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Using the Agilent NovoCyte Flow Cytometer for Immune Suspension Cell Normalization in Agilent Seahorse XF Assays

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Abstract

Metabolism has emerged as a critical driver of immune cell fate and function. Metabolic reprogramming of T cells can be used as a strategy to improve the antitumor efficacy of adoptive T cell therapies. When combined with the specific metabolic modulators, a complete assessment of the bioenergetic metabolic profile of different cell populations can be obtained. The Agilent NovoCyte flow cytometer enables rapid interrogation of cell numbers in a cell suspension while the Agilent Seahorse XF analyzers enable measurement of glycolytic and mitochondrial activity in live cells simultaneously. The optimized workflow using NovoCyte flow cytometry rapidly delivers total and viable cell numbers for the samples to be analyzed in the Seahorse XF platform. This type of quantitative measurement can be utilized in the design and development of more potent antitumor T cell therapies.

Introduction

Metabolism has emerged as a critical driver of immune cell fate and function. It has been demonstrated that metabolic reprogramming of T cells can be used as a strategy to improve the antitumor efficacy of adoptive T cell therapies. Agilent Seahorse XF analyzers allow you to measure glycolytic and mitochondrial activity in live cells simultaneously. In addition, when combined with the specific metabolic modulators, you can obtain a complete assessment of the bioenergetic metabolic profile of different cell populations. This type of quantitative measurement can be applied to the design and development of more potent antitumor T cell therapies.

Normalization of XF data is a critical component during data analysis to ensure accurate and consistent interpretation of results. Whether comparing different cell types, genetic modifications, or compound treatments, the data must be normalized to a commonly shared parameter for correct comparison and interpretation of results. Various methods can be used to normalize XF assays, including cell number, genomic DNA, and total cellular protein. However, normalizing by live cell number is the most widely used method for most XF applications.

Unlike adherent cells that can be seeded and grown in regular XF plates, XF studies with suspension cells such as T cells require seeding using the Seahorse XF PDL cell culture microplates or miniplates. Suspension cells are immobilized via gentle centrifugation and remain attached to the bottom of the well during the XF assay. To obtain robust XF data, suspension cells need to be seeded in an optimal density range. This range must allow oxygen consumption rates (OCR) and extracellular efflux rates (ECAR) or proton efflux rates (PER) 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. Variation occurs because some cells cannot attach to the bottom of the well, accumulating in the outside ring of the well, or being pushed aside during compound injection (Figure 1).

Another important aspect to consider when normalizing XF data is the relationship between cell number and cell viability, i.e., what percentage of the cells in each well or treatment group are viable.

Given the previous considerations, a method that ensures consistent cell counting of total and viable cells in suspension cell preparations before seeding in the XF plates is critical for achieving consistent assay results. In addition, in cases of limited sample material, using a method that minimizes the waste of samples during counting is also a critical consideration for these assays.

The method described here uses the Agilent NovoCyte flow cytometer combined with the Agilent NovoCyte autosampler, which allows for rapid determination of cell numbers in the cell suspension. This optimized workflow rapidly delivers total and viable cell numbers for the samples to be analyzed in the Seahorse XF platform. The method allows for adjustments in cell density before seeding in case the initial suspension density is outside the recommended range. This workflow also uses the live cell count from the final suspension that is used to seed plates for XF assay result normalization.
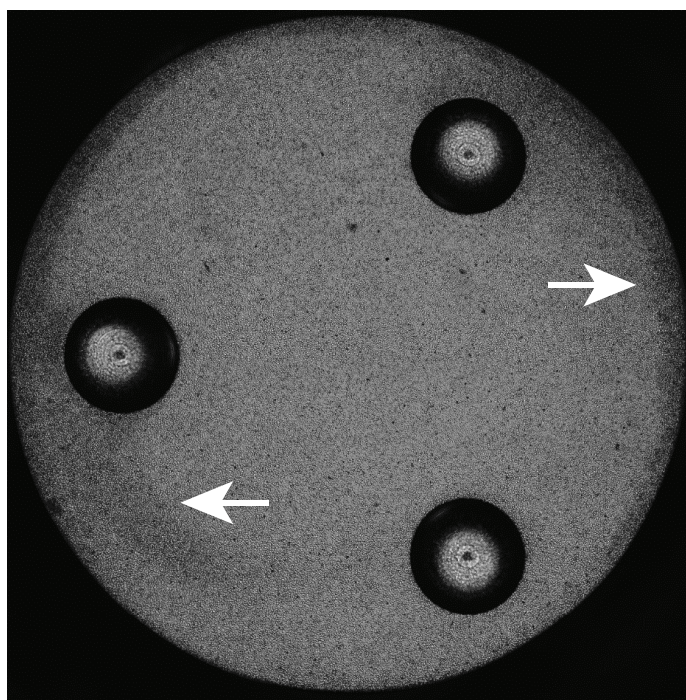

Figure 1. Example of an overloaded well (1.6×10^5 cells/well) showing T cells expanded in culture media for 10 days after activation with anti-CD3/CD28 dyna beads for 3 days. Note the cells aggregated on the edge of the well (white arrows).

Experimental

Human T cell culture

Human peripheral blood pan-T cells (Stemcell Technologies, part number 70024) were thawed in RPMI-1640 media (Thermo Fisher Scientific, part number A10491-01) supplemented with 10% FBS (HyClone, part number SH30070.03) and 0.05 mM beta-mercaptoethanol (Gibco, part number 21985). Cells were thawed and incubated overnight in culture media. The next day they were activated with anti-CD3/28 dyna beads (Thermo Fisher Scientific, part number 11132D) using a bead-to-cell ratio of 2 in the previously mentioned complete RPMI-1640 media. Three days after activation, beads were removed, and media was refreshed with complete RPMI-1640 supplemented with 300 U/mL human IL-2 (STEMCELL Technologies, part number 78036.3). From then (day 4), culture media and cytokine were refreshed every other day to maintain a cell density of around 1×10^6 cells/mL.

For the arginine preconditioning experiment, arginine (Sigma, part number A8094) was added in complete RPMI-1640 media to give a final concentration of 7.15 mM with pH adjusted to 7.4, and high Arg-RPMI media was used during T cell activation and expansion (supplemented with 300 U/mL human IL-2) in the arginine group.

Preparation of samples for XF assay and NovoCyte flow cytometer cell count

1. Turn on the NovoCyte flow cytometer according to instrument user guide.

2. Prepare Seahorse XF RPMI assay medium by supplementing the required volume of Agilent Seahorse XF RPMI medium, pH 7.4 with Agilent Seahorse XF glucose, pyruvate, and glutamine solutions (10 mM, 1 mM, and 2 mM final concentrations in assay medium, respectively). These are the recommended initial conditions. However, the assay medium composition can be modified if desired.

3. Pipette cell culture samples to mix and ensure a homogenous suspension. Take 50 µL of cell suspension for each sample to be analyzed, mix with 50 µL of viable cell dye like DAPI or 7-AAD (Biolegend, part numbers 422801 and 420404, respectively prepared as 2X stocks) in tubes or directly in an Agilent 96-well round bottom plate (part number 204600-100), and count 50 µL of cells according to the NovoCyte cell counting protocol.

Note: Remember to consider cell suspension dilution 1:2 due to addition of dye when exporting absolute cell counts.

4. Calculate the total number of cells needed to run the XF assay (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.

5. Transfer the appropriate volume of cell suspension from the culture vessel to a conical tube. Use multiple tubes if needed, then pool the cells after resuspending cells following centrifugation. Preferentially, use 1.5 or 15 mL conical tubes for best cell recovery.

6. Centrifuge cells at room temperature at 200 × g for 10 minutes. Calculate the volume required to resuspend the cell pellet. Using a volume 20% smaller than the calculated volume to avoid overdiluting the sample is recommended.

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

8. Resuspend cells in warm assay medium (see XF T Cell Metabolic Profiling kit user guide for additional details). Take a 50 µL sample, add live-cell dye, and perform cell counting again in the NovoCyte flow cytometer to confirm cell density is in the desired range. Adjust the volume to achieve the desired cell density if needed.

9. Carefully pipette 50 µL of the final cell suspension in prewarmed XF 96-well PDL cell culture plate. It is recommended to have at least 3 replicate wells per cell condition. Follow the XF T Cell Metabolic Profiling kit user guide for detailed XF assay procedures.

Note: Seeding volume is critical for achieving uniform distribution of cells within a well. It is not recommended to seed cells in a volume higher than what is recommended for each plate type before the centrifugation step. Similar protocol can be applied to XFp and HS miniplates, following the recommended seeding volumes for each plate type.

10. Centrifuge the PDL microplate gently (e.g., 200 × g for 1 to 2 minutes) to allow cells to attach to the bottom of the wells.

11. Gently add warm XF assay medium to achieve the optimal total starting volume, which varies for different assay types and plate types (typically 175 to 200 µL).

12. Incubate the microplate at 37 °C in a non-CO_2 incubator for 45 to 60 minutes before the assay.
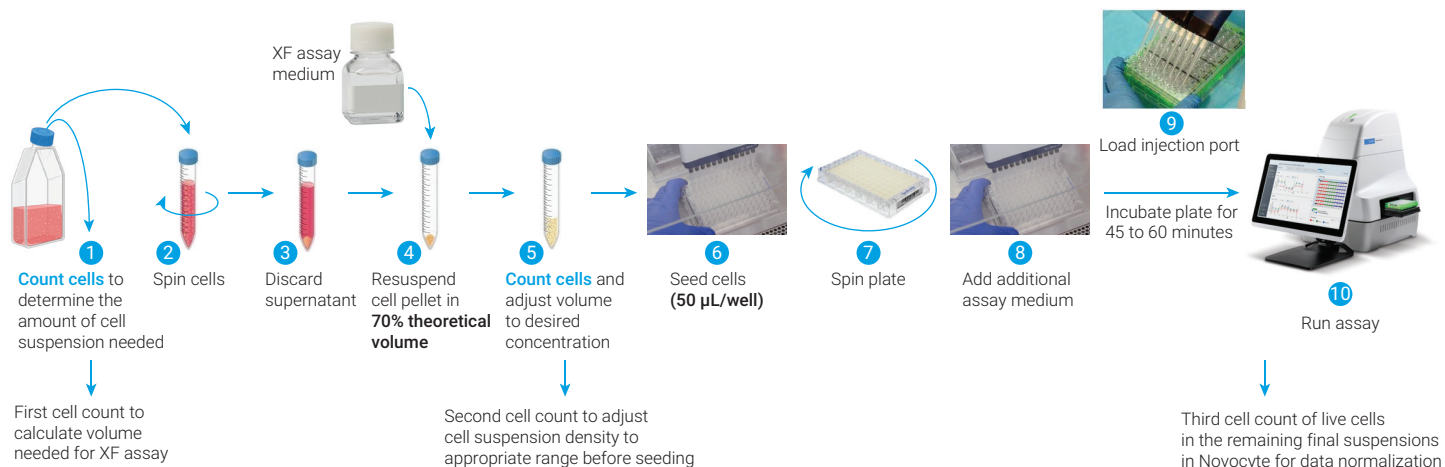
Figure 2. Schematic representation of the steps to perform an XF assay using T cells according to the XF T Cell Metabolic Profiling kit user guide.

13. Run the XF assay according to the corresponding assay user guide.

14. While the assay is running, count the number of live cells in the remaining sample of the final suspension (sample used for seeding the XF plate in Step 9). Avoid diluting samples for counting to reduce potential error and increase the accuracy of the normalization count. Cell counts (per mL) can be obtained from the statistical table view in NovoCyte software.

15. Calculate the exact number of cells seeded per well in the XF assay by multiplying live cell density obtained for each sample in the NovoCyte (cells/mL) by the volume seeded in the XF plate. For example, 50 µL (volume seeded in the XF plate) $\cdot$ 1.82 × 10^6 live cells/mL = 9.1 × 10^4 cells/well.

16. Live cell numbers per well obtained from NovoCyte can be added in the Normalization tab of Wave Desktop or Wave Pro software after the assay finishes (Figure 3A) or in Seahorse Analytics (Figure 3B) after uploading the assay file. If replicate samples were used for counting, use the average value to normalize all the wells from the same sample. Normalization values can be added manually or pasted from files containing the data in the same plate map layout. Adjust normalization units to display the axis of normalized data as well as the normalization scale factor. If using Seahorse Analytics, remember to click the option "apply to all wells". For more details, see Seahorse Wave Pro Software User Manual.

Note: This method allows for normalization to compare between cell samples but will not account for well-to-well variability in the replicate wells from the same sample. It is important to mix the final suspension well before seeding and check proper homogenous seeding in the well by visual inspection under the microscope or brightfield imaging.
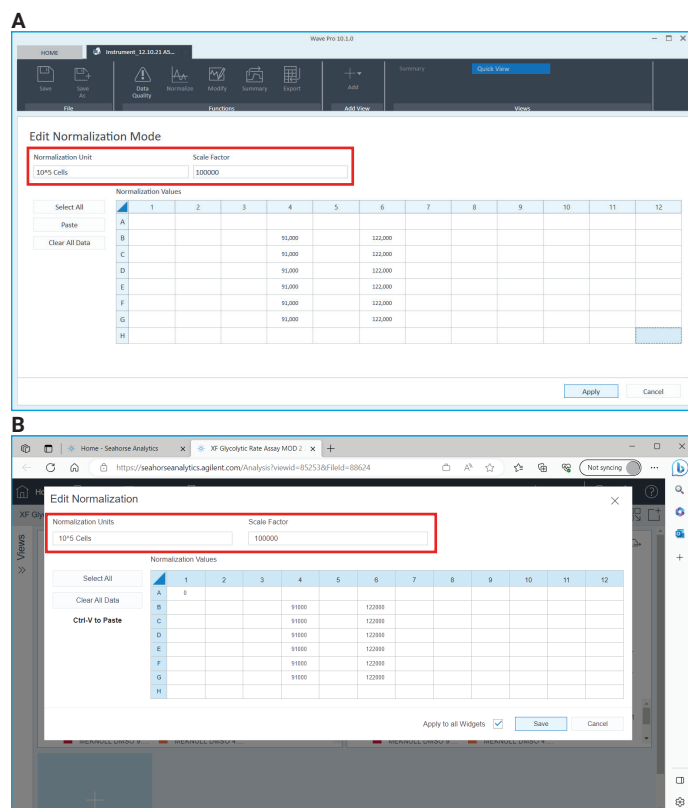

Figure 3. Screenshots of (A) normalization tabs of Wave Pro and (B) Seahorse Analytics.

Results and discussion

Performance of NovoCyte counting system

The NovoCyte flow cytometer delivers precise cell counting in a wide range of cell densities with high reproducibility. To confirm the performance of the cell counting function of the NovoCyte flow cytometer in the range of cell densities recommended for T cells seeding in the Seahorse XF plates, sequential dilutions of a T cell suspension (starting from > 4×10^6 cells/mL) were prepared. Cell counting was performed in triplicate for each sample tested. In addition, cells were stained with the viability dye 7-AAD to account for total and viable cells in the samples. Both counts are needed for determining the optimal seeding density and data normalization during XF assays. As shown in Figure 4 and Table 1, cell counting with the NovoCyte flow cytometer in the analyzed range has high precision with CVs <5% when counting at high sampling speed. Similar performance was also obtained at medium speed. In addition, there is a tight correlation between the cell number counted versus expected, considering the dilution factor of the starting cell suspension.

Table 1. The % CV of triplicate counts at different acquisition speeds for data from Figure 2.

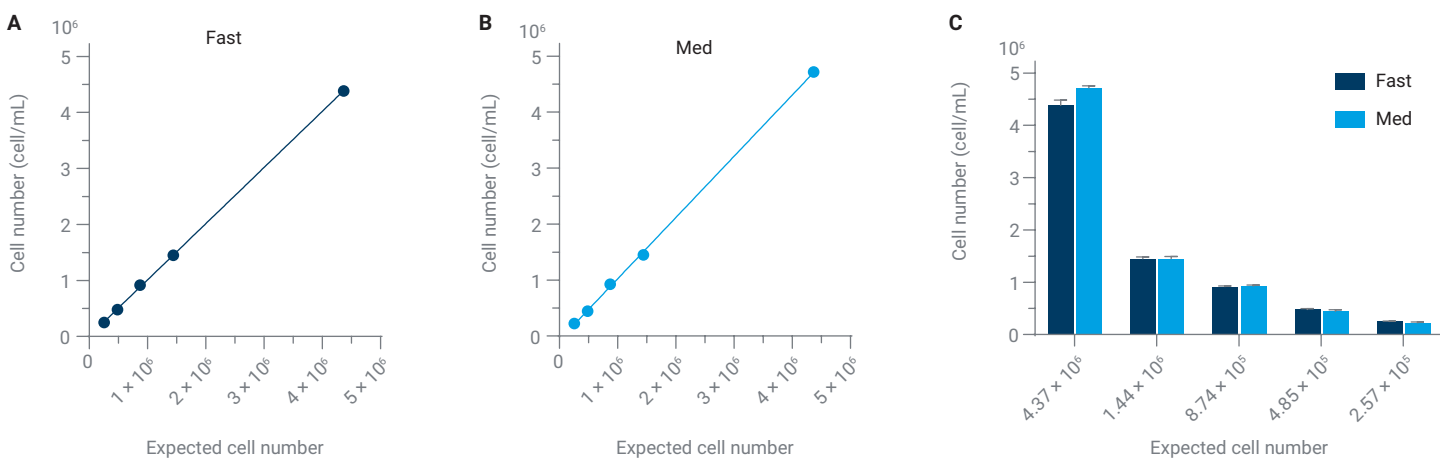| Expected Cell Number (Cells/mL)	% CV	
	Fast	Med
4.4×10^6	2%	1%
1.5×10^6	2%	3%
8.7×10^5	2%	2%
4.9×10^5	4%	6%
2.6×10^5	4%	8%

Figure 4. Comparison of expected cell number according to dilution performed and counted cell number using the Agilent NovoCyte Quanteon flow cytometer system at two different acquisition speeds. Table 1 indicates % CV values obtained for the triplicate measurements of the same cell suspension at the different acquisition speeds.

Correlation between T cell seeding number obtained using NovoCyte flow cytometer counts and XF assay parameters

Due to the high performance, reduced time, and small sample size requirements, use of the NovoCyte flow cytometer for cell counting as a preseeding step was evaluated. This allows for adjustment of the cell density of the cell suspensions when running XF assays using T cells or other immune suspension cells. In addition, the number of viable cells in the final suspension used for cell seeding was evaluated as a method to normalize XF data.

To demonstrate the applicability of NovoCyte cell counting function as a tool to normalize seahorse XF data, human pan-T cells were activated with anti CD3/28 dyna beads and expanded in RPMI-1640 culture media supplemented with 300 U/mL human IL-2. Culture media and cytokines were refreshed every other day, and cell culture density was maintained at around 1×10^6 cells/mL during the expansion period. Samples of naïve pan-T cells and T cells expanded to days 6, 9, and 14 after activation were collected and the culture media was discarded. Next, the cells were resuspended in the appropriate volume of XF assay media to a desired maximal seeding density and a series of dilutions was prepared afterward from each sample. Six replicate wells of each cell dilution were seeded in XF96/XF Pro PDL coated plate (part number 103799-100) and the T cell metabolic fitness assay was performed in the Agilent Seahorse XF Pro analyzer according to the user guide. In addition, 50 µL aliquots of each cell suspension in XF assay medium were collected in a standard 96 well round bottom microplate (part number 204600-100) by duplicate and stained with a viability dye. Total and viable cell counts were obtained using the NovoCyte cell counting protocol at medium or high speed. Figure 5 shows an example of the results obtained for naïve T cells seeded at seven different densities.
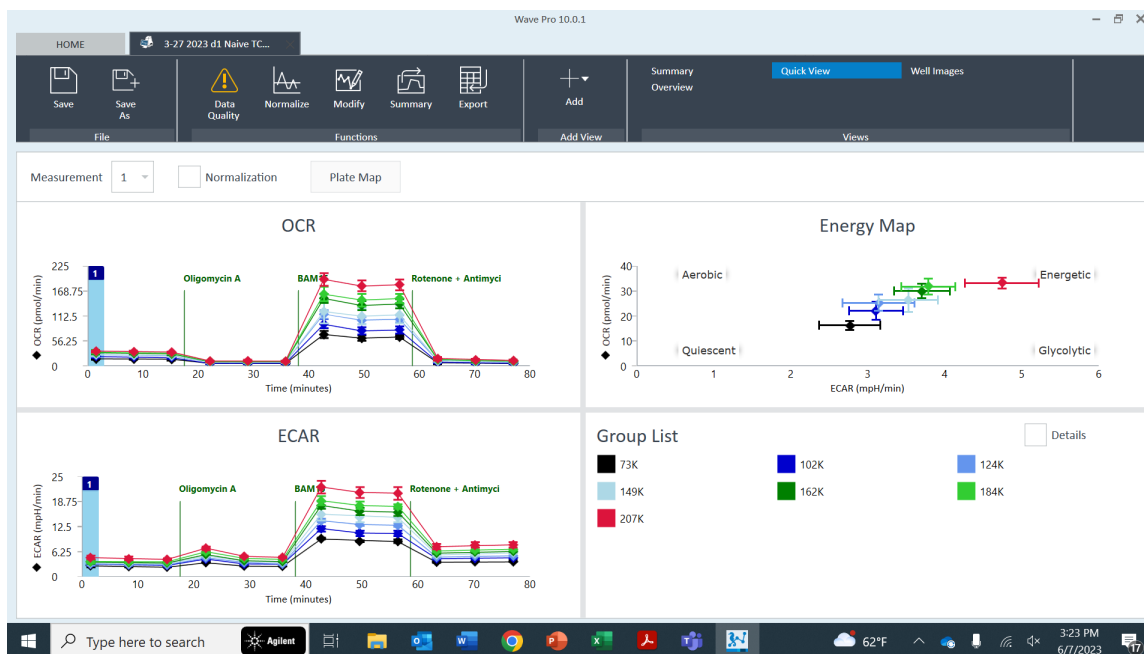

Figure 5. Screenshot of WavePro quick view window of the XF T cell metabolic fitness assay with naïve T cells seeded between 0.9×10^5 and 2.5×10^5 total cells per well (corresponding to 0.7×10^5 to 2.1×10^5 live cells, as shown in the Group List).

As shown in Figure 5, oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) obtained are proportional to the number of cells in the well. This data highlights the importance of properly normalizing the XF data for correct data interpretation, especially if different cell populations are compared.

To confirm that the cell number counts of the cell suspension used for preparing the XF plate correlate with the rates obtained in the XF assay, three of the critical assay measurements of the XF assay were plotted. Those measurements, namely basal OCR, basal PER, and maximal OCR, versus the absolute cell number of T cells seeded in the well were plotted. These data were previously obtained by counting the cell density of the cell suspension samples using the NovoCyte flow cytometer for the naïve T cells and T cells activated and expanded for 6, 9, or 14 days (Figure 6).
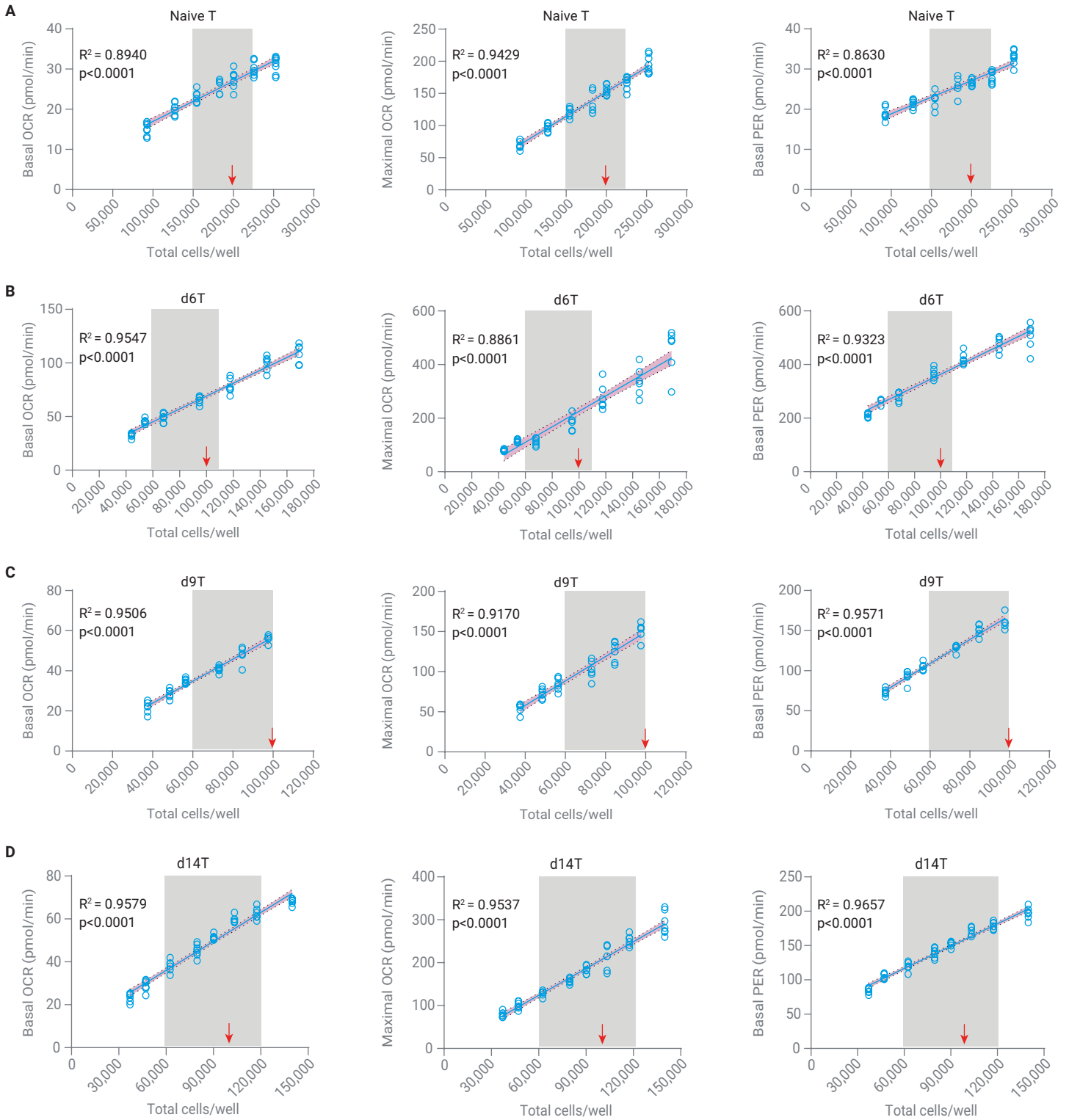
Figure 6. Examples of correlation between basal OCR, maximal OCR, and basal PER vs. total cell number seeded per well for human naïve pan-T cells, or human pan-T cells activated and expanded for 6, 9, and 14 days. Samples corresponding to day 6 (Figure 6B) and day 9 (Figure 6C) were frozen, thawed, and recovered in culture medium overnight before performing the XF assay, with ~ 75% viability after overnight incubation. Samples corresponding to day 14 (Figure 6D) were collected and an immediate XF assay was performed and had 95% viability. Red arrows indicate the recommended seeding density of T cells when performing XF assays and gray area are acceptable ranges for the corresponding examples.

As shown in Figure 6, the three XF parameters were plotted and highly correlate with the number of cells seeded according to previous counting in the NovoCyte flow cytometer. As expected, the optimal performance is obtained around the recommended seeding density (indicated by the red arrow), with rates above the lower limit of detection of the instrument and low well-to-well variability. The shaded area of the graphs represents the acceptable range of seeding density for these particular cell samples. However, the optimal range can vary slightly depending on the metabolic profile of the cell sample and cell size. Different culture conditions, medium compositions, fresh versus frozen, cell viability, etc., can affect the metabolic characteristics of the cells. Seeding low cell numbers can result in rates under the recommended range for optimal instrument performance. Seeding cell densities higher than recommended usually result in overloaded wells and higher well-to-well variability due to low attachment to the cell plate or data outside the range of linear response of rate versus cell number.

Since the cell viability is the same across the different seeding densities for each sample, only total cells/well are shown. Still, an identical linear correlation is observed when XF rates are plotted against live cells/well.

Table 2 shows the recommended seeding density ranges and cell size for different T cell populations that can be used as a reference when optimizing assay preparation.

As indicated before, these values are only a reference as some cells with a more quiescent or glycolytic phenotype must be seeded in the upper zone of the range.

Table 2. Recommended seeding density of naïve human pan-T cells, and human pan-T cells activated and expanded for 6, 9, and 14 days.

	Human Naïve T cell	Day 6 T	Day 9 T	Day 14 T
Diameter in RPMI-1640 Cell Culture Medium (µm)	~ 8 to 9	~11 to 12	~10	~8 to 9
Recommended Seeding Density (Cells/Well)	150 K to 225 K	60 K to 100 K	60 K to 100 K	60 K to 120 K

Homogenous distribution of cells in the well is a critical factor for obtaining high quality data. Acquisition of brightfield images or visual inspection of the XF plate under a microscope helps to perform a qualitative assessment and optimizes seeding densities for different cell types. Figure 7 shows the brightfield images of naïve T cells seeded at 1.3×10^5, 2×10^5, and 2.5×10^5 cells/well. Brightfield image of the well with the highest cell density (Figure 7C) clearly shows cell accumulation at the edge of the well. This uneven cell distribution in the well surface will result in higher variability and underestimation of OCR and PER measurements.
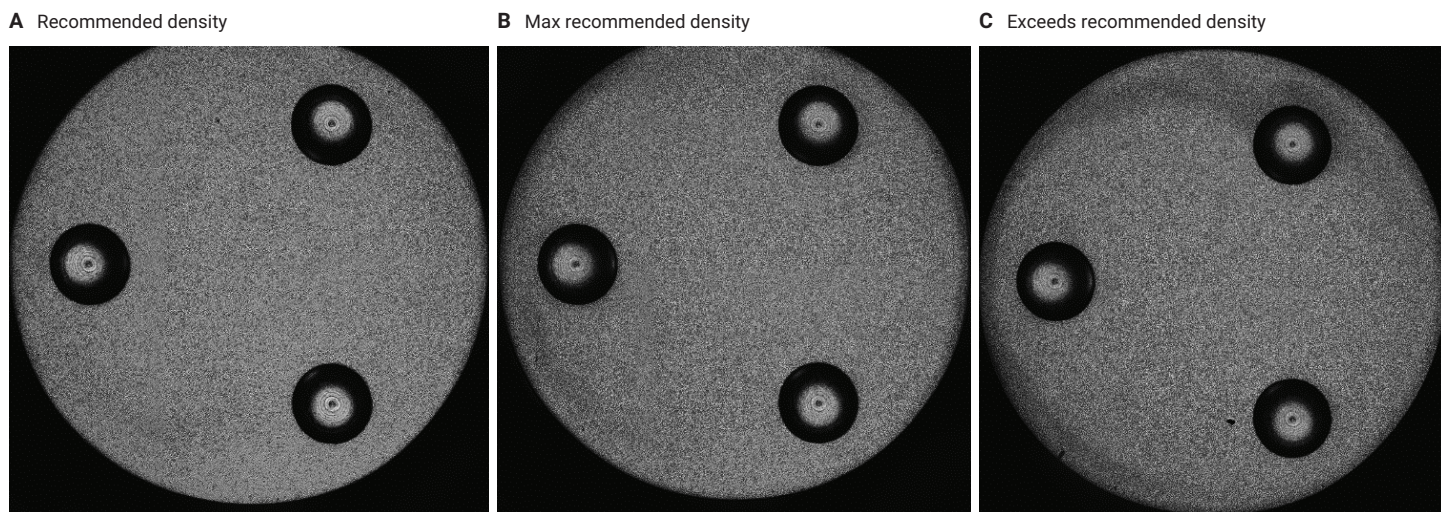
A Recommended density

B Max recommended density

C Exceeds recommended density

Figure 7. Brightfield images of human naïve T cells seeded in XF plates at increasing densities (A) 1.5×10^5 cells/well, (B) 2×10^5 cells/well, (C) 2.5×10^5 cells/well.

Normalization of XF assays with T cells using NovoCyte viable cell counting

While total cell number is used to determine optimal seeding density and especially to avoid well overload, when comparing different cell populations, it is important to normalize XF data to the number of live cells in the well. To facilitate live cell counting, a viability dye can be easily added to the cell samples used for NovoCyte counting, and the number of total and live cells can easily be obtained from the same cell count (See the NovoCyte Protocol).

– Precount of total cell number is needed to adjust the density of each cell sample to the appropriate range.

– Live cell count must be determined in the final cell suspension, the same one that is used for seeding XF plates to avoid introducing errors during dilutions, and is used for data normalization.

Once numbers of live cells for each of the samples are obtained in the NovoCyte flow cytometer, they can be easily incorporated into the XF data. Add the cell counts obtained to the normalization tab in Wave Pro software or Seahorse Analytics to rapidly obtain normalized XF rates as well as other parameters delivered using the Seahorse Analytics companion views (Figure 3).

As shown in Figure 8, after adding the cell number seeded per condition to XF software, the normalized OCR and ECAR kinetic profile of the 4 samples with densities that fall within our recommended seeding range (150 K to 225 K total cells per well, corresponding to 124 K to 184 K live cells per well) perfectly overlap. These data highlight the robustness of using the live cell count obtained in the NovoCyte flow cytometer for normalization of XF data.
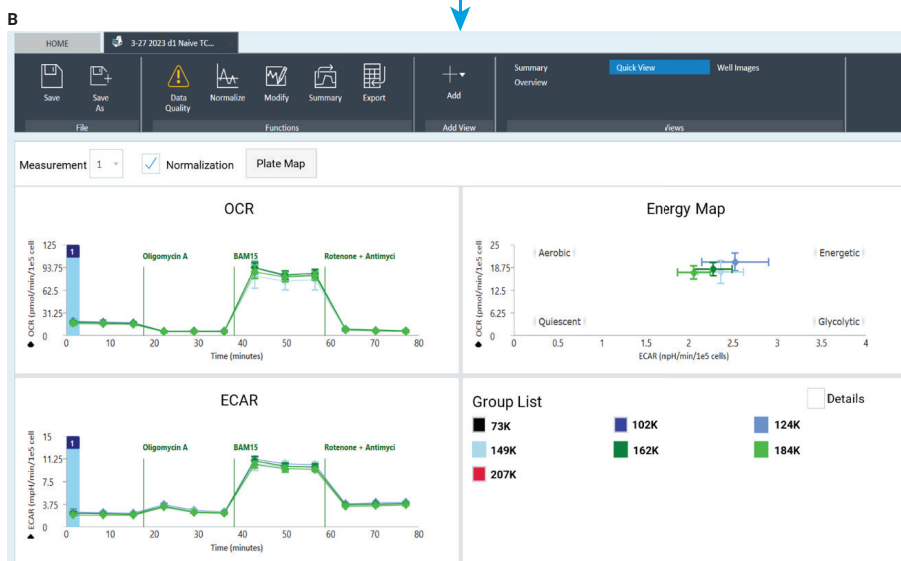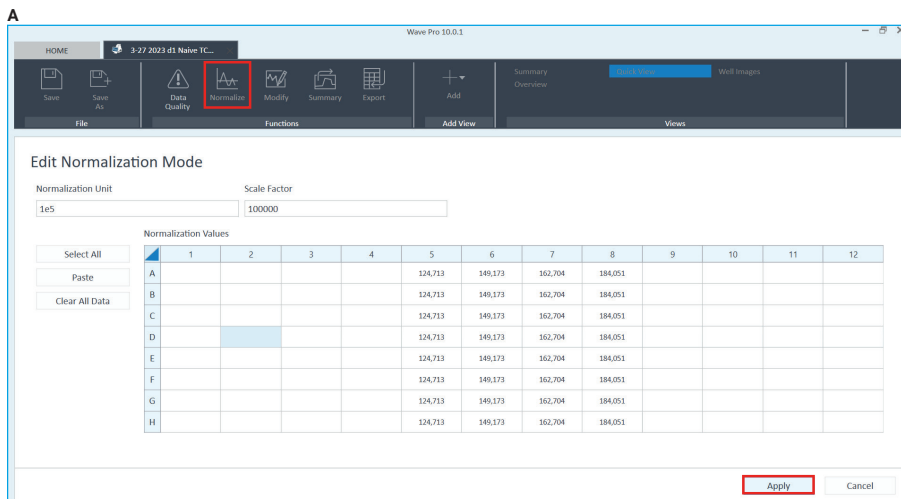

Figure 8. Screenshots of Wave Pro software normalization data input tab and normalized OCR/ECAR data from an XF T cell fitness assay with naïve T cells seeded according to the recommended range in Table 2.

Importance of normalization of XF parameters when comparing different cell populations.

Human pan-T cells were activated and expanded to day 9 in RPMI-1640 media containing standard (1.15 mM, control group) or high (7.15 mM, arginine group) concentrations of arginine. After the expansion period, the two cell populations were collected, culture media discarded, and cells resuspended in XF RPMI pH 7.4 supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate (XF assay media) at approximately 1.5×10^5 cells/mL. Then, 50 µL of each sample were seeded in XF96/XF Pro PDL coated plates, and the T cell metabolic fitness assay was performed according to user guide. In parallel, cell suspensions in XF assay media were analyzed for quantification of total and live cell numbers using the NovoCyte flow cytometer. The XF assay was completed and live cell numbers were obtained for the two samples, control and arginine, were added in the XF Pro normalization tab. (**Note**: Live cell counts can be added directly in XF Pro software at the end of the assay or later in the normalization tab of Seahorse Analytics during data analysis).

XF data were analyzed using the T cell Metabolic Fitness companion view in Seahorse Analytics (https://seahorseanalytics.agilent.com). When data were analyzed before normalization, the control group (black line) showed apparently higher basal and maximal OCR compared to the arginine group, and higher total ATP production as well (Figure 9).

However, after normalizing the data by considering the number of live cells per well (Figure 10, note the change in axis units), the results are reversed. The Arg group presents higher basal and maximal OCR compared to the control, as well as higher total ATP production rate. The percentage of ATP generated from glycolysis is lower in the arginine group compared to the control and is unaffected by normalization as it is a ratiometric parameter independent of cell number.
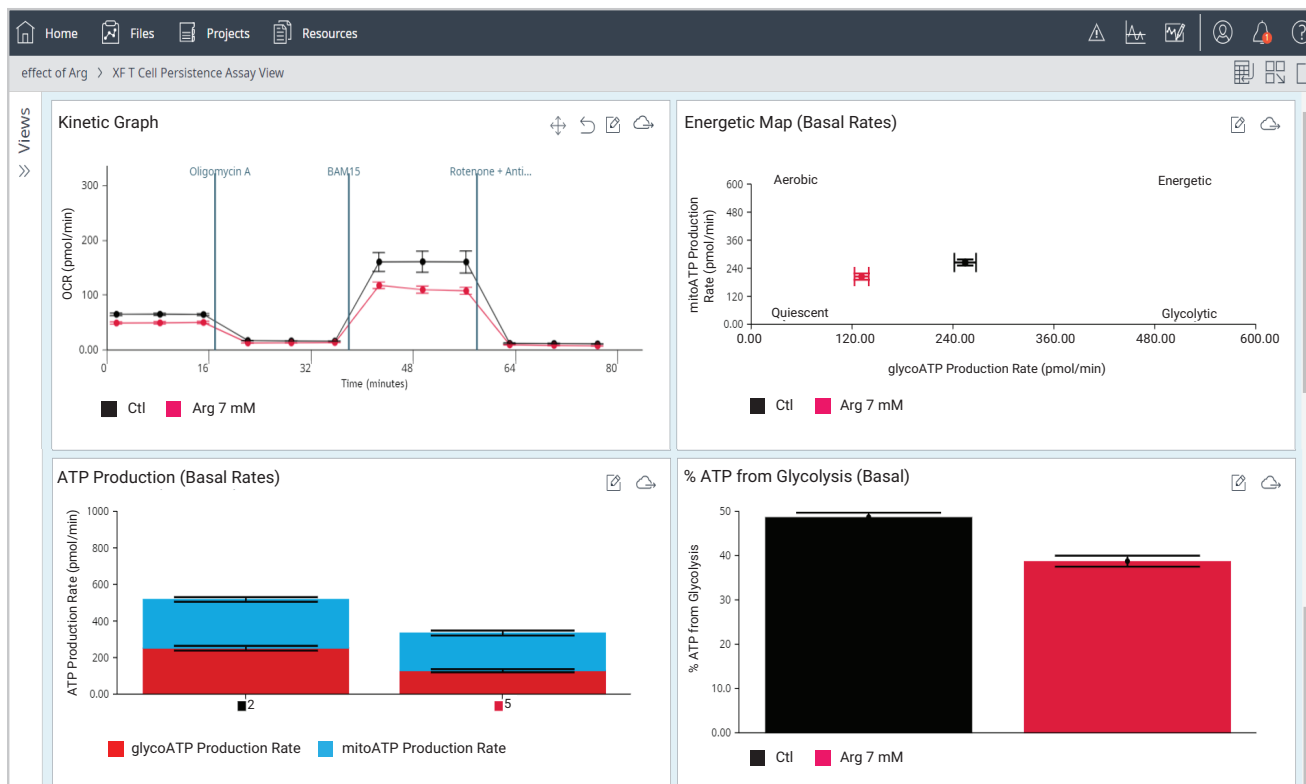

Figure 9. Screenshot of Seahorse Analytics report showing non-normalized OCR kinetic graph and ATP production rate in the T cell metabolic fitness assay performed in T cells activated and amplified for 9 days in regular RPMI-1640 media or RPMI-1640 media supplemented with additional arginine. Note the units for non-normalized data.
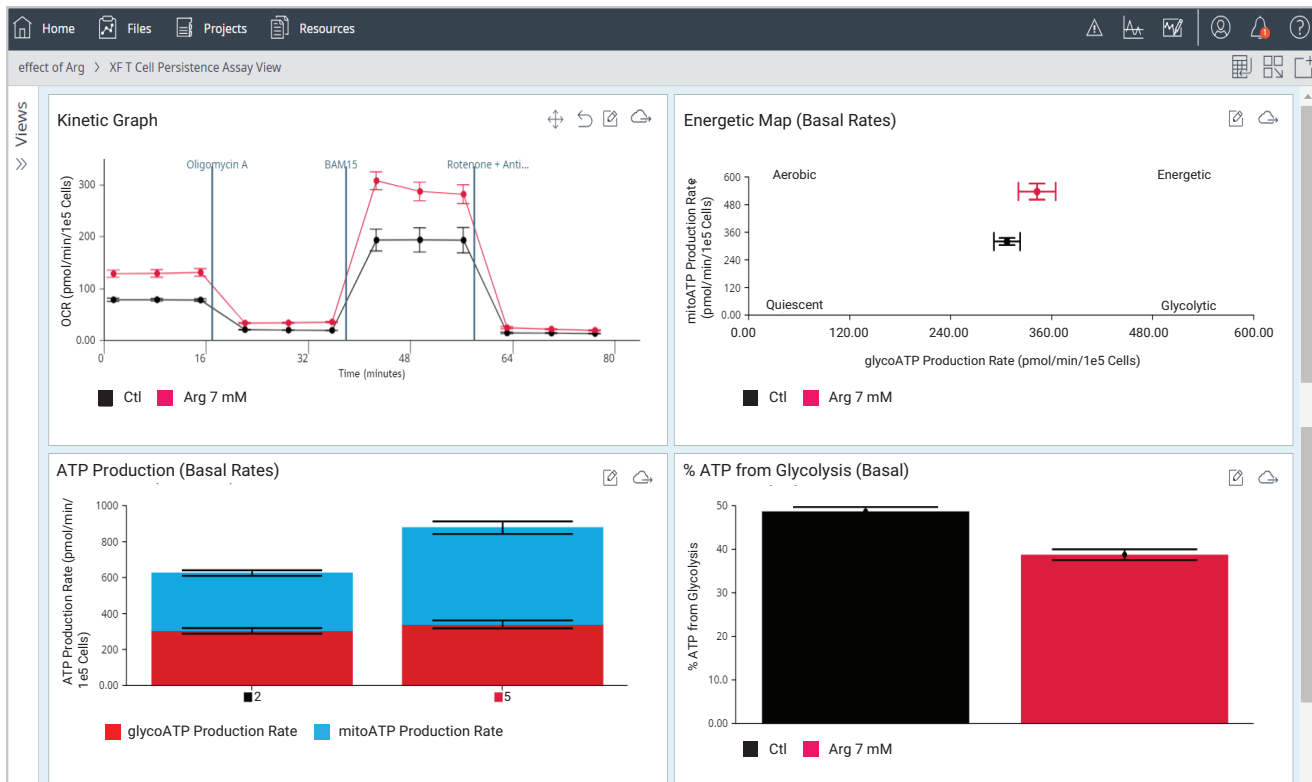
Figure 10. Screenshot from the Seahorse Analyzer report showing normalized data of Figure 9. Note the units for normalized data.

Conclusion

In conclusion, normalization of Agilent Seahorse XF data is a practice that should be incorporated in every XF assay, especially when comparing different cell populations for correct data interpretation. For XF assays using suspension cells, where samples are seeded in the plate immediately before running the experiment (not expecting cell growth during the assay), the number of live cells seeded per well provides a suitable parameter for data normalization when seeding densities around the recommended ranges. This normalization method allows proper comparison between different cell samples but does not account for well-to-well variability of replicates of the same sample. For this reason, it is important to perform cell counting in the same cell suspension used for preparing XF plates. Also, ensure thorough mixing of cell samples prior to counting cells, and careful seeding when preparing the XF plate (including visual inspection of the plates) to ensure homogenous distribution of the cells in the well.

Agilent NovoCyte flow cytometers offer an easy solution to acquire live cell numbers in suspension cell populations without requiring complicated calibrations of the fluidics system or the use of reference beads, and with minimal carry over between samples. Using a small sample volume, NovoCyte provides accurate cell counts for total and live cells. These counts can be used for the preparation of cell samples in the appropriate density range and easily added to the XF data file for obtaining normalized data. In addition, combined with the NovoSampler Q, the system provides an increased throughput with minimal hands-on time required, allowing rapid counting and preparation of multiple samples while saving time.

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