User Guide

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Seeding Adherent Cells in Agilent Seahorse XF96 Tissue Culture Microplates

Introduction

Agilent Seahorse XF Assays are performed in a Seahorse XF96 tissue culture microplates in conjunction with the Agilent Seahorse XFe96 Sensor Cartridge. Each microplate is formatted in a typical 96-well design; however, the surface area of each well is 0.106 cm^2, 40% the area of a typical 96-well plate. This procedure describes recommendations for seeding adherent cells for best results when performing Seahorse XFe96 assays.

Basic Cell Seeding Workflow

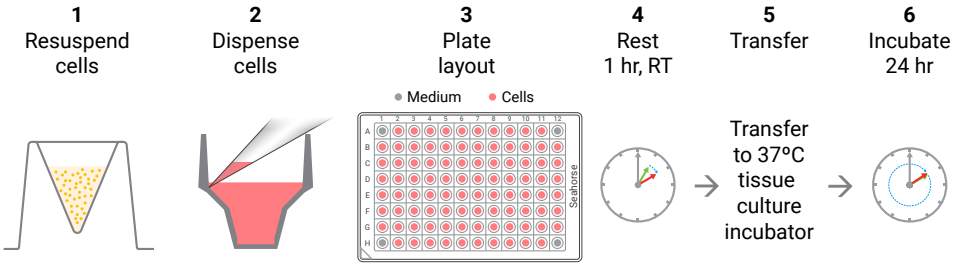


Figure 1. XF Tissue Culture Microplate Cell Seeding Procedure Workflow

1. Harvest and re-suspend the cells to desired final concentration to seed in 80 µL of growth medium. Please refer to the chart below for preparation of common cell seeding densities. Either, 1) dilute cell suspension to 1 x 10^6 cells per mL and dilute again to the desired density based on the table below, or 2) dilute cell suspension to the desired cell density using $Cells^{stock}$ x $Volume^{stock}$ = $Cells^{final}$ x $Volume^{final}$ where Cells is the number of cells and Volume is in mL.
2. Seed 80 µL of cell suspension per well (as shown in Figure 1); do not seed cells in background correction wells (A1, A12, H1, H12).
3. Add medium only (no cells) in the background correction wells.
4. IMPORTANT: Allow plate to rest at room temperature in the tissue culture hood for one hour.
5. After the 1 hour rest, transfer the cells to a properly humidified 37°C CO_2 incubator*.
6. Allow the cells to grow overnight in a tissue culture incubator. Monitor growth and health of cells using a microscope.

Detailed Procedure

Choosing a seeding density

Optimal cell seeding numbers vary widely based on cell type, though are typically between 0.5 - 4.0 x 10^4 cells per well and must be determined empirically.

Example: one desires to seed one XF96 plate at 2.0 x 10^4 cells per well, and a stock solution of 4.20 x 10^6 cells per mL is obtained. Since one plate is needed, a total volume of 10mL diluted cell suspension at a density of 2.5 x 10^4 cells/mL is required (i.e. 2.0 x 10^4 cells/well / 0.08 mL/well = 2.0 x 10^4 cells/well).

Using the equation from above: (4.20 x 10^6 cells) x (X mL) = 2.5 x 10^5 cells) x (10 mL).

Solving for X, one obtains 0.595 mL, and thus add 0.595 mL of stock cell suspension to 9.405 mL of appropriate cell culture media to obtain 10 mL of 2.5 x 10^4 cells/ml that provides a final cell density of 2.0 x 10^4 cells per well in 80 µL of cell culture media.

For further information on optimal cell density, please see the Agilent Seahorse XF Assay Learning Center including Related Support and Reference Materials: Cell Reference Database.

Harvesting and resuspending cells

1. A single-cell suspension is optimal for producing a consistent cell monolayer when cells are seeded. It is beneficial to break up any aggregated cells prior to seeding. It is also important to ensure that cells are thoroughly resuspended before counting and before seeding into XF96 Tissue Culture Microplates. For detailed information regarding best practices for cell culture and seeding cells in XF Cell Culture Microplates, see Knowing your Cells of Interest: Advice and Suggestions for a Successful XF Experience

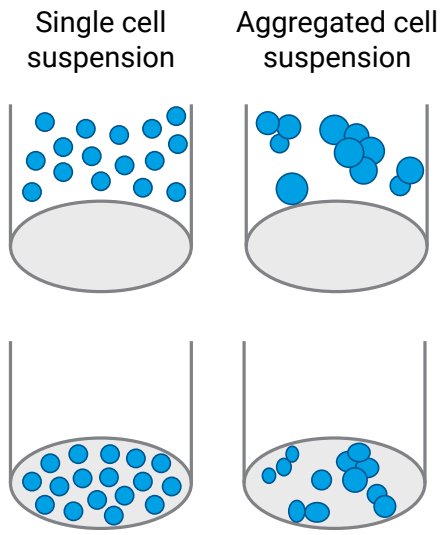


Figure 2.Single cell *vs* Aggregated cell suspension and resulting distribution of cells within the well.

Desired Cell Density per Well (# cells /80 µl media)	Stock Cell Suspension (1 x 10^6 cells/mL)	Growth Media	Minimal volume for one XF96 microplate
0.5 x 10^4	0.5 mL	7.5 mL	8.0 mL
1.0 x 10^4	1. mL	7.0 mL	8.0 mL
1.5 x 10^4	1.5 mL	6.5 mL	8.0 mL
2.0 x 10^4	2.0 mL	6.0 mL	8.0 mL
2.5 x 10^4	2.5 mL	5.5 mL	8.0 mL
3.0 x 10^4	3.0 mL	5.0 mL	8.0 mL
4.0 x 10^4	4.0 mL	4.0 mL	8.0 mL

Table 1. XFe96/XF96 Cell Seeding Dilution Table

2. When diluting cells to the desired seeding density, mix the full volume 2-3 x via pipet to ensure a homogeneous suspension.

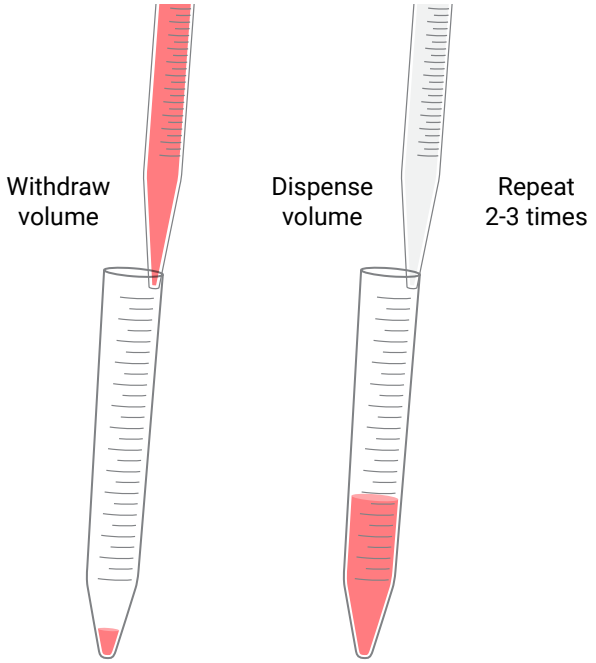


Figure 3. Dilute and mix cells thoroughly.

3. Transfer diluted and mixed cells to a pipetting reservoir.
4. Cells may subsequently begin to settle in the reservoir after mixing, especially for larger cell types. It is therefore recommended to gently mix the remaining cells in the reservoir during the seeding procedure; usually 1-2 additional mixes via pipet are sufficient when seeding a single plate.
   a. If the same suspension of cells is being used to seed multiple plates, the cell suspension should also be remixed between seeding each plate.

**A properly controlled incubator is one in which the temperature, % humidity and % CO_2 are maintained according to the model's manual, doors are kept closed when the incubator is not being accessed and time doors are open is minimal.*

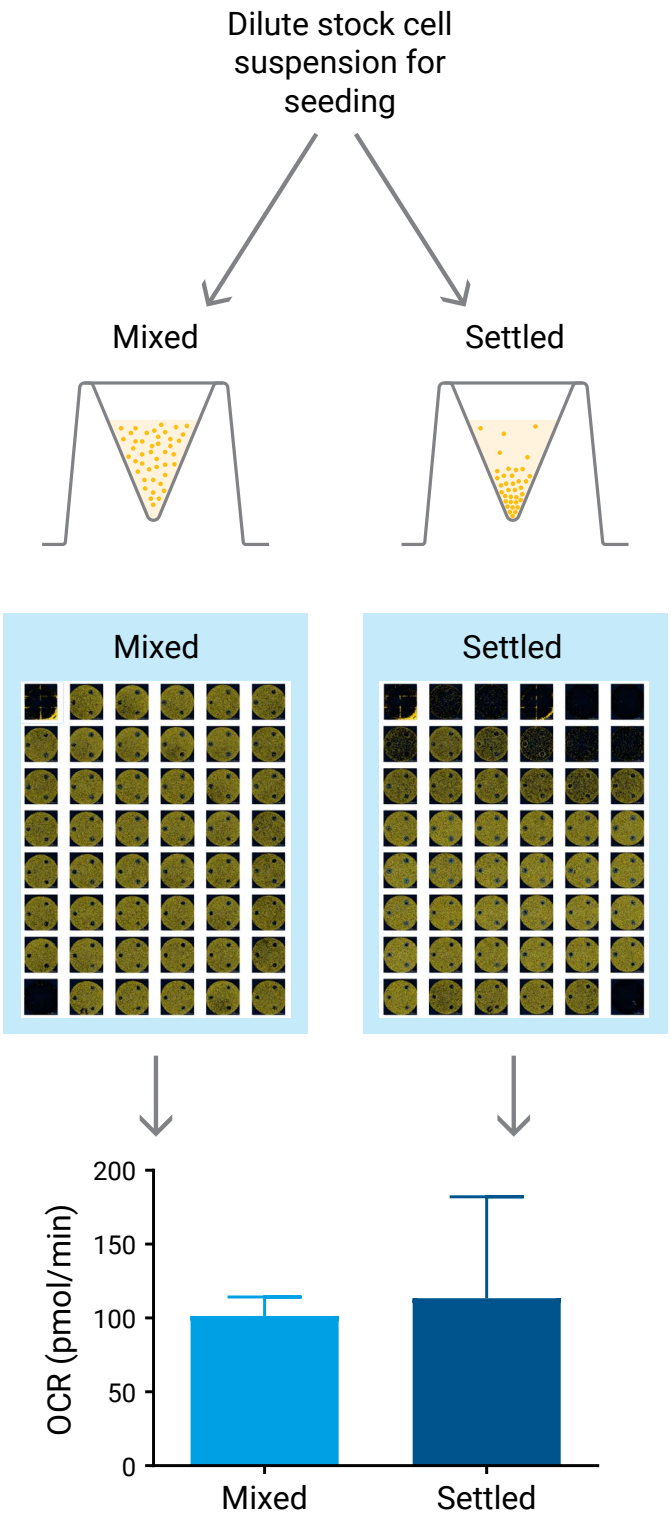


Figure 4. Well Mixed vs Settled cells and resulting distribution across wells. A549 cells were seeded at 1.5 x 10^4 cells per well in an XF96 Cell Culture Microplate. The left half of the microplate was seeded with cells that were mixed in the reservoir directly prior to seeding. The right half of the microplate was seeded after a brief period (≈2 min) during which cells settled in the reservoir. Note the uneven distribution (some wells received more cells than intended while others received fewer cells than intended) of cells in the Settled group compared to the Mixed group. This variation is reflected in the respiration rates (OCR values), with the Mixed Group showing a significantly lower coefficient of variation (CV) compared the Settled group.

Dispensing the cells into the XF Cell Culture Microplate

1. When dispensing cells into the XF96 Cell Culture Microplate, use of an 8- or 12-channel 20-200 µL multipipette is recommended for convenience, speed and consistency among wells and/or plates.
2. The pipet tips should be at ~45 degrees approximately halfway down the side wall, and should touch the wall of the well (Figure 5). The pipet should be held at the same angle for all wells.
   a. The tips should be slightly submerged in the media after the cells are dispensed.
   b. An electronic pipet may improve the consistency of dispensing volume.

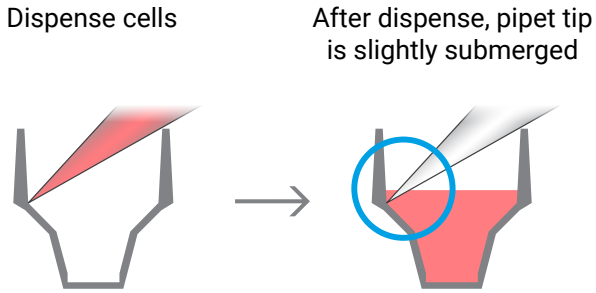
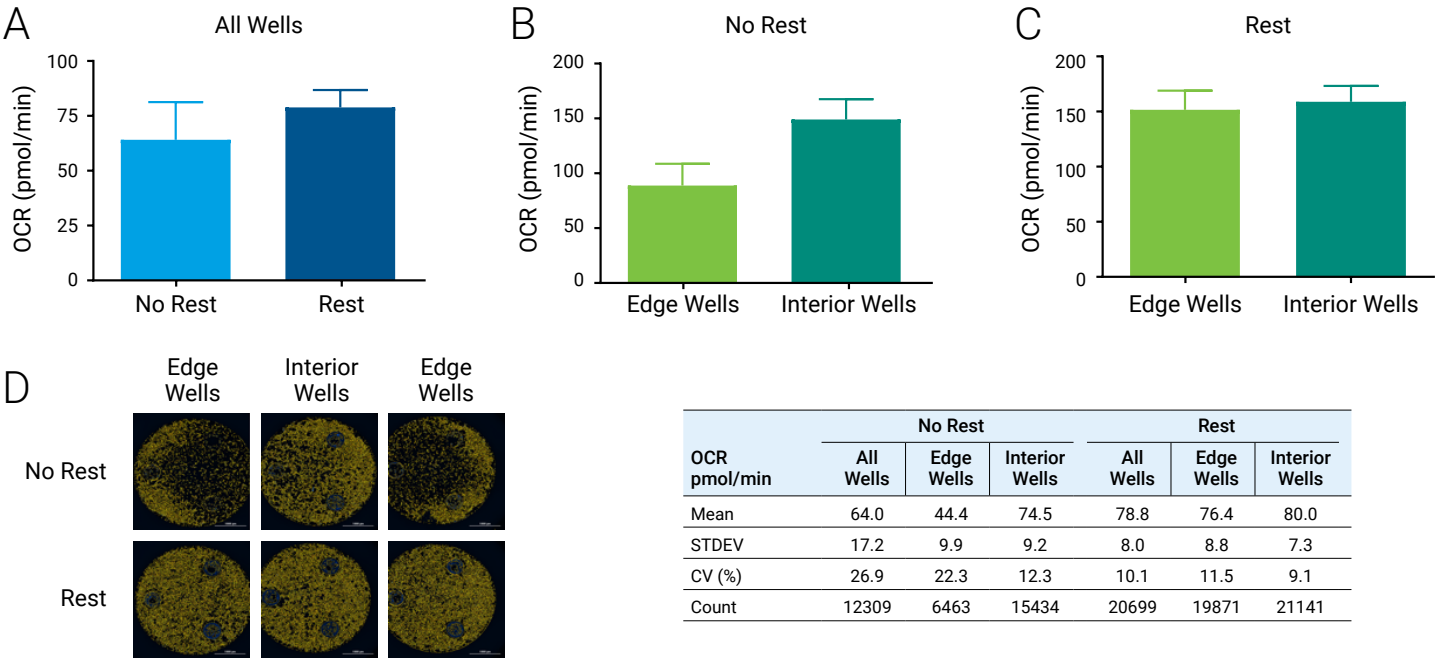


Figure 5. Proper pipetting technique for dispensing cells into XF Tissue Culture Microplates.



OCR pmol/min	No Rest			Rest		
	All Wells	Edge Wells	Interior Wells	All Wells	Edge Wells	Interior Wells
Mean	64.0	44.4	74.5	78.8	76.4	80.0
STDEV	17.2	9.9	9.2	8.0	8.8	7.3
CV (%)	26.9	22.3	12.3	10.1	11.5	9.1
Count	12309	6463	15434	20699	19871	21141

Figure 6. Effects of No Rest and Rest Methods on A549 cell monolayers and respiration rates. A549 cells were seeded at 1.5 x 10^4 cells/well in replicate XF96 tissue culture microplates. One plate was immediately transferred to a tissue culture incubator at 37°C (No rest). The other was left to Rest for 1 hour at ambient temperature (i.e. room temperature). After the 1 hour Rest at ambient conditions, this cell plate was then transferred to the same tissue culture incubator. Cells were cultured for an additional 24 hours before imaging wells with the Biotek Cytation 1 and measuring basal respiration rates (OCR) in an XFe96 Analyzer. OCR was measured and data is divided as follows: A) All wells grouped, No Rest vs. Rest conditions; B) and C) Edge wells vs Interior wells. D) Example images from Edge and Interior wells, No Rest vs. Rest conditions. Note the edge wells and interior wells show nearly identical respiration rates under Rest conditions, resulting in lower average coefficients of variation (CVs, 10.1% vs 26.9%) when all wells (i.e. edge + interior) are in a single group.

Rest and incubation of the cells

1. Allow the cells to rest at room temperature (RT) in the cell culture hood for 1 hour after seeding. Note that cell plates may be gently moved to the rear portion of the biosafety cabinet for the rest step if desired.

   A rapid change in temperature immediately after cell seeding can cause significant edge growth effects in an XF96 Cell Culture Microplate. Transferring cell plates to the tissue culture incubator immediately after seeding can result in significant edge effects with respect to cell growth. For detailed information on edge effects, please see Methods for Reducing Cell Growth Edge Effects in Agilent Seahorse XF Cell Culture Microplates.

2. Place cells in the tissue culture incubator

   a. The incubator should be properly humidified to prevent excess evaporation from the wells. Ensure the water pan in the tissue culture incubator is at the recommended volume.

   b. Place XF96 Cell Culture Microplates toward the back of the incubator to minimize exposure to temperature and humidity changes when the incubator is accessed.

   c. If resources allow, an incubator used only for the incubation of assay plates may help reduce the impact of frequent incubator door opening and closing.

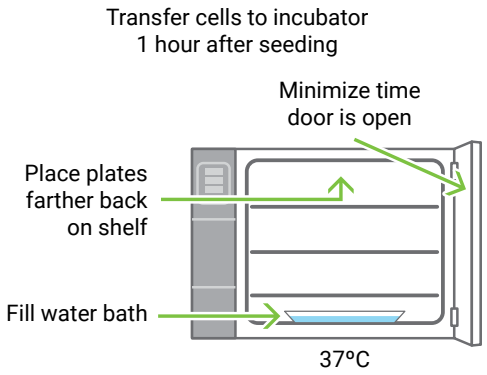


Figure 7. Transfer cells to a humidified incubator.

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