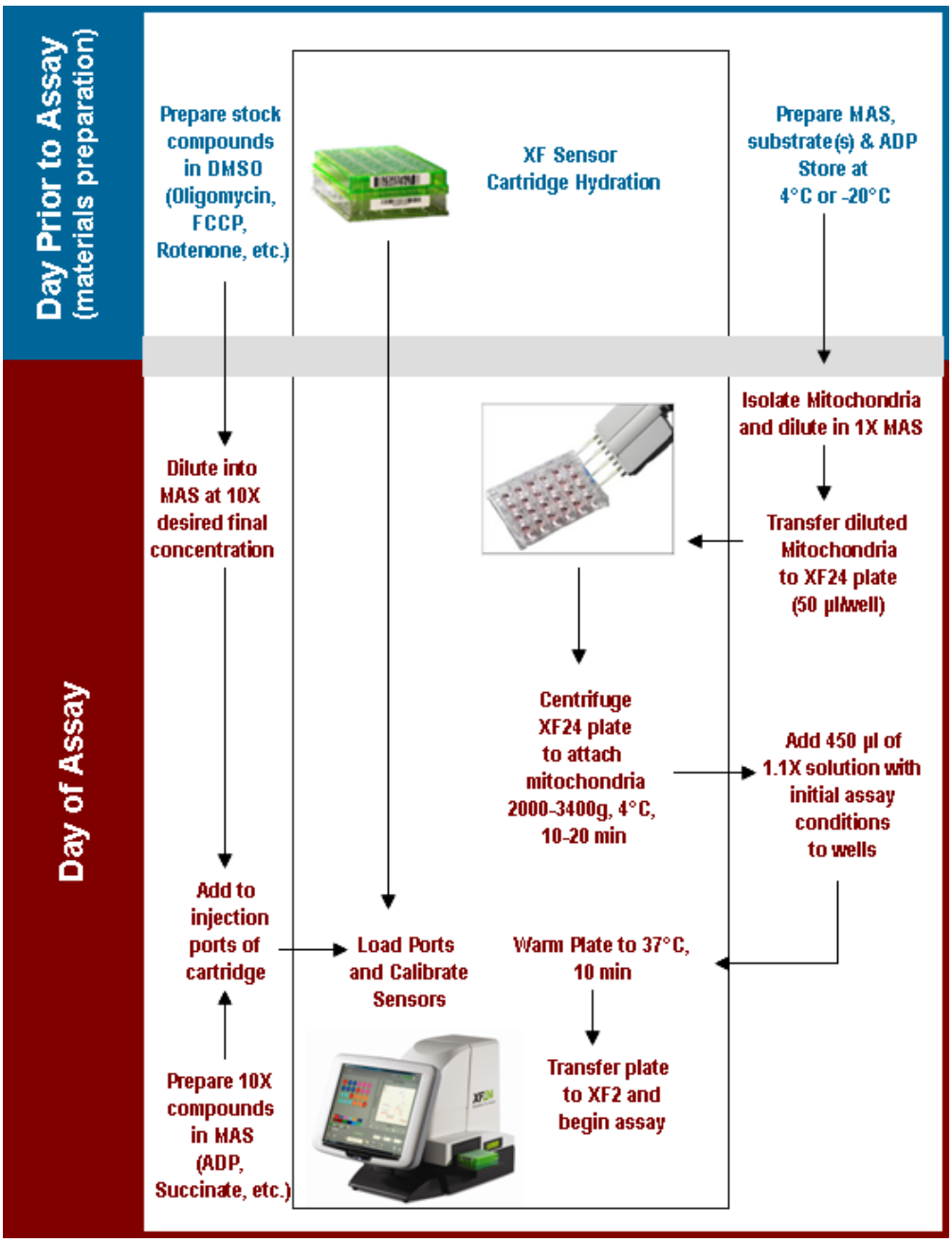
實際操作的時候，操作者可以考慮將探針校正的步驟跟細胞貼附的步驟整合，以加強實驗的順暢度。

1. 將分析用培養基確實回溫至37°C。
2. 以血球計數器計算所要使用的細胞數量
3. 將細胞離心，除去一般培養用培養基，並同時在背景控制組的孔槽中加入100 ul的分析用培養基
4. 將離心後細胞去上清液，重新懸浮於分析用培養基之中，注意體積跟細胞濃度的控制，並且於剩下的孔槽之中加入100ul含有細胞的培養基
5. 將含有細胞與培養基的培養盤置於37°C無二氧化碳的培養箱鐘培養25-30分鐘，原則上在經過25分鐘之後，細胞因該會貼附在容器上，但是他的型態因該不會有太大的差異，操作者可同時間開始進行探針校正的動作。
6. 緩慢且溫和的在每個孔槽中再補入500ul的分析培養基
7. 觀察細胞確認細胞貼附的狀況
8. 再置入無二氧化碳的培養箱中配養20-25分鐘
9. 待探針完成校正工作後，將細胞置入儀器中進行分析，其餘部分與貼附性細胞無異。

Isolated Mitochondria Assay using the XF24 Analyzer (revision 091104)

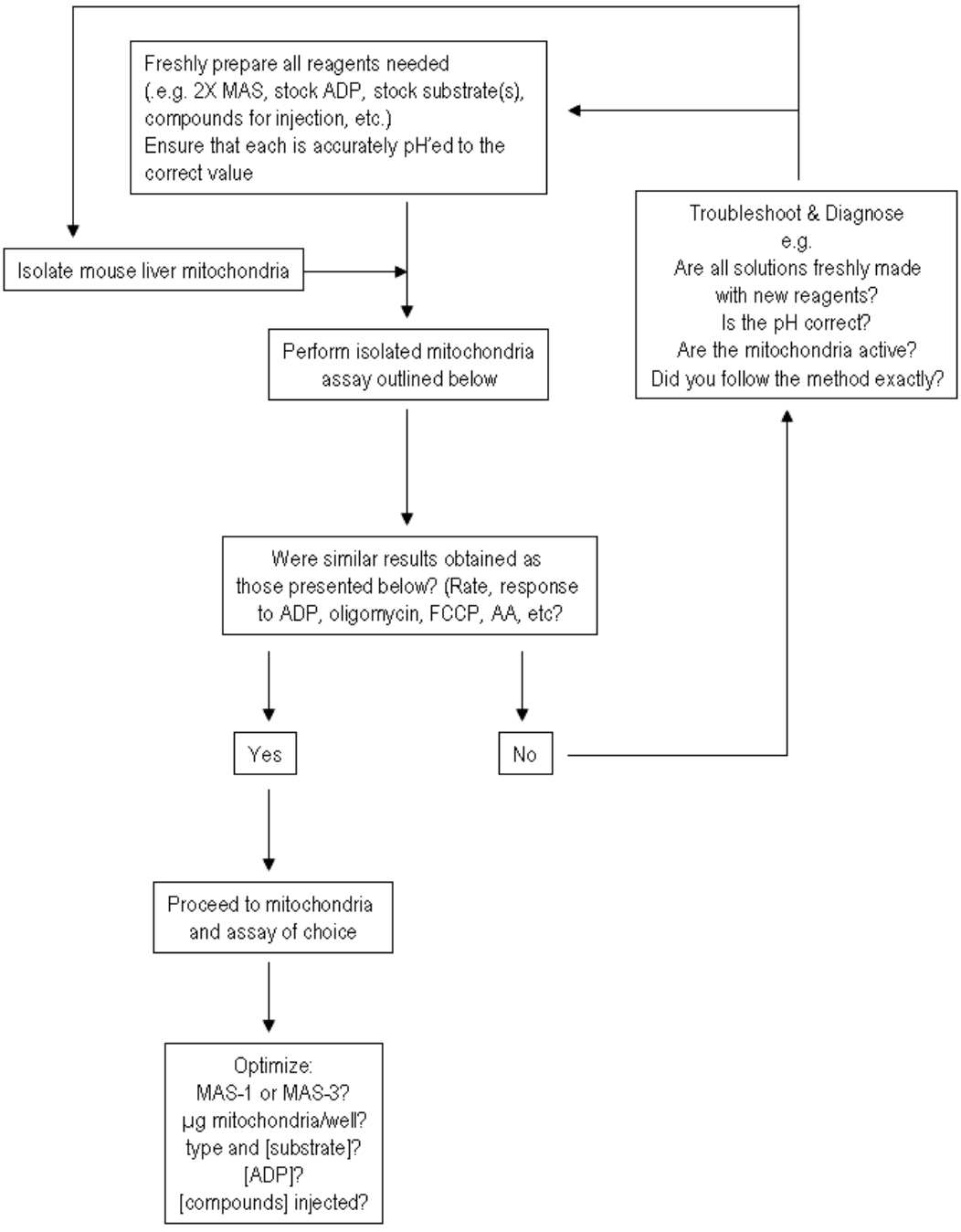
The XF Analyzer instruments were originally designed for use with intact cells attached to a tissue culture plate. Since many researchers routinely utilize isolated mitochondria to answer fundamental questions about mitochondrial function, bioenergetics and metabolism, Seahorse Bioscience (in conjunction with collaborators) has developed a protocol that expands the application of the XF24 analyzer to include isolated mitochondria. The advantages over traditional methods (e.g. Clarke Electrode Apparatus) include higher throughput (20 samples per assay) and smaller amounts of isolated mitochondria required (as low as 5 µg total mitochondrial protein per well).

Isolated Mitochondria Assay Flow Chart



Suggested Workflow for optimizing conditions using isolated mitochondria in the XF24

The methods presented on the following pages have been optimized for mitochondria isolated from mouse liver. Even if you are an experienced user of isolated mitochondria, Seahorse Bioscience strongly suggests taking a bit of time to reproduce the results shown in this protocol. This will ensure that all reagents prepared and used are working properly and provides the user with practice of the methodology on the XF24. Once the user has become familiar with this process and has validated the quality of all reagents used, then these methods can be translated to perform the desired experiments with the isolated mitochondria of choice. *Keep in mind that the protocol n the following pages provides a starting point only, and that users will be required to optimize the conditions for their specific application and experimental design.*



I. Reagents, Materials and Preparation of MAS and Injected Compounds

Two different Mitochondrial Assay Solutions (MAS) are provided below. Please use the buffer that will be optimal for your mitochondrial system of choice and your intended research goals.

MAS-1: Sucrose/Mannitol based MAS: Seahorse Bioscience has used this buffer with success for mitochondria purified from mouse liver and rat liver. Due to its high content of sucrose/mannitol, this buffer tends to be more protective of isolated mitochondria.

MAS-3: KCl based MAS: Seahorse Bioscience has used this buffer with success for mitochondria purified from mouse liver and rat liver. This buffer is more representative of the physiologic conditions *in vivo*.

Experiments performed by Seahorse Bioscience have shown that comparable results have been obtained using either MAS with mitochondria isolated from mouse liver.

Note that there are a number of Mitochondrial Assay Solutions reported in the literature that can vary in both the concentration and identity of components (e.g. some solutions include EDTA). Seahorse Bioscience recommends freshly preparing, testing and optimizing your solution of choice if different than those listed below.

I.1. Components/Formulation of Mitochondrial Assay Solution-1 (MAS-1)

Compound	Brand	Catalog Number	MW or Molar Concentration	Final Concentration (1X)	Grams or ml for 500 ml of 1X MAS	Grams or ml for 250 ml of 2X MAS
Sucrose	Sigma	S9378	342.30	70 mM	11.98 g	11.98 g
Mannitol	Sigma	M9647	182.2	220 mM	20.04 g	20.04 g
KH_2PO_4	Sigma	P9791	136.09	5 mM	0.34 g	0.34 g
$MgCl_2$	Sigma	M1028	1.0 M	5 mM	2.5 ml	2.5 ml
HEPES	Sigma	H0887	1.0 M	2 mM	1.0 ml	1.0 ml
EGTA*	Sigma	E4378	100 mM	1 mM	5.0 ml	5.0 ml
FA-free BSA	Sigma	A7511	66,430	0.2%	1.0 g	1.0 g
* Please make a 100 mM stock solution of EGTA and ensure the pH is ~ 7.2				MAS-1 may be aliquoted and stored at -20°C. Smaller volumes may be made by scaling down reagents.		

Dissolve components listed in table above into ~350 ml dH_2O for a 1X solution or ~150 ml dH_2O for a 2X solution. Warm solution to 37°C. Adjust solution to pH 7.2 using KOH. Add dH_2O to the appropriate final volume (500 ml or 250 ml for 1X and 2X solutions, respectively.)

I.2. Components/Formulation of Mitochondrial Assay Solution-3 (MAS-3)

Compound	Brand	Catalog Number	MW or Molar Concentration	Final Concentration	Grams or ml for 500 ml of 1X KHE	Grams or ml for 250 ml of 2X MAS-3
KCl	Sigma	P9333	74.56	115 mM	4.29 g	4.29 g
KH_2PO_4	Sigma	P9791	136.09	10 mM	0.68 g	0.68 g
$MgCl_2$	Sigma	M1028	203.31	2 mM	0.204	0.204
HEPES	Sigma	H0887	1.0 M	3 mM	1.5 ml	1.5 ml
EGTA*	Sigma	E4378	100 mM	1 mM	5.0 ml	5.0 ml
FA-free BSA	Sigma	A7511	66,430	0.2%	1.0 g	1.0 g
* Please make a 100 mM stock solution of EGTA and ensure the pH is ~ 7.2				MAS-3 may be aliquoted and stored at -20°C. Smaller volumes may be made by scaling down reagents.		

Dissolve components listed in table above into ~400 ml dH_2O for a 1X solution or ~200 ml dH_2O for a 2X solution. Warm solution to 37°C. Adjust solution to pH 7.2 using KOH.

Add dH_2O to the appropriate final volume (500 ml or 250 ml for 1X and 2X solutions, respectively.)

I.3. Components/Formulation of compounds to affect mitochondrial function

It is recommended that all compounds to be added or injected are diluted with the appropriate MAS. It is convenient and suggested to prepare a 2X MAS to use for dilution of compounds and substrates.

Compound	Brand	Catalog Number	Final Concentration	Dissolve in:
Glutamate	Sigma	G8415	5 mM	50 mM stock, MAS
Malate	Sigma	M6413	5 mM	50 mM stock, MAS
Succinate	Sigma	S2378	5 mM	50 mM stock, MAS
ADP	Sigma	A2754	0.25 – 2.0 mM	2.5 - 20 mM stock, MAS
Rotenone	Sigma	R8875	2 µM	Stock 1000X (1 mM) in DMSO. Dilute to 10X in MAS
Oligomycin	Sigma	O4876	2 µM	Stock 10000X (20 mM) in DMSO. Dilute to 10X in MAS
FCCP	Sigma	C2920	0.4 to 4 µM	Stock 10000X (4-40 mM) in DMSO Dilute to 10X in MAS
Antimycin A	Sigma	A8674	4 µM/1.5 µg/ml	Stock 1000X (40 mM/1.5 mg/ml) in DMSO. Dilute to 10X in MAS
Note: ADP can be made as a 100 mM stock in dH_20, pH to 7.0. Succinate, malate and glutamate may be prepared from either their respective free acids or sodium salt derivatives. All may be prepared as 500 mM stocks in dH_20, pH to 7.0. Be aware that sodium ions can influence isolated mitochondria function depending on the species and organ used for preparation. ADP and substrates may be stored at -20°C. Oligomycin, FCCP and Antimycin A should be freshly diluted in MAS for each experiment. Stock solutions in DMSO or ethanol (95%) may be stored at -20°C.				

I.4. Other items needed

XF24 Biosensor Cartridge
XF24 Tissue Culture Plate
Calibration buffer (Seahorse Bioscience)
Multi-channel pipettes and tips, centrifuge with plate adaptors, Eppendorf and Falcon tubes

II. Preparation of XF Assay Templates, XF Cartridges and Mitochondria

II.1. Prepare an XF assay template (via the Assay Wizard) using the XF24 operation manual as a guide and incorporating proper experimental design. Upload the assay template to the XF24 Analyzer before starting the assay. The experiment outlined below is an example of how to obtain the various mitochondrial respiration states using the XF24.

Use the following table as a guide to program the Mix, Wait, Measure and Injection protocol.

Command	Time	Port
Calibration	-	-
Equilibrate	12 min*	
Mix	25 sec	
Measure	4 min	
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject	-	A
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		B
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		C
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		D
Mix	25 sec	
Measure	4 min	

* Default Equilibrate command consists of 2 min Mix, 2 min Wait repeated 3X.

† The mixing commands AFTER measurement commands are optional and facilitate the sensors returning to ambient O2 concentration. These steps are useful if the basal respiration rate (OCR) is above 200 pmol/min.

Note that the Measure times indicated are guidelines only. Typical Measurement times are 3-5 minutes, and can be longer to allow for ADP exhaustion be depleted from the transient micro-chamber. It is advised to empirically determine the optimal Mix and Measure times for your desired application and experiment.

II.2 Prepare the XF sensor cartridge

II.2.1. Hydrate the XF sensor overnight in XF Calibration Buffer at 37°C, no CO_2.

II.2.2. Before calibration, load the XF sensor cartridge injection ports with following compounds listed in the table below.

Injection Ports	Volume	Concentration in Port	Final Concentration in Well
A: ADP	50 µl	2.5 – 20 mM	0.25 – 2 mM
B: Oligomycin	55 µl	20 µM	2 µM
C: FCCP	60 µl	40 µM	4 µM
D: Antimycin A	65 µl	40 µM	4 µM
Note: Vigorous mixing of the stock 20 uM oligomycin is required to prevent precipitation			
Note: Other substrates (e.g. glutamate and/or malate) may be substituted for succinate. Please consider the goals of your experiment and the information to be derived from the assay to choose appropriately.			

II.2.3 Prepare isolated mitochondria by the standard protocol(s) used in your laboratory. Typical isolated mitochondrial suspensions yield ~ 20-100 mg/ml mitochondrial protein as measured by a Bradford or BCA Assay.

III. Mitochondria attachment to the bottom of the plate well

III.1 Calibrate the sensor cartridge (loaded with desired compounds) as described in the XF manual.

III.2. Once the calibration has been started, dilute an appropriate volume of isolated mitochondria in MAS to yield a final concentration of 200 µg of mitochondrial protein/ml (= 10 µg/50 µl). For example, if the isolated mitochondria are 60 mg/ml, then dilute 5 µl of mitochondria in 1.5 ml of MAS (200 µg/ml final). Note that due to large dilution factors and the mitochondria being a suspension, it is recommended that the mitochondria be first mixed by gentle pipetting, then diluted into a small volume (~100 µl) of 1X MAS. This is then added to the larger volume (1.4 ml) and pipetted gently several times to mix thoroughly.

III.3. Transfer 50 µl (10 µg total*) of the diluted mitochondria into each well of a V7 XF24 Tissue Culture Plate. For background correction wells (A1, B4, C3 and D6) use 50 µl of MAS *(no mitochondria!).*

** Note: The final amount of total protein/well will depend from which tissue the mitochondria were obtained, e.g. mitochondrial rich tissues will contain more mitochondria per µg total protein than less mitochondrial rich tissues. Further, the amount of mitochondria per µg total protein will also be dependent on the overall purity of the mitochondrial preparation.* ***It is strongly suggested to perform a titration experiment with the mitochondria to determine the optimal amount to use.***

III.4. Spin down mitochondria at 4°C for 10-20 minutes at 2000-3600g. *The time and speed may need to be tested for optimal mitochondrial activity*. When finished, visualize the mitochondria on the well surface using a 20X or 40X lens. Note any wells that do not appear to have a consistent "monolayer" of mitochondria adhered to the well bottom. Add 450 µl of 1.1X intitial media conditions (if applicable) to each well (see IV below). Place the plate at 37°C (no CO_2) for 8-10 minutes to warm (do not allow warming to go longer than 10 minutes).

IV. Preparing initial buffer conditions for the assay

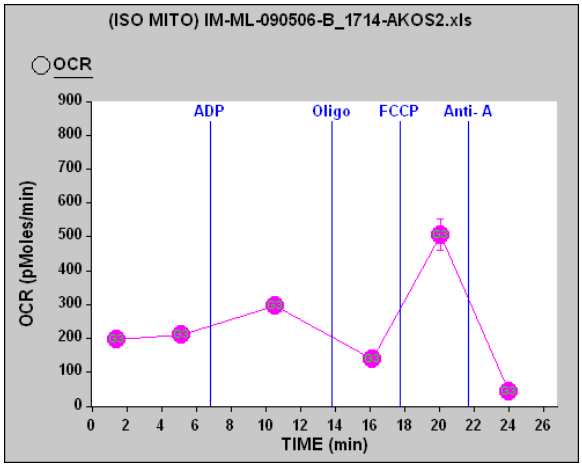
IV.1 During the time of centrifugation, prepare a 1.1X solution of succinate (5.5 mM) and Rotenone (2.2 µM) in 1X MAS.

IV.2. BEFORE the 10 minute warm up period, add 450 µl of 5.5 mM succinate and 2.2 µM rotenone in 1X MAS to each well (final volume is 500 µl, final concentrations of succinate and rotenone are 5 mM and 2 µM, respectively).

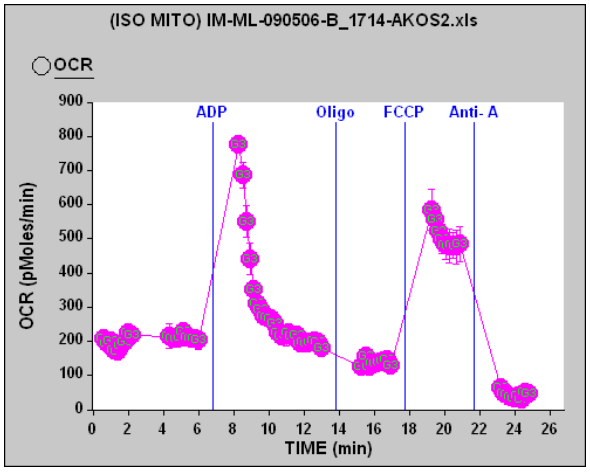
IV.3 After calibration is finished, follow the directions on the instrument controller and when prompted, exchange the calibration plate for the plate containing the mitochondria and continue to follow the directions on the instrument controller.

V. Examples of results and data analysis

The data shown below are typical results obtained using the methods outlined above.



OCR vs. time (2 wells), middle point mode

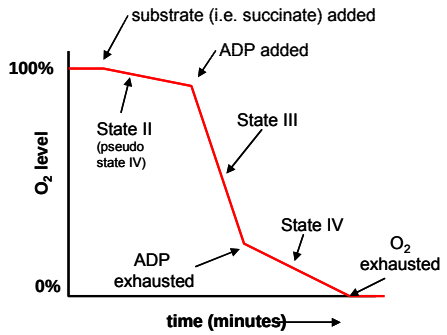


OCR vs. time (2 wells), point to point mode

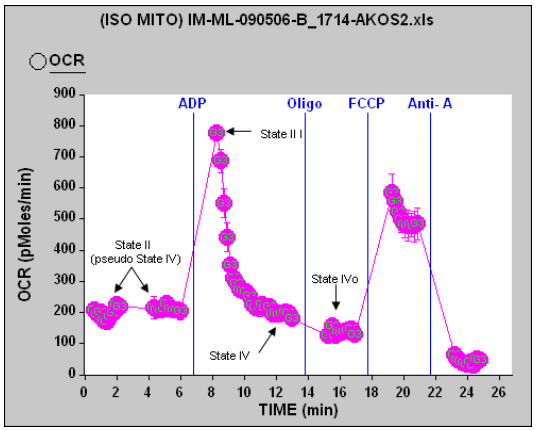
Conditions: MAS-1, 10 µg mouse liver mitochondria, intial assay conditions: 5 mM succinate, 2 uM rotenone, [ADP] = 0.25 mM, [Oligomycin] = 2 uM, [FCCP] = 4 uM, [Antimycin A] (AA) = 2 uM.

Note that the middle point mode (left) under-estimates the State III rate (Succ. + ADP) since the respiration rate changes rapidly during the course of this measurement period, which is evident in the point to point mode (right). The middle point mode accurately represents the other rates as these are relatively stable during the course of the measurement period.

The figure below shows examples of theoretical respiration states (if using a Clarke Electrode type apparatus, left) and actual respiration states obtained with the XF24 analyzer (right). The left panel shows [O_2] vs. time, while the right panel shows OCR (pmol/min) vs. time (point to point mode). Note that near the end of measurement 3 (after the injection of ADP), ADP exhaustion is apparent in the panel on the right as evidenced by the decrease in OCR during the course of the measurement.

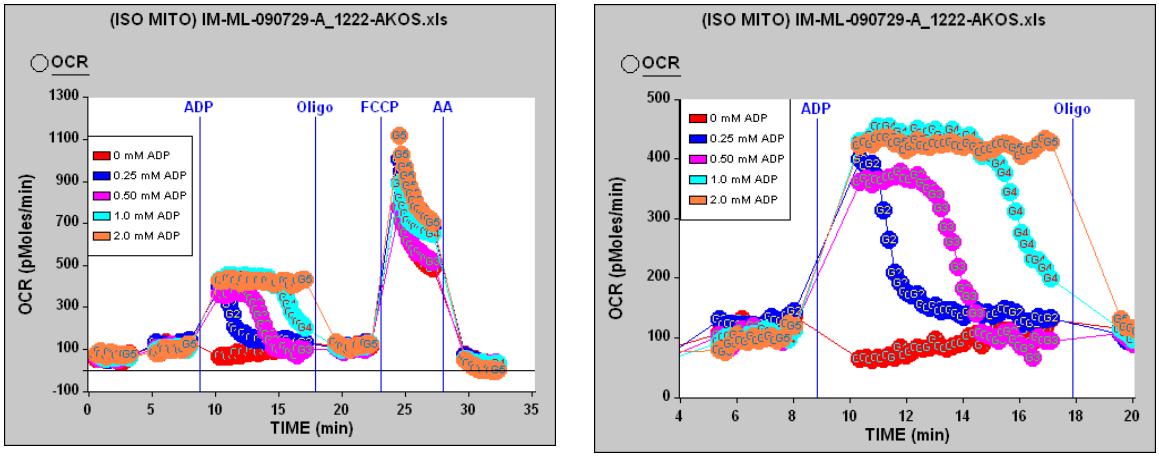


Theoretical respiration states
(Clarke Electrode apparatus)



Respiration states obtained with the XF24

VI. Example of ADP titration using mitochondria isolated from mouse liver



Example of ADP titration
Entire Assay Period (left) and zoom of period immediately after ADP injection (right).
(Error bars have been omitted for clarity)

Conditions: MAS-1, 10 µg mouse liver mitochondria, intial assay conditions: 5 mM succinate, 2 µM rotenone, [ADP] = 0 – 2 mM, Oligomycin = 2 µM, FCCP = 4 µM, Antimycin A (AA) = 2 µM.

Note that the concentration of ADP correlates directly with the length of time the mitochondria respire at State III.

VII. Obtaining State III and State IV respiration values and RCR values.

State III respiration is defined as ADP-stimulated respiration in the presence of saturating substrate. Isolated mitochondria are put into State III when ADP is added to intact mitochondria in the presence of excess substrate (typically glutamate + malate or succinate). This is the value of the slope of [O_2] vs. time, and is transformed by the XF software algorithm to OCR in units of pmol/min.

State IV respiration is defined as oxygen consumption by isolated mitochondria on a particular substrate, in the absence of ADP or any metabolic inhibitors. State IV respiration may be measured when the added ADP is exhausted (converted to ATP). Again, this is the value of the slope [O_2] vs. time, and is transformed by the XF software algorithm to OCR in units of pmol/min.

State IV may also be obtained by adding oligomycin to the mitochondria (State IVo), which inhibits Complex V and thus prevents the conversion from ADP to ATP. This method is commonly used by researchers to determine State IV. This is because an accurate State IV is difficult to achieve since there may be contaminating ATPases in the crude mitochondrial preparation which convert newly formed ATP back to ADP, and thus prevent a true "ADP exhausted" state. As with State III and IV, this is the value of the slope of [O_2] vs. time, and is transformed by the XF software algorithm to OCR in units of pmol/min.

Often, researchers also make a distinction between respiration rates with respect to the O_2 consumption rate before any ADP is added (substrate only) and after the added ADP is exhausted (converted to ATP). The former is referred to as *State II respiration or Resting Respiration*, and sometimes used as a "pseudo" State IV measurement.

The ratio of the State III rate to the State IV rate is called the *Respiratory Control Rate* (RCR) and indicates the tightness of the coupling between respiration and phosphorylation. With isolated mitochondria the coupling is not perfect, probably as a result of mechanical damage during the isolation procedure.

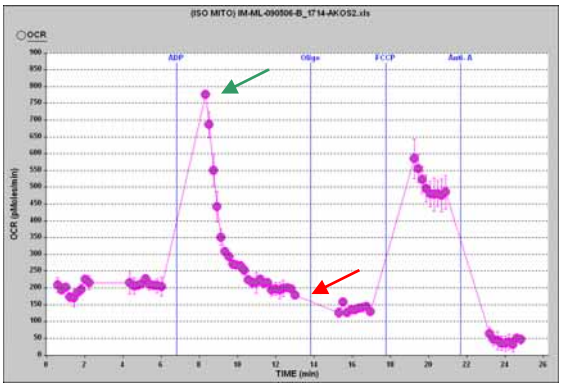
Note there are several methods to calculating an RCR value:

1) State III/State IV (using ADP exhaustion to determine the State IV rate)
2) State III/State II (State II is used as a "pseudo State IV" measurement)
3) State III/State IVo (State IV is induced by adding oligomycin to inhibit Complex V)

Please choose the method that is most suitable for your experiments and research goals. Examples of the 3 methods to calculate RCR values using XF data are illustrated below.

Method 1: State III/State IV (using ADP exhaustion to determine the State IV rate)

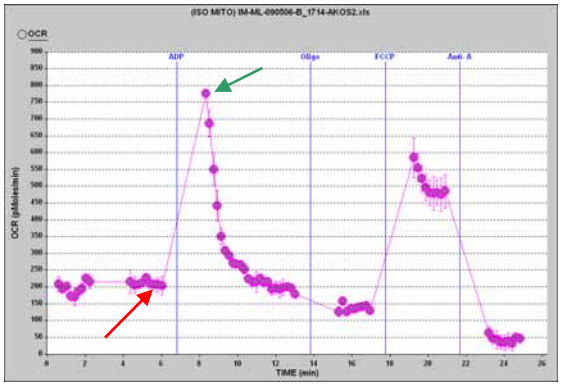
One can quickly estimate the RCR value from the graph by looking at the maximal and minimal rates obtained immediately after adding Succinate + ADP (green and red arrows, respectively). In this example, the State III rate is ~ 775 and the State IV rate is ~ 200, and thus the RCR is ~3.9.



Respiration states obtained with the XF

Method 2: State III/State II (State II is used as a "pseudo State IV" measurement)

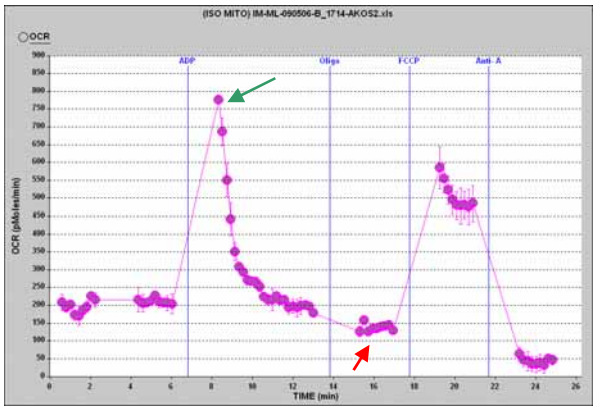
As above, one may estimate the RCR value from the graph by looking at the rate before succinate and ADP is added (State II, red arrow) and the maximal rate obtained immediately after adding Succinate + ADP (green arrow). In this example, the State III rate is ~ 775 and the State II rate is ~ 210, and thus the RCR is ~3.7.



Respiration states obtained with the XF

Method 3: State III/State IV (State IV is induced by adding oligomycin to inhibit Complex V)

Alternatively, one may estimate the RCR value from the graph by looking at the maximal rate obtained immediately after adding Succinate + ADP (green arrow), and the rate after oligomycin is added (State IVo, red arrow). In this example, the State III rate is ~ 775 and the State IVo rate is ~ 135, and thus the RCR is ~5.7.



Respiration states obtained with the XF

One may obtain exact rate values by viewing the group boxes in plate layout in the XF software if using the middle point mode (i.e. the rate is constant across the measurement period). Rate values from either the middle point or point to point graph modes may be obtained by right clicking on the graph in the XF software and choosing the "Save Graph Data…" function. This will generate a new tab in the excel worksheet which will display all the individual rate values (and associated error) of the data shown on the graph in a columnar format.

VI. Notes, Suggestions and Comments

The methods described above have been used successfully with isolated mouse liver mitochondria. Isolated mitochondria from other tissues and species can be used, however, the tissue, species (including age and sex), and method of isolation will contribute to the overall activity, degree of coupling and other variables associated with the isolated mitochondria.

Starting values, ranges, and optimization: it is recommended that the following parameters be explored and optimized depending on the overall goal(s) of the experiment and research topic.

- amount of mitochondria used (will be dependent on tissue type and purification method)
- the concentration of substrates and compounds injected
- Mix and Measure times
- MAS buffer type (1 or 3)

VII. References:

Please see Seahorse Bioscience's XF24 Training Course Workbook for a complete guide to operating and analyzing data using the Seahorse XF24 Flux Analyzer Instrument

For methods on isolating rat liver mitochondria and a introduction to State III and State IV respiration, please see: http://www.ruf.rice.edu/~bioslabs/studies/mitochondria/mitoprep.html

For a review of OXPHOS, Bioenergetics, and measuring respiration with isolated mitochondria, please see: http://www.bmb.leeds.ac.uk/illingworth/oxphos/

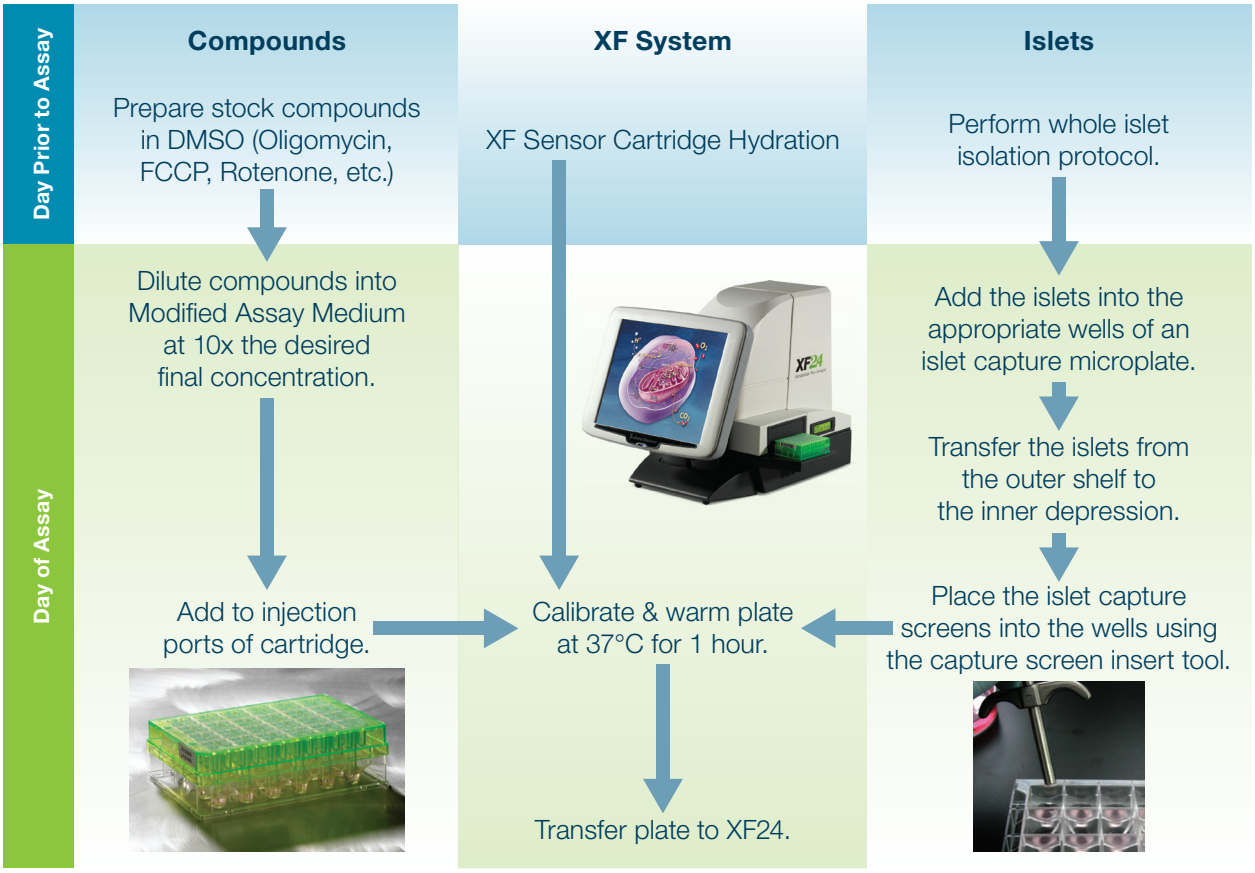
Please see the series of classical papers by B. Chance and G.R. Williams on the Respiratory Chain and Respiratory Enzymes in Oxidative Phosphorylation (1955-1956, various journals).

For an overall review of metabolism, including glycolysis, the TCA cycle, B-oxidation, the respiratory chain, oxidative phosphorylation, and much more, please see: "Metabolism at a Glance" 3rd Edition, by J.G. Salway, 2004, Blackwell Publishing, Ltd.

Guidelines for Assaying Islets with XF24 Analyzer & Islet Capture Microplate

Seahorse Bioscience, in collaboration with the Mitochondrial ARC (Advancing Research through Collaborations) at Boston University School of Medicine, has developed a protocol to assess whole islet bioenergetics in vitro with the XF24 Analyzer.

Islet Assay Flow Chart



Isolate Islets

1. Isolate islets from ~8 mice using your usual protocol the day before running an assay. [Approximately 1400 islets are required to load an XF24 Islet Capture Microplate.]
2. Culture islets overnight under standard conditions in a Petri dish.
3. Hydrate an XF24 sensor cartridge per the XF24 Protocol.

(continued on back side)

Seed Islets into XF24 Islet Plate

1. Aspirate islets from Petri dish and dispense into a 50 ml tube.
2. Wash 1X in Seahorse Assay Medium supplemented with 3mM glucose and 1% FBS. [islets turn sticky without serum.]
3. Remove supernatant and re-suspend in 2ml Seahorse XF Assay Medium.
4. While creating turbulence in the tube with a 20ul pipettor, take 20ul aliquots and place as a drop on 35mm culture dish – use 3 drops total to isolate ~3% of the islets for counting.
5. Count islets under a dissecting microscope to obtain an average number of islets per unit volume from which to estimate the total number of islets.
6. Determine the count of the islets, and adjust volume to ~70 islets per 100ul of media (700 islets/ml.)
7. Add 400ul of Seahorse XF Assay Medium to each well of the XF24 Islet plate.
8. Add 50ul of the islet suspension to each well, and repeat until well gets a total of 100ul of the islet suspension. Final volume should be 500ul per well.
9. When islets are seeded use a 20ul pipette to move all of the islets into the depressed chamber in the middle of the well. [This step can be tricky – use a dissecting microscope to be sure the islets are in the middle chamber.]
10. Prepare screens by pre-wetting them in XF Assay Medium in a small Petri dish to remove all air bubbles.
11. Carefully place the islet capture rings in the bottom of each well using the capture ring insert tool. Be sure the islets capture rings are stuck firmly at the bottom of the well. Confirm by gently pushing the screens down with a blunt pipette tip.
12. Caution: Be sure all wells contain an islet capture ring, even if ther are no cells in the well.

Run on the XF24 Analyzer

1. Place plate in 37°C Seahorse incubator with no CO_2.
2. The plate needs to stay in the incubator at least 1 hour to equilibrate tempurature and adjust islet metabolism to 3mM glucose.
3. While plate is incubating, prepare cartridge with desired injections.
4. After cartridge is filled with injections, load cartridge, start program and calibration.
5. When the XF24 calibration is complete, place the islet plate into the XF24 Analyzer.
6. After program is complete further you can normalize by counting the number of islets per well with the dissecting microscope. Some users have found that basal rates are sufficient for normalization.

The whole islet protocol is a modification of a standard XF24 protocol.

A detailed protocol is available at **www.seahorsebio.com/products/xf-islet-capture.php**
For Additional Technical Assistance, call:
+1-978-671-1600, Option #3 or
+1-800-671-0633 Option #3 (USA and Canada)