Conducting an XFe Assay in an Hypoxia Chamber at ≥3% O_2*

Technical Overview

Introduction

Agilent Seahorse XFe Analyzers calculate the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using a multiparameter algorithm[1] that accounts for oxygen diffusion in the transient microchamber assuming ambient environmental conditions. To calculate the rates correctly under low oxygen conditions, the parameters of the algorithm must be reset. Wave software (Version 2.0 and above) includes an integrated workflow and assay template specific for hypoxia.

Running an XFe assay under low oxygen conditions requires adapting standard assay procedures and workflows. Additionally, special equipment, reagents, and preplanning are needed. This Technical Overview describes procedures that are supplemental to, or different from, standard XF assay procedures, and requires familiarity with both XFe assay workflows and working within a hypoxia chamber.

This protocol is for XFe assays run in an hypoxia chamber set as low as 3 % oxygen. To run an assay below 3 % oxygen, please contact Agilent Technical Support to discuss additional steps required.

* Agilent recommends performing hypoxic assays at >3 % O_2 due to the potential O_2 exhaustion from the microhamber. Should assays at a lower O_2 % be warranted, the point-to-point OCR data and O_2 level data should be carefully inspected to ensure there is a linear reduction, and that the OCRs remain constant. Please contact Agilent Technical Support when planning hypoxia assays below 3 % O_2.

Agilent Technologies

Materials and Methods

Hypoxia chamber

- Calibrated and capable of maintaining a stable oxygen set-point
- Large enough to accommodate an XFe Analyzer (Dimensions: ≥28″ D × 42″ L × 26″ H; Examples: Coy Hypoxic Chambers for Seahorse XFe Analyzers; Ruskinn SCItive)
- Ports to connect the XFe Analyzer to the Controller. Required to connect the Agilent Seahorse XFe Analyzer to its external power source, and an access door or removable panels to keep the instrument upright during installation and removal.
- Temperature control (recommended, not required)
- Sodium sulfite, Na_2SO_3 (Sigma catalog #S0505)
- Hypoxia Mode for Wave (Agilent Seahorse Bioscience)
- Wave software (Version 2.0 or later) must be installed prior to installing Hypoxia Mode for Wave
- Download and install the Hypoxia Mode for wave on your instrument and desktop PC from the Agilent Seahorse website
- Plate warmer (recommended, not required) (for example, TAP Biosystems Plate Heater)

Installing the Agilent Seahorse XFe Analyzer in an Hypoxia Chamber

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

1. Position the Seahorse XFe Analyzer (not the controller) inside the chamber to allow sufficient workspace, and maintain the desired atmospheric settings. Avoid blocking inlet lines and inhibiting airflow.
2. Position the XFe 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 chamber temperature to 28 °C. **Note:** The Seahorse XFe Analyzer must be set at 37 °C for the assay. Agilent recommends setting the chamber temperature at least 9 °C lower than the intended assay temperature.
5. Once the instrument is installed in the chamber, test the operation of the system.
6. Bring the oxygen level in the hypoxia chamber to the target assay set-point (for example, 3 % O_2), and turn on the Seahorse XFe Analyzer.
7. Confirm the oxygen and temperature stability by running the chamber and instrument together for at least 2 hours.

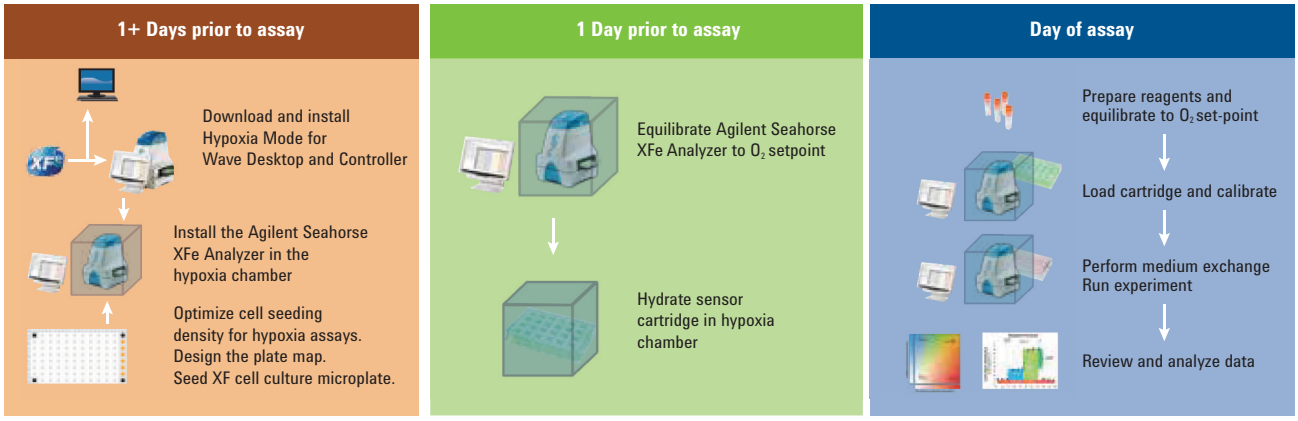

Figure 1. Hypoxia assay workflow.

Planning an XF Hypoxia Assay

Optimization

1. Optimize cell-seeding density parameters under hypoxia conditions. **Note:** An XF Hypoxia assay may require fewer cells per well to prevent anoxia during the measurement period. Density optimization under ambient environmental conditions may not apply.
2. Optimize XF Stress Test reagents. Refer to the Agilent Seahorse XF Cell Mito Stress Test and XF Glycolysis Stress Test Kit User Guides for detailed instructions on optimization assays.
3. Optimize mix-wait-measure times based on the chosen cell density.

Hypoxia chamber oxygen set-point and equilibration

1. Set the oxygen level of the hypoxia chamber to the assay set-point.
2. Allow the Seahorse XFe Analyzer to equilibrate to the oxygen set-point overnight (minimum 6 hours).
3. Equilibrate consumables and reagents to the oxygen set-point.

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) and keep overnight prior to the assay. Alternatively, place cells in the hypoxia chamber inside the Seahorse XFe Analyzer to equilibrate on the day of the assay (minimum 6 hours).

Table 1 summarizes the recommended equilibration times for all assay components.

Plate map

Hypoxia group wells

Note: An XF Hypoxia Assay requires the use of sodium sulfite (a chemical oxygen scavenger) to provide a "zero" oxygen reference. It is injected during the assay into wells containing XF Calibrant (Hypoxia Group wells).

1. The last column of wells (not being used as background wells) is always reserved as the Hypoxia group wells 0 % O_2 reference; see Figures 2 and 3).
2. Plan for hypoxia group wells to receive a final concentration of 100 mM sodium sulfite during the injections. **Note:** Do not seed cells in the hypoxia group wells.

IMPORTANT! Do not use sodium dithionite (also known as sodium hydrosulfite) in place of sodium sulfite. Sodium dithionite will release acidic hydrogen sulfide gas, which will acidify the assay media, and could damage the instrument.

Table 1. O_2 level equilibration guidelines.

Component	Procedure
Agilent Seahorse XF^e Analyzer	Equilibrate overnight (minimum 6 hours).
XF Sensor Cartridge and Utility Plate	Hydrate overnight in hypoxia chamber (Optional: hydrate in the TAP Biosystems Plate Heater set at 30 °C).
Cells	Equilibrate overnight in cell culture incubator set at assay oxygen level (alternatively, place in the Agilent Seahorse XFe Analyzer inside the hypoxia chamber for minimum 6 hours).
XF Calibrant	Equilibrate overnight (minimum 6 hours).
XF Assay or Base medium	Place in the hypoxia chamber for 1 hour prior to the medium exchange. Following medium exchange, the cell culture microplate can be incubated in either a plate heater (for example, TAP Biosystems Plate Heater) set at 37 °C, or in the XFe Analyzer while running the mix and wait cycles for the hour (that is, 4 minutes mix, 4 minutes wait).
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

Plan experimental group wells (blue wells) in Figures 2 and 3.

- Experimental group
- Background correction group wells (no cells)
- Hypoxia group wells (no cells; XF Calibrant only)

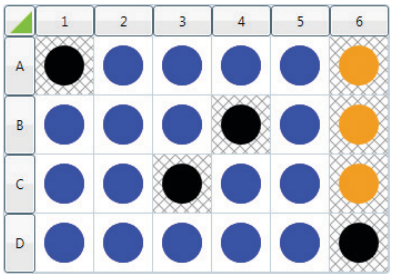

Figure 2. 24-well plate map.

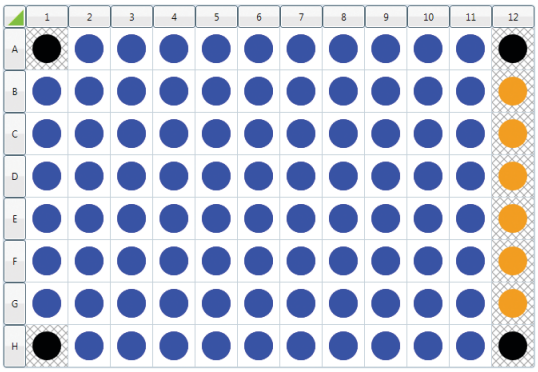

Figure 3. 96-well plate map.

Running an XF Hypoxia Assay

Steps done outside the hypoxia chamber

1. Create a new assay template in Wave Desktop Hypoxia Mode.

    - First use the drop-down to select Hypoxia Mode (1). Select the **Default** assay template (2), then click **Open** (3) (Figure 4).

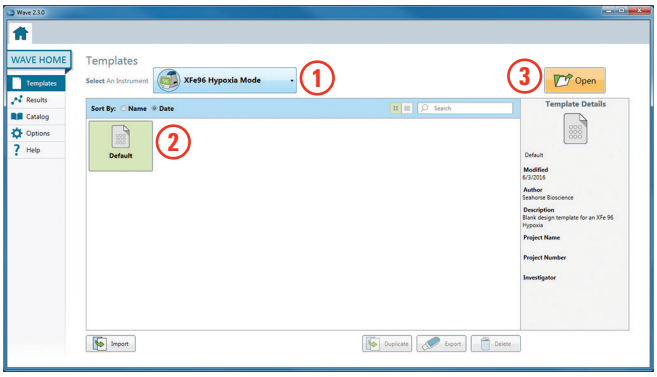

Figure 4. Assay template view in Wave Desktop Hypoxia Mode.

2. Design your XF assay

    a. After defining each group or condition that will be measured during the assay, add each group to the plate map.

    - **Plate Map tab:** Assay wells for Background group and Hypoxia group are automatically assigned to the plate map. The Hypoxia group and assay wells are locked from editing Hypoxia group is locked and prepopulated on the Plate Map (Figure 5).

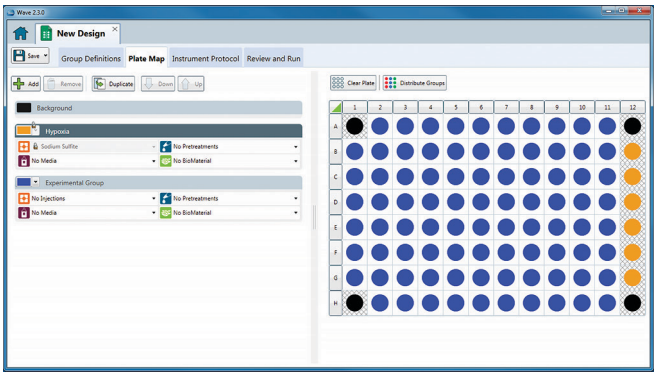

Figure 5. Plate Map tab in Wave Desktop Hypoxia Mode.

    b. Add the Instrument Protocol

    - **Instrument Protocol tab:** Use the **Measure** and **Injection** buttons to customize the Instrument Protocol for the assay. The Mix, Wait, and Measure times and cycles in Hypoxia Mode are pre-populated and differ from the standard XF assay Instrument Protocol.

    c. **Review and Run tab:** In Advanced Settings, define the O_2 % for the XF assay (Figure 6).

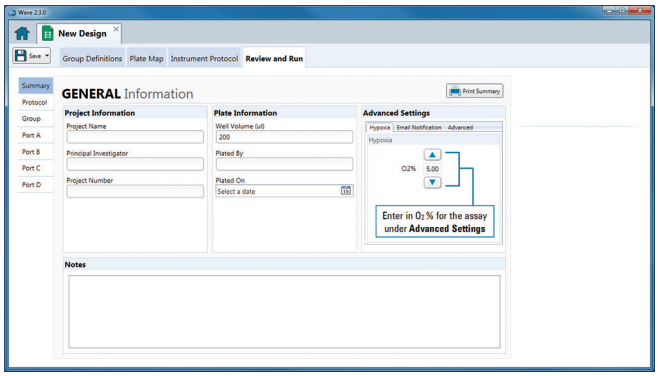

Figure 6. Advanced Settings on the Review and Run tab.

3. Warm assay medium to 37 °C, and adjust pH to 7.4.

4. Prepare injection compound stock solutions using warmed assay medium.

5. Prepare 1 M stock solution of sodium sulfite.

   a. Prepare fresh each day; begin the XF assay within 2 hours of preparation.

   b. Dissolve 0.189 g of sodium sulfite in 1.5 mL of XF Calibrant (189 mg/1.5 mL).

   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 all sodium sulfite has dissolved into solution and is well-mixed.

   e. Equilibrate for 1 hour in the hypoxia chamber.

Steps done inside the hypoxia chamber

1. Transfer assay medium to chamber, and equilibrate 25 mL in a single 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 the cartridge.

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

4. Perform the medium exchange using equilibrated XF Assay or Base Medium and XF Calibrant (Reminder: Hypoxia group wells receive only XF Calibrant).

   - For chambers without CO_2 control, or with CO_2 control set at 0 %: Option: Place the cell microplate in a plate heater (for example, TAP Biosystem plate heater) set at 37 °C during the 1 hour equilibration.

5. 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

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

7. Transfer the data file to a desktop or laptop computer, and use Wave Desktop for data review and analysis.

Review and analyze data

Validate sodium sulfite performance

Review OCR data for the wells in the hypoxia group.

a. OCR after injection must be higher than before injection (Figure 7).

b. If one of the wells in the group did not respond, omit it from the calculation.

c. If two or more wells in the hypoxia group did not respond, repeat the assay.

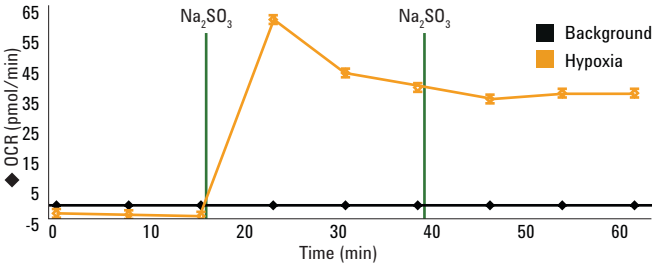

Figure 7. Typical profile of OCR in Hypoxia group wells before and after sodium sulfite injection.

Examine temperature stablility and oxygen level data

1. Review the oxygen level data.

   a. Hypoxia group wells should be zero (±5 mm Hg) (Figure 8).

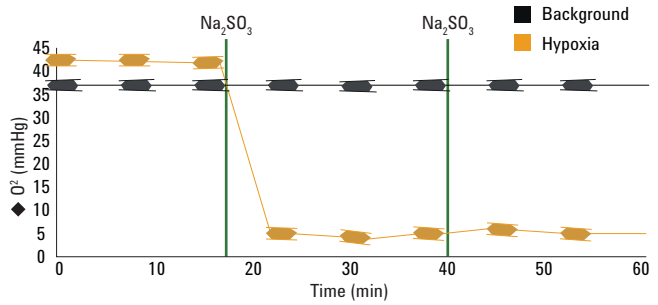

Figure 8. Hypoxia group O_2 levels after recalculation 0 ±5 mm Hg O_2.

b. Check experimental group wells for a linear reduction in oxygen. A nonlinear reduction during measurement indicates an anoxic microchamber, as indicated by the purple trace in Figure 9.

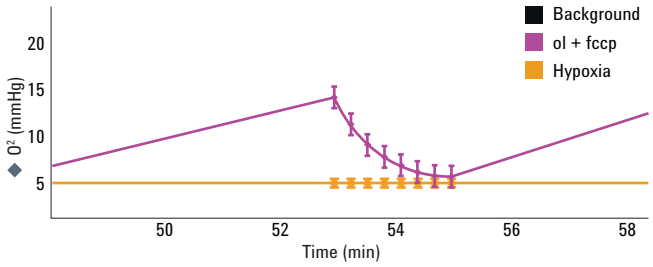

Figure 9. This experimental group well begins to show a nonlinear reduction in the O_2 level during a measurement.

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

d. If the experimental group wells demonstrate a failure to recover to baseline oxygen between measurements or a nonlinear decrease in the oxygen level (Figure 10), these wells may be developing anoxic conditions in the microchamber. Consider seeding fewer cells and repeating the assay.

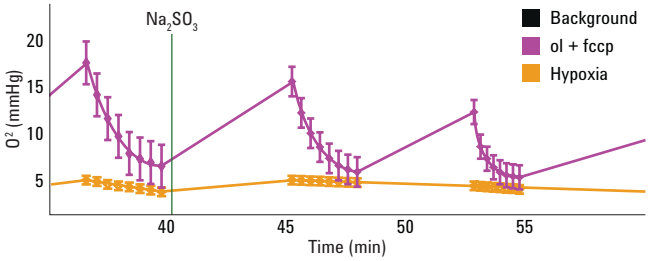

Figure 10. This group does not recover to baseline oxygen levels between measurements.

2. Review the temperature of the wells and environment recorded by the Seahorse XFe Analyzer during the assay.

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

Examine extracellular acidification rate (ECAR) data

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

Reference

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

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