



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 or NK cells.

Notices

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Manual Part Number

5994-3494EN Rev. C

Edition

Second edition, March 2024

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Boulevard
Santa Clara, CA 95051



Manufactured by:
Agilent Technologies, Inc.
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Chicopee, MA 01022

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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 and NK cell populations. These measurements can be associated with increased antitumor function of cell therapy products. They are especially valuable in therapy development targeting to improve cell persistence and avoid exhaustion in a tumor microenvironment.^{1,2} The new kit allows you to obtain more accurate measurements of the maximal respiratory capacity of T cells and NK 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 and NK cells). BAM15 also surpasses FCCP in its exhibition of a wider range of concentrations that induce maximum respiration, minimizing the needs of uncoupler optimization.³

The kit supports multiple 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.³ This assay can be used to evaluate different construct designs, engineering strategies, selection of starting material, or metabolic conditioning during the manufacturing 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**.

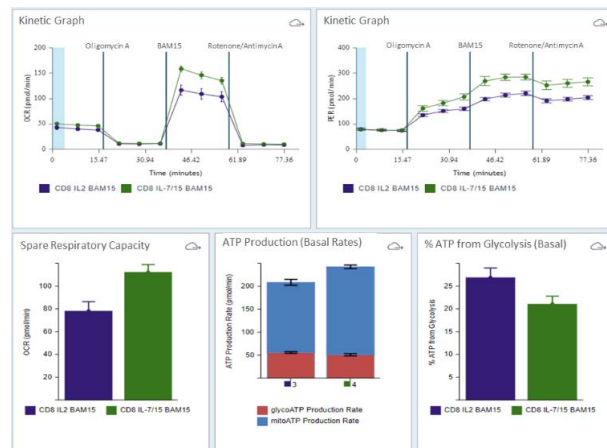


Figure 1. Typical Agilent Seahorse XF T Cell Persistence assay profile and data output.

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), by replacing glucose with 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**.

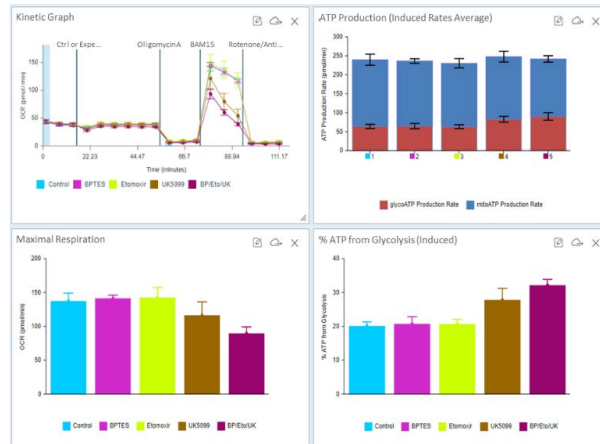


Figure 2. Typical Agilent Seahorse XF T Cell Fitness assay profiles and data output.

XF NK Cell Metabolic Profiling assay

Cell metabolism and especially glycolysis are important drivers for NK cell anti-tumor function. Using this kit, superior data and a comprehensive metabolic profile can be obtained for NK cells. An injection prior to the kit reagents allows for evaluation of acute interventions, like fuel inhibitors or metabolic modulators. The typical assay profile and data output is shown in **Figure 3**.

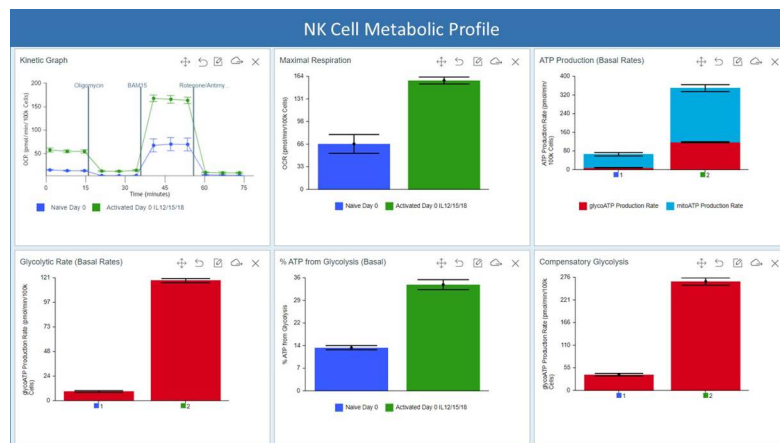


Figure 3. Typical Agilent Seahorse XF NK Cell Metabolic Profiling assay.

Templates are provided on the **XF T Cell Metabolic Profiling kit** website. You can download the templates and upload them 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 4**.

1 Introduction

XF NK Cell Metabolic Profiling assay

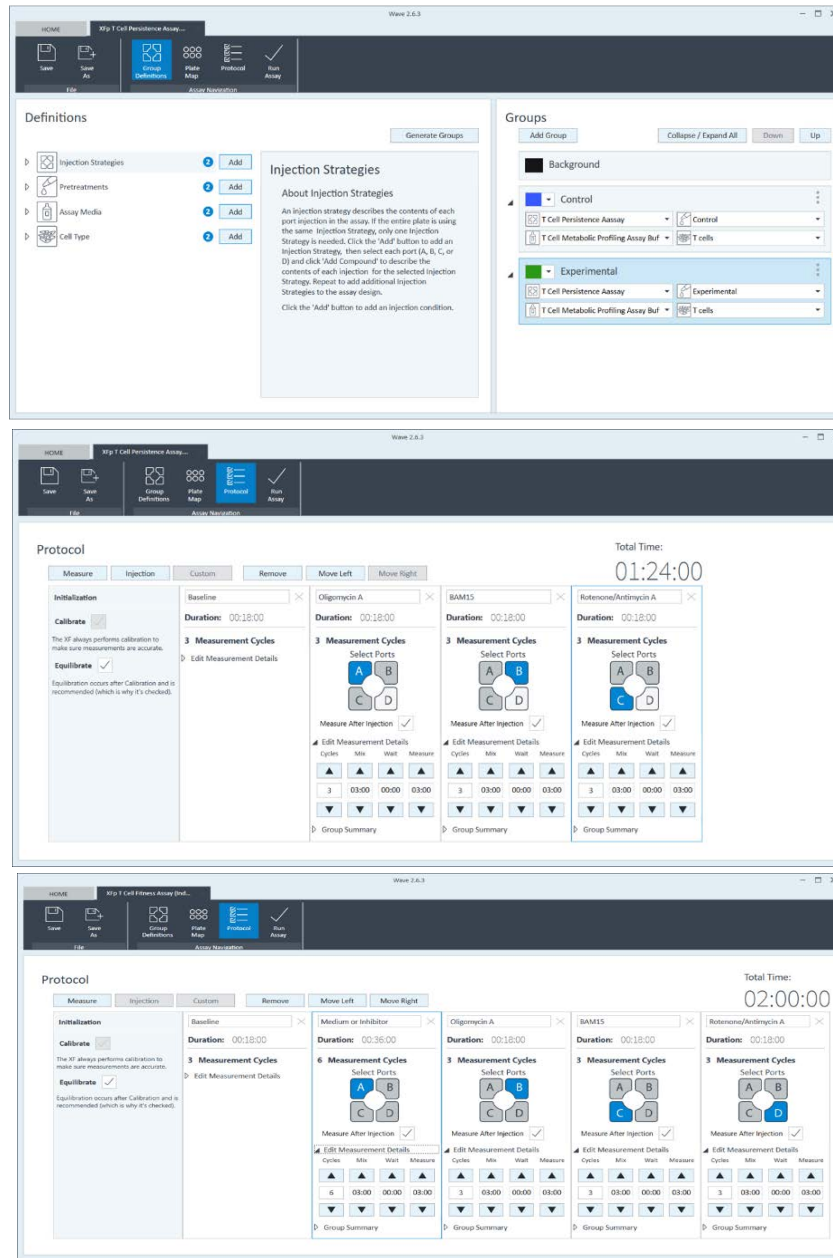


Figure 4. Template designs for assays using the Agilent Seahorse XFp T Cell Metabolic Profiling kit.

Data analysis and reporting results can easily be performed using Agilent Seahorse Analytics. A valid assay kit is 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 and NK cells, analyze assay data, and generate result reports.

Glossary

- **Compensatory glycolysis:** The rate of glycolysis in cells following the addition of mitochondrial inhibitors, driving compensatory changes in the cell to use glycolysis to meet the cells' energy demands.
- **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.*
- **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.
- **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.*
- **Percent of ATP from glycolysis (basal):** The ratio of glycoATP and totalATP production rates.
- **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 metabolic 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: $(OCR_{Max} - OCR_{Rot/AA}) \div (OCR_{Basal} - OCR_{Rot/AA})$.
- **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.

References

- 1 Kawalekar, O. U. Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells. *Immunity* **2016**, *44*, 380–390.
- 2 Van Bruggen, J. A. C. *et al.* Chronic Lymphocytic Leukemia Cells Impair Mitochondrial Fitness in CD8⁺ T Cells and Impede CAR T Cell Efficacy. *Blood* **2019**, *134(1)*, 44–58.
- 3 Walls, J. *et al.* Assessing T Cell Bioenergetic Poise and Spare Respiratory Capacity Using Extracellular Flux Analysis. *Agilent Technologies application note*, publication number: 5994-4494EN.

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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.

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

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 18 months 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 18 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 2 Additional required items.

Item	Part number
Agilent Seahorse XF HS Mini analyzer or XFp analyzer	
Agilent Seahorse XFp FluxPak (PDL miniplates)*	103721-100
Agilent Seahorse XF HS Mini FluxPak (PDL miniplates)*	103724-100
Agilent Seahorse XF RPMI Medium, pH 7.4†	103576-100
Agilent Seahorse XF 1.0 M Glucose Solution	103577-100
Agilent Seahorse XF 100 mM Pyruvate Solution	103578-100
Agilent Seahorse XF 200 mM Glutamine Solution	103579-100
Agilent Reservoir, 12 column, polypropylene or Agilent Reservoir, 12 column, polypropylene, irradiated	201280-100 or 204365-100

* HS PDL miniplates are suitable for experiments with limited cells. XFp PDL miniplates are suitable for experiments with abundant cell availability.

† Agilent Seahorse XF RPMI medium can also be purchased together with the supplements listed in this table as bundled products (part number 103681-100).

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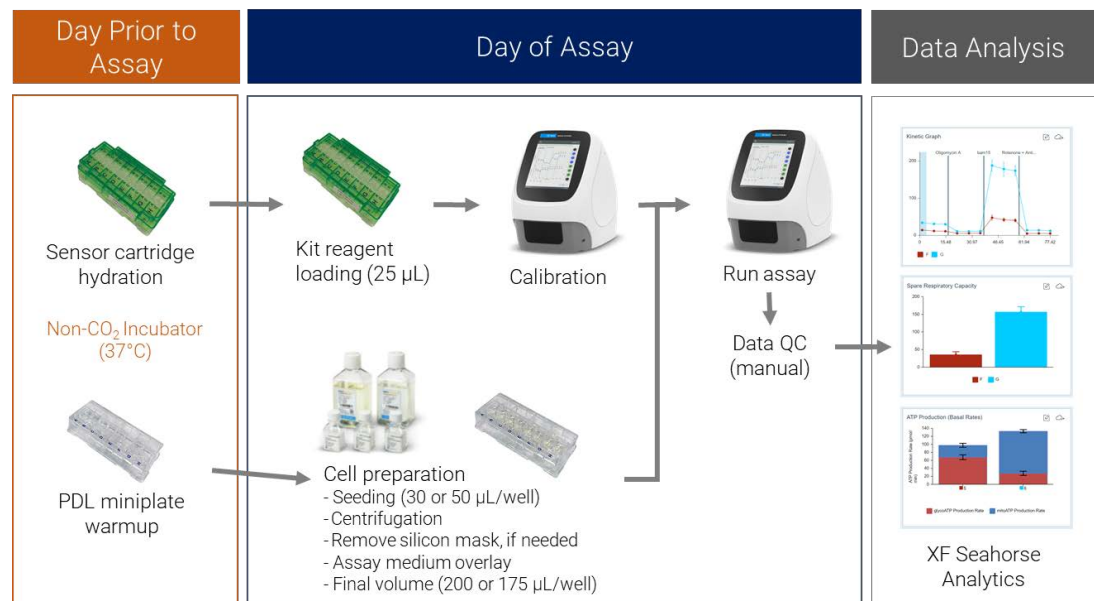


Figure 5. Agilent Seahorse XF T Cell Metabolic Profiling Kit with the Agilent Seahorse XF HS Mini and XFp Analyzer.

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 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 μ L 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 μ L (**Figure 6**). This will result in a 400 μ L 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 μ L of water.



Figure 6. An eight-channel P200 pipettor is used to fill the moat on the utility plate.

- 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.
- 9 Remove a precoated Agilent Seahorse XF HS or Seahorse 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.

3 Assay Workflow for XF HS Mini and XFp Analyzer

Design and upload the assay template to the XF HS Mini or XFp analyzer

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 μ L 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 0.2 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 3 Agilent Seahorse XF RPMI assay medium.

Component	Volume (mL)	Final Concentration (mM)
XF RPMI Medium, pH 7.4	19.4	–
XF 1.0 M Glucose Solution	0.2	10.0
XF 100 mM Pyruvate Solution	0.2	1.0
XF 200 mM Glutamine Solution	0.2	2.0

- 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 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, counting cells, and normalizing data, 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.

NOTE

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

- 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. See **Using the Agilent NovoCyte Flow Cytometer for Immune Suspension Cell Normalization in Agilent Seahorse XF Assays** for more detailed information.

NOTE

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 4 Starting cell seeding conditions for the different types of cells and plates.

Plate Type	Cell Type	Cell Suspension (Total Cells/mL)	Seeding Volume (µL/Well)	Final Cell Density (Total Cells/Well)
HS PDL Miniplate	Naïve T Cell	1.7×10^6 to 2.3×10^6	30	5.0×10^4 to 7.0×10^4
	Activated T Cell	6.7×10^5 to 1.2×10^6	30	2.0×10^4 to 3.5×10^4
	NK Cell	1.7×10^6 to 2.7×10^6	30	5.0×10^4 to 8.0×10^4
	Stimulated NK Cell	6.7×10^5 to 1.2×10^6	30	2.0×10^4 to 3.5×10^4
XFp PDL Plate	Naïve T Cell	3.0×10^6 to 4.0×10^6	50	1.5×10^5 to 2.0×10^5
	Activated T Cell	1.2×10^6 to 2.0×10^6	50	6.0×10^4 to 1.0×10^5
	NK Cell	3.0×10^6 to 5.0×10^6	50	1.5×10^5 to 2.5×10^5
	Stimulated NK Cell	1.0×10^6 to 2.0×10^6	50	5.0×10^4 to 1.0×10^5

3 Assay Workflow for XF HS Mini and XFp Analyzer

Prepare and seed cells in the Seahorse HS or XFp PDL miniplates

- 7 Remove the warm XF HS PDL miniplate or XFp PDL miniplate from the incubator.
- 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 7**). **Do not** add cells to wells A and H.

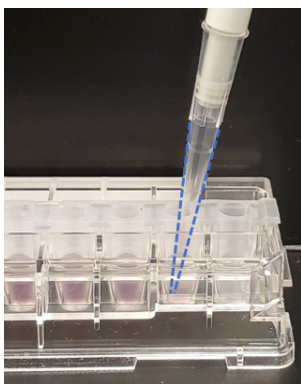


Figure 7. The pipette tip (outlined in blue) is inserted to the bottom of each well to dispense cell suspension.

- 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 5**.

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).

- 11 Centrifuge gently (e.g., 200 $\times g$ 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.

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

Assay Type	Plate Type	Seeding Volume ($\mu\text{L}/\text{Well}$)	Additional Assay Medium Volume ($\mu\text{L}/\text{Well}$)	Total Starting Volume ($\mu\text{L}/\text{Well}$)
Assay Without an Acute Injection	HS PDL miniplate	30	170	200
	XFp PDL miniplate	50	150	200
Assay with an Acute Injection	HS PDL miniplate	30	145	175
	XFp PDL miniplate	50	125	175

- 15 Incubate the miniplate at 37 $^{\circ}\text{C}$ in a non- CO_2 incubator for 45 to 60 minutes prior to the assay.

3 Assay Workflow for XF HS Mini and XFp Analyzer

Prepare the compound solutions and load the injection ports

Prepare the compound solutions and load the injection ports

Important: It is not necessary to perform titration experiments for the compounds provided in the kit when T cells or NK cells are used. This is based upon testing with a panel of NK cell and T cell types.³ 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.

Compound	Cap Color	Volume to Add (mL)	Solution Concentration (μM)
Oligomycin A	Blue	0.5	13.5
Bam15	White	0.5	25
Rotenone/Antimycin A	Red	0.5	5.5

- 4 Load 25 μL of compound to each injection port, according to **Table 7**. For the location of the ports, refer to **Figure 8**.

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

Compound	Port Concentration	Assay Without Acute Injection		Assay With an Acute Injection		Final Concentration
		Port	Loading Volume	Port	Loading Volume	
Starting Volume	–	200 μL		175 μL		–
Test Compound	8 X	–	–	Port A	25 μL	1 X
Oligomycin A	9 X	Port A	25 μL	Port B	25 μL	1.5 μM
BAM15	10 X	Port B	25 μL	Port C	25 μL	2.5 μM
Rotenone/ Antimycin A	11 X	Port C	25 μL	Port D	25 μL	0.5 μM each

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.

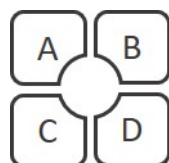


Figure 8. Location and numbering of injection ports on Agilent Seahorse XF sensor cartridges.

Running the 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.

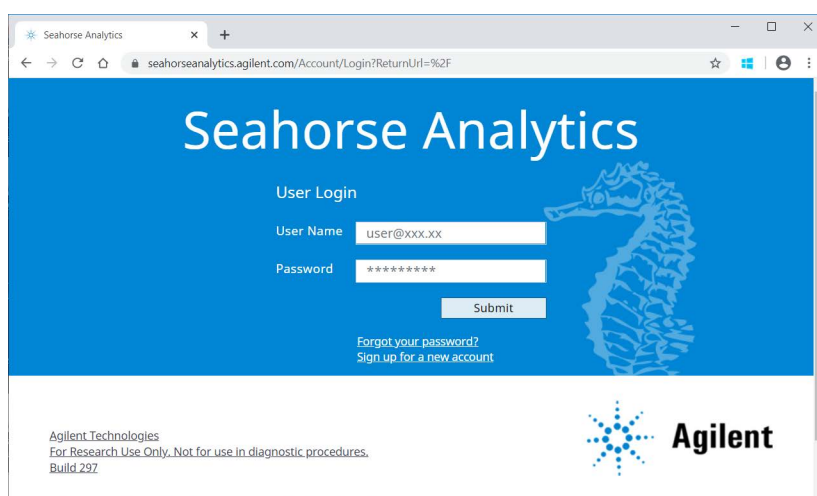
Data Analysis

Seahorse Analytics is a web-based software platform that provides a simple, streamlined data analysis workflow.

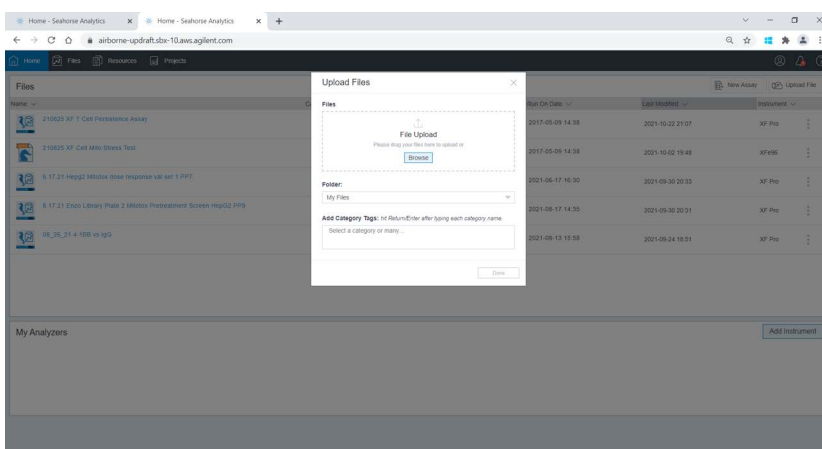
After obtaining assay result files, it is recommended to perform a data quality review with either Wave Desktop software or in Seahorse Analytics, before moving further to analyzing the data. A data quality review is performed by manually examining the O₂ and pH level data for each well. Outliers, if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

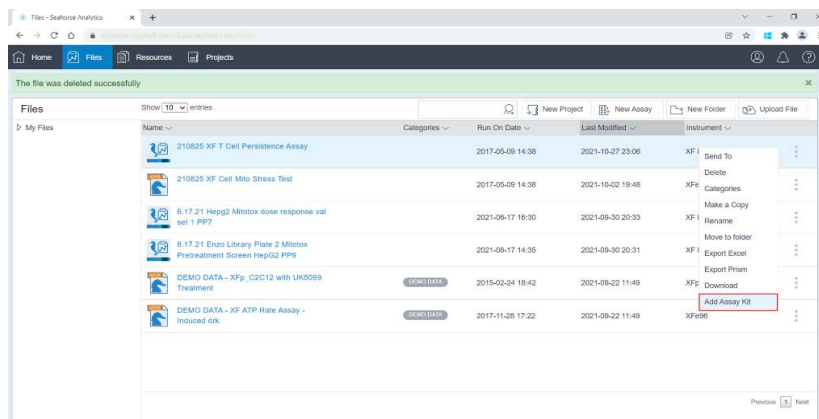


- 3 Import the assay result file to your account.

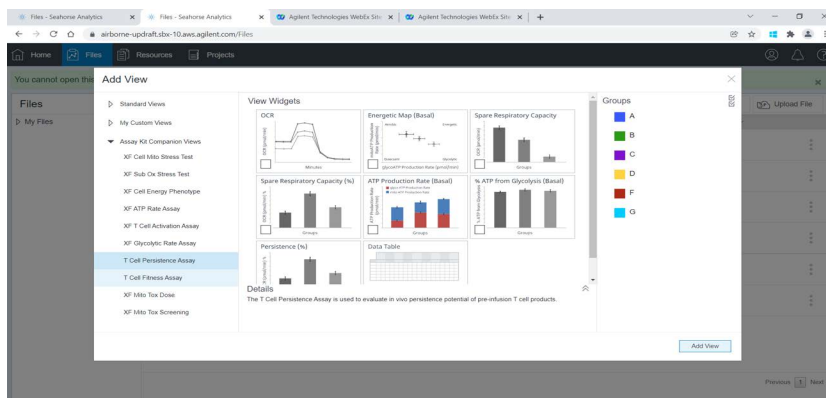


- 4 Click the three-dot menu to the right of the file on the Files view (only). Click **Add Assay Kit**.

3 Assay Workflow for XF HS Mini and XFp Analyzer Data Analysis



- 5 In the pop-up dialog box, enter assay kit information (kit part number, lot number, and SW ID). Click **Validate**. This unlocks the 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, the **XF T Cell Fitness Assay** analysis view, or the **XF NK Cell Metabolic Profile** analysis view.
- 8 Select the desired analysis view(s) in the View Widgets window, then click **Add View**.



For additional information about using Seahorse Analytics, visit the [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). See [Using the Agilent NovoCyte Flow Cytometer for Immune Suspension Cell Normalization in Agilent Seahorse XF Assays](#) for more detailed information.
- Count cells twice: before centrifugation, and after resuspending cells in assay medium. The second counting in step 5 in [Figure 9](#) is especially important to ensure accurate seeding density, and should not be omitted because some cells can be lost when discarding spent medium.

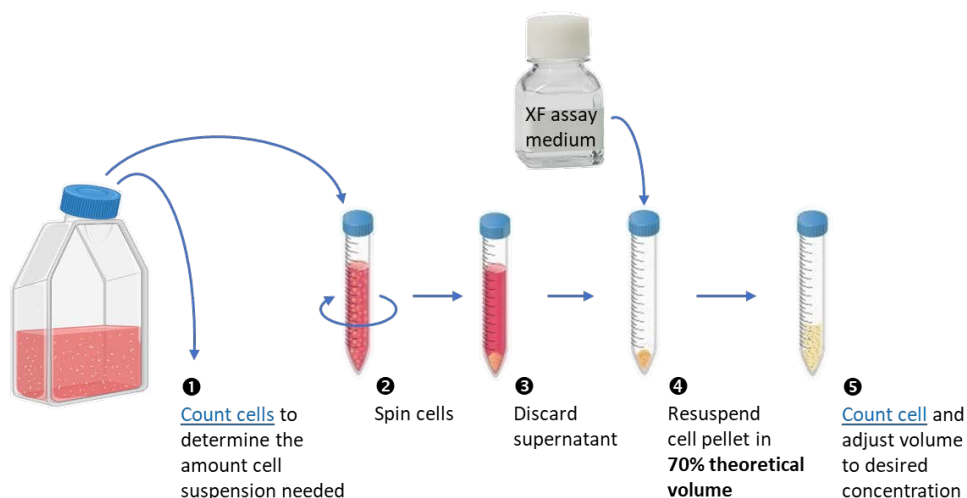


Figure 9. Suggested cell-preparation steps for cell seeding with T cells.

- 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 the 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.

4 Frequently Asked Questions

- 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 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 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 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	3.0×10^4 cells in 30 μ L
Total number of cells needed for an experiment with 30 wells plus 50% extra	$(3.0 \times 10^4 \text{ cells} \times 6 \text{ wells}) \times 1.5 = 2.7 \times 10^5$ cells
Target cell density for the final cell suspension in assay medium (to be used in seeding plate)	$3.0 \times 10^4 \text{ cells} / 30 \mu\text{L} = 1.0 \times 10^6$ cells/mL
Cell count for cell suspension in growth medium (total cells)	
	1.5×10^6 cells/mL
Amount of cell suspension needed	
	$1.0 \times 10^6 \text{ cells} / 1.5 \times 10^6 \text{ cells/mL} = 0.67 \text{ mL}$
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)	
	$(1.0 \times 10^6 \text{ cells}) / 1.0 \times 10^6 \text{ cells/mL} \times 70\% = 0.7 \text{ mL}$
Cell count for cell suspension in assay medium (use 100 μ L aliquot, dilute if needed)	
	1.3×10^6 cells/mL
Cell viability	
	92%
Total cells (live + dead) in 0.6 mL cell suspension (amount left after 100 μ L is used for cell counting)	
	$(1.3 \times 10^6 \text{ cells/mL}) \times 0.6 \text{ mL} = 0.78 \times 10^6$ cells
Additional assay medium needed to achieve target cell density for the final cell suspension	
	$(0.78 \times 10^6 \text{ cells} / 1.0 \times 10^6 \text{ cells}) - 0.6 \text{ mL} = 0.18 \text{ mL}$
Transfer 50 μ L to each well	
Total cells per well (live + dead)	
	3.0×10^4 cells
Cell number to use for normalization	
	$(3.0 \times 10^4 \text{ cells}) \times 0.92 = 2.76 \times 10^4$ 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 10](#)). The optimal cell density after the centrifugation step should show a cell distribution similar to [Figure 10A](#), or slightly higher, and should never be more than 100% confluent (not denser than what is shown in [Figure 10B](#)).

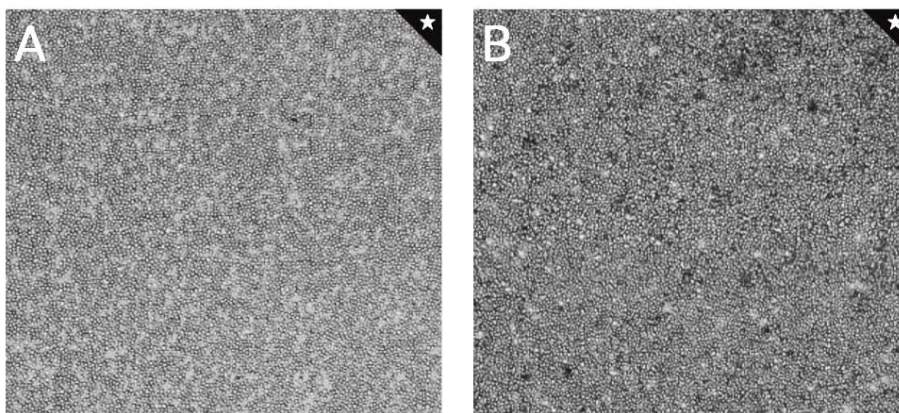


Figure 10. Microscopic view of optimal cell seeding densities: (A) Low end of optimal cell density. (B) High end of optimal seeding density.

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 and NK cells from separate donors, as well as mouse T cells and NK cells. The results show that the recommended compound concentrations work well for all T cell types and NK cell types. Therefore, it is not necessary to perform concentration optimization experiments when working with T cells or NK cells.

Can I use the XF T Cell Metabolic Profiling kit for other types of cells (non-T cells or NK 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.

Compounds	Assay Medium per Vial (mL)	Stock Concentration (μM)	Loading Solution			Fold in Port	Final Concentration (μM)
			Stock Solution (μL)	Assay Medium (μL)	Concentration (μM)		
Oligomycin A	250	27	40	80	4.5	9x	1.0
			60	60	13.5		1.5
			80	40	18.0		2.0
BAM15	416	30	40	80	10.0	10x	1.0
			60	60	15.0		1.5
			80	40	20.0		2.0
			100	20	25.0		2.5
			120	0	30.0		3.0
Rotenone/ Antimycin A	250	11	30	90	2.75	11x	0.25
			60	60	5.5		0.5
			90	30	8.25		0.75

Why is it important to ensure an optimal seeding density? How can I normalize my suspension cell data between experiments?

To obtain robust XF data, suspension cells need to be seeded in an optimal density range. This range must produce measurements within the recommended range of the instrument, and also provide an even distribution of the cells on the surface of the cell culture plate. Lower than optimal seeding densities may yield a signal that is below the limit of detection of the instrument, while higher than optimal seeding densities can result in increased well-to-well variation of the assay results. Using the Agilent NovoCyte Flow Cytometer to count live cells at several points during the cell seeding workflow allows for optimal seeding and for normalization of XF data between experiments.

See [Using the Agilent NovoCyte Flow Cytometer for Immune Suspension Cell Normalization in Agilent Seahorse XF Assays](#) for more detailed information.

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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RA44560.4251041667

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Second edition, March 2024

5994-3494EN Rev. C

