Conducting an XF Assay in a Hypoxia Chamber (≥3 % O_2)

Technical Overview

Introduction

The oxygen consumption and extracellular acidification rates (OCR and ECAR) reported by Agilent Seahorse XF Analyzers are calculated using a multiparameter algorithm[1]. This algorithm accounts for oxygen diffusion in the transient microchamber under ambient environmental conditions. To calculate the rates correctly under low oxygen conditions, the parameters of the algorithm must be reset. The XF Analyzer requires the use of a secondary program, the XF Hypoxia Rate Calculator Program, to reset these parameters.

XF assays may be run in hypoxia chambers set as low as 3 % oxygen. Running an XF assay under low oxygen conditions also requires adapting standard XF assay procedures and workflows. Additionally, special equipment and reagents are needed, along with preplanning, to conduct these assays successfully. This Technical Overview describes procedures that are supplemental to, or different from standard XF assay procedures, and requires familiarity with both XF assay workflows and working within controlled atmosphere environments.

Agilent Technologies

Materials and Methods

Hypoxia chamber

- Calibrated and capable of maintaining a stable oxygen set-point
- Temperature control (recommended, not required)
- Large enough to accommodate an Agilent Seahorse XF Analyzer (Example: Coy Hypoxic Chambers for Seahorse XF Analyzers)
- The chamber must have sufficient ports (to connect the XF Analyzer to the Controller, and connect the XF Analyzer to its external power source)

Sodium sulfite, Na_2SO_3 (Sigma Catalog #S0505)

This chemical oxygen scavenger will be injected into wells containing XF Calibrant during the assay to provide a "zero" oxygen reference parameter for the software algorithm.

Cell culture incubator

Set at the target oxygen set-point for the assay (recommended, not required)

XF Hypoxia Rate Calculator Software

(Available from Agilent Seahorse Bioscience)

Installing the Agilent Seahorse XF Analyzer in a Hypoxia Chamber

These are general guidelines; specific instructions may vary depending on the type of chamber. Work with the chamber manufacturer and Agilent Technical Support when planning to install the Seahorse XF Analyzer.

1. Position the Seahorse XF Analyzer to allow sufficient workspace inside to set up the XF Assay Plate, and maintain the desired atmosphere settings (that is, avoid blocking inlet lines and inhibiting airflow).
2. Position the Seahorse XF Analyzer Controller outside the chamber.
3. If the chamber is equipped with CO_2 control, set the CO_2 level to 0 % (to ensure that CO_2 will not interfere with ECAR measurements).
4. If the chamber is equipped with temperature control, set the temperature to 28 °C to maintain 37 °C temperature during the XF assay. Agilent recommends setting the chamber temperature 9 °C lower than the intended assay temperature.
5. Once the instrument is installed in the chamber, test the operation of the system.

   Bring the hypoxia chamber to the target assay set-point, and turn the Seahorse XF Analyzer on. Leave them running together for at least 2 hours to ensure that oxygen and temperature levels are stable.

Planning an XF Hypoxia Assay

Optimization

1. Agilent recommends optimization of cell-seeding density parameters under hypoxia conditions, even if this cell type has previously been optimized under ambient environmental conditions.
   XF Hypoxia assays may require fewer cells per well to prevent anoxia during the measurement period.
2. XF Stress Test reagents may also require optimization. Refer to the Agilent Seahorse XF Cell Mito Stress Test and XF Glycolysis Stress Test kit manuals for detailed instructions to design optimization assays.
3. Mix-wait-measure times may need to be optimized based on the cell density. Agilent recommendations are outlined in *Running the XF Hypoxia Assay* (Figure 1)

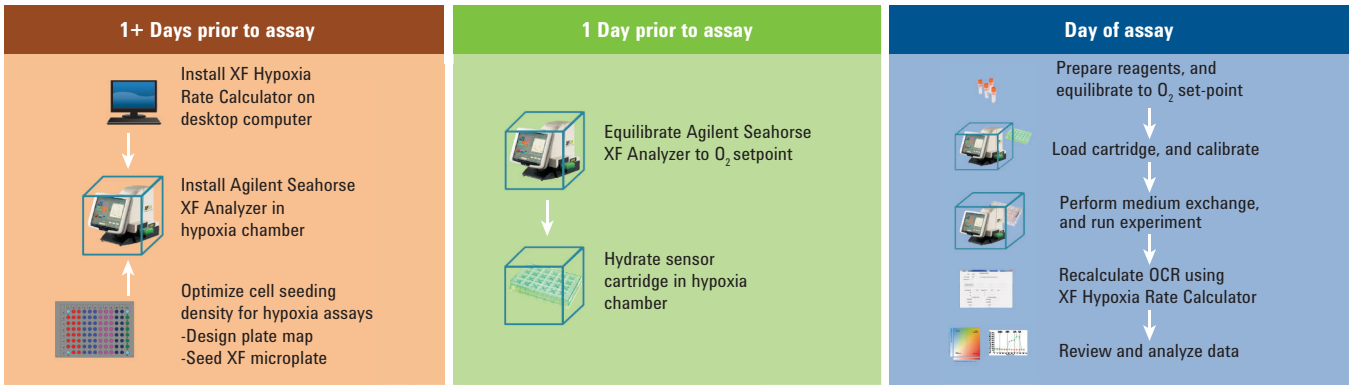


Figure 1. Hypoxia assay workflow.

Oxygen set-point and equilibration

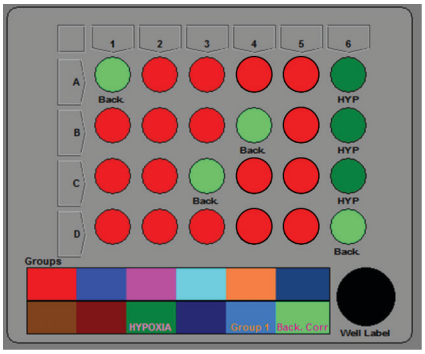
1. Set the oxygen level of the hypoxia chamber to the assay set-point (minimum 3 %).
2. Allow the Seahorse XF Analyzer to equilibrate to the oxygen set-point overnight (minimum 6 hours).
3. Equilibrate the Seahorse XF Analyzer and reagents to the oxygen set-point. Equilibration occurs within the chamber according to Table 1.

Plan plate map

- Reserve the last column of wells (minus background wells) for the hypoxia (sodium sulfite injection) group (0 % O_2 reference). See Figure 2 and Figure 3.
- Do not seed cells in background correction wells or hypoxia group wells.
- Hypoxia wells contain XF Calibrant (not XF Assay Medium).
- If growing cells at a different oxygen level than the assay set-point, move the cells to an incubator set at the assay oxygen level overnight before the assay. Alternatively, place cells in the hypoxia chamber with the XF Analyzer to equilibrate on the day of the assay (minimum 6 hours).

Table 1. O_2 level equlibration guidelines.

Component	Procedure
Agilent Seahorse XF Analyzer	Equilibrate overnight (minimum 6 hours)
XF Flux Assay Kit (Sensor Cartridge and Utility Plate)	Hydrate overnight in hypoxia chamber
Cells	Equilibrate overnight in cell culture incubator set at assay oxygen level (Alternative: place in Agilent Seahorse XF Analyzer inside the hypoxia chamber for minimum 6 hours)
XF Calibrant	Equilibrate overnight (minimum 6 hours)
XF Assay Medium	Place in the hypoxia chamber for 1 hour prior to the medium exchange
Injection compounds (including sodium sulfite)	Prepare stock concentrations Place volume required for the assay into a reservoir in the hypoxia chamber for 1 hour prior to loading the cartridge



Experimental group wells

Background correction group wells (no cells)

Hypoxia group wells (no cells; XF Calibrant)

Figure 2. 24-Well plate map.

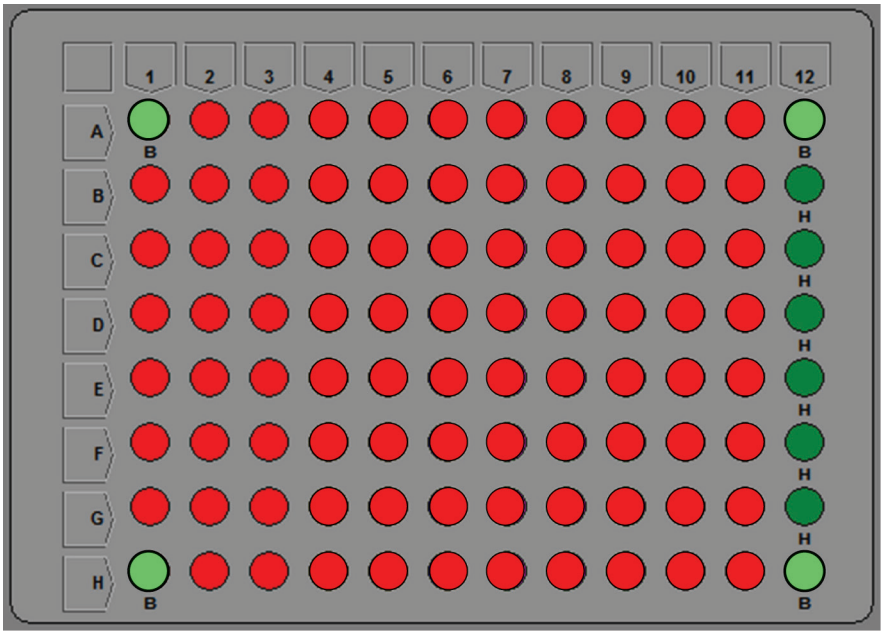


Figure 3. 96-Well plate map.

Plan injection strategy

1. Experimental groups and background wells receive user-defined injections according to the XF assay design. See Table 3 for an example.

2. Hypoxia group wells receive 100 mM sodium sulfite injections in ports that correspond to the assay design injections.

Table 3. Example injection strategy.

Experimental groups and background wells	Hypoxia group
Port A: 25 µL Glucose	Port A: 25 µL 1M sodium sulfite stock solution
Port B: 25 µL Oligomycin	Port B: 25 µL 1M sodium sulfite stock solution
Port C: 25 µL 2-DG	Port C: 25 µL 1M sodium sulfite stock solution

Running an XF hypoxia assay

Working outside the hypoxia chamber

1. Create an XF assay template in the XF software using the Assay Wizard (See Table 4).

Table 4. Recommended Mix-Wait-Measure times for assays run at ≥3 % oxygen.

Agilent Seahorse XF24	
Mix	5 minutes
Wait	1 minutes
Measure	2 minutes
Agilent Seahorse XF96	
Mix	5 minutes
Wait	0 minutes
Measure	2 minutes

2. Warm XF Assay Medium to 37 °C and pH to 7.4.

3. Prepare stock solutions of compounds for injections using warmed XF Assay Medium.

4. Prepare 1 M stock solution of sodium sulfite.

   a. Prepare fresh each day; begin assay within two hours of preparation.

   b. Add 1.26 g sodium sulfite to 10 mL XF Calibrant (in a 15 mL conical tube, 20 mL glass bottle, and so forth). with a tight-fitting lid.

   c. Vortex or shake vigorously to dissolve the bulk of the sodium sulfite.

   d. Invert solution intermittently over the next 5–10 minutes to ensure that all sodium sulfite has gone into solution and is well-mixed.

Working inside the hypoxia chamber

1. Transfer XF Assay Medium to the chamber, and equilibrate 25 mL in a 10-cm petri dish for at least 1 hour prior to media change.

2. Transfer stock solutions required for injections to reservoirs, and allow them to equilibrate for 1 hour prior to loading cartridge.

3. Transfer cells from the separate low oxygen incubator to the hypoxia chamber in an airtight hypoxia box or bag.

4. Perform the medium exchange using equilibrated XF Assay Medium and XF Calibrant (**Reminder:** hypoxia group receives XF Calibrant).

   - Chambers without CO_2 control, or with CO_2 control set at 0 %: cells may degas in chamber (minimum one hour).

5. Transfer sodium sulfite stock solution to the hypoxia chamber.

6. Load compound injection ports.

   a. **Hypoxia group:** 1 M sodium sulfite stock in all compound injection ports

   b. **All other groups (including background):** Desired assay compounds

7. Start the XF assay, and allow it to run to completion.

8. Save the XF data file, and transfer it to a desktop or laptop computer to review and analyze the XF data.

Recalculating the OCR using the XF Hypoxia Rate Calculator

1. Open the XF Hypoxia Rate Calculator program.
2. Click **Read .xls File** (1, Figure 4).

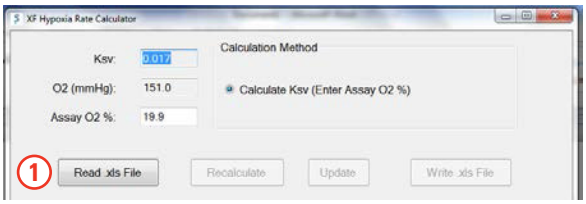


Figure 4. **Read .xls File** command.

3. Browse to the XF data file and open it. The Calculator will display a warning (Figure 5).

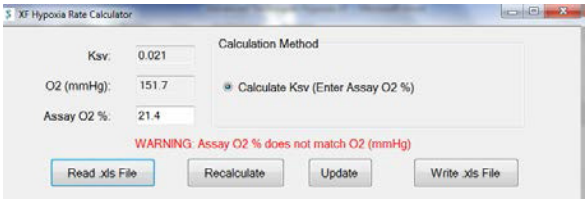


Figure 5. Warning message.

4. Adjust the **First Rate** and **Last Rate** fields (2, Figure 6): if the sodium sulfite injection was after measurement 3, then the First Rate under **Assay O_2 %** should be 1, and the Last Rate should be 3. The First Rate for the sodium sulfite column will be rate 4, and the Last Rate will be the final rate measurement of the assay.

   If one of the wells in the sodium sulfite group did not respond correctly to the injections, uncheck the box associated with that rate to omit it from the calculation.

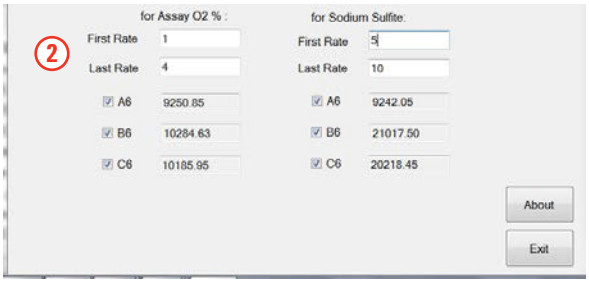


Figure 6. Identifying rate measurements and wells that did not respond to sodium sulfite.

5. Enter the assay oxygen percentage in the **Assay O_2 %** field (3, Figure 7).

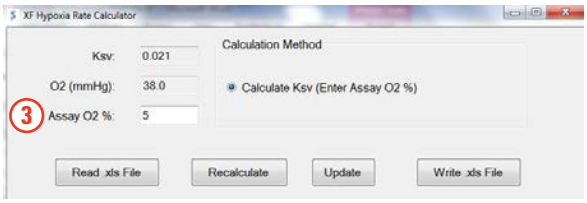


Figure 7. Indicate assay oxygen set-point.

6. Click **Recalculate** (4, Figure 8).
7. Click **Update** (5, Figure 8).
8. Click **Write .xls File** (6, Figure 8).

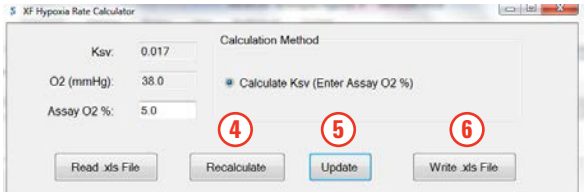


Figure 8. **Recalculate**, **Update**, and **Write .xls File** commands.

9. A new file will be created in the same folder as the original file.
10. The new file will have the same name as the original file, but the word *hypoxia* will be appended at the end (Figure 9).

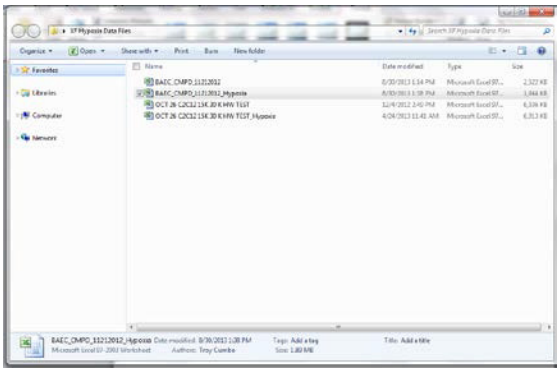


Figure 9. New file with recalculated rate data.

11. Open the new *hypoxia* file to examine the data.

Reviewing and analyzing the XF data

Validate sodium sulfite performance

1. Review OCR data for the hypoxia group.

   a. OCR after injection must be higher than before injection (Figure 10).

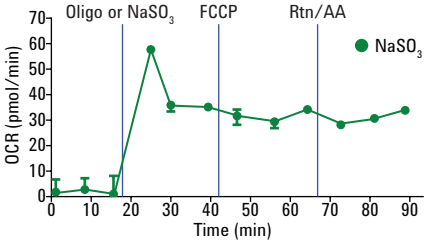


Figure 10. Typical profile of OCR before and after sodium sulfite injection.

   b. If one of the wells in the group did not respond, make a note: you will be able to omit it from the calculation later.

   (**Note:** If more than one well in the hypoxia group did not respond, repeat the assay).

Examining temperature stability and oxygen level data

1. Review the oxygen level data.

   a. Hypoxia group wells should be zero (±5 mm Hg) (Figure 11).

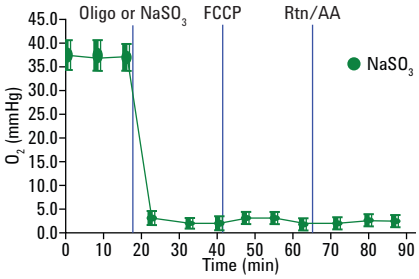


Figure 11. Hypoxia group O_2 levels after recalculation are 0 ±5 mm Hg.

   b. Check experimental group wells for a linear reduction in oxygen (Figure 12).

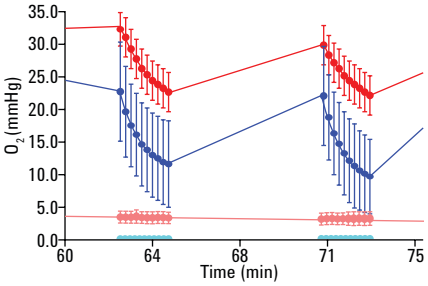


Figure 12. Blue trace (50k cells/well) shows nonlinear reduction in O_2 levels during measurement period, red trace is linear (25k cells/well).

   c. Check that experimental group wells recover to oxygen baselines after a measurement.

Although the trace associated with the experimental group wells may not overlap with the trace for the hypoxia group wells, if the experimental group wells demonstrate a failure to recover to baseline oxygen between measurements, or a nonlinear decrease in the oxygen level, these these wells may be developing anoxic conditions in the microchamber. Consider seeding fewer cells.

2. Review the onboard temperature of the XF Analyzer during the assay.

   - If the temperature of the instrument increases by more than 1 °C during the assay, contact Agilent Technical Support.

   Support representatives will be able to describe methods that manage temperature stability during a hypoxia assay

Examine ECAR data

ECAR results will not be affected by the calculation changes required to report OCR under reduced oxygen levels

Review your experimental data

Data analysis, including application of normalization parameters, should be conducted after the rates have been recalculated using the XF Hypoxia Rate Calculator.

Reference

1. Gerencser, A. A.; *et al.* Quantitative Microplate-Based Respirometry with Correction for Oxygen Diffusion. *Anal. Chem.* **2009**, *81*, 6868–6878.

www.agilent.com/chem

Published in the USA, December 1, 2016
5991-7267EN

Agilent Technologies