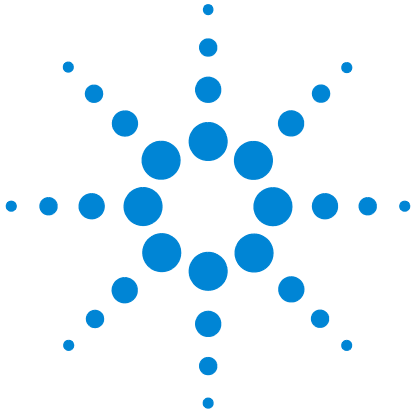

Agilent Seahorse BHI Report Generator

User Guide

Agilent Technologies

Notices

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Contents

1 Introduction

Alterations in cellular bioenergetic function are associated with the progression of many diseases, and scientists' understanding of this connection continues to increase at an impressive pace. The Agilent Seahorse XF Cell Mito Stress Test is a standard method for assessing mitochondrial bioenergetic function. The Bioenergetic Health Index (BHI) is a new concept that builds upon this standard, and distills multiple metrics of mitochondrial function into a single value. Now researchers can easily determine an overall value for cellular bioenergetic health. The information in this User Guide describes how BHI is calculated, and how to use the BHI Report Generator to perform this calculation from Seahorse XF Cell Mito Stress Test data.

Figure 1 Workflow diagram

Assay Parameter Calculations

This section describes how the assay parameters are calculated for the Seahorse XF Cell Mito Stress Test. Values are calculated as absolute oxygen consumption rate (OCR) in pmol O_2/min, or absolute extracellular acidification rates (ECAR) in mph/min, and use the AVERAGE value of the selected wells within a group to calculate a specific parameter (that is, the Seahorse XF Stress Test Report Generators do not use Area-Under-the Curve (AUC) calculations). Contact Agilent Seahorse Technical Support with any questions.

Agilent Seahorse XF Cell Mito Stress Test parameters

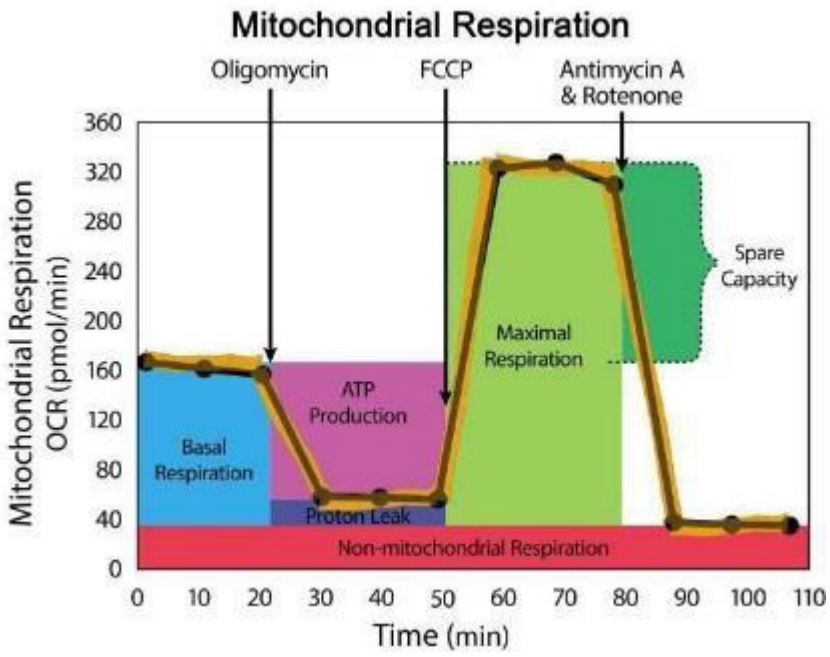


Figure 2 Agilent Seahorse XF Cell Mito Stress Test Parameters

Table 1 Agilent Seahorse XF Cell Mito Stress Test Parameters

Parameter	Rate measurement equation used by Agilent Seahorse XF Stress Test Report Generator
Non-Mitochondrial respiration	Minimum rate measurement after Rotenone/antimycin-A injection
Basal respiration	(Last rate measurement before first injection) – (Non-Mitochondrial respiration rate)
Maximal respiration	(Maximum rate measurement after FCCP injection) – (Non-Mitochondrial respiration)
H+ (Proton) leak	(Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial respiration)
ATP Production	(Last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection)
Spare respiratory capacity	(Maximal respiration) – (Basal respiration)
Spare respiratory capacity %	(Maximal respiration) / (Basal respiration) ×100
Acute response	(Last measurement rate before Oligomycin injection) – (Last rate measurement before acute injection)
Coupling efficiency	(ATP Production rate) / (Basal respiration rate) ×100

BHI Background Information

The Bioenergetic Health Index was developed by Dr. Victor Darley-Usmar at the University of Alabama at Birmingham. Figure 3 displays the equation on how to calculate BHI values from Seahorse XF Cell Mito Stress Test assay results. For more information, see Dr. Darley-Usmar's publication in *Clinical Science* (2014) 127, 367â€“373 doi: 10.1042/CS20140101

$$BHI = \frac{(\text{Spare Respiratory Capacity})^a \times (\text{ATP Production})^b}{(\text{Non-Mitochondrial Respiration})^c \times (\text{Proton Leak})^d}$$

The exponents in this calculation are set to a value of 1 in this report generator.

Figure 3 BHI Equation

2 How To

Configure Excel to Enable Macros

The BHI Report Generator requires Microsoft Excel 2007, 2010, or 2013 for use.

Note: Excel must be configured to allow macros to run. On the yellow information bar, select **Enable Editing** if prompted to do so when opening the Report Generator file for the first time.

To Enable macros:

1. Launch Excel
2. Select **File> Options> Trust Center> Trust Center Settings> Macro Settings> Enable all macros** or **Disable all macros with notification** or **Disable all macros except digitally signed macros**
3. Select **Message Bar Settings> Show the message bar in all applications when active content, such as ActiveX controls and macros, has been blocked**

NOTE

The BHI Report Generator requires the "**Measure after Injection**" box to remain checked in the assay design file (which is the default setting) to yield correct parameter calculations.

Save Wave Assay Result File in Excel format (*.xlsx)

1 Open the Seahorse Cell Mito Stress Test Assay results file (*.asyr) in Wave Desktop.

NOTE For Seahorse XF24 and XF96 users, convert *.xfd files to *.asyr before using the XF Cell Energy Phenotype Test Report Generator.

To convert files:

a Open **Wave**

b Browse, and select the **.xfd result file**.

c Click **Open**.

d Click **Save as...** and select **Assay Analyze** (*.asyr) as the file type.

e Rename (if necessary), and select a location to save the file.

f Save the .xfd file as an *.asyr, and proceed to Step 2.

2 Click **Save As...** and select **Excel 2007/2010 (*.xlsx)** as the file type.

3 Follow the prompts to select a name and location for the Excel output file.

Load Excel File into BHI Report Generator

1 Double-click on the desktop icon to open the BHI Report Generator.

2 Click **Load New Data File** on the **Summary Report** to import XF assay data into the Report Generator (see Figure 4).

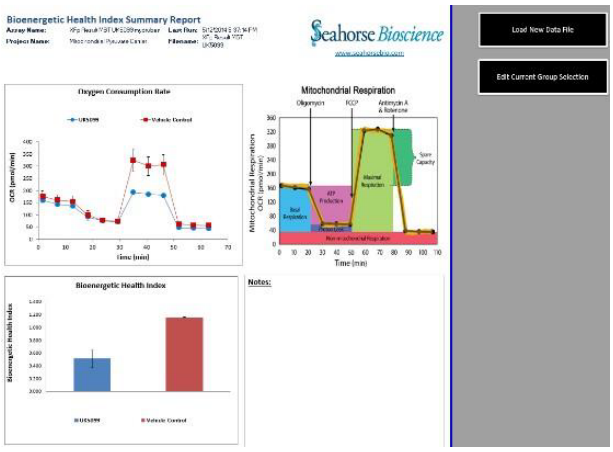


Figure 4 Summary Report screen

3 Browse and select the Excel file (from Section II), and click **Open**.

4 In the **Display Options** window, select one or two groups to display in the report as well as the error bar type (see Figure 5).

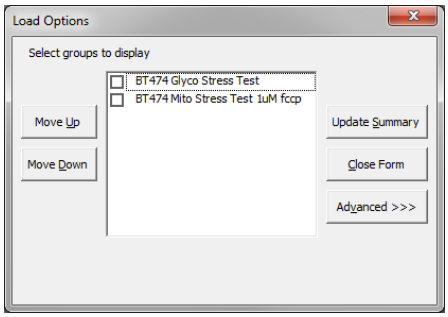


Figure 5 Update Summary

5 Click **Update Summary**, and when the **Success!** Window appears, click **OK.**

Optional normalization steps:

1 Select normalization data to apply to Report Generator data.

   a From **Wave Desktop** - Open an Assay Result file (.asyr) and from the **Overview** page, select **Modify> Normalization**.

   b From **Microsoft Excel** – Open an Excel file, select data to input, right-click, and select **Copy**.

2 Click **Select All**.

3 In the **Report Generator**, select the **Normalization** tab at the bottom of the program.

4 Right-click, and select **Paste**.

   - Normalization data may also be manually entered into the Normalization Table. Input the Normalization Unit prior to clicking **Normalize Data**.

5 Click **Normalize Data**.

6 Select the groups to apply the normalization data, and view in the report generator.

7 Click **Update Summary**.

8 Click **OK**.

NOTE Background wells must have a "1" entered into the Normalization Table. Values of "0" are not supported.

Customize and Review the BHI Report

The report includes five tabs: Summary Printout, Bar Charts, Normalize, Measures Sheet, and "How to..."

- **Summary Printout**: One-page graphical summary of the OCR kinetic graph, BHI Bar Charts and Notes section.
- **Bar Charts**: Individual Seahorse Cell Mito Stress Test parameter calculations data represented as bar charts.
- **Normalize**: Input Normalization Data and a Normalization Unit to normalize XF assay data to cellular or mitochondrial parameters.
- **Measures Sheet**: Displays the kinetic graphs of both OCR and ECAR, the raw values for each bar chart on the Bar Charts page, and a list of all excluded wells from the Report Generator.

NOTE For any individual well, a negative value for any individual parameter (for example, ATP linked, Spare Respiratory Capacity, or Non-mitochondrial Respiration) results in exclusion of the well.

- **How to...**: Instructions on how to use the BHI Report Generator.

Customize the charts if desired, then click **Save As**. Rename and select a location to save the file. Refer to "Assay Parameter Calculations" on page 5 for Assay Parameter Calculation discussion.

NOTE Saving the Report Generator as any file other than the default (*.xlsm) will render the Excel Macro inoperable, thus data cannot be imported again. If this occurs, simply locate the original Report generator file or download again from the Seahorse website.

3 Advanced Options

The BHI Report Generator uses the data in the Excel Wave Output file to automatically populate the Advanced Options fields. If a fourth injection is detected, the Report Generator will display a **Load Options** window with a **Supplemental Injection** selection. If there are four injections in the assay protocol, the BHI Report Generator will assume the 4th injection is an Acute injection, but have the option to select **Post** when selecting groups in the Load Options box. (see Figure 6).

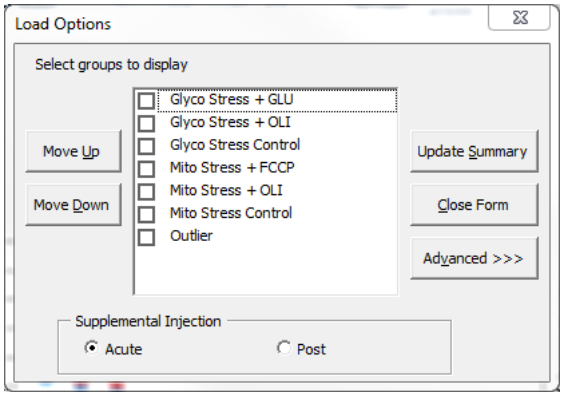


Figure 6 Acute and Post Injection Options

Acute Injection

An injection that occurs in the assay protocol following the basal measurement, but before oligomycin in the Seahorse XF Cell Mito Stress Test and must be injected in Port A.

Note: Acute injections of test compounds in B, C, or D will result in inaccurate XF stress test parameter calculations.

Post Injection

An injection that occurs in the assay protocol following the completion of the Seahorse XF Cell Mito Stress Test. Must be injected in Port D.

Note: Rates from the Post Injection measurements are not displayed in the BHI Report Generator. These measurements are not related to any parameter as described in Table 1 on page 6 for the Seahorse XF Cell Mito Stress Test, and are not part of the BHI calculation.

Number of Basal and Injection Measurements

The number of measurements before and after each injection is automatically assigned in the report generator based on the Instrument Protocol set up in the assay design. Users may re-assign the number of measurements in the **Advanced** section of **Load Options** as necessary (see Figure 7).

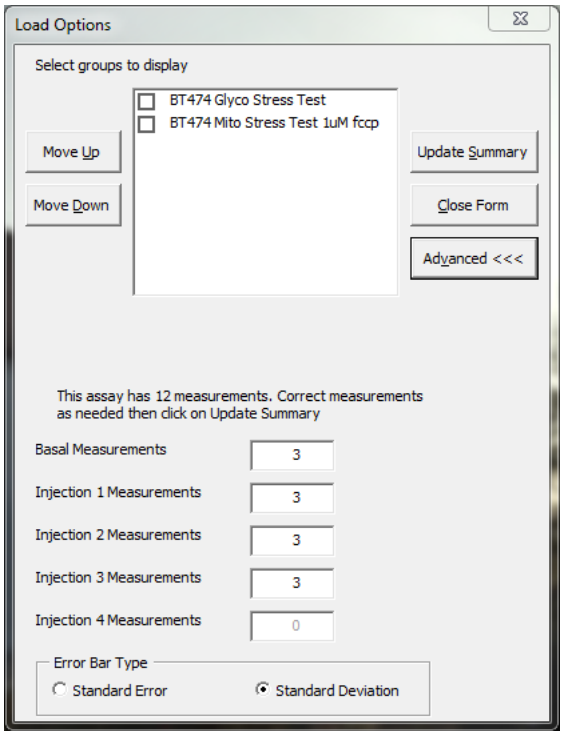


Figure 7 Measurement Adjustment

Error Bar Information:

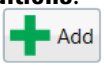
- Standard Deviation is selected by default. Users may also choose Standard Error of the Mean.
- Standard Deviation is calculated using the Microsoft Excel function.
- Standard Error of the Mean is calculated using the equation:

$$\frac{(Standard\ Deviation\ of\ Group)}{\sqrt{(Number\ of\ Wells\ in\ Group)}}$$

- Error Bars for the Seahorse XF Cell Mito Stress Test Parameters shown on the Bar Chart tab are calculated from each replicate of the rate measurement used to determine the stress test parameter (See Table 1 on page 6).
- BHI Error Bars are calculated from each replicate of the rate measurement.
- Each individual well per group will have a BHI value calculated which has no error. The error is then calculated based on the standard deviation of the BHI per well within the group.

Outlier Wells

Outliers or unwanted wells may be excluded from the BHI Report Generator parameter calculations by reassigning them from their original group in Wave before saving the file to Excel.

1. From the **Wave Overview Page**, click **Modify** in the upper right corner, then select **Groups/Conditions**.
2. In the **Groups/Conditions** view, click 

to add a new group and name it **Outliers**. Switch to the Plate Map view, and select the wells to be included in the Outliers group
   a. Click **Apply**. Select **Save As**, and set the file type to **Excel 2007/2010 (*.xlsx)**.
   b. Load the Wave Excel output file into the XF Stress Test Report Generator.

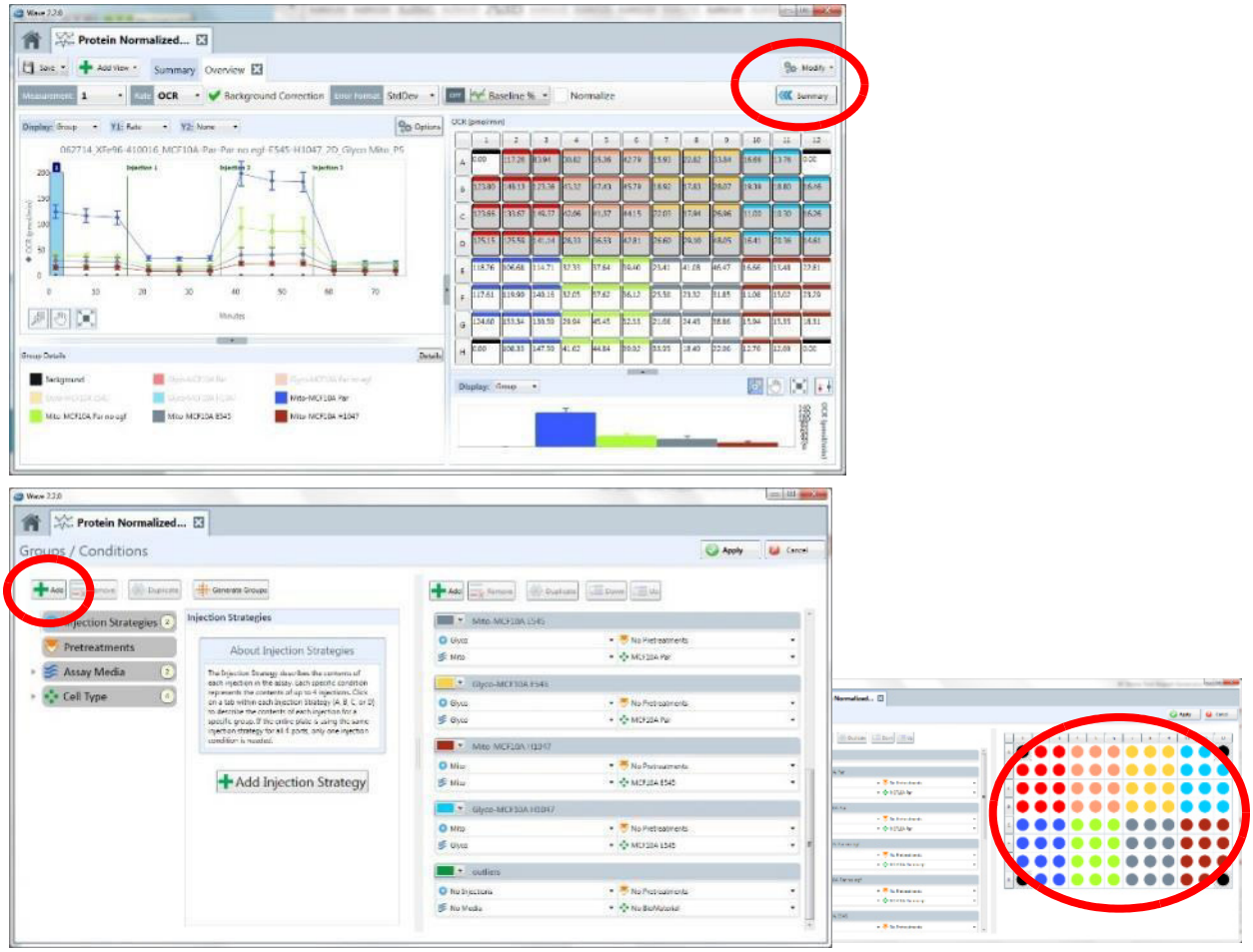


Figure 8 Outlier wells

3 The Wave Excel output file will list the newly created Outlier group in the Load Options window.

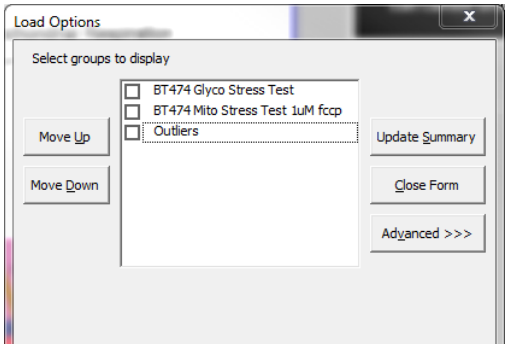


Figure 9 Outlier Group

NOTE Ensure the Outliers group is unchecked in the Load Options window so the outliers are omitted from the calculations.

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