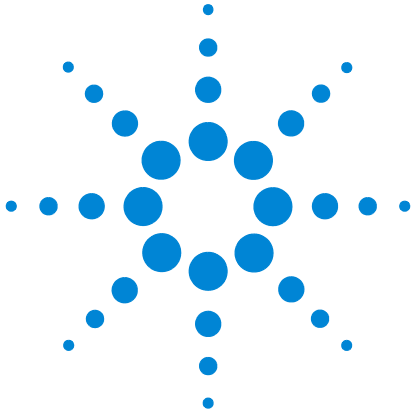

Agilent Seahorse XFe96 Spheroid Microplate and Agilent Seahorse XFe96 Spheroid Flux Pak

User Guide

Agilent Technologies

Notices

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Contents

1 Introduction

Recommended Use

The Agilent Seahorse XFe96 Spheroid Microplate is designed for researchers currently working with spheroid cultures, to perform metabolic assays in single isolated spheroids.

Introduction

In traditional two-dimensional (2D) monolayer cell culture, cells are grown on a flat plastic surface, have unrestricted access to growth media, and are uniformly exposed to drugs during treatment. In contrast, cells *in vivo* exist in three-dimensional (3D) configurations that promote, regulate, and support their physiology, including cell signaling, gene expression, protein production, differentiation, and proliferation. The 2D culture is a limitation of many toxicology screens due to the lack of transferable data from 2D to 3D culture. The importance of 3D architecture is also relevant for tumor development *in vivo*. For example, interactions between tumor cells and between tumor and stromal cell populations are necessary to define and maintain their dedifferentiated, highly proliferative state. Culturing of cells as spheroids mimics this 3D architecture.

The 2D and 3D cultures can have significantly different rates of metabolism. HCT116 colon carcinoma cells grown as a 2D monolayer culture (Figure 1A on page 6), and as spheroids (Figure 1B on page 6). Compared to the 2D monolayer, the spheroids have a dramatically increased spare respiratory capacity. This is consistent with a shift from the highly proliferative nature of 2D cell culture to the 3D state where nutrient supply gradients contribute to balanced proliferation, differentiation, and cell death. The metabolic requirements of these cells are notably different, contributing to the difference observed in spare respiratory capacity. These data were produced on an Agilent Seahorse XFe96 Analyzer with an Agilent Seahorse XF Cell Mito Stress Test Kit. (See Figure 1 on page 6.)

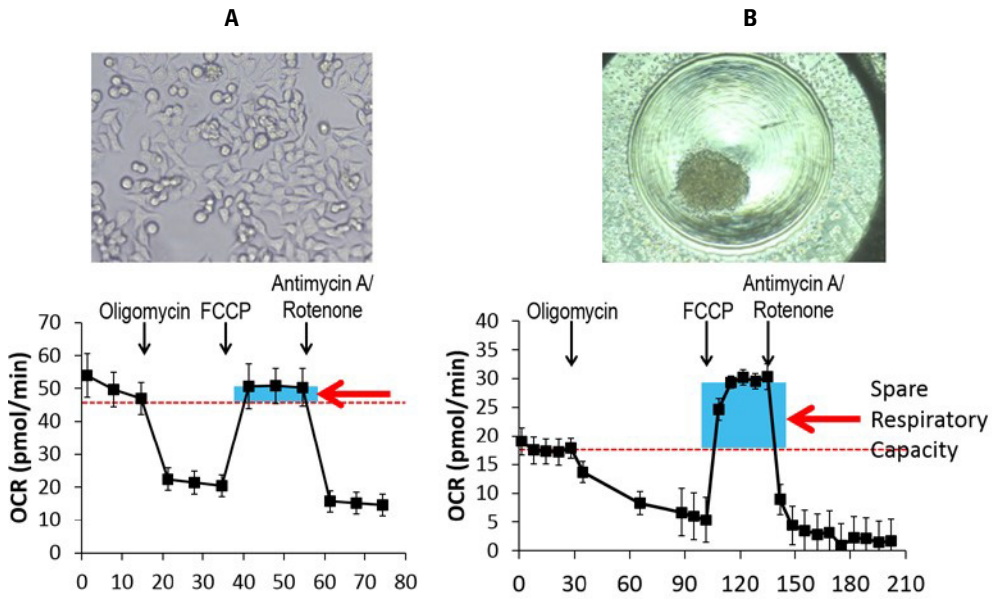

Figure 1 2D and 3D cultures significantly different rates of metabolism

Agilent Seahorse has developed a 96-well microplate with an innovative design for assaying individual spheroids with the Agilent Seahorse XFe96 Analyzer. The design of the microplate allows for placement of individual spheroids in the position necessary to perform gold standard metabolic assays. This product uniquely enables highly sensitive, real-time, metabolic measurement of single spheroids, delivering a robust method to assay metabolic function. This microplate is compatible with Agilent Seahorse XFe96 cartridges. The Agilent Seahorse XF Cell Mito Stress Test and Agilent Seahorse XF Glycolysis Stress Test Kits have been validated for use with spheroids in the Agilent Seahorse XFe96 Spheroid Microplate.

Materials

- Spheroid culture materials (InSphero GravityPLUS™ 10× Kit (96-well) #CS-06-001)
- Poly-D-lysine hydrobromide (Sigma #P7280 or #P6407) or Corning® Cell-Tak™ Cell and Tissue Adhesive (1 mg p/n 354240)
- Agilent Seahorse XF Base Medium (Agilent Seahorse #102353-100) and desired supplements
- Agilent Seahorse XFe96 Assay Cartridge and Agilent Seahorse XFe96 Spheroid Microplates (Agilent Seahorse XFe96 Spheroid FluxPak, #102905-100)
- Agilent Seahorse XFe96 Analyzer with compatible tray and correct plate type file (see Figure 3 on page 11)
- Non-CO_2 incubator
- Dissecting or inverted microscope (for verifying correct positioning of spheroids)

2
Spheroid Microplate Assay Workflow

Planning a Spheroid Assay

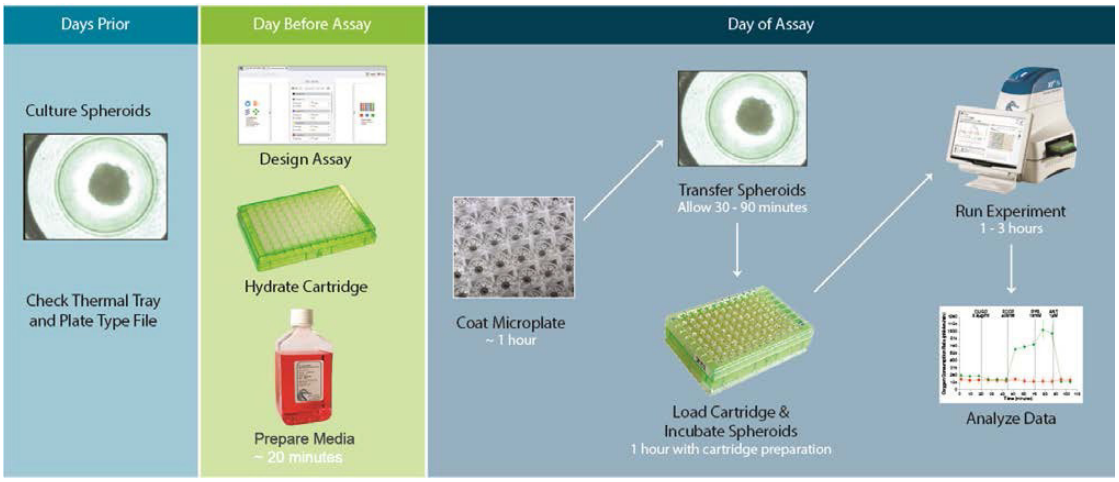

Figure 2 Agilent Seahorse XFe96 Spheroid Microplate Assay Workflow

Spheroid dimensions

Spheroids between 200 and 500 µm generate oxygen consumption, and extracellular acidification rates are within the dynamic range of the oxygen and pH sensors. Spheroid size can be controlled by varying the starting cell number, number of days in culture, and the growth media used during culture. Using spheroids larger than 500 µm is not recommended due to the size limitations of the measurement chamber.

Thermal tray

Confirm that your Agilent Seahorse XFe96 Analyzer has the correct thermal tray and plate type file (see Figure 3 on page 11).

Before using the Agilent Seahorse XFe96 Spheroid Microplate, it is essential to determine if the correct thermal tray and plate type file have been installed.

- The posts in the Agilent Seahorse XFe96 Spheroid Microplate-compatible thermal tray do not touch one another. The posts in the original thermal tray are connected in vertical rows. See Figure 3 on page 11 (Side View of 3A and 3B).
    - If unsure, contact Agilent Technical Support. Ensure the instrument serial number is available.

- If there is no record of the plate type installation, contact Agilent Technical Support to obtain the file and instructions for installation. Reinstalling the plate type file will not create any adverse effect.

Figure 3 Agilent Seahorse XFe96 Spheroid Microplate-compatible Thermal Tray and Correct Plate Type File

Coating the microplate

Prior to transfer of spheroids to the Agilent Seahorse XFe96 Spheroid Microplate, the microplate must be coated with cell adhesive to maintain spheroids in the proper location (see "Steps to Coat the Microplate" on page 15). Not coating the microplate with cell adhesive may result in spheroid movement during an assay. This will be apparent in the data as a rapid, unexpected drop in OCR (see "Technical Considerations" on page 17).

Time to transfer spheroids

Depending on operator experience, individual spheroid transfer time can range from 30 minutes to > 60 minutes, and may require visual aids including glasses or a dissecting microscope to more easily see smaller spheroids. Practicing the transfer process will significantly reduce the time required for this step, and is strongly recommended prior to running the first assay (see "Transferring Spheroids" on page 13).

Drug concentration

The 3D nature of spheroids will slow the diffusion of some compounds into the cells. To account for possible differences, reagents may require titration to assure appropriate concentration for your cells. It has been observed that the maximal inhibition of oxygen consumption in spheroids with oligomycin takes longer than a typical 2D experiment (see Figure 6 on page 18). In addition, the concentration of oligomycin required to effectively inhibit Complex V has been shown to differ from that used in 2D culture of the same cell line. Performing cell and compound characterization experiments ensures that you are using a drug concentration that effectively penetrates your spheroids.

Temperature equilibration and Cartridge calibration

Following the transfer, a 1 hour equilibration is recommended in a 37 °C non-CO_2 incubator to ensure uniform temperature of the samples. The Agilent Seahorse XFe96 assay cartridge can be loaded and calibrated during this incubation period. Following calibration, and when requested by the software, the Agilent Seahorse XFe96 Spheroid Microplate is placed into the instrument, and the assay performed.

Transferring Spheroids

Prior to transfer, the Agilent Seahorse XFe96 Spheroid Microplate should be coated to prevent spheroid movement and warmed to 37 °C. A single spheroid should be transferred into each well of the Agilent Seahorse XFe96 Spheroid Microplate. Single spheroids should be produced in a 96-well format.

NOTE Agilent Seahorse recommends the GravityPLUS™ culture system for producing spheroids of optimal size and ease of transfer.

1. Using a sufficiently large bore pipette tip to accommodate your spheroid size, place the tip against the bottom of the well containing the spheroid and aspirate.
2. Slowly remove the tip, and visually confirm that you have aspirated the spheroid.
3. Move the tip containing the spheroid to a well of the Agilent Seahorse XFe96 Spheroid Microplate.
4. Place the tip against the bottom of the well directly in the center, and hold the pipette steady for about 20 seconds.

The spheroid should float out of the tip, to the bottom of the well by gravity alone, without ejection of media, and attach to the cell adhesive. See Figure 4.

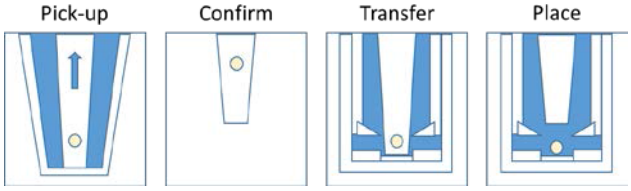

Figure 4 Transferring spheroids

Standard technique for transferring spheroids

1. Place the pipette tip against the bottom of the culture well containing a single spheroid.
2. Aspirate a single spheroid up to 20 µL, slowly remove the tip, visually verify the spheroid tip.
3. Place the tip against the bottom center of the Agilent Seahorse XFe96 Spheroid Microplate well, and hold steady.
4. Allow the spheroid to float out of the tip. This may take 20 seconds.
5. Visually verify that spheroids are near the center of the well.

Visual aid

A dissecting scope can be used to observe the spheroid in the original culture plate while aspirating, and to position the spheroid in the microplate. However, this technique can add time to the process, and may not improve spheroid transfer accuracy.

Faster technique

For faster transfer time, spheroids can be aspirated and placed using the first technique with a multichannel pipette. This technique requires practice, and proper placement of spheroids in the microplate depends on accurate alignment of the tips.

Media ejection

If you need to eject media to release the spheroid, do so slowly and carefully in an effort to dispense a consistent volume of less than 10 µL in each well. Use a dissecting or inverted microscope to ensure the spheroid is placed in the center of the well. If an entire spheroid can be seen in the circular well under a microscope, data will be consistent between wells. If spheroids are only partially visible, or in a channel of the well insert, data may be compromised.

Steps to Coat the Microplate

Poly-D Lysine 100 µg/mL coated Agilent Seahorse XFe96 Spheroid Microplates

(Sigma P7280 or P6407)

1. Add 50 mL of sterile tissue culture grade water to a 5 mg bottle.
2. Add 30 µL of poly-D-lysine per well, ensuring that the detent in the plate is covered.
3. Remove air bubbles, if present.
4. Cover the plate, and let sit for 20 minutes.
5. Aspirate poly-D-lysine from wells.
6. Wash the wells two times with 200 µL sterile water.
7. Aspirate after final wash, and air dry for 30 minutes, minimum.
8. Warm the plate for 30 minutes in a 37 °C non-CO_2 incubator.
9. Add 37 °C media, and return the plate to a 37 °C non-CO_2 incubator until ready to load spheroids.
10. Aliquots of poly-D-lysine can be stored frozen at -20 °C, and thawed in a 37 °C water bath.

Corning® Cell-Tak™ Cell and Tissue Adhesive

(Corning® Cat # 354240)

Formulation comes at approximately 2 mg/mL in 5% acetic acid, and is stored at 4 °C.

1. Prepare 0.1 M sodium bicarbonate pH 8.0, and filter sterilize.
2. Add 200 µL Cell-Tak™ to 2.8 mL of 0.1 M sodium bicarbonate.
3. Add 30 µL of Cell-Tak™ mix per well of Agilent Seahorse XFe96 Spheroid Microplate.
4. Ensure that the bottom of each well is covered, and remove air bubbles.
5. Incubate the plate in a 37 °C non-CO_2 incubator for 1 hour.
6. Aspirate Cell-Tak™ from the plate, and wash two times with 400 µL of sterile 37 °C water.
7. Allow to air dry, or use immediately.

If using immediately, add sterile 37 °C media, and return plate to a 37 °C non-CO_2 incubator until ready to load spheroids.

Running the Assay

1. Prepare assay media by supplementing Agilent Seahorse XF Base Medium with desired substrates.
2. Warm assay media to 37 °C, and adjust the pH to 7.4. Filter sterilize prepared media using a 0.2 µm filter. Maintain media at 37 °C.
3. Add 175 µL of assay media to each well of an empty Agilent Seahorse XFe96 Spheroid Microplate.
4. Remove the culture plate that contains spheroids from the cell culture incubator.
5. Transfer individual spheroids from the culture well to the Agilent Seahorse XFe96 Spheroid Microplate containing prewarmed assay media (refer to "Transferring Spheroids" on page 13).
6. Warm the microplate at 37 °C in a non-CO_2 incubator for 1 hour.
7. Load the desired compounds into the appropriate injector ports in the assay cartridge, and perform calibration.
8. When prompted by software, exchange the utility plate containing calibrant for the Agilent Seahorse XFe96 Spheroid Microplate containing spheroids, and run the assay.
9. At the completion of the assay, examine the position of the spheroids in each well using a dissecting or inverted microscope. This is to confirm that all spheroids remained in the center of the well during the assay and within correct proximity of the sensor. The importance of the spheroid position in data analysis is discussed in "Technical Considerations" on page 17.

3 Technical Considerations

Spheroid Movement

To ensure detection of OCR and ECAR, the entire spheroid should be visible in the well, before and after the assay. After the assay has completed, inspect data from individual wells for a sudden decrease of OCR and ECAR, which is indicative of spheroid movement and can be confirmed by microscopy. The use of cell adhesive significantly reduces the likelihood of spheroid movement.

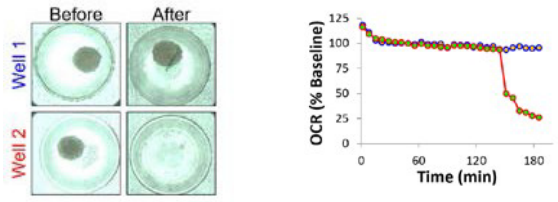

Figure 5 Baseline measurement for two wells

Well 1, shown in blue, maintains a steady OCR reading over time. Well 2, shown in red, has a sudden drop in OCR. This suggests that the spheroid moved out of the center of the well. The images were taken before the assay began, and after it completed. The spheroid in well 1 remained in place, while the spheroid in well 2 can no longer be seen in the center of the well. Plate imaging is a useful QC step, and can be used to confirm that an outlier was due to movement of the sample.

Compound Penetration Into a Spheroid

The response to injected compounds can be different due to the geometric differences between a 3D spheroid and 2D monolayer culture. For example, because oligomycin typically requires more time to penetrate the entirety of the spheroid, ensure that you obtain a stable inhibition consisting of at least three measurements, indicating that the chosen concentration is optimal.

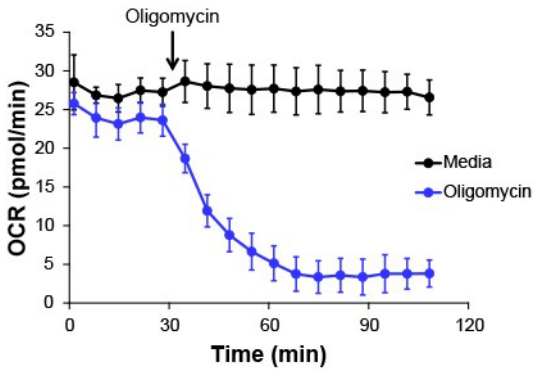

Figure 6 Penetration of Oligomycin into Spheroid

Penetration of oligomycin into a spheroid. In this example, oligomycin (a fairly large compound) takes much longer to reach its full effect. Compare this with the almost immediate response that occurs when oligomycin is added to 2D culture.

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