Agilent Seahorse XFP T Cell Metabolic Profiling Kit

User Guide
Kit 103771-100

For use with Agilent Seahorse XF HS Mini or XFP Extracellular Flux analyzers and with T cells.
Notices

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300 Griffith Rd,
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Assay Background

The Agilent Seahorse XFp T Cell Metabolic Profiling kit is designed to enable the acquisition of robust bioenergetic measurements of both glycolytic and mitochondrial activity in T cell populations. These measurements can be associated with increased antitumor function of T cell therapy products. They are especially valuable in therapy development targeting to improve T cell persistence, or avoid exhaustion postactivation in a tumor microenvironment. The new kit allows you to obtain more accurate measurements of the maximal respiratory capacity of T cells, by using the uncoupler BAM15. BAM15 is less cytotoxic than other protonophore uncouplers, such as FCCP, and is especially suitable for sensitive cell types (such as naïve T cells). BAM15 also surpasses FCCP in its exhibition of a wider range of concentrations that induce maximum respiration, minimizing the needs of uncoupler optimization.

The kit supports two assay workflows.

XF T Cell Persistence assay

The Agilent Seahorse XF T Cell Persistence assay is developed to assess the ability of T cell products (such as CAR-T) to develop durable immune response after infusion, and to generate memory-like T cells. Several publications indicate that T cells with a highly oxidative basal metabolic phenotype (mitoATP/glycoATP production rate) and high spare respiratory capacity (SRC) exhibit increased persistence after adoptive transference. This assay can be used to evaluate different construct designs, engineering strategies, selection of starting material, or metabolic conditioning during the manufacture expansion process, to drive a metabolic phenotype that correlates with persistent T cells. The typical assay profile and data output is shown in Figure 1.

![Figure 1. Typical Agilent Seahorse XF T Cell Persistence assay profile and data output.](image)
XF T Cell Fitness assay

The Agilent Seahorse XF T Cell Fitness assay is developed to evaluate if T cells can meet the metabolic demand under altered or restrictive nutrient conditions. In this way, it assesses the capacity of T cells to maintain metabolic fitness in a tumor microenvironment. The nutrient-restrictive conditions can be created by using substrate pathway inhibitors (such as etomoxir, UK5099, BPTES), replacing glucose by other carbon sources, or by directly altering levels of metabolic substrates in the assay medium. In this assay, the basal ATP production rates from both respiration and glycolysis (metabolic poise), as well as the maximal respiratory capacity, are reported. The typical assay profile and data output are shown in Figure 2.

![Figure 2. Typical Agilent Seahorse XF T Cell Fitness assay profiles and data output.](image)

Templates for performing both XF T Cell Persistence and Fitness assays are provided on the XF T Cell Metabolic Profiling kit website. You can download the templates and upload to your Agilent Seahorse XF HS Mini or XFp analyzer. Examples of assay and controls groups, as well as instrument protocols, are shown in Figure 3.
Figure 3. Template designs for assays using the Agilent Seahorse XFp T Cell Metabolic Profiling kit.
Data analysis and reporting results of the XF T Cell Persistence and Fitness assays can easily be performed using Agilent Seahorse Analytics. A valid assay kit are required for use of these analysis views. Otherwise, relevant analysis views will not be accessible.

This user guide details how to use the XFp T Cell Metabolic Profiling kit with T cells, analyze assay data, and generate result reports.

Glossary

- **mitoATP production rate**: The rate of ATP production (expressed in pmol/min) associated with oxidative phosphorylation in the mitochondria. It is calculated by multiplying the oxygen consumption rate (OCR) signal between basal and oligomycin addition, with a P:O ratio. For details of the calculation, refer to the Agilent white paper by Romero et al.
- **glycoATP production rate**: The rate of ATP production (expressed in pmol/min) associated with the conversion of glucose to lactate in the glycolytic pathway. For details of calculation, refer to the Agilent white paper by Romero et al.
- **totalATP production rate**: The sum of mitochondrial and glycolytic ATP production rates in live cells under a specific assay condition. This parameter represents the energy demand or requirement for the cells under the conditions being tested.
- **Percent of ATP from glycolysis (basal)**: The ratio of glycoATP and totalATP.
- **Spare respiratory capacity**: The difference between the maximal OCR signal (obtained after exposure to an uncoupler), and the basal OCR signal. This measurement indicates the potential (capacity) of a cell to respond to increased energetic demand, as well as how closely the cell is respiring at its theoretical maximum. This parameter can be an indicator of cell fitness or flexibility.
- **Spare respiratory capacity (%)**: The ratio of the maximal respiration, obtained with the signal after exposure to an uncoupler and the signal after rotenone and antimycin A (Rot/AA) addition; and the basal respiration, obtained with the basal OCR signal and the signal after Rot/AA addition. The formula is: \( \frac{OCR_{\text{Max}} - OCR_{\text{Rot/AA}}}{OCR_{\text{Basal}} - OCR_{\text{Rot/AA}}} \).
- **Maximal respiration**: The difference between the maximal OCR (obtained after exposure to an uncoupler) and the OCR signal after Rot/AA addition. The uncoupler mimics a physiological “energy demand” by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, and amino acids) to meet this metabolic challenge. It shows the maximum rate of respiration that the cell can achieve.

References

Kit Information

Kit Contents

The Seahorse XFp T Cell Metabolic Profiling kit includes six foil pouches, and each contains reagents sufficient for a full-plate assay in an eight-well format. Every pouch includes one vial of the following compounds: oligomycin A, BAM15, and a mixture of rotenone/antimycin A (see Table 1).

Table 1  Agilent Seahorse XFp T Cell Metabolic Profiling kit contents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Function</th>
<th>Cap Color</th>
<th>Quantity Per Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomycin A</td>
<td>ATP synthase inhibitor</td>
<td>Blue</td>
<td>6.75 nmol</td>
</tr>
<tr>
<td>BAM15</td>
<td>Mitochondrial membrane uncoupler</td>
<td>White</td>
<td>12.5 nmol</td>
</tr>
<tr>
<td>Rotenone/Antimycin A</td>
<td>Complex I and III inhibitor (respectively)</td>
<td>Red</td>
<td>2.75 nmol each</td>
</tr>
</tbody>
</table>

Kit Shipping and Storage

The XFp T Cell Metabolic Profiling kit can be shipped at ambient temperature and stored at room temperature. The product is stable for one year from the date of manufacture. The actual expiration date is printed on the label of the assay kit box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 3 to 12 months.
Additional Required Items

The products in Table 2 are also required for performing Seahorse XFp T Cell Metabolic Profiling assays, but are not supplied with the kit.

<table>
<thead>
<tr>
<th>Item</th>
<th>Part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Seahorse XF HS Mini analyzer or XFp analyzer</td>
<td></td>
</tr>
<tr>
<td>Agilent Seahorse XFp FluxPak (PDL miniplates)†</td>
<td>103721-100</td>
</tr>
<tr>
<td>Agilent Seahorse XFp PDL Miniplates†</td>
<td>103722-100</td>
</tr>
<tr>
<td>Agilent Seahorse XF HS Mini FluxPak (PDL miniplates)†</td>
<td>103724-100</td>
</tr>
<tr>
<td>Agilent Seahorse XF HS PDL Miniplates†</td>
<td>103727-100</td>
</tr>
<tr>
<td>Agilent Seahorse XF RPMI Medium, pH 7.4‡</td>
<td>103576-100</td>
</tr>
<tr>
<td>Agilent Seahorse XF 1.0 M Glucose Solution</td>
<td>103577-100</td>
</tr>
<tr>
<td>Agilent Seahorse XF 100 mM Pyruvate Solution</td>
<td>103578-100</td>
</tr>
<tr>
<td>Agilent Seahorse XF 200 mM Glutamine Solution</td>
<td>103579-100</td>
</tr>
<tr>
<td>Agilent Reservoir, 12 column, polypropylene or</td>
<td>201280-100</td>
</tr>
<tr>
<td>Agilent Reservoir, 12 column, polypropylene, irradiated</td>
<td>204365-100</td>
</tr>
</tbody>
</table>

* Part numbers 103721-100 and 103724-100 contain Agilent Seahorse XFp cartridges and XFp or HS PDL miniplates required to perform XF assays.

† Part numbers 103722-100 and 103727-100 do not contain XFp cartridges, which are also required to perform XF assays. These part numbers are only needed when extra PDL miniplates are needed for optimizing seeding density.

‡ Agilent Seahorse XF RPMI medium can also be purchased together with the supplements listed in this table as bundled products (part number 103681-100). For a full list of all medium types and our recommendation for each assay kit, please refer to the Agilent Seahorse XF Media Selection Guide.
Optimal cell seeding density should be empirically determined for your cell type prior to the assay. Please refer to the “Frequently Asked Questions” section for recommendations. Additional information can also be found on the Agilent Cell Analysis Learning Center website.

The Agilent Life Science Publication Database is an excellent resource for finding information published in peer-reviewed research articles.
One Day Prior to Assay (Day 1)

Power up the XF HS Mini or XFp Analyzer to allow the temperature to stabilize overnight (minimum of 18 hours).

Prepare the sensor cartridge and HS or XFp PDL miniplate

1. Remove a three pack of the Agilent Seahorse XFp Extracellular Flux assay kit from the box. Remove the foil seal on the tub.
2. Place the sensor cartridge upside down next to the utility plate.
3. Fill each well of the utility plate with 200 µL of sterile, tissue culture-grade water.
4. Fill the moats around the wells (both sides) with water, using an eight-channel pipettor set to 200 µL (Figure 5). This will result in a 400 µL volume in each chamber (two tips can fit into each chamber). If no multichannel pipette is available, fill each chamber of the moat with 400 µL of water.

5. Lower the sensor cartridge onto the utility plate, submerging the sensors in the calibrant or water.
6. Verify that the calibrant or water level is high enough to keep the sensors submerged.
7. Place the cartridge/utility plate assembly in a 37 °C, non-CO₂ incubator overnight.
8. Transfer approximately 4 mL of calibrant solution to a 15 mL conical tube. Place it in a 37 °C, non-CO₂, nonhumidified incubator overnight to warm up the plate.
9. Remove a precoated Agilent Seahorse XF HS or XFp PDL miniplate from the box and place it in a 37 °C, non-CO₂, nonhumidified incubator overnight to warm up the plate.

NOTE

The XF HS PDL miniplate consists of a raised “ring” element in the center of each well, and is pre-assembled with a silicone cell-seeding mask. This allows performance of XF assays with significantly fewer cells per well, facilitating functional analysis of non-proliferative or limited amounts of cells. Please review the document Seeding Suspension Cells in Agilent Seahorse XF HS PDL Miniplates, prior to performing any XFp assays using the XF HS PDL miniplate, for detailed information about this miniplate.
Design and upload the assay template to the XF HS Mini or XFp analyzer

1. On a computer with internet access, download the default template(s) on the XF T Cell Metabolic Profiling Kit website.
2. Unzip the folder to retrieve the templates. If necessary, you may modify the templates using Wave Desktop.
3. Transfer the templates to a USB drive. Templates must reside on the root of the USB flash drive and not in a subfolder.
4. Insert the USB drive into the front USB port on the Seahorse XF HS Mini or XFp analyzer.
5. Click Settings, then click Template Management.
6. Click the USB tab, and locate the assay template(s) to upload.
7. Select the check box next to each assay template to be imported.
8. Click Import.
9. All imported assay templates are now available for selection from the Local tab.
Day of Assay (Day 2)

Prepare the cartridge

Complete hydration of the sensor cartridge by removing the water and adding 200 µL of prewarmed calibrant to each well in the utility plate. Incubate in a 37 °C, non-CO₂ incubator for 60 minutes.

Prepare the assay medium

1. Prepare standard Seahorse XF RPMI assay medium by supplementing 19.4 mL of Seahorse XF RPMI Medium, pH 7.4 with 1.0 mL each of Agilent Seahorse XF glucose, pyruvate, and glutamine solutions (10 mM, 1 mM, and 2 mM final concentrations in assay medium, respectively), as shown in Table 3. These are the recommended initial substrate conditions. However, the desired assay medium composition can be modified depending on the user needs.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XF RPMI Medium, pH 7.4</td>
<td>19.4</td>
<td>–</td>
</tr>
<tr>
<td>XF 1.0 M Glucose Solution</td>
<td>0.2</td>
<td>10.0</td>
</tr>
<tr>
<td>XF 100 mM Pyruvate Solution</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>XF 200 mM Glutamine Solution</td>
<td>0.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

2. Warm up to 37 °C.

**NOTE**

No pH adjustment to the assay medium is necessary when nonsaturating concentrations of substrates, listed in Table 3, are used for XF assay medium.
Prepare and seed T cells in the Seahorse HS or XFp PDL miniplates

Important: The procedure described below is also known as the Seahorse XFp assay suspension cell protocol. Before starting any experiments, please read the information in the “Frequently Asked Questions” section, which contains information about the best practices for handling cells, as well as examples of calculations, to ensure that optimal results are achieved.

1. Take a small sample of cell suspension and count the cells.

2. Calculate the total number of cells needed (desired number of cells per well multiplied by the number of wells to be seeded).

   For example: 100,000 cells per well × 6 wells = 6.0 × 10^5 cells).

3. Transfer the appropriate volume of cell suspension from the culture vessel to a conical tube. Preferentially, use 15 mL conical tubes for best cell recovery. Use multiple tubes if needed, then pool the cells after resuspending them following centrifugation.

4. Centrifuge the cells at room temperature at 200 ×g for 10 minutes. Calculate the volume required to resuspend the cell pellet. We recommend using a volume 20% smaller than the calculated volume to avoid over-diluting the sample.

5. Remove the supernatant carefully from the centrifuged conical tube using a pipette (do not aspirate, to minimize cell loss).

6. Resuspend the cells in warm assay medium. Take a small sample and perform the cell counting again. Adjust the volume to achieve the desired cell concentration as indicated in Table 4, for different types of cells and miniplates. In general, the XF HS PDL miniplate will usually accommodate one-third the number of cells that an XFp PDL miniplate requires.

7. Remove the warm XF HS PDL miniplate or XFp PDL miniplate from the incubator.

**NOTE**

It is recommended to allow at least 50% extra cells to account for the potential loss of cells during centrifugation and other steps.

Table 4 lists the potential starting cell densities as tested in Agilent laboratories. It is recommended to perform density optimization when you start working with your cells to ensure optimal results (also see the “Frequently Asked Questions” section).

<table>
<thead>
<tr>
<th>Plate Type</th>
<th>Cell Type</th>
<th>Cell Suspension (Total Cells/mL)</th>
<th>Seeding Volume (µL/Well)</th>
<th>Final Cell Density (Total Cells/Well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS PDL Miniplate</td>
<td>Naïve T Cell</td>
<td>2.3 × 10^6</td>
<td>30</td>
<td>7.0 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Activated T Cell</td>
<td>1.0 × 10^6</td>
<td>30</td>
<td>3.0 × 10^4</td>
</tr>
<tr>
<td>XFp PDL Miniplate</td>
<td>Naïve T Cell</td>
<td>4.0 × 10^5</td>
<td>50</td>
<td>2.0 × 10^5</td>
</tr>
<tr>
<td></td>
<td>Activated T Cell</td>
<td>2.0 × 10^5</td>
<td>50</td>
<td>1.0 × 10^5</td>
</tr>
</tbody>
</table>
3 Assay Workflow for XF HS Mini and XFp Analyzer
Prepare and seed T cells in the Seahorse HS or XFp PDL miniplates

8 Pipette 30 µL or 50 µL of cell suspension to wells B through G for XF HS or XFp PDL miniplates, respectively, using a single channel pipettor. The pipette tip must be placed at the bottom of the well and in the inner ring element of the wells on the XF HS PDL miniplate, to dispense properly (Figure 6). Do not add cells to wells A and H.

![Figure 6. The pipette tip (outlined in blue) is inserted to the bottom of each well to dispense cell suspension.](image)

9 Add 30 µL or 50 µL of assay medium to wells A and H (background wells) for XF HS or XFp PDL miniplates, respectively.

10 Fill the moats around the wells (both sides) with warm water or PBS, using an eight-channel pipettor set to 200 µL, as demonstrated in Figure 4.

11 Centrifuge gently (e.g., 200 ×g for 1 minute) to allow the cells to attach to the bottom of the wells. It is recommended to use a centrifuge break.

12 If XF HS PDL miniplate is used, follow the steps described in the document Seeding Suspension Cells in Agilent Seahorse XF HS PDL Miniplates to remove the silicone mask.

13 The final number of cells per well varies depending on the types of miniplates and cells used. (See Table 4.)

14 Gently add additional warm XF assay medium to achieve the optimal total starting volume, according to Table 5, which varies for different assay and plate types.

15 Incubate the miniplate at 37 °C in a non-CO₂ incubator for 45 to 60 minutes prior to the assay.

### Table 5  Standard cell seeding conditions for the different types of T cells and plates.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Plate Type</th>
<th>Seeding Volume (µL/Well)</th>
<th>Additional Assay Medium Volume (µL/Well)</th>
<th>Total Starting Volume (µL/Well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Without an Acute Injection</td>
<td>HS PDL miniplate</td>
<td>30</td>
<td>170</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>XFp PDL miniplate</td>
<td>50</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>Assay with an Acute Injection</td>
<td>HS PDL miniplate</td>
<td>30</td>
<td>145</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>XFp PDL miniplate</td>
<td>50</td>
<td>125</td>
<td>175</td>
</tr>
</tbody>
</table>

**NOTE** Seeding volume is critical for achieving a uniform distribution of cells within a well. Do not seed cells in a volume higher than what is recommended for each plate type before the centrifugation step (See Table 4).
Prepare the compound solutions and load the injection ports

**Important:** It is not necessary to perform titration experiment for the compounds provided in the kit when T cells are used. This is based on the results from the extensive tests performed with a wide panel of T cell types. Check the "Frequently Asked Questions" section for more guideline on working with other types of cells. Use the compounds on the same day that they are reconstituted. Discard any remaining compound solutions. Do not refreeze and reuse.

1. Remove one foil pouch from the Agilent Seahorse XFp T Cell Metabolic Profiling kit box.
2. Open the pouch. Remove all three vials with a gloved hand.
3. Add 0.5 mL of XF assay medium to each vial to resuspend the content (see Table 6). Vortex for 30 seconds, if needed, to ensure that the content dissolves completely.

**Table 6** Compound solutions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cap Color</th>
<th>Volume to Add (mL)</th>
<th>Solution Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomycin A</td>
<td>Blue</td>
<td>0.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Bam15</td>
<td>White</td>
<td>0.5</td>
<td>25</td>
</tr>
<tr>
<td>Rotenone/Antimycin A</td>
<td>Red</td>
<td>0.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

4. Load 25 µL of compound to each injection port, according to Table 7. For the location of the ports, refer to Figure 7.

**Table 7** Assay type, injection port, and volume recommendations for eight-well miniplates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Port Concentration</th>
<th>Assay Without Acute Injection</th>
<th>Assay With an Acute Injection</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Port</td>
<td>Loading Volume</td>
<td>Port</td>
<td>Loading Volume</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Port</td>
<td>Loading</td>
<td>Concentration</td>
</tr>
<tr>
<td>Test Compound</td>
<td>8 X</td>
<td>–</td>
<td>–</td>
<td>Port A</td>
</tr>
<tr>
<td>Oligomycin A</td>
<td>9 X</td>
<td>Port A</td>
<td>25 µL</td>
<td>Port B</td>
</tr>
<tr>
<td>BAM15</td>
<td>10 X</td>
<td>Port B</td>
<td>25 µL</td>
<td>Port C</td>
</tr>
<tr>
<td>Rotenone/Antimycin A</td>
<td>11 X</td>
<td>Port C</td>
<td>25 µL</td>
<td>Port D</td>
</tr>
</tbody>
</table>

**NOTE** Proper port-loading techniques can be found in Basic Procedure "Loading the Sensor Cartridge with Compounds", on the Agilent Cell Analysis Learning Center website. Please read the information prior to loading compounds. Ensure that the sensor cartridge is properly hydrated prior to use.

Figure 7. Location and numbering of injection ports on Agilent Seahorse XF sensor cartridges.
Running the XF T Cell Persistence or Fitness assay on the XF HS Mini analyzer or XFp analyzer

**NOTE**
If your assay template is not uploaded to the XF HS Mini or XFp analyzer, refer to section “Design and upload the assay template to the XF HS Mini or XFp analyzer” on page 13 to upload the assay template.

1. On the XF HS Mini analyzer or XFp analyzer Home view, click **Start** to display the list of available assay templates.
2. Select the assay template from the list of available templates on the **Local** tab. Click to open the template.
3. Review groups (cell types or conditions), plate layout map, and instrument protocol by using the menu on top of the XF HS Mini analyzer display, or by touching the arrow on the lower right corner on XFp analyzer display.
4. You can edit the assay result file name, add notes about the assay, and review alert notifications on the review and run display on XF HS Mini analyzer, or by clicking the arrow on the lower right corner on XFp analyzer display. You can also enter an email address to receive automated notifications from your analyzer when user interaction is required, and a copy of the assay result file when the assay has completed. An active internet connection is required for this feature.
5. Click **Start Run** when ready to start the assay.
6. When prompted, remove the cartridge lid and place the loaded sensor cartridge with the utility plate on the thermal tray on the analyzer. Ensure correct plate orientation and that the cartridge lid has been removed. Then, click **I'm Ready**. Calibration takes approximately 15 to 20 minutes.
7. After completing calibration, the screen will display the Load Cell Plate dialog. Click **Open Tray** to eject the utility plate and load the cell plate. *Ensure that the lid is removed from the cell plate before loading.*
8. Click **Continue** to run the assay.
Data Analysis

Seahorse Analytics is a web-based software platform that provides a simple, streamlined data analysis workflow for analyzing the XF T Cell Persistence and XF T Cell Fitness assay data.

After obtaining assay result files, it is recommended to perform a data quality review with either Wave Desktop software or in Seahorse Analytics, before moving further to analyzing the data. A data quality review is performed by manually examining the O₂ and pH level data for each well. Outliers, if any, can be excluded at this step.

Follow the steps below to use the relevant assay widgets in Seahorse Analytics:

1. After the assay is completed, transfer your assay result file to your office or personal computer using a USB drive or network drive.

2. To register or log in to your Seahorse Analytics account, visit: https://seahorseanalytics.agilent.com.

3. Import the assay result file to your account.

4. Click the three-dot menu to the right of the file on the Files view (only). Click Add Assay Kit.
5 In the pop-up dialog box, enter assay kit information (kit part number, lot number, and SW ID). Click **Validate**. This unlocks the XF T Cell Persistence Assay and XF T Cell Fitness Assay analysis views.

6 Click the result file to display the **Add View** window. If a data quality review has not been performed, it can be done by using the **Standard Graph** widgets. Outliers, if any, can be excluded at this step using the **Modify** function. For more information about this function, please visit the **Agilent Cell Analysis Learning Center**.

7 After the data quality review, open the **Add View** window again. Click the **XF T Cell Persistence Assay** analysis view or the **XF T Cell Fitness Assay** analysis view found under the Assay Kit Companion Views menu.

8 Select the desired analysis view(s) in the View Widgets window, then click **Add View**.

For additional information about using Seahorse Analytics, visit **Agilent Cell Analysis Learning Center** website.
Frequently Asked Questions

How should T cells be handled to maximize the chance of a successful assay?

Cell preparation is one of the most critical steps to ensure consistent and robust response. Here are some tips for you to consider:

- Never put your cells on ice and never spin cells in a cold centrifugation chamber. Metabolism is strongly affected by temperature changes. In general, lowered temperature will slow or alter metabolism and exert stress on cells, leading to reduced metabolic rates and higher assay variation.

- Warm up the PDL cell culture plate overnight prior to use. Plastic plates can absorb O₂ from the air. When the temperature increases, the O₂ absorbed in the plates is slowly released into the wells. This process can take hours to reach equilibrium. If cells are plated onto a cold PDL plate, the O₂ in the plate is then slowly released into the assay medium during the assay. This can interfere with assay signals, especially in the case when the OCR signal is in the low detection range.

- Ensure consistent cell counting by counting both total cells and viable cells. This is critical for achieving consistent assay results. Ensure that you obtain both the total cell number, as well as the viable cell number (or a percentage). A cell-counting protocol using the Agilent NovoCyte Quanteon flow cytometer has been developed. For more information, please contact the Agilent technical support team at: cellanalysis.support@agilent.com.

- Count cells twice: before centrifugation, and after resuspending cells in assay medium. The second counting in step 5 in Figure 8 is especially important to ensure accurate seeding density, and should not be omitted because some cells can be lost when discarding spent medium.

- Use total cell number (live + dead cells) to calculate the number of cells needed for each well. Both live and dead cells occupy space at the bottom of wells. Therefore, you should use total cell count to calculate medium volume and dilution factor for cell seeding, not using viable cell count. This is especially important when you have a sample with less than 90% viable cells. In this case, if you only consider viable cells, you may end up seeding too many cells per well, leading to high assay variability. Viable cell count is only useful in performing normalization of signals.

Figure 8. Suggested cell-preparation steps for cell seeding with T cells.
- Minimize the number of centrifugation steps during cell preparation. We recommend spinning cells only once during cell preparation. This means performing cell counting first to obtain the total number of cells you have, before spinning the cells. This will give you an idea about the volume of assay medium you will need to resuspend the pellet without over-diluting your cell suspension; avoiding spinning the cells again.

**Can I thaw frozen T cells and seed directly to XF PDL culture plates to perform XF assays on the same day?**

When working with frozen cells, it is recommended to thaw the cells in T cell maintenance medium overnight before using the cells to perform XF assays. This is because cellular metabolism takes time to recover. The overall signal can be low if overnight recovery is not implemented. If it is desired to obtain data from the cells right after thawing, it is recommended to perform a second assay with the same batch of cells after recovering in T cell maintenance medium overnight.

**Do you provide a tool for calculating the cells required at each of the cell-preparation steps?**

We do not provide a tool for this purpose. However, you can use Table 8 as an example of how to calculate the number of cells you will need for your assay.

**Table 8 Example calculations for preparing a cell suspension to seed six wells in an Agilent Seahorse XF HS PDL miniplate.**

| Desired seeding density and volume per well | 3.0 x 10^4 cells in 30 µL |
| Total number of cells needed for an experiment with 30 wells plus 50% extra | (3.0 x 10^4 cells x 6 wells) x 1.5 = 2.7 x 10^5 cells |
| Target cell density for the final cell suspension in assay medium (to be used in seeding plate) | 3.0 x 10^4 cells / 30 µL = 1.0 x 10^6 cells/mL |
| Cell count for cell suspension in growth medium (total cells) | 1.5 x 10^6 cells/mL |
| Amount of cell suspension needed | 1.0 x 10^6 cells / 1.5 x 10^6 cells/mL = 0.67 mL |
| Spin and carefully remove cell culture medium | |
| Recommended assay medium volume to resuspend cell pellet (= 70% of the theoretical volume assuming there is no loss of cells) | (1.0 x 10^6 cells) / 1.0 x 10^6 cells/mL x 70% = 0.7 mL |
| Cell count for cell suspension in assay medium (use 100 µL aliquot, dilute if needed) | 1.3 x 10^6 cells/mL |
| Cell viability | 92% |
| Total cells (live + dead) in 0.6 mL cell suspension (amount left after 100 µL is used for cell counting) | (1.3 x 10^6 cells/mL) x 0.6 mL = 0.78 x 10^6 cells |
| Additional assay medium needed to achieve target cell density for the final cell suspension | (0.78 x 10^6 cells / 1.0 x 10^6 cells) - 0.6 mL = 0.18 mL |
| Transfer 50 µL to each well | |
| Total cells per well (live + dead) | 3.0 x 10^4 cells |
| Cell number to use for normalization | (3.0 x 10^4 cells) x 0.92 = 2.76 x 10^4 cells/well |
Do I need to perform a cell density optimization experiment, and how?

Yes. We recommend a test, such as microscopic evaluation of the monolayer, to optimize cell-plating density when you first start performing XF assays. The seeding densities recommended in Table 4 serve only as a reference point, and you should plan testing with a couple of seeding densities above and below the recommended numbers. This is because different cell-counting methods used by different users or in different labs may yield different counting results, which could lead to suboptimal seeding density on the XF cell culture microplates. If cell density is below optimal, it may yield reduced signal level. In contrast, if a well is overloaded with cells, some of the cells cannot attach to the bottom of the well and can be pushed aside during the assay when compounds are injected. This can result in high well-to-well variation in assay results. In general, cells should be distributed evenly as a monolayer at the bottom of the plate after the centrifugation step, with some space between the cells (see Figure 9). The optimal cell density after the centrifugation step should show a cell distribution similar to Figure 9A, or slightly higher, and should never be more than 100% confluent (not denser than what is shown in Figure 9B).

![Figure 9](image-url)  
Figure 9. Microscopic view of optimal cell seeding densities: (A) Low end of optimal cell density. (B) High end of optimal seeding density.

Do I need to perform experiments to optimize the concentrations of injection compounds for different types of T cells?

Extensive tests have been performed with different types of human T cells (such as T naïve, T effector, or T memory) from separate donors, as well as mouse T cells. The results show that the recommended compound concentrations work well for all T cell types. Therefore, it is not necessary to perform concentration optimization experiments when working with T cells.

Can I use the XF T Cell Metabolic Profiling kit for other types of cells (non T cells)?

In theory, the kit should work for all types of cells. However, verification and optimization experiments (such as seeding density and modulator concentration) are required to use the kit with non T cells. Table 9 shows a suggested concentration range for preparing reagents, to perform a concentration optimization experiment. For information on how to optimize seeding density, refer to the Agilent Seahorse XF Learning Center.
Table 9  Recommended concentration range for injection compound optimization experiments.

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<th>Compounds</th>
<th>Assay Medium per Vial (mL)</th>
<th>Stock Concentration (µM)</th>
<th>Loading Solution</th>
<th>Fold in Port</th>
<th>Final Concentration (µM)</th>
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<td>Concentration (µM)</td>
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I cannot access Seahorse Analytics. What should I do?

If you experience any issues using Seahorse Analytics, please contact the Agilent Cell Analysis Support team, at: cellanalysis.support@agilent.com, to report your issue. You should also double-check your internet connection and/or web browser. Seahorse Analytics works best with Google Chrome, Safari (Apple), Mozilla Firefox, and Microsoft Edge. For any other issues preventing you from using Seahorse Analytics for data analysis, you can export your data to Microsoft Excel and GraphPad Prism to quantify and plot maximum responses and area under the curve data. For more information on how assay parameters are calculated, refer to the Agilent application note, 5994-4494EN.³
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