Agilent

Agilent Seahorse XF Hu T Cell Activation Assay Kit

User Guide

Agilent Seahorse XF Hu T Cell Activation Assay Kit, 200 tests (p/n 103759-100)
Agilent Seahorse XF Hu T Cell Activation Assay 96-Well Pack, 200 tests (p/n 103766-100)

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Notices

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Contents

1 Introduction

Assay Background

A rapid increase in glycolytic activity is an early signature event of T cell activation and is required for subsequent T cell propagation and differentiation.[1-3] It has been demonstrated that cellular glycolytic activity is a robust measure of T cell activation.[4] Agilent Seahorse XF technology is a live cell analysis platform for measurement of cellular bioenergetic metabolism *in vitro*. With the XF platform, cellular glycolytic activity is assessed by measuring proton efflux rates (PER).[5]

The Agilent Seahorse XF T Cell Activation Assay is designed to measure T cell activation by monitoring glycolytic responses induced by activators. This rapid assay presents an early window to discriminate activation responses that typically cannot be achieved by orthogonal methods that measure gene expression, cell proliferation, or cytokine production.[1,3] The Seahorse XF T Cell Activation Assay also provides kinetic information for monitoring and modulating of T cell activation in real-time with live cells, and can be used to compare different T cell subtypes and assess pharmaceutical or genetic interventions. In the Seahorse XF T Cell Activation Assay, T cell activation is achieved by the co-stimulation of CD3 and CD28 surface ligands, a commonly used method for *in vitro* T cell activation (**Figure 1**).

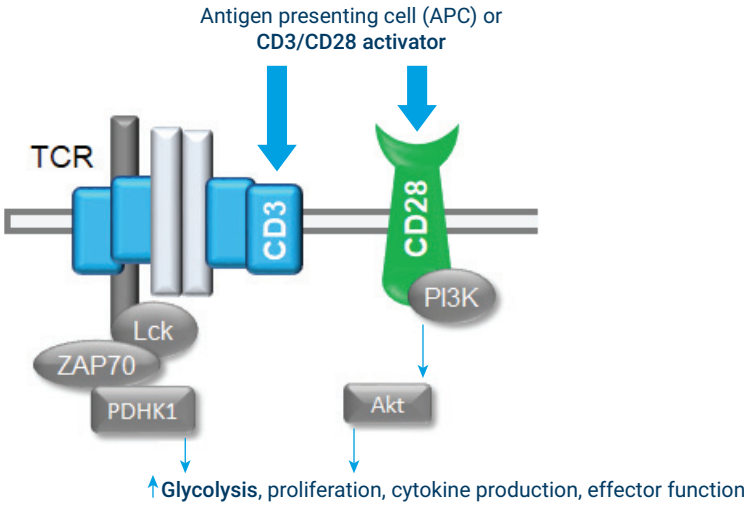


Figure 1. Glycolysis increase induced by T cell activation.

The Seahorse XF Hu T Cell Activation Assay Kit is designed for use with human (Hu) T cells and contains two reagents: ImmunoCult Human CD3/CD28 T Cell Activator and 2-deoxy-D-glucose (2-DG). **ImmunoCult Human CD3/CD28 T Cell Activator** (STEMCELL Technologies, Inc.) consists of soluble tetrameric antibody complexes that bind to CD3 and CD28 cell surface ligands, providing the required primary and co-stimulatory signals for T cell activation. 2-DG is a glucose analog that interrupts glycolysis via competitive inhibition, which abrogates the T cell activation-associated glycolysis increase. It serves as a negative assay control in the Seahorse XF T Cell Activation Assay.

The XF T Cell Activation Assay supports two assay designs: the standard assay for testing T cell activation and the modulation assay for investigating immediate modulator effects (**Figure 2**). The standard assay includes two injections: the activator injection followed by a 2-DG injection (Figure 2A). It is typically used to compare activation potential among T cells from different donors or T cells with applied interventions (e.g., genetic manipulation and/or chronic compound exposure). The modulation assay incorporates an additional injection of test compound(s) prior to the activator injection (Figure 2B). This design allows testing and monitoring of the acute effects of test compound(s) on T cell activation potential.

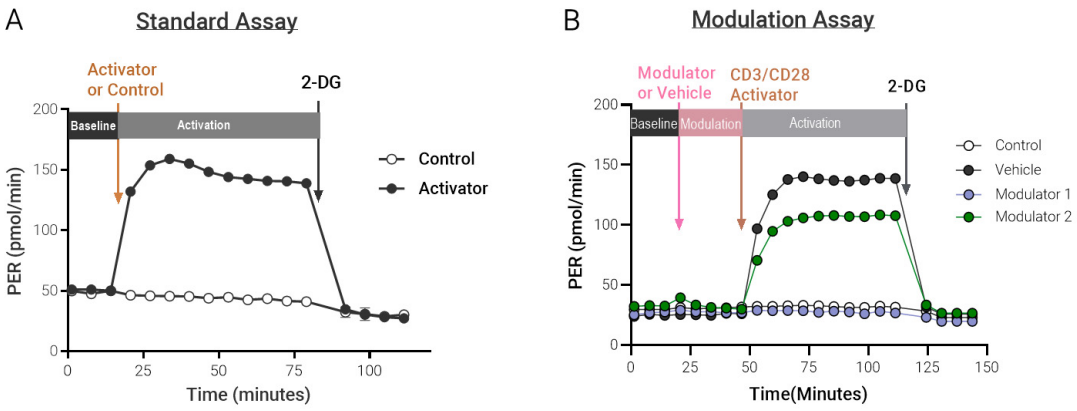
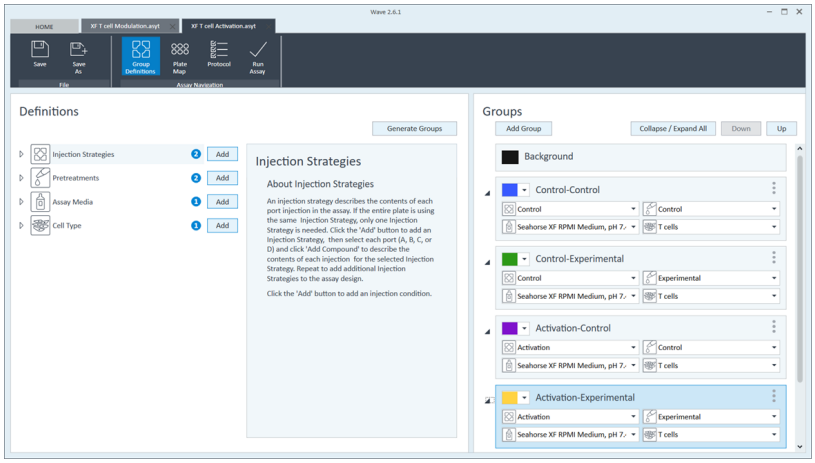


Figure 2. Example kinetic profiles of Agilent Seahorse XF Hu T Cell Activation Assay illustrating the workflows for the standard assay (2A) and the modulation assay (2B). The activation signal is expressed as absolute PER (pmol/min) or the percent of PER relative to the basal rate right before the activator or compound injection. The % PER signal can help to normalize potential variation in cell numbers between wells and provide consistent results.

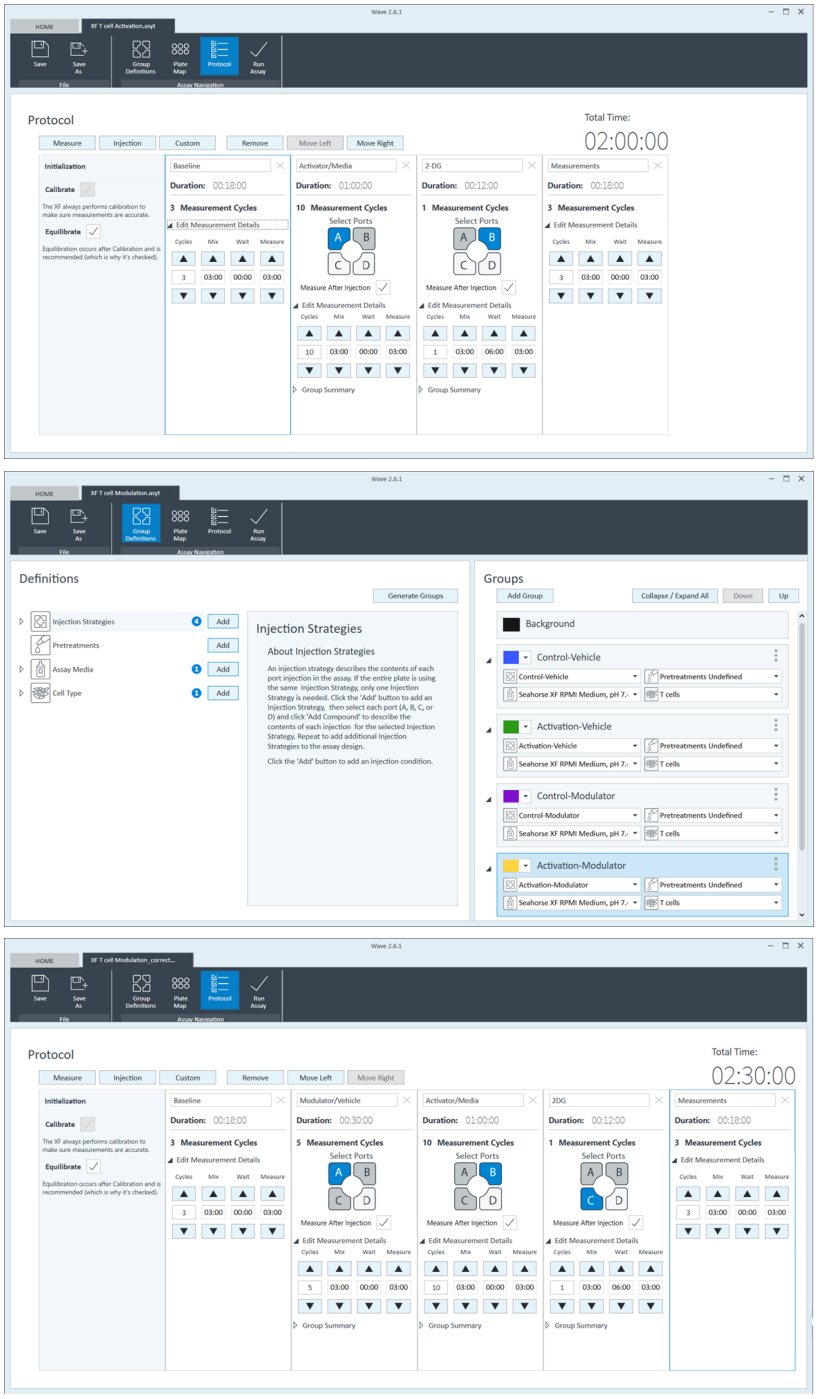
Both standard and modulation assays can be performed using the respective **XF T Cell Activation Assay template** designs, shown in **Figure 3**. These default template designs for standard assay (Figure 3A and 3B) and modulation assay (Figure 3C and 3D) measure PER for 10 cycles after the activator injection and 4 cycles after 2-DG injection. After the injection of 2-DG, a six-minute wait period is included in the instrument command protocols prior to the subsequent measurement command, which prevents any potential transient perturbation in ECAR measurements associated with the injection of 2-DG.



A

Starting group definitions in the standard assay template for XFe96 analyzer.

Here, the user can define the type of cells or pretreatments for control and activation groups.



B

Instrument protocol in the standard assay template for XFe96 analyzers and can be adapted for XF96 analyzers.

C

Starting group definitions in the modulation assay template for XFe96 analyzers.

Here, the user can define the modulators for controls and/or activation groups.

D

Instrument protocol in the modulation assay template for XFe96 analyzers and can be adapted for XF96 analyzers.

Figure 3. Assay template designs for Agilent Seahorse XF T Cell Activation Assays.

Agilent Seahorse XF T Cell Activation Assay signal can be quantified by two methods using Seahorse Analytics. The first method considers the difference between the basal PER signal and maximal PER signal. The second method considers the area under the curve (AUC) between the time point where the CD3/CD28 activator is injected, and the time point where 2-DG is injected.

This user guide details how to perform the Agilent Seahorse XF T Cell Activation Assay and how to quantify assay results.

Glossary

CD3/CD28 activator: A physical complex of two antibodies designed to stimulate CD3 and CD28 ligands simultaneously mimicking antigen-presenting cell.

Glycolysis: The process of converting glucose to lactate.

Proton efflux rate (PER): The number of protons exported by cells into the assay medium over time, expressed as pmol/min.

2-DG: A glucose analog inhibiting glucose uptake competitively, which results in suppression of glycolysis.

Basal PER rate: The last measurement of proton efflux rate before the injection/addition of reagents.

Baseline: The last measurement of proton efflux rate before the injection of the CD3/CD28 activator.

Standard assay: An assay design that involves injection of a T cell activator followed by injection of 2-DG. It is used to compare the activation potential between cell populations.

Modulation assay: An assay design that includes an acute injection of a test compound prior to the injection of the activator. It is used to investigate modulators of T cell activation.

Maximum rate: A quantitative estimation of T cell activation potential from the best fitting curve by using the least square method.

Area under the curve (AUC): A quantitative measurement of T cell activation potential by calculating the cumulative effect between the times when the activator is injected and when the assay is terminated by 2-DG injection.

References

1 Gubser, P. M. *et al.* Rapid Effector Function of Memory CD8+ T Cells Requires an Immediate-Early Glycolytic Switch. *Nat. Immunol.* **2013**, *14*, 1064-1072.

2 Menk, A. V. *et al.* Early TCR Signaling Induces Rapid Aerobic Glycolysis Enabling Distinct Acute T Cell Effector Functions. *Cell Rep.* **2018**, *22*, 1509-1521.

3 Jones, N. *et al.* Akt and STAT5 Mediate Naïve Human CD4+ T-Cell Early Metabolic Response to TCR Stimulation. *Nature Comm.* **2019**, *10*, 152-160.

4 Kam, Y. *et al.* **"Real-Time Detection and Modulation of Human T Cell Activation Using Agilent Seahorse XF Hu T Cell Activation Assay Kit"**. Agilent Technologies application note, publication number: 5994-1983EN.

5 Romero, N. *et al.* Quantifying Cellular Glycolytic Rate Using CO_2 -Corrected Extracellular Acidification. Agilent Technologies white paper, publication number: 5991-7894EN-D4, **2017**.

2 Kit Information

Kit Contents

The Agilent Seahorse XF Hu T Cell Activation Assay Kit contains one vial (2 mL) of ImmunoCult human CD3/CD28 T cell activator (STEMCELL Technologies, Inc.) and two vials of 2-DG. Each kit provides sufficient materials for 200 tests, which is equivalent to two full plate assays in 96-well format.

The Agilent Seahorse XF Agilent Seahorse XF Hu T Cell Activation Assay 96-Well Pack is a bundled product that contains one XF Hu T Cell Activation Assay Kit, two XFe96 FluxPak with PDL cell culture microplates, and 100 mL calibrant. See **Table 1**.

Table 1 Seahorse XF Hu T Cell Activation Assay Kit and Assay Pack Contents

Component	Cap Color	Quantity
Seahorse XF Hu T Cell Activation Assay Kit contents (p/n 103759-100)		
ImmunoCult human CD3/CD28 T Cell Activator	Clear	1 × 2 mL
2-Deoxy-D-Glucose	Green	2 × 1.5 mmol
Seahorse XF Hu T Cell Activation Assay 96-Well Pack contents (p/n 103766-100)		
ImmunoCult human CD3/CD28 T Cell Activator	Clear	1 × 2 mL
2-Deoxy-D-Glucose	Green	2 × 1.5 mmol
XFe96 Sensor Cartridge	-	2
XFe96 PDL Cell Culture Microplate	-	2
Calibrant	White	100 mL

Kit Shipping and Storage

The Agilent Seahorse XF Hu T Cell Activation Assay Kit is shipped in an insulated container with gel packs to minimize the exposure to elevated temperatures. Upon arrival, the kit should be immediately placed in a refrigerator and stored at 4 °C. The Seahorse XFe96 FluxPak with PDL cell culture plates and calibrant are shipped at ambient temperature and stored at room temperature.

All products are stable for one year from the date of manufacture. The expiration date is printed on the label of the product packaging box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 3 and 12 months.

Additional Required Items

The following products are also required for performing Seahorse XF T Cell Activation Assays, but not supplied with the kit. For a complete list of materials required to perform an XF assay, please visit the **Agilent Cell Analysis Learning Center** website.

Table 2 Additional Required Items

Item	Vendor	Part Number
Seahorse XF XFe96/XF96 Analyzers	Agilent Technologies	-
Seahorse XF FluxPaks*	Agilent Technologies	-
Seahorse XF RPMI Medium, pH 7.4†	Agilent Technologies	103576-100
Seahorse XF 1.0 M Glucose Solution	Agilent Technologies	103577-100
Seahorse XF 100 mM Pyruvate Solution	Agilent Technologies	103578-100
Seahorse XF 200 mM Glutamine Solution	Agilent Technologies	103579-100

* Seahorse XF Hu T Cell Activation Assay Kit (p/n 103759-100) does not include any FluxPaks necessary for performing XF T cell activation assay. If Seahorse XF Hu T Cell Activation Assay Kit is purchased, relevant XF FluxPaks need to be purchased separately in order to perform the assay.

† XF RPMI medium can also be purchased together with the supplements listed in this table as a bundled product (p/n 103681-100).

3 Assay Workflow

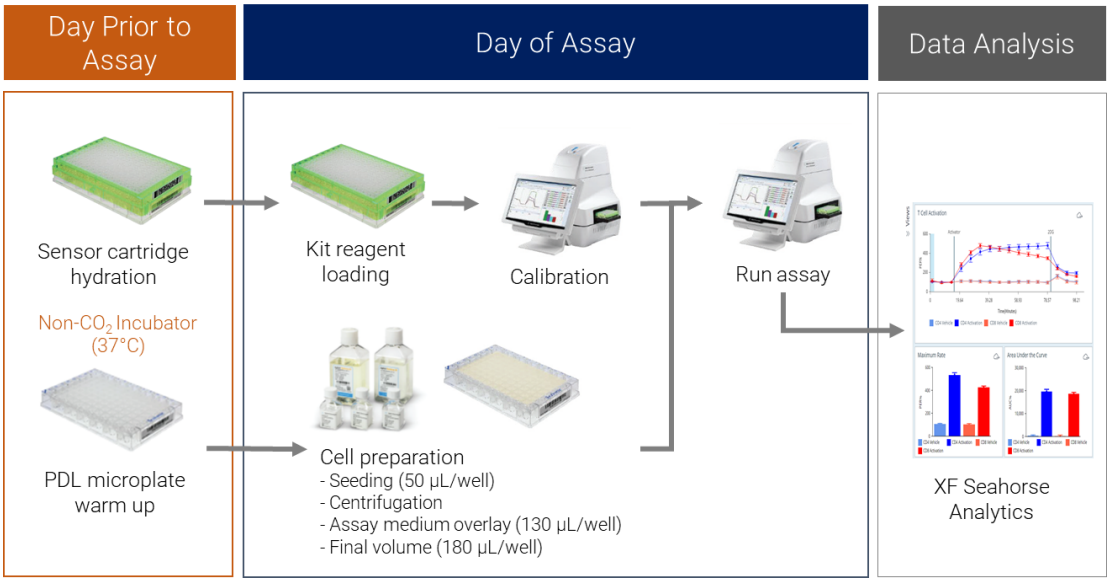


Figure 4. Agilent Seahorse XF T Cell Activation Assay workflow.

NOTE

Optimal cell seeding density should be empirically determined for your cell type prior to the assay. Please refer to Table 4 and FAQ section for recommendations. Additional information can also be found on the Agilent Cell Analysis Learning Center website.

The Cell Line Reference Database is an excellent resource for finding information published in peer-reviewed research articles.

One Day Prior to Assay (Day 1)

Power up the Seahorse XFe96/XF96 Analyzer to allow the temperature to stabilize overnight. Follow the following steps to hydrate sensor cartridges, warm XF cell culture microplates, and design assay templates:

1 Open the XFe96 Extracellular Flux Assay Kit and remove a sensor cartridge.

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 Lower the sensor cartridge onto the utility plate, submerging the sensors in the water.

5 Verify that the water level is high enough to keep the sensors submerged.

6 Place in a 37 °C, **non-CO_2** incubator overnight.

7 Remove a precoated XFe96 PDL cell culture plate from the box and place in a 37 °C **non-CO_2** incubator without humidity control overnight to warm up the plate.

8 Create an assay template file with Wave Desktop software using the *Assay Template*. Refer to **Figure 3** on page 8 for recommended assay settings. Make any necessary group modifications to the template for your specific assay design.

NOTE

For more information, refer to the basic procedure on the Agilent Cell Analysis Learning Center website.

Day of Assay (Day 2)

Complete hydration of the sensor cartridge by removing water and adding **200 µL** of prewarmed XF calibrant to each well and incubate in a 37 °C, non-CO_2 incubator for 60 minutes.

Prepare assay medium

1 Prepare T cell activation assay medium by supplementing 97 mL of Seahorse XF RPMI Medium, pH 7.4 with 1.0 mL each of XF Glucose, XF Pyruvate, and XF Glutamine (10, 1, and 2 mM final in assay media, respectively). These are the recommended initial conditions.

Table 3 Standard XF T Cell Activation Assay Media

Component	Volume (mL)	Final Concentration (mM)
Seahorse XF RPMI Medium, pH 7.4	97	-
XF 1.0 M Glucose Solution	1.0	10
XF 100 mM Pyruvate Solution	1.0	1
XF 200 mM Glutamine Solution	1.0	2

2 Warm to 37 °C.

NOTE

No pH adjustment to the assay media is necessary when recommended XF supplement concentrations are used.

Seeding T cells on the Seahorse XFe96 PDL Cell Culture Microplate

1 Centrifuge T cells out of their growth medium and resuspend in prewarmed assay medium.

2 Count cells and dilute to 4.0 ×10^6 cells/mL for naïve and memory T cells or 2.0 × 10^6 cells/mL for effector T cells.

3 Transfer 50 µL of the cell suspension to each well, leaving the four corner wells (A1, A12, H1, H12) empty for use as background correction wells.

4 Centrifuge gently (e.g., 200×g for 1 minute) to adhere the cells to the plate. The final respective cell densities expected are at 2.0 × 10^5 cells/well for naïve and memory T cells or 1.0 × 10^5 cells/well for activated T cells (i.e., effector T cells). See **Table 4**.

5 Gently add 130 µL/well of the assay medium to each well containing cells. Total well volume should be 180 µL/well.

6 Add 180 µL of assay medium to each of the four corner wells (A1, A12, H1, H12) for use as background correction wells.

7 Incubate the plate in a 37 °C, non-CO_2 incubator for 45 to 60 minutes before the assay.

Table 4 Standard Cell Seeding Conditions for XF T Cell Activation Assays

Cell Type	Cell Suspension (cells/mL)	Seeding Volume (µL/well)	Expected Final Cell Density (cells/well)
Naïve T cell	4.0 × 10^6	50	2.0 × 10^5
Activated T cell	2.0 × 10^6	50	1.0 × 10^5

Prepare 10× working solutions for reagents (see Table 5)

1 Remove one vial of 2-DG from the kit box and resuspend the contents with 3 mL assay medium. Vortex to ensure that the compound goes into solution completely. This solution is ready to be loaded to the injection ports.

NOTE

Resuspended 2-DG solution can be stored as aliquots at –20 °C for up to 6 months.

2 In a cell culture hood, aliquot 1 mL of CD3/CD28 T cell activator to a new vial. Place the original vial with the remainder of the activator solution at 4 °C.

3 Add 1 mL assay medium to the aliquoted activator solution (1:1 dilution). This solution is ready to be loaded to injection ports.

4 If performing the modulation assay, prepare the modulator solutions at 10× of the desired final concentration in the assay medium.

NOTE

If the stock solution of the modulator contains DMSO, it is recommended that the DMSO concentration in the 10× work solution is less than 1%.

Table 5 Preparation of 10x working solutions

Reagent	10x Working Solution
Activator	1:1 mixture with assay medium
2-DG	Resuspend in 3 mL assay medium
Test compound	10x concentration in assay medium

Load the injection ports on sensor cartridge

Proper port loading techniques can be found under the **Set Up Your XF Assay → Loading Solutions** section on the **Agilent Cell Analysis Learning Center** website. Please read the information before loading compounds.

Ensure that the sensor cartridge is properly hydrated before use.

For the designation of the ports, please refer to **Figure 5** below.

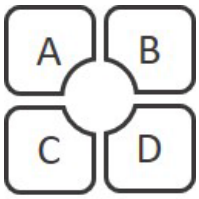


Figure 5. Layout of injection ports on sensor cartridges.

Standard assay No injection before the CD3/CD28 T cell activator.

Modulation assay Injection of test compound(s) prior to the CD3/CD28 T cell activator.

Refer to **Table 6** for loading volumes and port designations to load solutions for each type of assay.

Table 6 Assay type, injection port, and volume recommendations. Starting assay medium volume is 180 µL per well for 96-well cell plates.

Port	Standard Assay		Modulation Assay	
	10× solution	Loading volume	10x solution	Loading volume
Port A	Activator	20 µL	Test compound	20 µL
Port B	2-DG	22 µL	Activator	22 µL
Port C	-	-	2-DG	25 µL

Load assay template onto the Seahorse Analyzer

NOTE

If templates(s) are already present on the analyzer, skip step 1 through 6 below.

1. Insert USB drive in front USB port and wait ~10 seconds.
2. Click **Import** (bottom of the *New Assay* view).
3. Locate the assay template file to import on the USB or network drive.
4. Click **Open** in the Windows dialogue box. The imported assay template will be available for selection from the *Templates* view on the XFe96/XF96 Analyzer.
5. Repeat for next template, if applicable.
6. The imported assay template(s) will now be available for selection in the list of available templates.

Running the XF T Cell Activation Assay

1. Select one of the **T Cell Activation Assay** templates (standard or modulation) or user-created template from the list of available templates and click **Open File** (or double-click the template).
2. Group Definitions: confirm or modify the default groups and conditions for your assay.
3. Plate Map: confirm or modify the plate layout map for your assay.
4. Protocol: No action required - confirm or modify the instrument protocol for additional measurements cycles during the assay.
5. Run Assay: Click **Start Run** when ready.
6. When prompted, remove the cartridge lid and place the loaded sensor cartridge with the utility plate on the thermal tray of the Seahorse XF Analyzer. Ensure correct plate orientation and the cartridge lid has been removed. Then, click **I'm Ready**. Calibration takes approximately 15 to 30 minutes.
7. After completing calibration, Wave Controller will display the Load Cell Plate dialog. Click **Open Tray** to eject utility plate and load the cell plate. Ensure that the lid is removed from cell plate before loading.
8. Click **Load Cell Plate** to run the assay.

Data Analysis Using Agilent Seahorse Analytics

Agilent Seahorse Analytics is a web-based software platform that provides a simple, streamlined data analysis workflow for analyzing the XF T Cell Activation Assay results. Using this tool, XF T cell activation kinetic data can be quantified via two approaches: the maximum PER achieved and AUC as an accumulated glycolytic activity after the activation event (**Figure 6**).

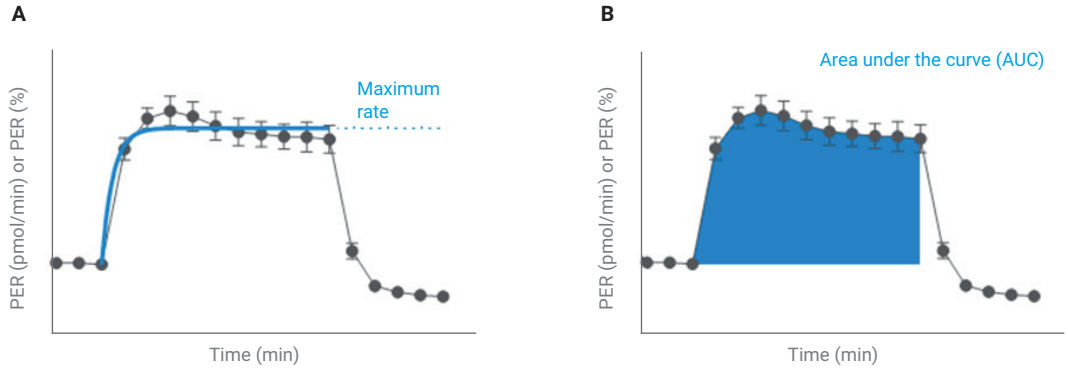
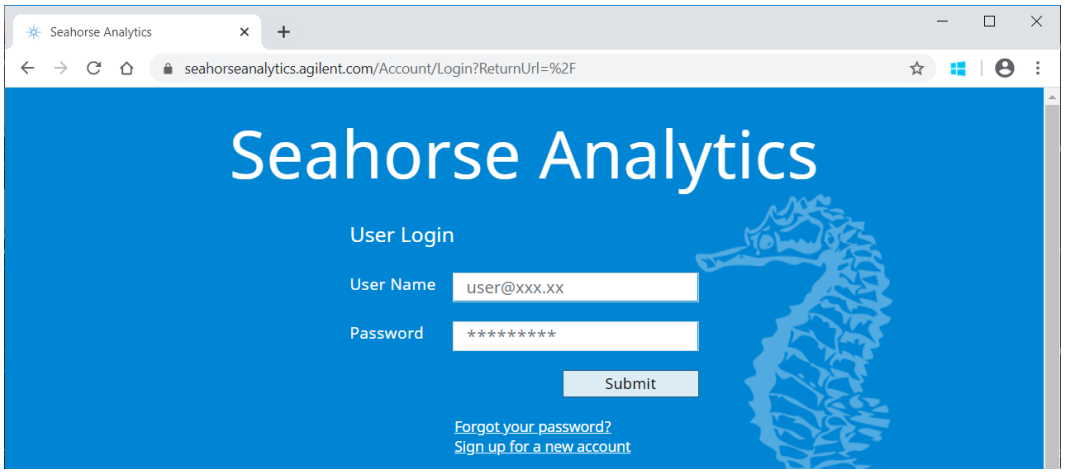


Figure 6. Schematic illustrations of data analysis methods for maximum PER (A) and AUC between CD3/CD28 activator and 2-DG injection timepoints (B).

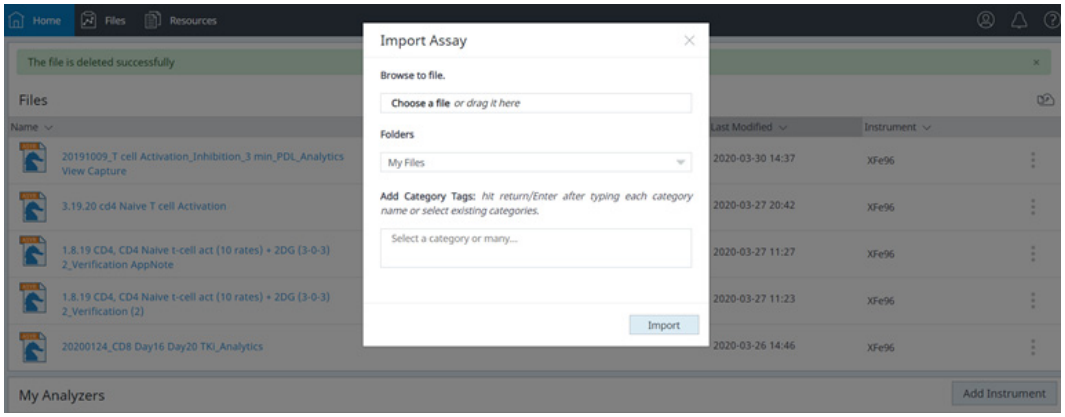
The assay data are provided in four views: a kinetic view, a bar chart with maximal PER estimated by curve fitting, a bar chart with area under the curve (AUC), and a data table with mean and standard deviation for each group. All four views shown in **step 5** can be presented with or without baseline normalization.

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

2 Go to **https://seahorseanalytics.agilent.com** to register or log in to your Seahorse Analytics account.

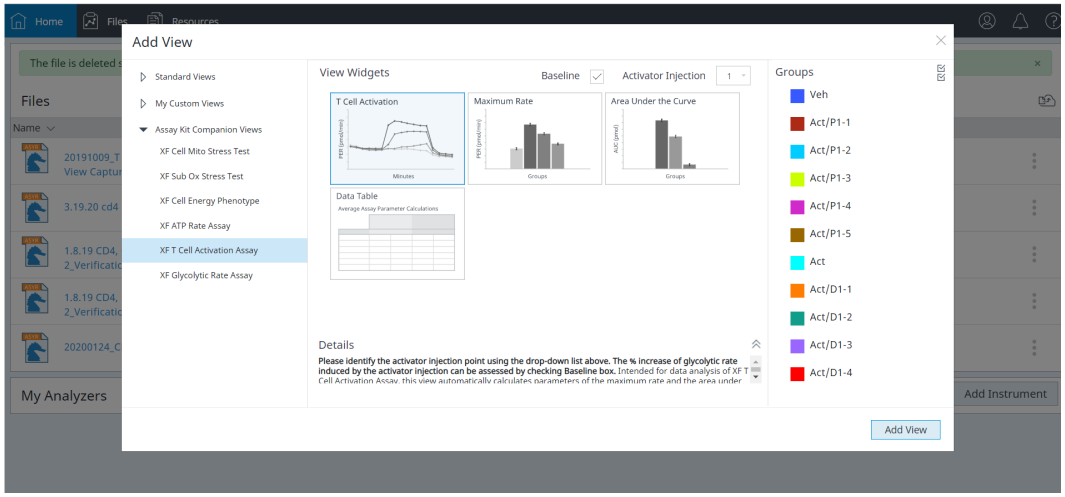


3 Import the assay result file to your account.



4 Open the assay result file and select the XF T Cell Activation Assay analysis view found under the assay kit companion views menu.

5 Select groups to add to the analysis view, then click **Add View**.



4 Guideline for HS Mini, XFp and XFe24 Analyzers

Use with HS Mini and XFp Analyzers

The Agilent Seahorse XF Hu T Cell Activation Assay Kit (p/n 103759-100) can be used to perform the assay on both HS Mini and XFp Analyzers using the same workflow or steps described for use with XFe96/XF96 Analyzers in Section 3. Because the well geometries for Seahorse XFp Cell Culture Miniplates and Seahorse XFe96 Cell Culture Microplates are identical, the cell seeding density and volume (**Table 4** on page 17) and the injection volume for each well (**Table 6** on page 18) are exactly the same for performing assays using XFp Cell Culture Miniplates and XFe96 Cell Culture Microplates.

Because each XFp Cell Culture Miniplates contains only eight wells, the amount of assay medium and 10× working solution needed for each plate is reduced. In general, 1/10 of the amount (10 mL assay medium, 100 µL of the T cell activator solution, and 300 µL of 2-DG) is sufficient for each XFp Cell Culture Miniplate (**Table 7**).

Table 7 Standard XFp T Cell Activation Assay Media

Component	Volume (mL)	Final Concentration
Seahorse XF RPMI Medium, pH 7.4	9.7	-
XF 1.0 M Glucose Solution	0.1	10 mM
XF 100 mM Pyruvate Solution	0.1	1 mM
XF 200 mM Glutamine Solution	0.1	2 mM

Agilent offers precoated XFp PDL Cell Culture Miniplates (p/n 103722-100) and XFp FluxPak (PDL Plates) (p/n 103721-100). These ready-to-use products provide convenience by eliminating the time and labor involved in manually coating microplates. They can also reduce data variation commonly caused by manual coating processes. When performing the XF Hu T Cell Activation Assay, these precoated miniplates are recommended.

Use with XFe24 Analyzer

The Agilent Seahorse XF Hu T Cell Activation Assay Kit (p/n 103759-100) has not been validated for use with the XFe24 Analyzer. Therefore, Agilent does not support the use of the kit with XFe24 Analyzer currently.

5 Frequently Asked Questions

Can Agilent Seahorse XF Hu T Cell Activation Assay Kit be used for non-human T cells? No. This kit is only for human primary T cells or human T cell lines.

What is in the activator reagent that comes with the XF Hu T Cell Activation Assay Kit? The activator reagent contains the soluble ImmunoCult Human CD3/CD28 T Cell Activator in PBS (STEMCELL Technologies, Inc.) and does not contain bovine serum albumin (BSA). The ImmunoCult Human CD3/CD28 T Cell Activator is a tetrameric antibody conjugate that can bind to CD3 and CD28 cell surface ligands and stimulate T cell activation. It is a ready-to-use reagent and compatible with the specially-designed injection ports on Seahorse XF cartridges, reducing assay preparation time and improving assay reliability.

Can I use a different type of assay medium to perform the XF T Cell Activation Assay? The XF T cell activation assay is validated and optimized using the XF RPMI medium, pH 7.4 (part number 103576-100) supplemented with 10 mM XF Glucose, 1 mM XF Pyruvate, and 2 mM XF Glutamine (part numbers 103577-100, 103578-100 and 103579-100, respectively). If other types of media are used or the supplement concentrations are modified, the PER responses may change, and a titration of activator may be needed.

Do I need to perform cell density optimization experiment and how? Yes. We recommend a test (e.g., microscopic evaluation of monolayer) to optimize cell plating density when you first start performing the XF T Cell Activation Assay. The seeding densities recommended in **Table 4** on page 17 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 activation signal level or uninterpretable data. In contrast, if a well is overloaded with cells, some of the cells cannot attach to the bottom of the wells and can be pushed aside during assay when activators are injected, resulting 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 cells (see **Figure 7** below). The optimal cell density after the centrifugation step should show cell distribution similar to Figure 7A or slightly higher and should not be denser than what is shown in Figure 7B.

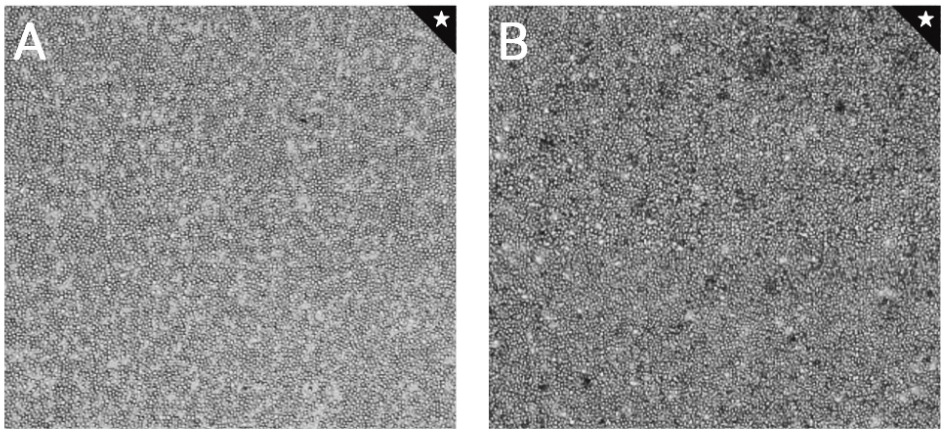


Figure 7. Microscopic view of examples of optimal cell seeding densities.
A) Image shows low end of optimal cell density.
B) Image shows high end of optimal seeding density.

How many measurements are recommended for each condition and can I change the length of the assay? In general, three basal measurements are recommended before the first reagent injection to get a reliable basal rate and four measurements are recommended after 6 minutes waiting period following 2-DG injection. The measurement duration after T cell activator injection is usually 1 hour or 10 measurements and can be extended up to 3 hours depending on the experimental needs. The measurement duration after modulator injection varies from 30 minutes to 1 hour depending on the experimental needs.

What can I expect with regards to the T cell activation potential with the XF Hu T Cell Activation Assay Kit? T cell activation potential is determined by the basal PER signal and maximal PER signal and they vary depending on cell types. **Table 8** provides a summary of the typical PER levels among different cell types, to use as a reference point. There are other factors that may affect T cell activation potential, such as donors, how cells are handled during collection, and storage conditions (see next question).

Table 8 Typical Proton Efflux Rates (PER) in Different Types of T Cells

	$T_{naïve}$	$T_{effector}$	T_{memory}	Jurket cells
Basal signal range (PER in pmol/min)	20 to 40	100 to 200	30 to 60	500 to 1,000
Activation potential (PER in pmol/min)	50 to 120 (200 to 300%)	120 to 250 (20 to 40%)	60 to 200 (200 to 300%)	600 to 1,200 (20 to 40%)

What should I do if my cells don't respond? The activation potential can vary depending on the donor, how cells are handled during collection, and storage conditions. A positive control using responsive cells is always recommended to ensure that the assay works properly. If positive control shows good response, but testing cells does not show any response, it is recommended to revisit the cell collection procedure or storage conditions. Orthogonal functional methods such as cytokine production can be used to confirm the activation potential of your cells.

I cannot access Agilent Seahorse Analytics. What should I do? If you experience any issues using Seahorse Analytics, please contact the **Agilent Cell Analysis Support team** to report your issue. You should also double-check your internet connection. To rule out any issue with internet browser, Seahorse Analytics works best with Chrome, Safari, Firefox and 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 Agilent application note: **Modulation and Detection of Human T Cell Activation Through Real-Time Glycolysis Measurement Using Seahorse XFe96 Analyzer**.

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