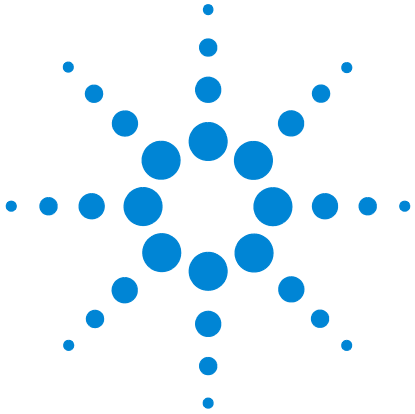

Agilent Seahorse XFp Glycolysis Stress Test Kit

User Guide
Kit 103017-100

Agilent Technologies

Notices

Manual Part Number

103017-400

Kit Part Number

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Contents

1 Introduction

The Agilent Seahorse XF Glycolysis Stress Test is the standard assay for measuring glycolytic function in cells. By directly measuring the extracellular acidification rate (ECAR) (Figure 1 on page 7), the Agilent Seahorse XF Glycolysis Stress Test used on the Agilent Seahorse XFp Extracellular Flux Analyzer provides a standard and comprehensive method to assess the key parameters of glycolytic flux: Glycolysis, Glycolytic Capacity, Glycolytic Reserve, as well as Non-glycolytic Acidification. (Refer to “” on page 7 for more details.)

Introduction

Glycolysis and oxidative phosphorylation are the two major energy-producing pathways in the cell. Most cells possess the ability to switch between these two pathways, thereby adapting to changes in their environment. Glucose in the cell is converted to pyruvate (referred to as glycolysis), and then converted to lactate in the cytoplasm, or CO_2 and H_2O in the mitochondria. The conversion of glucose to pyruvate, and subsequently lactate, results in a net production and extrusion of protons into the extracellular medium (Figure 2 on page 7). The extrusion of protons results in the acidification of the medium surrounding the cell. The Agilent Seahorse XFp Analyzer directly measures the acidification rate, and reports this as ECAR.

The assay workflow is as follows. First, cells are incubated in the glycolysis stress test medium without glucose or pyruvate, and the ECAR is measured. The first injection is a saturating concentration of glucose (10 mM). The cells utilize the glucose injection and catabolize it through the glycolytic pathway to pyruvate, producing ATP, NADH, water, and protons. The cofactor NAD+, regenerated in the cytosol by the conversion of pyruvate and protons to lactate, is secreted by the cell. The extrusion of protons into the surrounding medium causes a rapid increase in ECAR. This glucose-induced response is reported as the rate of glycolysis under basal conditions.

The second injection is oligomycin, an ATP synthase inhibitor. Oligomycin inhibits mitochondrial ATP production, and shifts the energy production to glycolysis, with the subsequent increase in ECAR revealing the cellular maximum glycolytic capacity. The final injection is 2-deoxy-glucose (2-DG, 50 mM), a glucose analog, which inhibits glycolysis through competitive binding to glucose hexokinase, the first enzyme in the glycolytic pathway. The resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis. The difference between glycolytic capacity and glycolysis rate defines glycolytic reserve. ECAR, prior to glucose injection, is referred to as non-glycolytic acidification; caused by processes in the cell other than glycolysis.

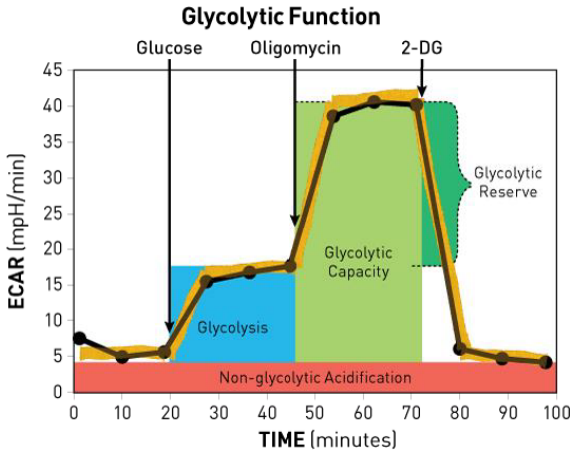

Figure 1 Agilent Seahorse XF Glycolysis Stress Test profile of the key parameters of glycolytic function. Sequential compound injections measure glycolysis, glycolytic capacity, and allow calculation of glycolytic reserve and nonglycolytic acidification.

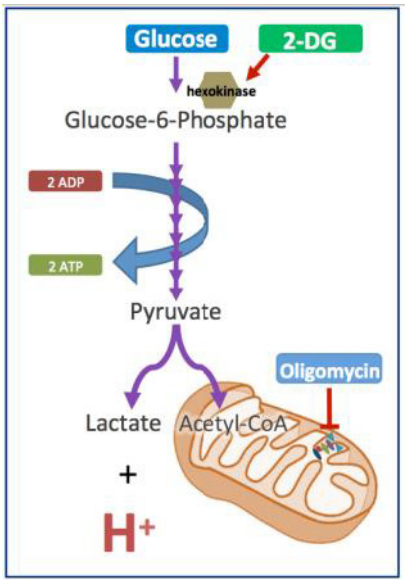

Figure 2 Agilent Seahorse XF Glycolysis Stress Test Modulators of Glycolysis. This diagram illustrates a simplified version of glycolysis and the sites of action of the kit components. Glucose fuels glycolysis. Oligomycin inhibits ATP synthase in the mitochondria resulting in an increased dependence on glycolysis. 2-DG is a competitive inhibitor of glucose and functions to shut down glycolysis.

Table 1 Agilent Seahorse XFp Glycolysis Stress Test Kit Reagents (in order of injection).

Compound(s)	Target	Effect on ECAR
Glucose	Glycolysis	Increase
Oligomycin*	ATP Synthase Complex V	Increase
2-DG†	Glycolysis	Decrease

* Oligomycin is a mixture of Oligomycin A, B, and C with Oligomycin A ≥ 60%.

† 2-DG may appear clear, opaque (white), or as a mix of white solid and clear liquid. Appearance does not affect performance.

Glossary

- **Glycolysis:** The process of converting glucose to pyruvate. The Agilent Seahorse XF Glycolysis Stress Test presents the measure of glycolysis as the ECAR rate reached by a given cell after the addition of saturating amounts of glucose.
- **Glycolytic capacity:** This measurement is the maximum ECAR rate reached by a cell following the addition of oligomycin, effectively shutting down oxidative phosphorylation and driving the cell to use glycolysis to its maximum capacity.
- **Glycolytic reserve:** This measure indicates the capability of a cell to respond to an energetic demand as well as how close the glycolytic function is to the cell's theoretical maximum.
- **Nonglycolytic acidification:** This measures other sources of extracellular acidification that are not attributed to glycolysis.

2
Kit Information

Kit Contents

The Seahorse XF Glycolysis Stress Test Kit includes six foil pouches. Each pouch contains reagents sufficient for an assay in a full 96- or 24-well XF Cell Culture Microplate. Every pouch includes one tube of each of the following compounds: glucose, oligomycin, and 2-DG. See Table 2.

Table 2 Agilent Seahorse XF Glycolysis Stress Test Kit contents.

Compound	Cap color	Quantity per tube
Glucose	Blue	30 µmol
Oligomycin	Light blue	14.4 nmol
2-DG	Green	150 µmol

Kit Shipping and Storage

Product ships at ambient temperature, and should be stored at room temperature.

Additional Required Items

The following items are also required for performing Seahorse XF Glycolysis Stress Tests. But they are not supplied with the kits

Table 3 Additional required items.

Item	Supplier	Part number
Agilent Seahorse XFp analyzers	Agilent Technologies	
Seahorse XFp FluxPak (cartridges, miniplates, and calibrant)	Agilent Technologies	103022-100
XF Base Medium (100 mL or 500 mL)*	Agilent Technologies	103193-100, 103334-100
XF 200 mM Glutamine Solution	Agilent Technologies	103579-100

* For a full list of all medium types and our recommendation for each assay kit, please refer to the Seahorse XF Media Selection Guide. http://www.agilent.com/cs/library/selectionguide/public/5991-7878EN.pdf

3 Assay

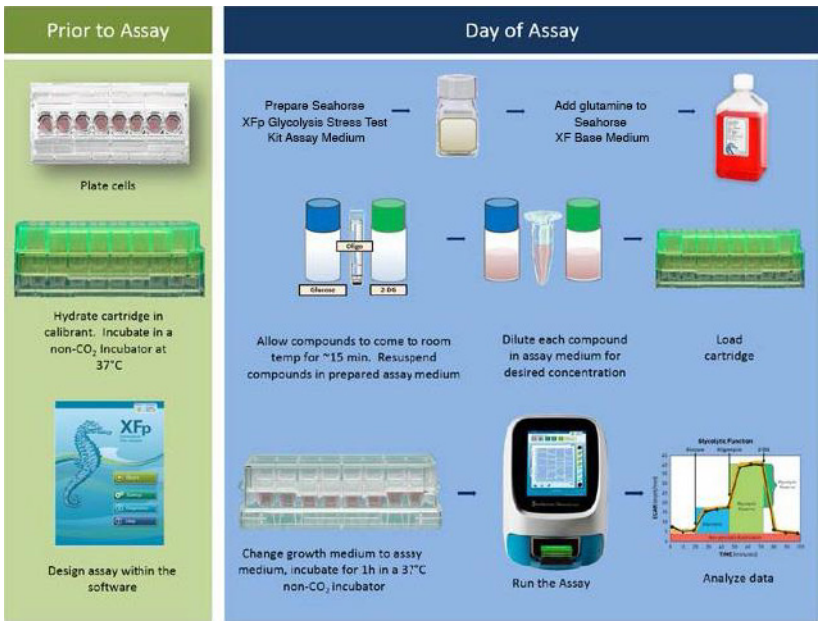

Figure 3 Agilent Seahorse XFp Glycolysis Stress Test Workflow.

Day Prior to Assay

1. Hydrate an XFp sensor cartridge in XF Calibrant at 37 °C in a non-CO_2 incubator overnight. (Refer to *Hydrating the Sensor Cartridge for the XFp Analyzer* in the Basic Procedures section, https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay
2. For adherent cells, plate cells in the XFp Cell Culture Miniplate at the desired density using the appropriate cell culture growth medium. Add sterile water or PBS to the moat chambers to prevent evaporation of the culture medium. (Refer to *Cell Characterization Data Table* and *Seeding Adherent Cells in XFp Cell Culture Miniplates* in the Basic Procedures section, https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay
3. For suspension cells, determine the desired density, then plan to seed cells on the day of the assay. (Refer to S*eeding Suspension Cells in XFp Cell Culture Miniplates* in the Basic Procedures section, https://www.agilent.com/en/products/cell-analysis/cell-analysis-software

Day of Assay

Prepare assay medium

1. Prepare assay medium by supplementing Seahorse XF Base Medium. It is best to use 2 mM glutamine, as a starting point; however, desired medium composition can be varied depending on cell type or *in vitro* culture conditions.
2. Warm assay medium to 37 °C.
3. Adjust pH to 7.4 with 0.1 N NaOH (Note: Seahorse recommends sterile filtration following pH adjustment).
4. Keep at 37 °C until ready to use.

Prepare stock compounds

Important: Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.

1. The Seahorse XFp Glycolysis Stress Test Kit includes:
   - Six foil pouches each containing oligomycin
   - Six vials containing glucose
   - Six vials containing 2-DG

   The kit reagents are sufficient for six complete Seahorse XF Glycolysis Stress Test assays in the Seahorse XFp Miniplate.
2. Open the foil pouches containing oligomycin, and remove one vial containing glucose and one vial containing 2-DG from the kit box.
3. Resuspend each component with prepared assay medium in volumes described in Table 3 with a p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds. Vortex the 2-DG for approximately. 1 minute to ensure that it goes into solution.

Hold the tube in gloved hand, and roll thumb in forward motion over cap to loosen or, using the decapping tool provided, insert the tooth of a decapper into the inner lip of the cap, and gently rotate the tool backwards.

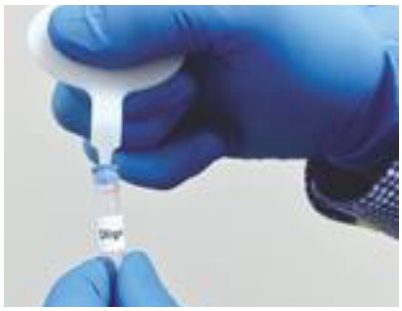

Figure 4 Removing reagent caps.

Table 4 Stock solution.

Compound	Volume of assay medium	Final stock concentration
Glucose	300 µL	100 mM
Oligomycin	288 µL	50 µM
2-DG	300 µL	500 mM

Prepare compounds for loading in sensor cartridge

There are two approaches to loading the injection ports of the sensor cartridge:

- **Constant loading volume/variable compound concentration:** This approach entails loading a constant volume of compound in each injection port and requires that each compound be prepared at a different concentration
- **Constant compound concentration/variable loading volume:** This approach entails preparing the compounds at a constant concentration and requires that a different volume of each compound be loaded in the injection port.

Table 5 on page 15 describes how to prepare to load the cartridges using both options. If using the constant volume option, media can be added directly to the glucose vial. If using the constant concentration option, no additional media is necessary. For oligomycin (with either loading option) pipette the stock volume into a microfuge tube, and add the given volume of media. No media addition is necessary for 2-DG when running a standard assay.

Table 5 Compound preparation for loading sensor cartridge ports.

Agilent Seahorse XFp Analyzer		Constant volume				Constant concentration			
		Starting well volume: 175 µL assay medium				Starting well volume: 180 µL assay medium			
Port A Glucose	[Final well] (mM)	Stock volume (µL)	Media volume (µL)	8X [Port] (mM)	Add to port (µL)	Stock volume (µL)	Media volume (µL)	10X [Port] (mM)	Add to port (µL)
	10	300	75	80	25	300	0	100	20
Port B Oligomycin	[Final well] (µM)	Stock volume (µL)	Media volume (µL)	9X [Port] (iM)	Add to port (µL)	Stock volume (µL)	Media volume (µL)	10X [Port] (mM)	Add to port (µL)
	1.0	54	246	9	25	60	240	10	22
Port C 2-DG	[Final well] (mM)	Stock volume (µL)	Media volume (µL)	10X [Port] (mM)	Add to port (µL)	Stock volume (µL)	Media volume (µL)	10X [Port] (mM)	Add to port (µL)
	50	300	0	500	25	300	0	500	25

Seahorse recommends 1 µM oligomycin; however, this can be varied if necessary given the specific sample conditions.

Load sensor cartridge

Load compounds into the appropriate ports of a hydrated sensor cartridge.

- **Standard Assay:** No additional injection:

  Port A: Glucose

  Port B: Oligomycin

  Port C: 2-DG

- **Modified Assay:** Additional injection included prior to glucose injection.

  Port A: Test compound

  Port B: Glucose

  Port C: Oligomycin

  Port D: 2-DG

Table 6 Compound injection volumes involving an acute injection.

Agilent Seahorse XFp Analyzer	Constant volume		Constant concentration	
	Starting well volume: 175 µL assay medium		Starting well volume: 180 µL assay medium	
Port	**Volume**	**Concentration**	**Volume**	**Concentration**
A	25 µL	8X	20 µL	10X
B	25 µL	9X	22 µL	10X
C	25 µL	10X	25 µL	10X
D	25 µL	11X	27 µL	10X

Prepare Agilent Seahorse XFp Cell Culture Miniplate for assay

1. Remove Seahorse XFp Cell Culture Miniplates from 37 °C CO_2 incubator and examine cells under microscope to confirm confluence.
2. Remove assay medium from water bath.
3. Change the cell culture growth medium in the cell culture miniplate to warmed assay medium using a multichannel pipette, and place the cell culture miniplate into a 37 °C non-CO_2 incubator for 45 minutes to 1 hour prior to the assay.
4. You are now ready to run the Seahorse XF Glycolysis Stress Test assay on the Seahorse XFp Analyzer.

Run the Agilent Seahorse XF Glycolysis Stress Test

1. Click **Start** and select the **Seahorse Glycolysis Stress Test** default template. Click the right arrow on the Groups page, and then on the Protocol page. Click **Start Assay**.
2. Place the utility plate with the loaded assay cartridge on the instrument tray, and click **Continue**. Calibration takes approximately 20 minutes.
   Note: Remove the cartridge lid and verify correct plate orientation.
3. When calibration has been completed, remove the utility plate, place the Seahorse XFp Miniplate on the tray, and press **Continue** to start the assay.
   Note: Remove miniplate lid, and verify correct plate orientation.

Data Analysis

The Seahorse XF Glycolysis Stress Test Report Generator automatically calculates the Seahorse XF Glycolysis Stress Test parameters from Wave data that has been exported to Excel. The Seahorse XF Stress Test Report Generator can be used with either a standard or modified stress test protocol, and provides a convenient, customizable, one-page assay summary.

The Seahorse XF Report Generator can be installed either alongside Wave or directly from the Agilent Technologies website. Visit http://www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-xf-report-generators to learn more about the Agilent Seahorse XF Stress Test Report Generators and download the User Guide.

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