User Guide

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Washing Adherent Cells in Agilent Seahorse XF96 Cell Culture Microplates

Introduction

Before performing an Agilent Seahorse XF Assay, cell growth medium must be replaced with a suitable XF assay medium (generally this means medium without bicarbonate buffer or serum and with low/no phenol red content).

This procedure reflects best practices for replacing the growth medium with assay medium for adherent cells grown in XF96 Cell Culture Microplates prior to being assayed using an Agilent Seahorse XFe96/XF96 Analyzer.

For guidance on choosing and preparing the appropriate XF assay medium for your experiment, please see Preparation of XF Assay Media.

The importance of good cell washing technique

To generate high-quality data, it is necessary to maintain a consistent distribution of cells in the wells of the XF96 Cell Culture Microplate. Note that the cell monolayer does not need to be entirely (100%) confluent, rather the cells need to be evenly distributed in the well. Inconsistency in the cell monolayer caused by scraping or washing cells from the wells can result in variable rate data. Also note that normalization will not be effective in correcting this variability in situations of significant cell loss, particularly in cases in which portions of the monolayer have been entirely dislodged.

Basic procedure

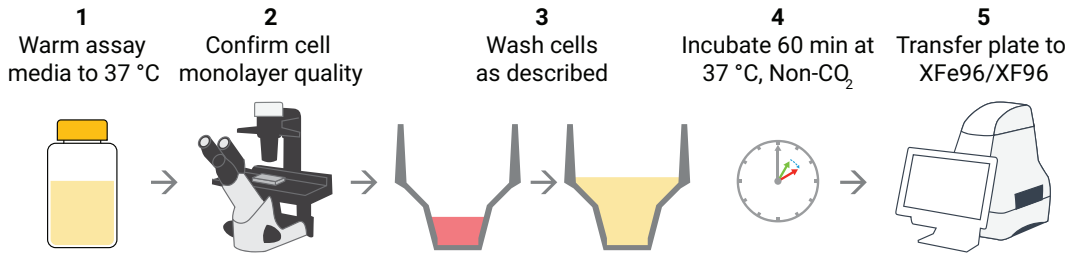


Figure 1. Basic procedure.

1. Prepare and warm the XF assay medium to 37 °C.
2. View the cells under the microscope to assess cell monolayer plating quality.
3. Remove all but 20 µL of the culture medium from each well using an 8- or 12-channel pipette (Figure 2).
   a. When removing media from the well, NEVER USE A VACUUM ASPIRATOR. It is difficult to control the volume removed and can easily damage the cell monolayer.
4. Wash cells two times (2x) with 200 µL of XF assay medium, leaving 20 µL behind after each wash.
5. After the second wash, add 160 µL of XF assay medium to each well for a final volume of 180 µL/well. Final volume in the well may vary depending on the XF Assay Kit/Reagent or application. Refer to the appropriate XF Assay Kit/Reagent or application guide.
   a. When performing the Agilent Seahorse XF Real-Time ATP Rate Assay and Agilent Seahorse XF Glycolytic Rate Assay, the second cell wash is delayed until cells have been incubated for 1 hour at 37 °C, non-CO_2, and immediately prior to starting the experiment (Figure 3).
   b. Note that this washing method can be applied to any XF assay.
6. View cells under the microscope to ensure that cells were not disturbed or washed away.
7. Incubate the cell plate at 37 °C without CO_2 for 60 minutes prior to the assay.

Standard cell washing method

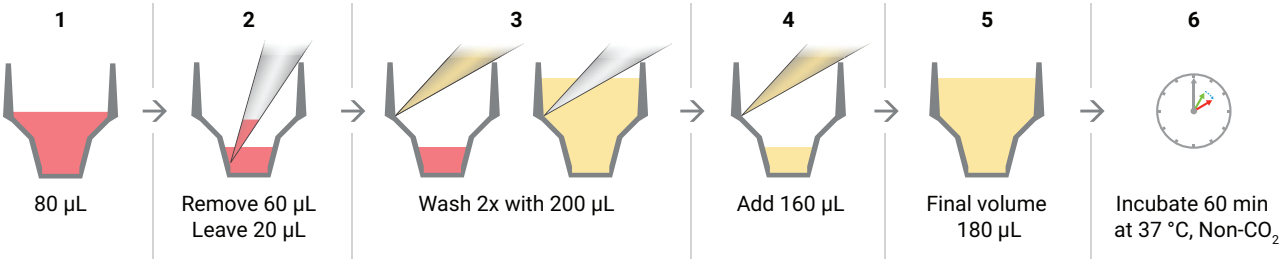


Figure 2. Cell wash workflow schematic – standard XF assay (not applicable to the XF Real-Time ATP Rate Assay and the XF Glycolytic Rate Assay. See Figure 3)

Modified cell washing method

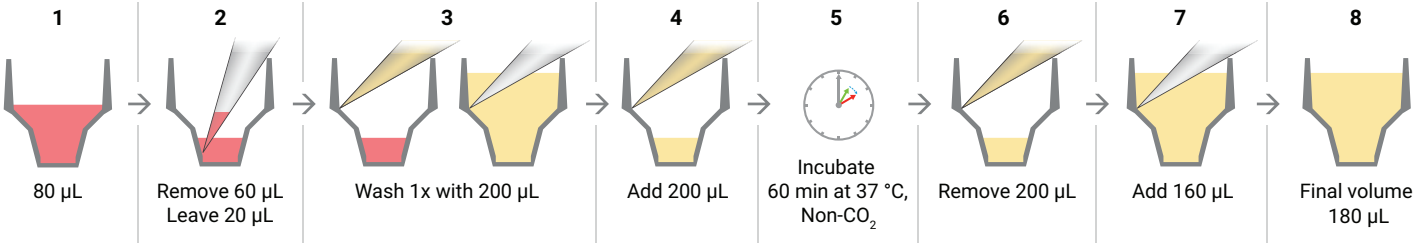


Figure 3. Cell wash workflow schematic – XF Real-Time ATP Rate Assay and XF Glycolytic Rate Assay.

Specific details and information

Prepare assay media

Warm the XF assay medium to 37 °C. For an XF96 plate, 100 mL of assay media is typically sufficient for cell washing and injection solution preparation. It is recommended to aliquot, supplement and heat only the amount of media required for the assay (Figure 4), as repeated warming of media can result in degradation of the components, especially glutamine.

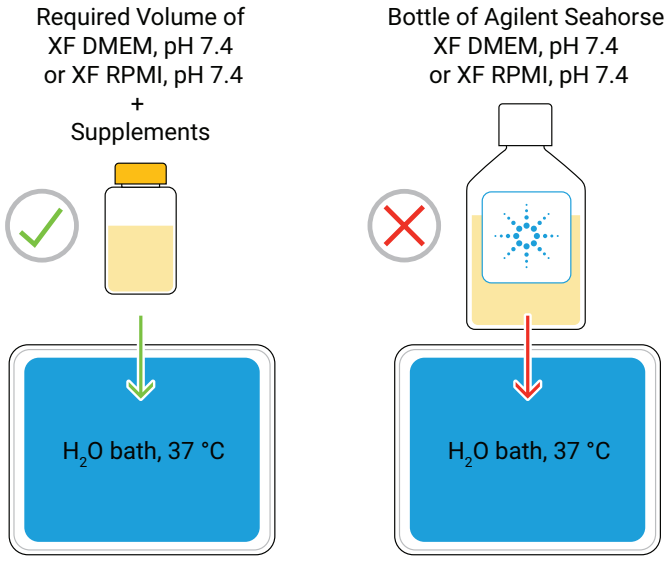


Figure 4. Incubation of prepared XF Assay Media.

Visual confirmation of cell quality

View the cells under the microscope to confirm cell monolayer quality.

1. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
2. Ensure cells are adhered, showing a consistent monolayer.
3. Make sure no cells were plated in the background correction wells. If cells are found in any of the background wells, be sure to un-assign them in the assay template.

If a situation arises in which ALL wells received cells, an attempt may be made to remove cells from wells A1, A12, H1, and H12 by scraping or trypsin treatment of these 4 wells only. Use a microscope to confirm complete removal of cells. Otherwise the plate should be discarded and a correctly seeded plate with correct background wells used.

Wash the cells – manipulating the plate

1. When removing media, ensure the cell plate rests on a stable surface, i.e., a lab bench or biosafety cabinet/tissue culture hood. This allows easy visualization of the wells and keeps the plate steady. Tilting the plate slightly may provide further control when removing media, as this results in the lowest, deepest point of each well to be at the well perimeter rather than the center of the well.
2. Gently slide the pipette tips down the wall to the bottom of the well to avoid bumping and scratching the monolayer. Gently touching the bottom at the edge is acceptable to ensure all tips are at the correct depth (Figure 5).

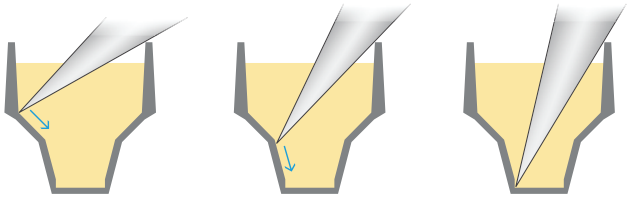


Figure 5. Slide pipette tips down the wall.

3. When adding media, the plate should rest on a flat surface. Hold the plate steady with one hand while operating the pipette.

Wash the cells - removing media

Slowly remove media. Removing the media at a slow or moderate pace:

1. Reduces the chances of introducing air bubbles.
2. Reduces the chances of exposing the cells to air.
3. Allows you to adjust if you find some tips were not placed deep enough into the well.

Hints

a. The minimum volume required to cover the well bottom and prevent exposure of cells to air is 20 µL (Figure 6). When removing media, ensure that the cells always remain covered. If the cells are exposed to air, gently dispense just enough media to cover the cell layer before discarding the remainder of the media.

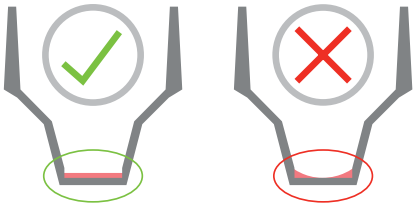


Figure 6. Minimum volume required is 20 µL.

b. A small amount of evaporation may occur with overnight incubation of a 96-well plate. This volume can be taken into account when removing growth media during the wash. For example, in an XF96 plate seeded as recommended using a volume of 80 µL/well, it can be assumed that after 24 hours ≈75 µL remain.

Seeding volume	80 µL
Evaporation volume	5 µL
Expected volume	75 µL
Volume to remove	55 µL

c. If bubbles are created during washing/dispensing, raise the pipette and slowly redispense the volume along the upper half of the well wall and repeat the aspiration step (Figure 7).

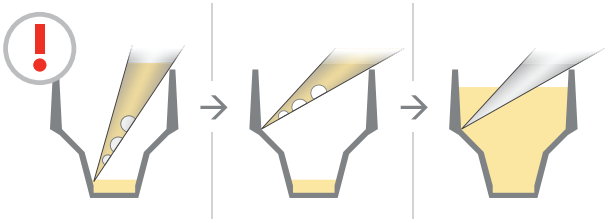


Figure 7. If bubbles are introduced during the washing/dispensing step.

d. When adding media, place the pipette tips high on the well wall and dispense gently (Figure 8). This minimizes disruption to the monolayer and reduces detachment of cells from the well surface, especially loosely adherent cells.

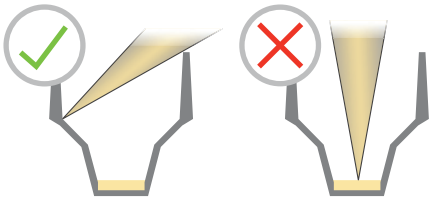


Figure 8. Pipette tip placement.

Check cells post-wash

Visualize cells under a microscope to ensure that cells were not disrupted or washed away. Note any wells with damaged monolayers to reference later if a data point is suspect. A BioTek Cytation can also be used to collect images during the pre-assay incubation and referenced later (Figure 9).

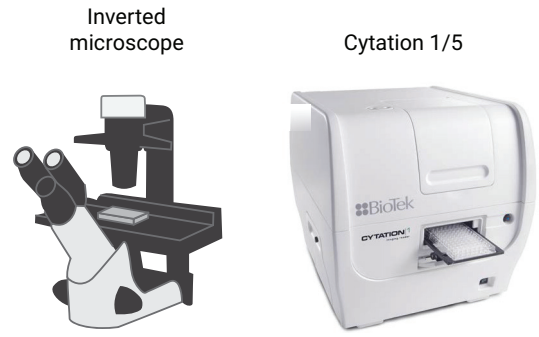


Figure 9. Post-wash inspection options.

For more information on using a Cytation with the XFe Analyzers to image well plates and to normalize XF data post assay see the Seahorse XF Imaging and Normalization System.

Incubate cells

Incubate the washed cells at 37 °C **without CO_2** for 60 minutes prior to the assay. This incubation period allows CO_2 outgassing from the XF Tissue Culture Microplate and is required for accurate ECAR measurements (Figure 10).

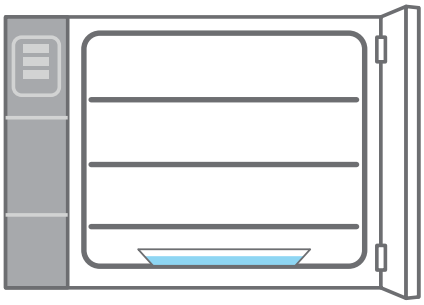


Figure 10. Incubate cells at 37 °C, 60 min, no CO_2.

Gentle Washing Option for Weakly Adherent Cells

For cells that are weakly adherent, partial media changes using smaller volumes for each wash can be performed to further protect the cell monolayer from damage. For example, remove no growth media before the first wash, leave a greater volume behind when aspirating and add additional rinses to dilute the original growth media. Example gentle washing methods are described in the workflow diagrams below (Figures 11 and 12).

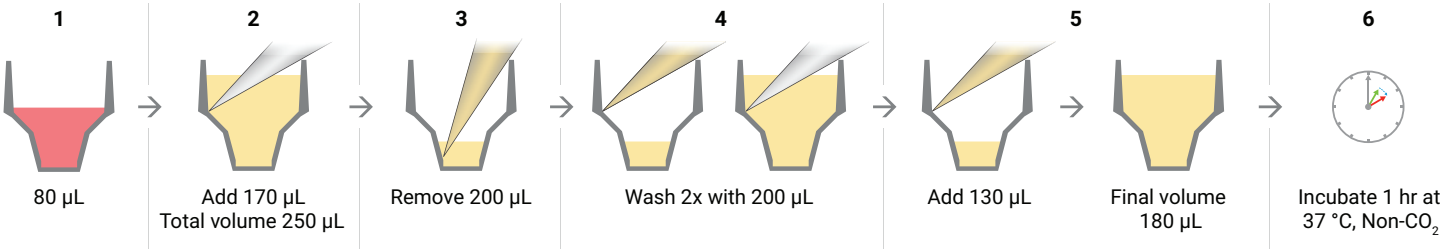


Figure 11. Gentle cell wash workflow schematic – standard assay. All except Real-Time ATP rate assay and Glycolytic Rate Assay.

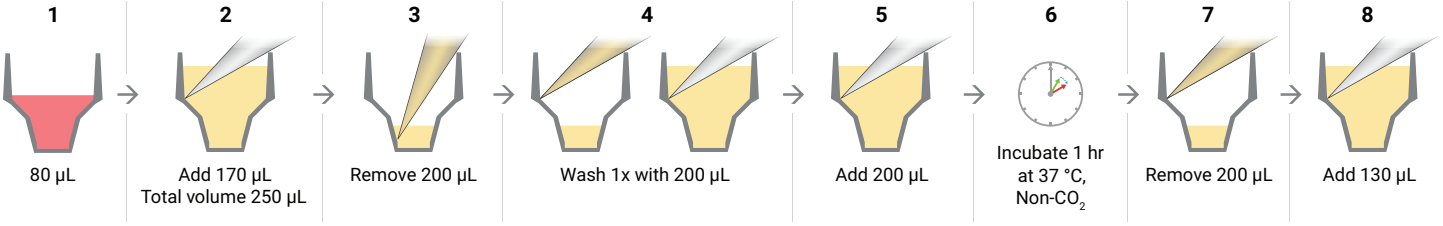
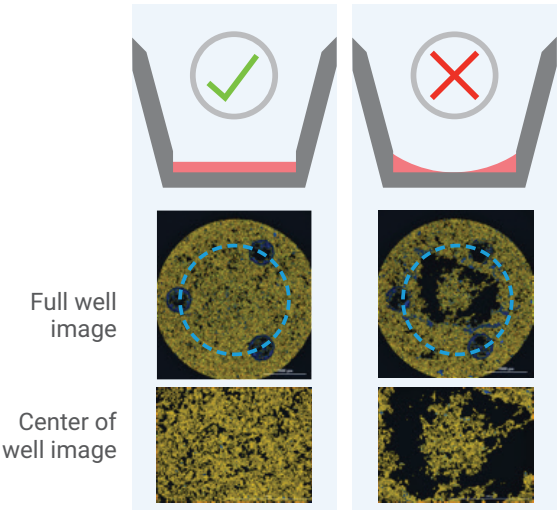
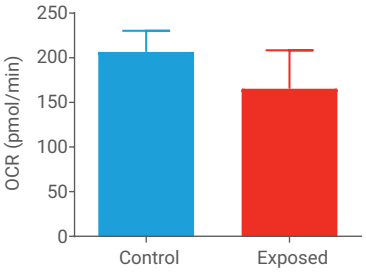


Figure 12. Gentle cell wash workflow schematic – Real-Time ATP rate assay and Glycolytic Rate Assay.

Examples: correct versus incorrect methods

1. Cells were exposed to air during the media change/wash.

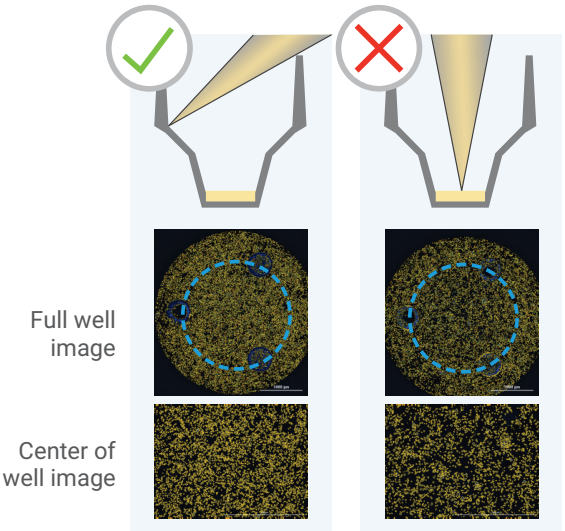


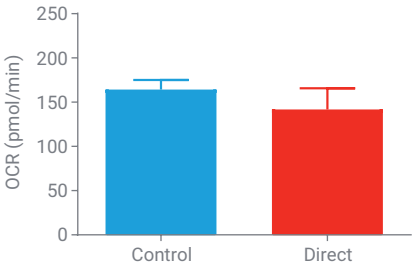


	Control	Exposed
Cell Count	42,031	31,220
OCR (pmol/min)		
Mean	207	166
STDEV	23.5	42.8
CV (%)	11.4	25.9

Figure 13. HCT116 seeded at a density of 2 × 10^4 cells per well, 24-hour incubation. When the cell monolayer was exposed during the cell wash, a significant number of wells suffered damage to the cell monolayer. As a result, the average OCR is lower and rates are significantly more variable compared to cells washed in which they were not exposed to air.

2. XF Assay media was added directly onto the monolayer (center of well) instead of along the side wall of the well.





	Control	Exposed
Cell Count	23,330	20,067
OCR (pmol/min)		
Mean	164.3	141.8
STDEV	10.8	24.0
CV (%)	6.6	16.9

Figure 14. H1975 seeded at a density of 2 × 10^4 cells per well, 24-hour incubation. When the XF Assay Media was added directly to the monolayer, weakly adherent HEK293 cells were washed off the well surface. As a result, the average OCR rates are significantly lower and more variable compared to cells washed using the proper pipetting technique.

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