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Measuring Mitochondrial Substrate Flux in Recombinant Perfringolysin O-Permeabilized Cells

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TITLE:

Measuring Mitochondrial Substrate Flux in Recombinant Perfringolysin O-Permeabilized Cells.

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KEYWORDS:

microplate respirometry; mitochondria; metformin; permeabilized cells; perfringolysin O; mitochondrial substrates

SUMMARY:

In this work, we describe a modified protocol to test mitochondrial respiratory substrate flux using recombinant perfringolysin O in combination with microplate-based respirometry. With this protocol, we show how metformin affects mitochondrial respiration of two different tumor cell lines.

ABSTRACT:

Mitochondrial substrate flux is a distinguishing characteristic of each cell type, and changes in its components such as transporters, channels, or enzymes are involved in the pathogenesis of several diseases. Mitochondrial substrate flux can be studied using intact cells, permeabilized cells, or isolated mitochondria. Investigating intact cells encounters several problems due to simultaneous oxidation of different substrates. Besides, several cell types contain internal stores of different substrates that complicate results interpretation. Methods such as mitochondrial

isolation or using permeabilizing agents are not easily reproducible. Isolating pure mitochondria with intact membranes in sufficient amounts from small samples is problematic. Using non-selective permeabilizers causes various degrees of unavoidable mitochondrial membrane damage. Recombinant perfringolysin O (rPFO) was offered as a more appropriate permeabilizer, thanks to its ability to selectively permeabilize plasma membrane without affecting mitochondrial integrity. When used in combination with microplate respirometry, it allows testing the flux of several mitochondrial substrates with enough replicates within one experiment while using a minimal number of cells. In this work, the protocol describes a method to compare mitochondrial substrate flux of two different cellular phenotypes or genotypes and can be customized to test various mitochondrial substrates or inhibitors.

INTRODUCTION:

Microplate-based respirometry has revolutionized mitochondrial research by enabling the study of cellular respiration of a small sample size[1]. Cellular respiration is generally considered as an indicator of mitochondrial function or 'dysfunction', despite the fact that the mitochondrial range of functions extends beyond energy production[2]. In aerobic conditions, mitochondria extract the energy stored in different substrates by breaking down and converting these substrates into metabolic intermediates that can fuel the citric acid cycle[3] (**Figure 1**). The continuous flux of substrates is essential for the flow of the citric acid cycle to generate high energy 'electron donors', which deliver electrons to the electron transport chain that generates a proton gradient across the inner mitochondrial membrane, enabling ATP-synthase to phosphorylate ADP to ATP[4]. Therefore, an experimental design to assay mitochondrial respiration must include the sample nature (intact cells, permeabilized cells, or isolated mitochondria) and mitochondrial substrates.

Cells keep a store of indigenous substrates[5], and mitochondria oxidize several types of substrates simultaneously[6], which complicates the interpretation of results obtained from experiments performed on intact cells. A common approach to investigate mitochondrial ability to oxidize a selected substrate is to isolate mitochondria or permeabilize the investigated cells[5]. Although isolated mitochondria are ideal for quantitative studies, the isolation process is laborious. It faces technical difficulties such as the need for large sample size, purity of the yield, and reproducibility of the technique[5]. Permeabilized cells offer a solution for the disadvantages of mitochondrial isolation; however, routine permeabilizing agents of detergent nature are not specific and may damage mitochondrial membranes[5].

Recombinant perfringolysin O (rPFO) was offered as a selective plasma membrane permeabilizing agent[7], and it was used successfully in combination with an extracellular flux analyzer in several studies[7–10]. We have modified a protocol using rPFO to screen mitochondrial substrate flux using XFe96 extracellular flux analyzer. In this protocol, four different substrate oxidizing pathways in two cellular phenotypes are compared while having sufficient replicates and the proper control for each tested material.

PROTOCOL:

1. One day before the assay

1.1. Preparation of reagents and substrates.

1.1.1. Mitochondrial assay solution (MAS): Prepare stock concentration of all reagents as described in **Table 1**. Warm the stocks of mannitol and sucrose to 37 °C to completely dissolve. Mix the reagents to prepare 2x MAS, then warm the mixture to 37 °C. Adjust the pH with 5N KOH to 7.4 (~7 mL), then add water to bring the volume up to 1 L. Filter sterilize and store the aliquots at -20 °C until the measurement day.

1.1.2. Bovine serum albumin (5% BSA): Dissolve 5 g of BSA in 90 mL of prewarmed sterile water on a magnetic stirrer, avoid shaking. Adjust pH to 7.4 with 5N KOH, then add water to bring the volume up to 100 mL. Filter sterilize and store the aliquots at -20 °C until the measurement day.

1.1.3. Mitochondrial substrates: Prepare 1 M stock concentration of sodium succinate, sodium pyruvate, and sodium glutamate in sterile water. Prepare 100 mM stock concentration of sodium malate. Use prewarmed sterile water at 37 °C to prepare a stock concentration of 10 mM of palmitoyl carnitine. Adjust pH of each substrate to 7.4 by 5N KOH and filter sterilize. Store the substrates at 2–8 °C. At the time of use, warm palmitoyl carnitine at 37 °C for 15 min to completely dissolve any precipitates. Aliquot and store all the substrates except pyruvate at -20 °C.

1.1.4. Mitochondrial inhibitors: Use dimethyl sulfoxide (DMSO) to prepare stock concentration of 25 mM oligomycin, 50 mM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 20 mM rotenone, and 20 mM antimycin A. Store the aliquots at -20 °C.

1.2. Seeding and treating the cells: As shown in **Figure 2**, seed the cells in columns 2–11. Columns 1 and 12 must be left empty to serve as background wells. For the results mentioned in this work, HepG2 and A549 cells are used.

1.2.1. Seed the cells at a density of 20 000 cells per well. Use a volume of 50–80 µL of cell culture medium for seeding.

1.2.2. Fill the blank wells with an equal volume of cell culture medium and incubate the cells at 37 °C in a humidified incubator with 5% CO_2. Allow the cells to attach for 3–4 h, then add 100 µL of cell culture medium to all wells.

1.2.3. With that final medium addition, perform the treatment. Use the columns from 7–11 to perform the treatment. In this work, the experimental group was treated with 1 mM metformin hydrochloride for 16 h, and the control group was treated with an equal volume of sterile distilled water as vehicle control.

1.3. Hydrating the sensors: Pipette 200 µL of sterile water per well in the utility plate, then carefully return the sensor cartridge while immersing the sensor in water. Incubate the cartridge in a CO_2-free incubator at 37 °C till the next day.

1.4. The assay protocol template: Switch on the analyzer (see **Table of Materials**) and the controller unit. Start the instrument control and data acquisition software and design the assay protocol as described in **Table 2**. Under **Group Definitions,** create four injection strategies where Port A differs according to the injected substrate (**Figure 3**). Name the strategies after the substrates or their abbreviations.

1.5. Load the ports B, C, and D with oligomycin, FCCP, and rotenone/antimycin A, respectively. Create eight groups and name them, as shown in **Figure 4**. Under **Plate Map** assign the groups to the corresponding wells, then save the protocol as a ready-to-use template. Leave the analyzer switched on to allow the temperature to stabilize overnight. Keep the analyzer in a place with a stable temperature to avoid sudden temperature changes.

2. The day of the assay

2.1. Replacing water with the calibrant: Discard the water from the utility plate and pipette 200 µL per well of prewarmed calibrant to the utility plate. Return the cartridge to the CO_2-free incubator until the time of the assay. To avoid rapid evaporation of the calibrant, maintain a source of humidity inside the incubator and turn off or reduce the fan speed to a minimum.

2.2. Preparing the working concentration of the reagents: Start by warming 2x MAS, 5% BSA, and sterile water to 37 °C. Meanwhile, allow the inhibitor stocks to reach room temperature. Use warm 2x MAS and sterile distilled water to prepare 5 mL of the working concentration of the substrates and inhibitors as described in **Table 3**.

2.3. Loading the injection ports: As shown in **Figure 3**, load 20 µL of the substrates into port A. Load succinate/rotenone mixture into port A of rows A and B. Load pyruvate/malate mixture into port A of rows C and D. Load glutamate/malate mixture into port A of rows E and F. Load palmitoyl carnitine/malate mixture into port A of rows G and H. For the whole plate, load port B with 22 µL of oligomycin preparation, port C with 25 µL of FCCP preparation, and port D with 27 µL of rotenone/antimycin A mixture.

2.4. Starting the calibration step: Under **Run Assay** tab, click on **Start Run** to start the assay. Insert the loaded sensor cartridge and start the calibration step. Wait for the calibration to complete before proceeding to the next step.

2.5. Preparation of the assay medium (MAS-BSA-rPFO): To prepare 20 mL of the assay medium, mix 10 mL of 2x MAS, 9.2 mL of sterile water, and 0.8 mL of 5% BSA in a 50 mL tube. Add 2 µL of rPFO and gently resuspend the mixture with gentle pipetting. Avoid shaking and do not use a vortex mixer for mixing. Incubate the tube at 37 °C until the time of use.

2.6. Washing the cells: Wash the cells and the empty blank wells two times using prewarmed calcium- and magnesium-free PBS solution. Use a multichannel pipette to wash. Avoid reusing the same tips to discard the cell culture medium and to add PBS solution. Perform this step

outside the laminar flow to protect the cells from drying out by the airflow.

2.7. Cell permeabilization in assay medium: Using a multichannel pipette, discard the PBS and replace it with 180 µL of the prewarmed assay medium (MAS-BSA-rPFO).

2.8. Starting the measurement: Immediately after permeabilization, replace the utility plate of the calibrated sensor cartridge with the cell plate containing permeabilized cells and start the measurement.

REPRESENTATIVE RESULTS:

Start by normalizing the results to the second measurement of baseline respiration to show values as oxygen consumption rate percentage (OCR%). The results of the assay are shown in **Figures 5, Figure 6, Figure 7,** and **Figure 8**. It is important to assign the proper background wells for each group and inactivate the background wells of other groups. **Figure 5** shows that the treatment group has a higher rate of succinate-induced respiration. The response of A549 cells to metformin treatment (**Figure 5A**) was higher than HepG2 (**Figure 5B**). The background control wells were only those from the same rows of the compared group, in this case, wells A1, B1, A12, and B12. **Figure 6** shows the changes in pyruvate/malate-induced respiration. **Figure 7** shows the changes in glutamate/malate-induced respiration, and **Figure 8** shows the changes in palmitoyl carnitine/malate-induced respiration.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic representation of citric acid cycle. The used substrates to test mitochondrial substrate flux are in red. Malate is not used alone but used in combination with pyruvate, palmitoyl carnitine, and glutamate. The role of malate in pyruvate/malate- and palmitoyl carnitine/malate-induced respiration is to provide oxaloacetate through the action of the malate dehydrogenase enzyme. In glutamate/malate-induced respiration, malate takes part in the malate-aspartate shuttle.

Figure 2: Illustration of the cell seeding plan in the cell culture microplate. Blank wells in columns 1 and 12 must be left empty without cells. Columns 7–11 are used to treat the experimental group.

Figure 3: Illustration of the injection strategy. Port A of rows A and B are loaded with succinate/rotenone mixture. Port A of rows C and D are loaded with pyruvate/malate mixture. Port A of rows E and F are loaded with glutamate/malate mixture. Port A of rows G and H are loaded with palmitoyl carnitine/malate mixture. For the whole plate, ports B, C, and D are loaded with oligomycin, FCCP, and rotenone/antimycin A, respectively.

Figure 4: Group names and plate map. Each group is named according to the phenotype (control or treated) and the substrate used to induce respiration. (S), succinate-induced respiration. (P/M), pyruvate/malate-induced respiration. (G/M), glutamate/malate-induced respiration. (CP/M), palmitoyl carnitine/malate-induced respiration.

Figure 5: Succinate-induced respiration. (**A**) A549 and (**B**) HepG2. The tested group was treated with 1 mM metformin hydrochloride for 16 h. Only the background wells A1, B1, A12, and B12 are used for correction. The results are shown as average OCR% ± SD. The graph and plate grid were created and exported as image files by the assay design, data analysis, and file management software.

Figure 6: Pyruvate/malate-induced respiration. (**A**) A549 and (**B**) HepG2. The tested group was treated with 1 mM metformin hydrochloride for 16 h. Only the background wells C1, D1, C12, and D12 are used for correction. The results are shown as average OCR% ± SD. The graph and plate grid were created and exported as image files by the assay design, data analysis, and file management software.

Figure 7: Glutamate/malate-induced respiration. (**A**) A549 and (**B**) HepG2. The tested group was treated with 1 mM metformin hydrochloride for 16 h. Only the background wells E1, F1, E12, and F12 are used for correction. The results are shown as average OCR% ± SD. The graph and plate grid were created and exported as image files by the assay design, data analysis, and file management software.

Figure 8: Palmitoyl carnitine/malate-induced respiration. (**A**) A549 and (**B**) HepG2. The tested group was treated with 1 mM metformin hydrochloride for 16 h. Only the background wells G1, H1, G12, and H12 are used for correction. The results are shown as average OCR% ± SD. The graph and plate grid were created and exported as image files by the assay design, data analysis, and file management software.

Table 1: Mitochondrial assay solution. Mix the indicated volume of each ingredient stock solution to prepare (2x MAS). Warm the solution at 37 °C, then adjust pH with 5N KOH to 7.4. Add distilled water to bring the volume up to 1 L. Filter sterilize and then store aliquots at -20 °C. Prepare the assay medium and working concentrations of mitochondrial substrates and inhibitors using 2x MAS.

Table 2: Commands of the assay protocol.

Table 3: List of mitochondrial substrates and inhibitors to be loaded into injection ports of the sensor cartridges as previously discussed in Figure 3. Mix the indicated volumes from stock solutions, 2x MAS, and distilled water to prepare the 5 mL of the working concentration of each substrate or inhibitor mixture. The final concentration is the concentration achieved in the wells after the injection process.

DISCUSSION:

This protocol is a modification of previously published studies[7–10] and the product user guide. In contrast to the manufacturer's protocol, 2x MAS is used instead of 3x MAS, since 2× MAS is easier to dissolve and does not form precipitations after freezing. Frozen 2x MAS aliquots can be stored up to 6 months and show consistent results. Another difference is including ADP in the components of 2x MAS and omitting BSA from the formula. Solutions containing BSA are more

difficult to inject and cause a larger possibility of errors and outliers. However, the presence of BSA is essential to reduce the amount needed of rPFO to achieve proper permeabilization. Therefore, BSA is added only to the assay medium (MAS-BSA-rPFO) that is used in the permeabilization step after cell washing.

To wash the cells from cell culture medium, this protocol uses PBS instead of MAS. PBS is isotonic and does not cause any change in cellular shape, in contrast to the sodium-free MAS that is rich in potassium and can alter cellular morphology. Another major difference is keeping the equilibration step in the assay protocol. The equilibration step lasts for 12 min, which is equal to 2 measurement cycles. The aim of keeping the equilibration step is to stabilize the temperature inside the instrument and, at the same time, allow the cells to oxidize any possible internal oxidizable stores, which is enhanced by the presence of ADP in the assay medium.

Some considerations concerning cell culture techniques should be given. In this work, the examined cells were seeded on the day before the assay. However, some cells require longer culture or treatment time. If the study design includes differentiated cells, freshly isolated, or non-adherent cells, a proper coating is required to fix cells into the cell culture microplate. This protocol is not suitable for cells in suspension, and the use of cell and tissue adhesive is recommended. Another limitation to this protocol is that this method is not suitable to conclude quantitative data. In other words, it is not possible by this method to estimate the actual amount of mitochondrial protein in each well before the measurement. Therefore, this method generates a quick screening of mitochondrial substrate flux without providing an accurate estimate of the phosphate/oxygen ratio (P/O ratio)[5]. However, it is possible to use this protocol for quantitative studies on small samples[11]. For this purpose, it is necessary to obtain freshly isolated mitochondria and use cell and tissue adhesive to fix mitochondria to the cell culture microplate.

For obtaining the best reproducible results from using this protocol, pay attention to the concentration of the used reagents. The temperature of the used solutions, incubators, and instrument should be stable. Ensure that all the solutions and substrates have an adjusted pH. As previously mentioned, it is not recommended to include BSA in the solutions planned to be injected. If the results show a wide error range, display the results in the well format instead of group format to look for and delete possible outliers.

In this work, we tried to achieve maximum use of a single measurement to simultaneously screen multiple mitochondrial substrate fluxes with proper background control and enough replicates in a relatively short time. As shown in the results, the method is useful in comparing two phenotypes created by treating one group with one concentration of a drug. It can be employed to compare different cell lines or genetically engineered cells. The protocol is versatile and different substrates, or inhibitors can be used to screen adaptation of mitochondrial substrate flux in any cellular model.

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DISCLOSURES:

The authors have no conflict of interest to declare.

REFERENCES:

1. Gerencser, A. A. et al. Quantitative microplate-based respirometry with correction for oxygen diffusion. *Analytical Chemistry*. **81** (16), 6868–6878. (2009).
2. Murphy. E. et al. Mitochondrial function, biology, and role in disease: A scientific statement from the American Heart Association. *Circulation Research.* **118** (12), 1960–1991 (2016).
3. Owen, O. E., Kalhan, S. C., Hanson, R. W. The key role of anaplerosis and cataplerosis for citric acid cycle function. *Journal of Biological Chemistry*. **277** (34), 30409–30412 (2002).
4. Nicholls, D. G., Ferguson, S. J. *Bioenergetics 3*. Academic press, London (2002).
5. Brand, M. D., Nicholls, D. G. Assessing mitochondrial dysfunction in cells. *Biochemical Journal*. **435** (2), 297–312 (2011).
6. Staňková, P. et al. Adaptation of mitochondrial substrate flux in a mouse model of nonalcoholic fatty liver disease. *International Journal of Molecular Sciences*. **21**(3), 1101 (2020).
7. Salabei, J. K., Gibb, A. A., Hill, B. G. Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. *Nature Protocols*. **9**(2), 421–438 (2014).
8. Divakaruni, A. S. et al. Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. *Proceedings of the National Academy of Sciences of the United States of America*. **110** (14), 5422–5427 (2011).
9. Divakaruni, A. S., Rogers, G. W., Murphy, A. N. Measuring mitochondrial function in permeabilized cells using the seahorse XF analyzer or a Clark-type oxygen electrode. *Current Protocols in Toxicology*. **60**, 25.2.1–25.2.16 (2014).
10. Elkalaf, M., Tůma, P., Weiszenstein, M., Polák, J., Trnka, J. Mitochondrial probe Methyltriphenylphosphonium (TPMP) inhibits the Krebs cycle enzyme 2-Oxoglutarate dehydrogenase. *PLoS One*. **11** (8), e0161413 (2016).
11. Rogers G. W. et al. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS One*. **6** (7), e21746 (2011).

Figure 1

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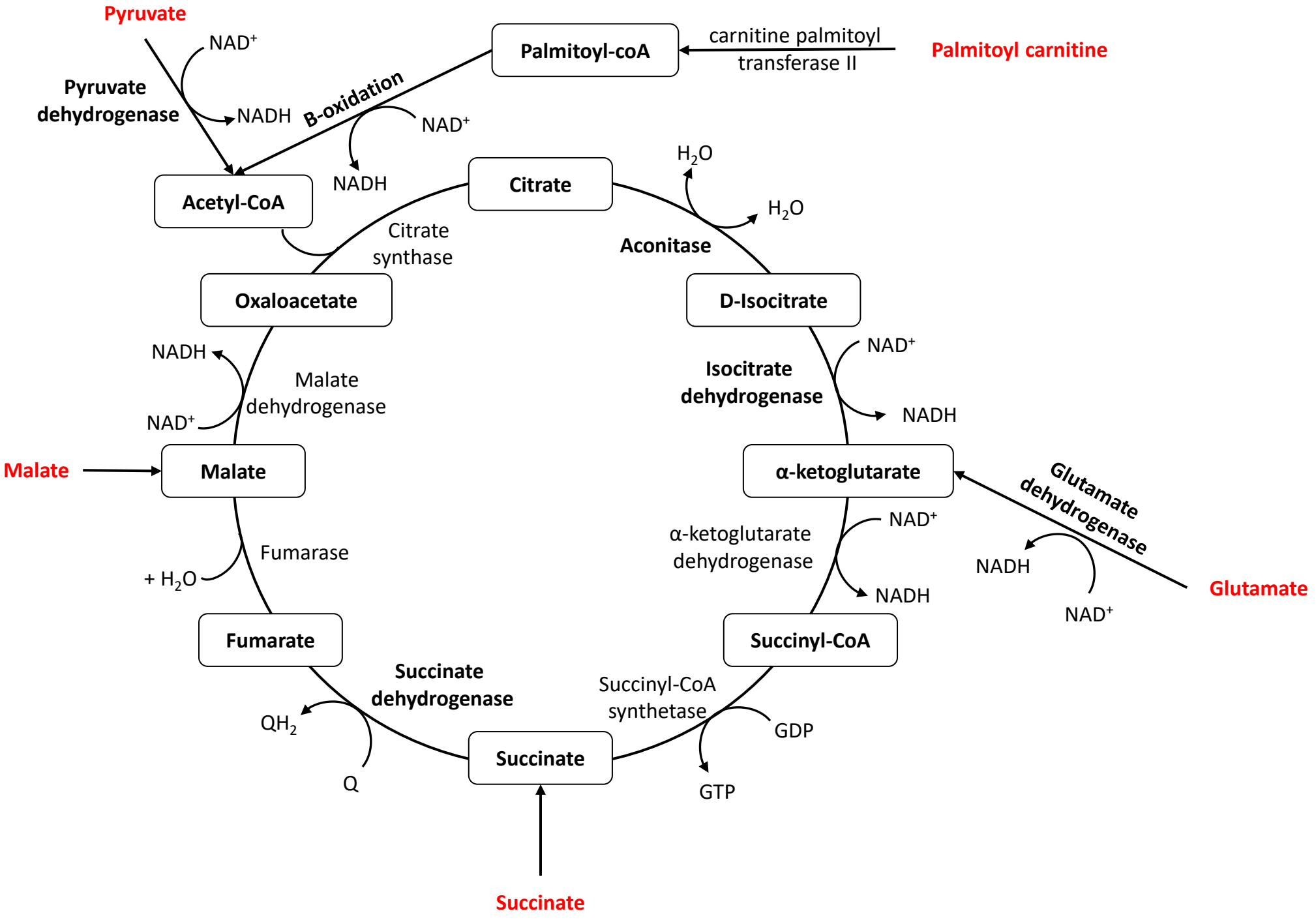
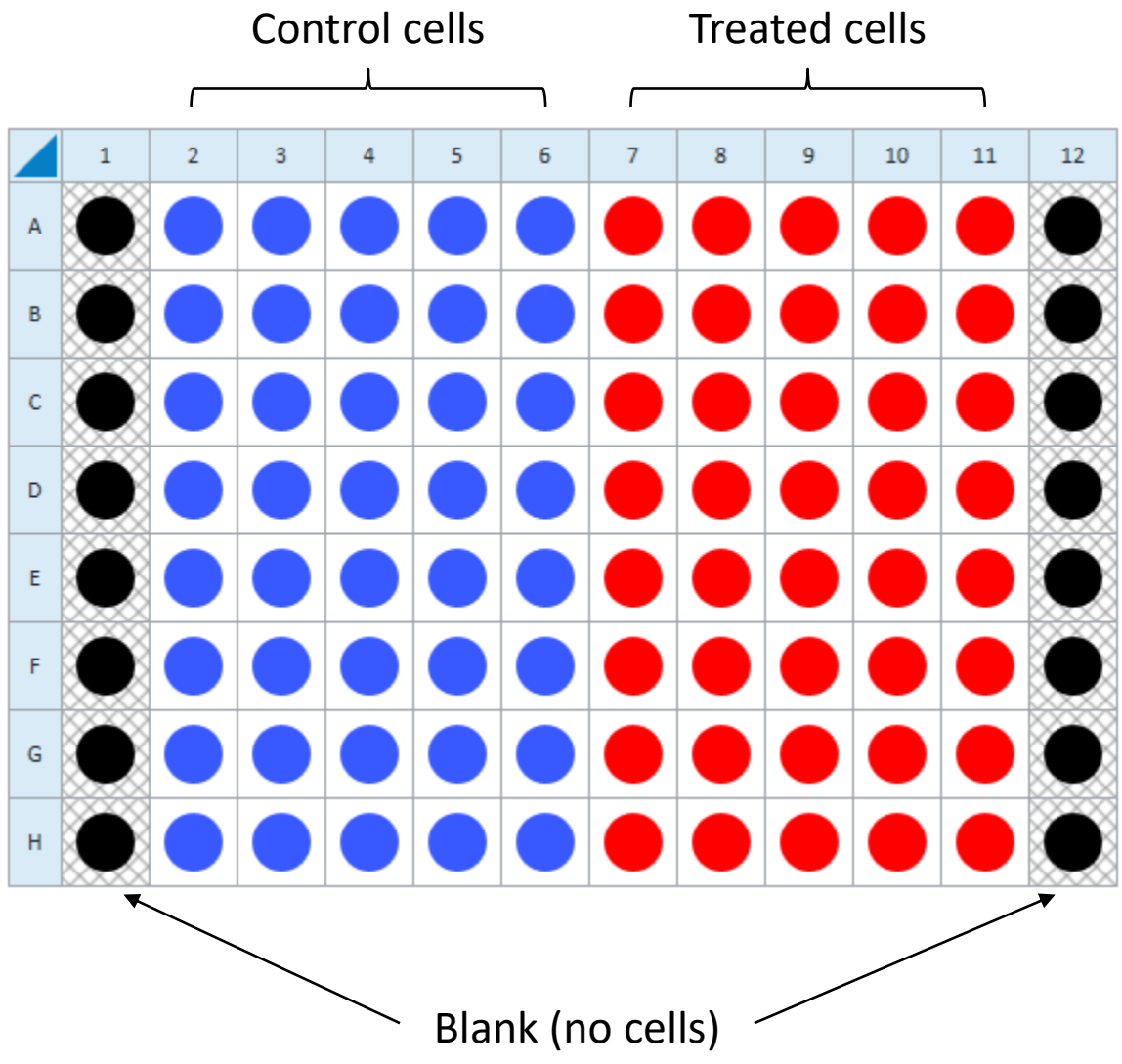


Figure 2



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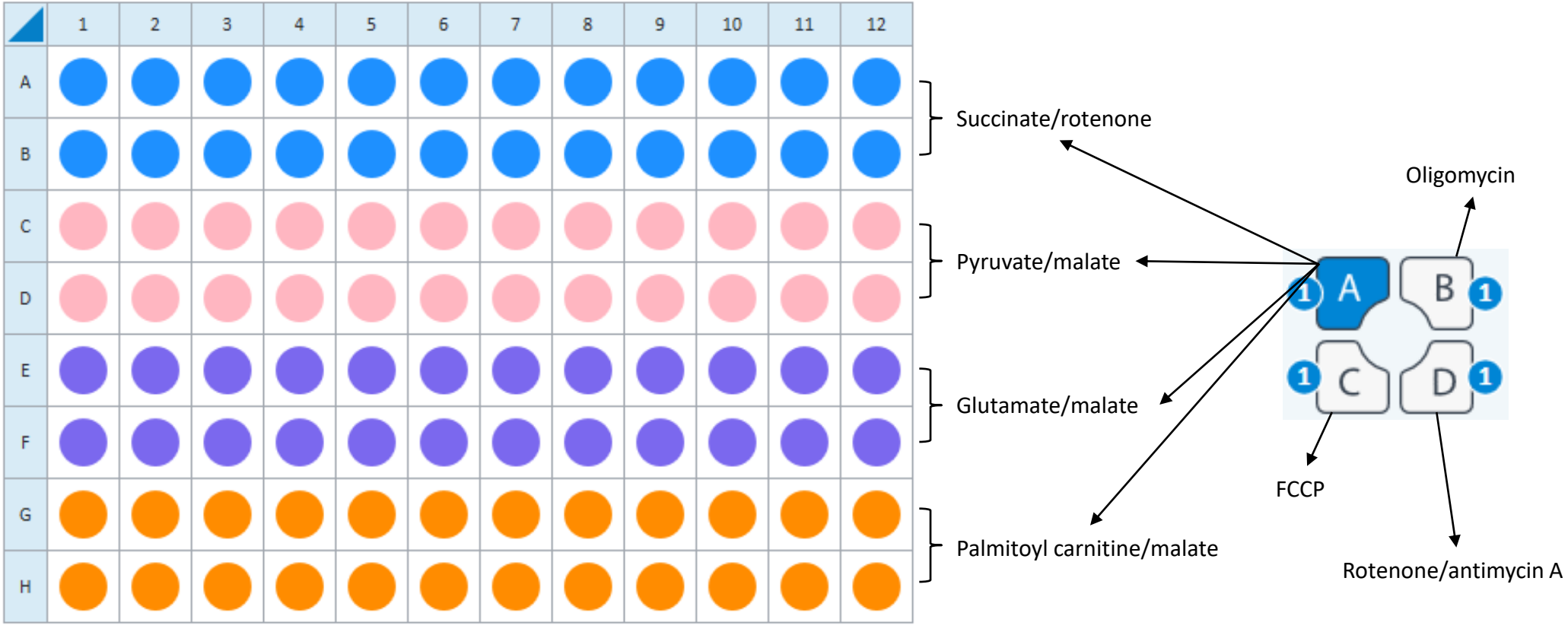

Figure 4

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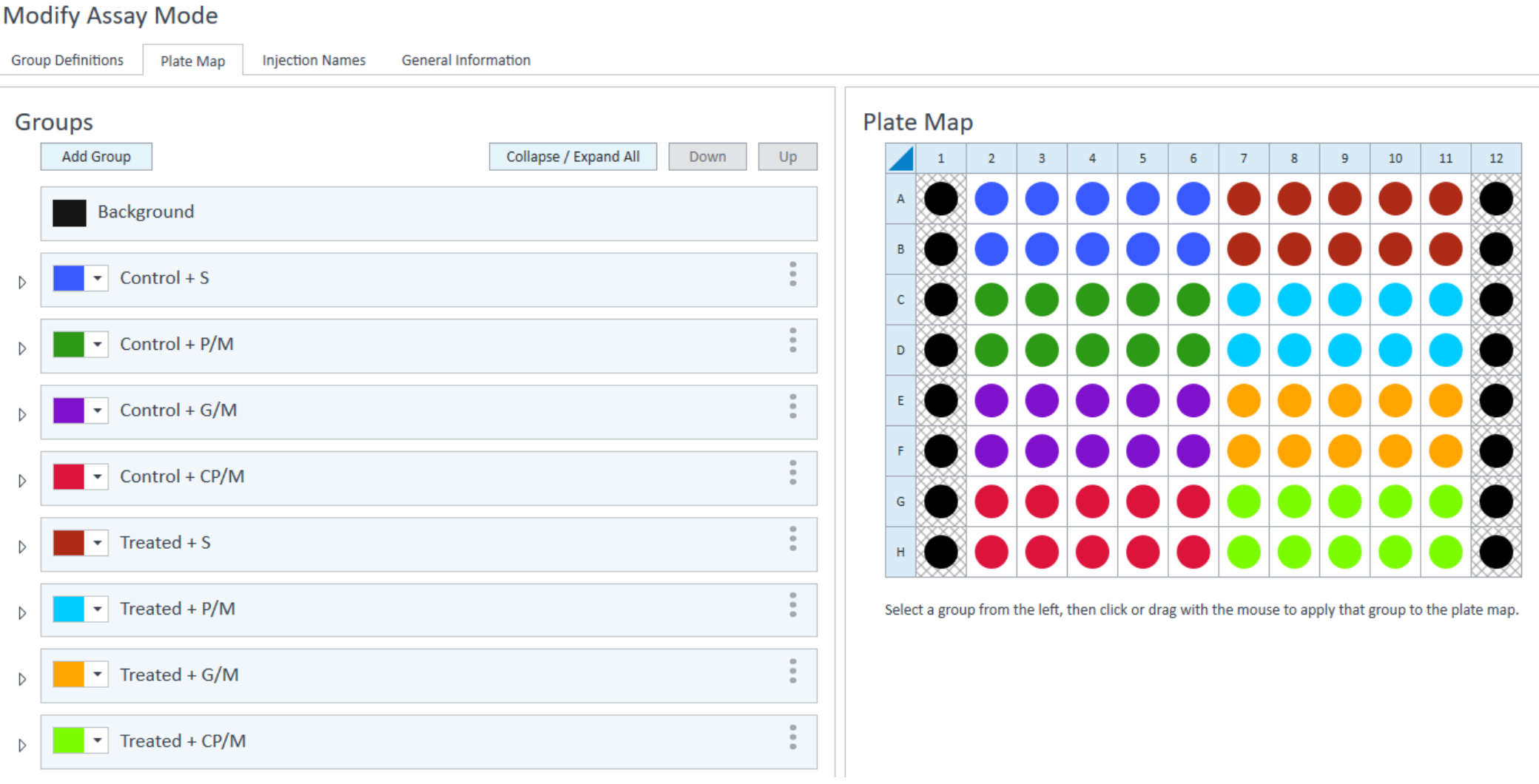

Figure 5

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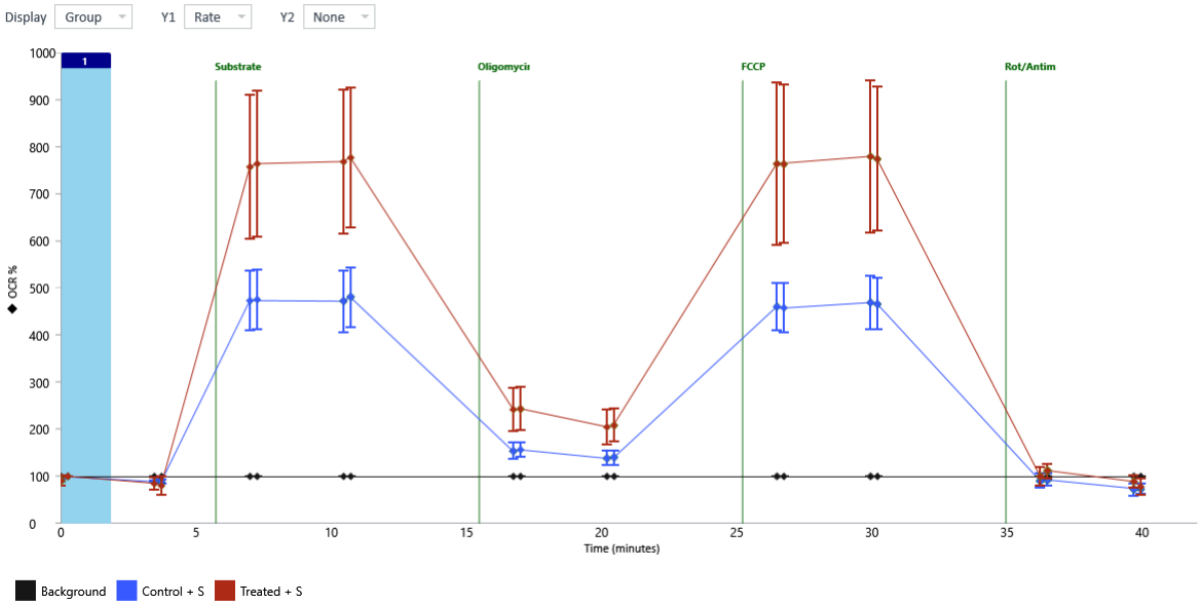
A



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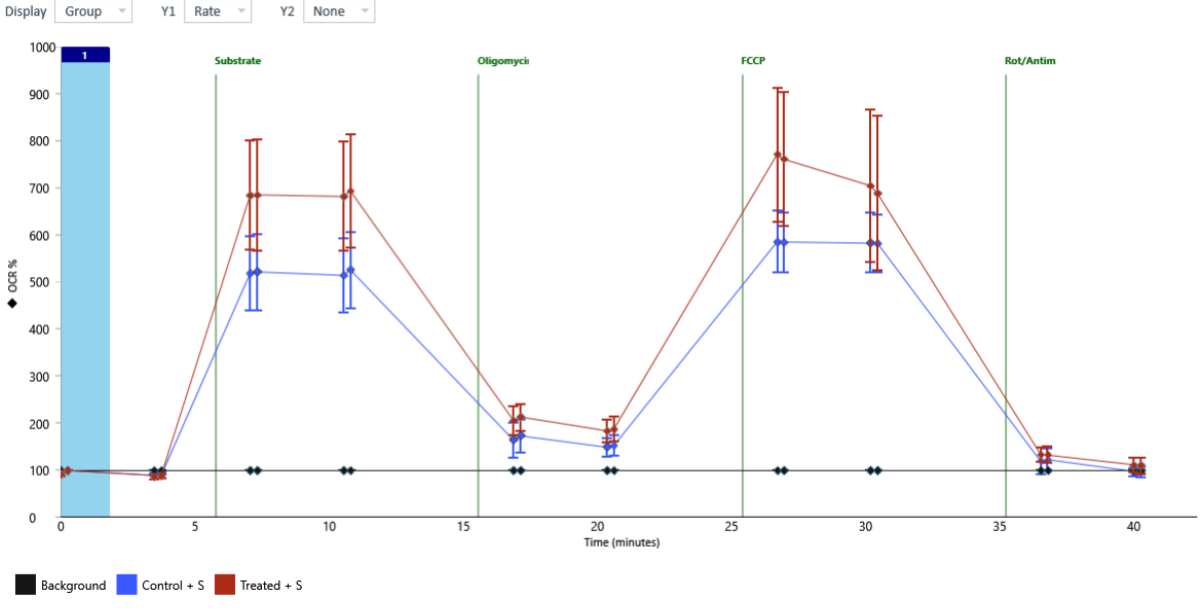
B	100.00	111.72	106.14	103.90	107.52	102.25	119.76	109.34	110.60	124.09	108.46	100.00
C	-15.99	101.98	109.61	107.47	105.63	115.24	111.74	110.50	125.14	133.82	111.09	198.45
D	3579.00	111.45	107.58	109.71	111.11	110.42	116.18	116.36	116.13	131.18	115.44	177.04
E	59.02	109.71	107.53	111.65	110.46	102.36	128.05	126.08	102.29	109.30	104.50	440.55
F	39.33	113.99	106.33	112.89	108.15	108.96	117.41	115.52	115.04	123.68	108.05	469.65
G	-10.64	116.11	107.31	113.36	111.96	110.57	115.23	118.55	96.92	112.61	105.94	3.02
H	728.39	107.84	95.87	102.12	99.53	104.05	128.35	156.68	124.00	110.23	96.60	109.79

Background Control + S Treated + S

B



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B	100.00	116.99	113.97	110.45	113.83	105.54	107.77	115.28	109.90	109.57	105.07	100.00
C	1332.35	**108.07**	**97.62**	**104.60**	**103.34**	**108.89**	112.07	114.89	109.51	105.63	118.54	1659.44
D	42.24	**110.17**	**108.47**	**109.84**	**103.91**	**103.52**	108.36	113.63	106.34	107.44	114.39	21.41
E	61.59	104.86	109.69	105.69	110.24	104.32	119.44	84.60	106.57	116.04	116.60	186.96
F	143467...	112.69	107.51	110.47	108.61	101.86	112.05	108.25	106.49	111.67	113.62	98.11
G	143.07	110.85	111.75	111.40	105.84	110.27	112.85	110.84	107.41	104.14	118.98	84.67
H	98.42	99.42	100.71	108.63	104.25	108.84	109.22	99.27	99.55	109.62	121.63	207.64

Background Control + S Treated + S

Figure 6

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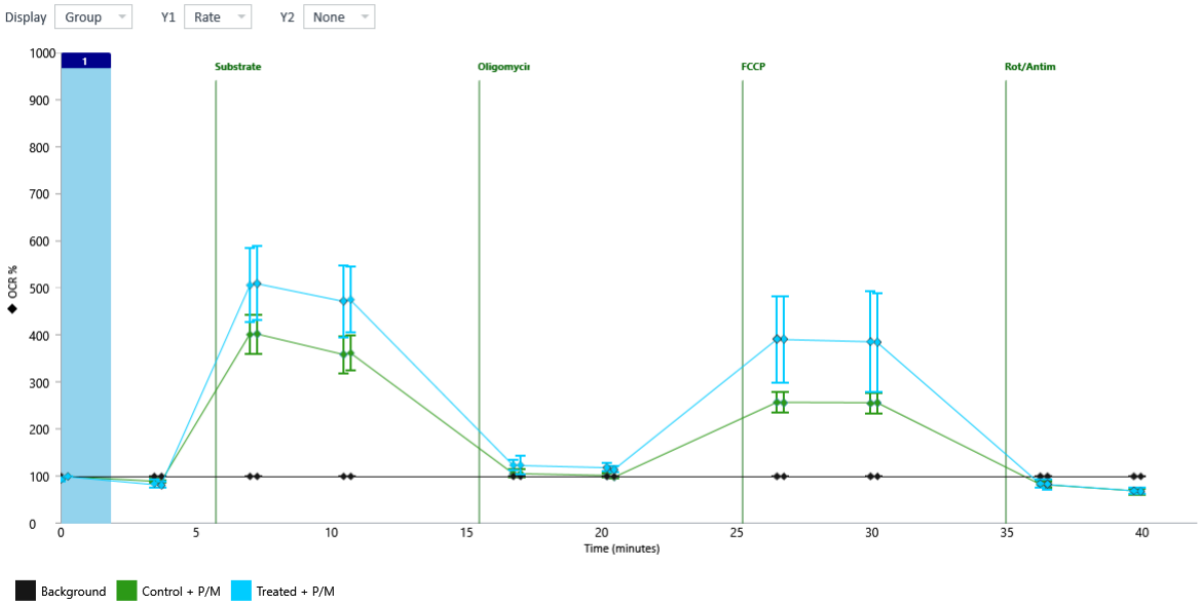

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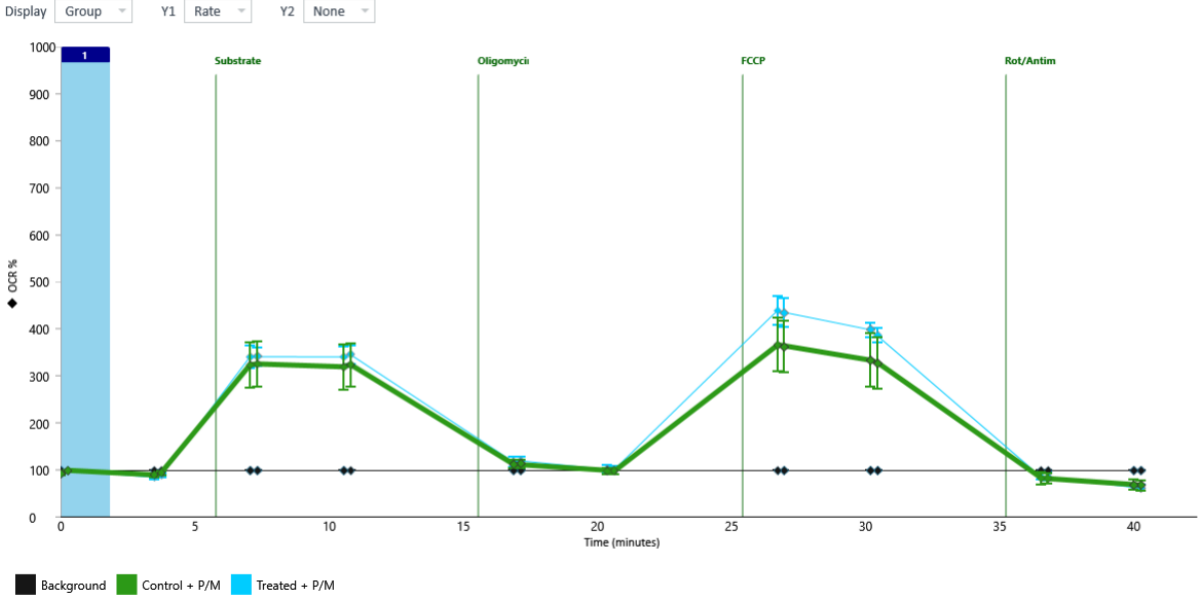
C	100.00	100.52	108.15	106.02	104.17	113.96	109.05	107.56	122.49	131.24	108.95	100.00
D	100.00	110.00	106.19	108.40	109.71	109.11	113.45	113.46	113.23	128.73	113.15	100.00
E	-12.26	108.14	106.10	110.34	109.04	101.03	125.47	123.62	99.57	106.30	103.05	284.05
F	5.98	112.55	105.00	111.53	106.71	107.62	115.41	113.21	112.58	121.20	107.11	291.13
G	-219.90	114.67	105.73	111.99	110.41	109.11	112.73	115.77	93.81	109.50	104.81	-139.00
H	254.46	105.96	93.58	99.69	97.19	102.01	123.19	152.26	116.78	105.43	91.86	59.91

Background Control + P/M Treated + P/M

B



OCR %

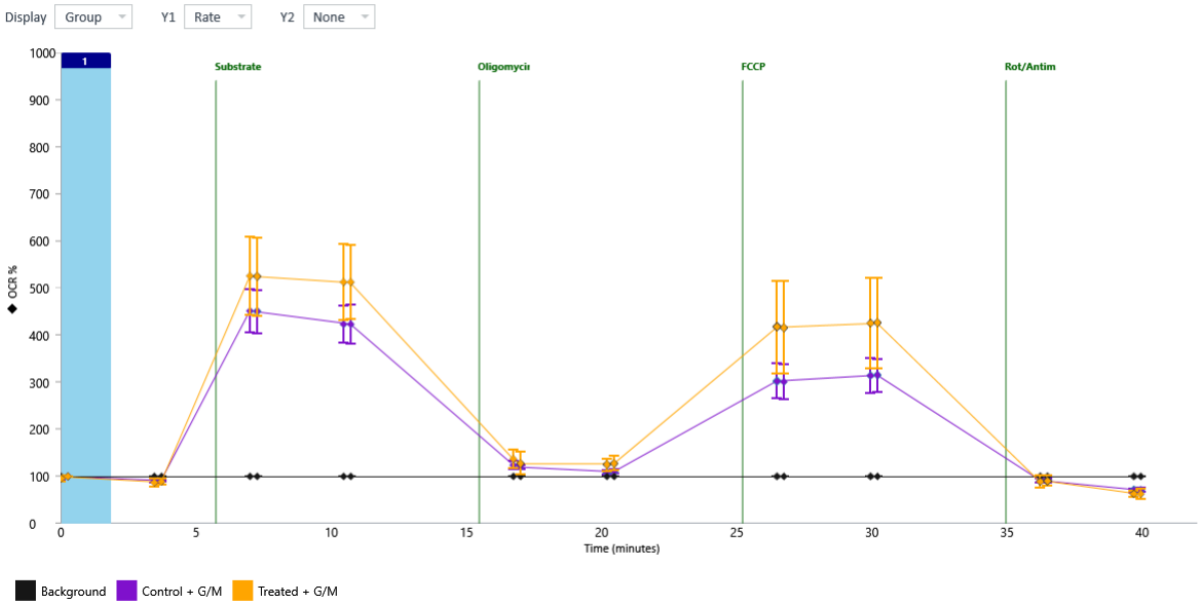
	1	2	3	4	5	6	7	8	9	10	11	12
A	107.37	119.00	106.70	111.59	92.35	97.95	109.40	108.85	98.96	117.04	105.23	18.29
B	193.80	115.79	112.66	109.28	112.61	104.26	105.84	114.05	108.50	108.18	103.62	85.45
C	100.00	**106.71**	**96.39**	**103.37**	**102.12**	**107.74**	110.10	113.25	108.03	103.99	117.01	100.00
D	100.00	**109.05**	**107.32**	**108.73**	**102.63**	**102.34**	106.35	112.19	104.64	105.68	112.82	100.00
E	10.51	103.54	108.58	104.31	109.14	103.20	117.42	82.28	103.79	114.15	115.18	187.33
F	807.01	111.39	106.32	109.10	107.48	100.57	110.07	106.73	104.52	109.68	111.48	-105.49
G	125.08	109.69	110.32	109.95	104.39	108.93	111.17	108.75	105.45	101.77	117.00	61.25
H	86.37	97.91	98.75	107.02	102.40	107.38	106.23	96.62	96.88	107.12	119.34	133.16

Background Control + P/M Treated + P/M

Figure 7

Click here to access/download;Figure;Figure 7.pdf

A

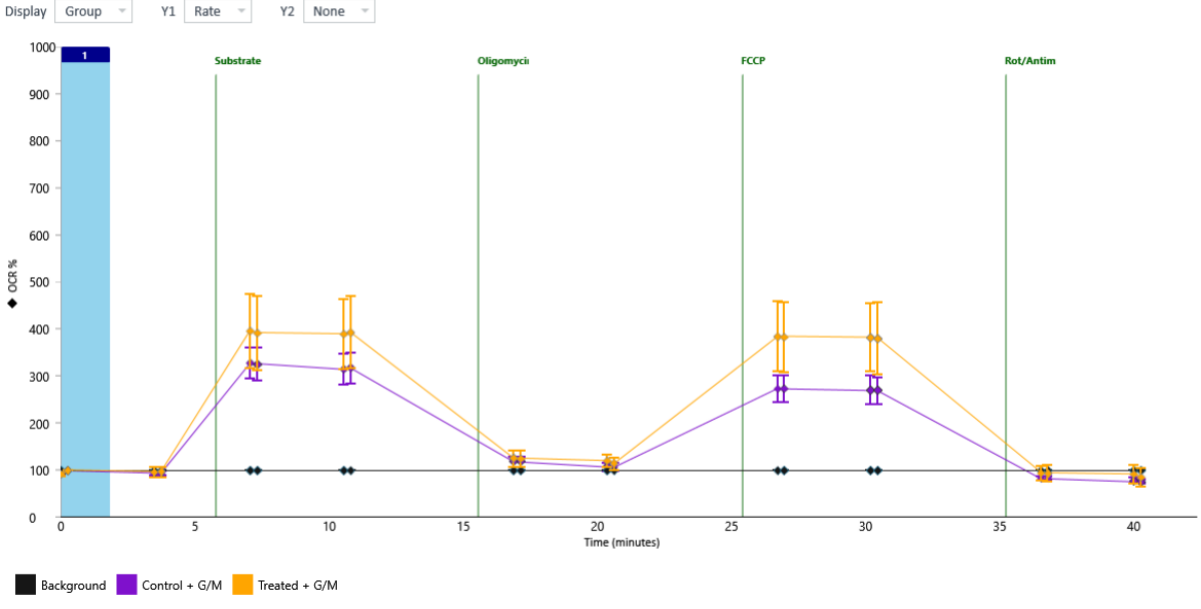


OCR %

	1	2	3	4	5	6	7	8	9	10	11	12
A	-541.19	108.24	100.80	106.11	112.30	102.03	116.70	157.95	121.47	73.53	96.57	-361.26
B	89.36	109.49	103.54	101.37	105.18	99.54	114.79	104.66	106.18	120.52	105.73	148.59
C	-210.69	99.81	107.47	105.33	103.47	113.37	107.77	106.15	121.28	130.10	107.94	121.69
D	208.52	109.32	105.53	107.78	109.05	108.49	112.16	112.09	111.85	127.64	112.08	137.31
E	100.00	107.41	105.42	109.73	108.37	100.38	124.30	122.51	98.25	104.85	102.36	100.00
F	100.00	111.88	104.37	110.89	106.03	107.00	114.48	112.14	111.42	120.06	106.67	100.00
G	-725.02	114.00	104.99	111.35	109.68	108.43	111.55	114.47	92.25	108.02	104.27	-357.89
H	124.37	105.07	92.45	98.50	96.05	101.02	120.75	150.50	113.19	103.06	89.44	49.01

Background Control + G/M Treated + G/M

B



OCR %

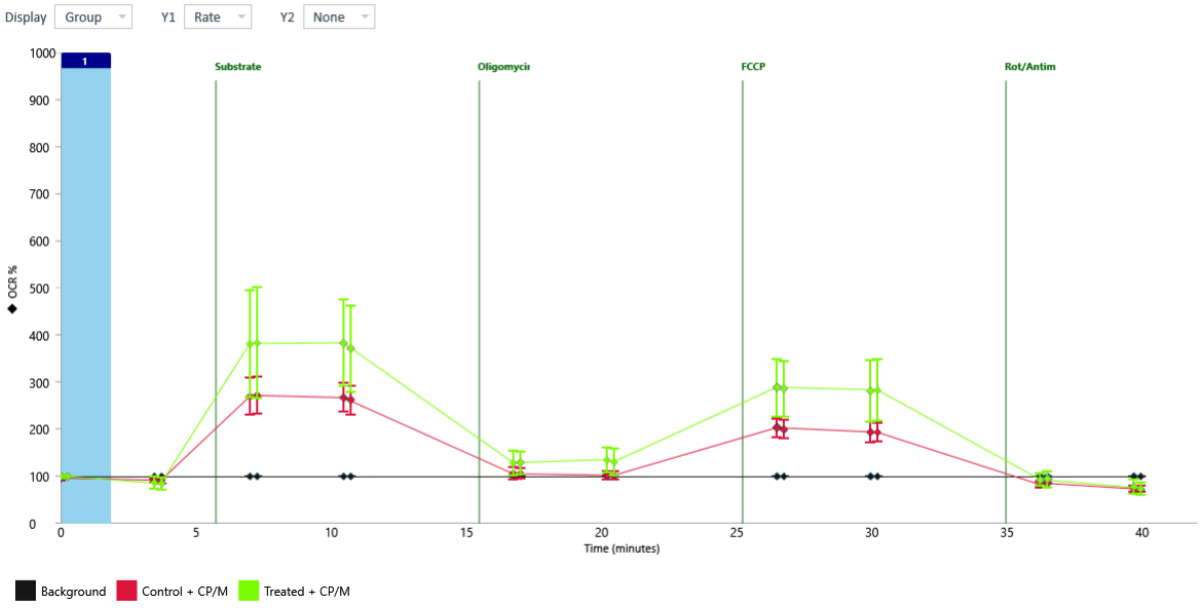
	1	2	3	4	5	6	7	8	9	10	11	12
A	92.15	114.74	100.95	107.04	86.82	92.38	102.02	101.17	91.47	112.15	99.69	5.85
B	67.82	111.61	108.18	105.39	108.47	100.06	99.30	109.80	103.80	103.54	98.83	64.99
C	95.92	**102.20**	**92.48**	**99.34**	**98.17**	**103.93**	103.27	107.53	103.06	98.53	111.59	92.60
D	-321.87	**105.35**	**103.51**	**105.04**	**98.49**	**98.54**	99.45	107.25	98.95	99.74	107.38	-532.51
E	100.00	99.20	104.87	99.78	105.50	99.58	110.08	74.97	94.06	107.48	110.23	100.00
F	100.00	106.96	102.39	104.46	103.74	96.40	103.19	101.67	97.85	102.80	103.93	100.00
G	-2276.81	105.85	105.46	105.02	99.62	104.41	105.41	101.53	98.79	93.74	109.85	-107.24
H	61.21	93.04	92.25	101.58	96.22	102.48	95.55	87.73	87.89	98.36	110.80	63.84

Background Control + G/M Treated + G/M

Figure 8

Click here to access/download;Figure;Figure 8.pdf

A

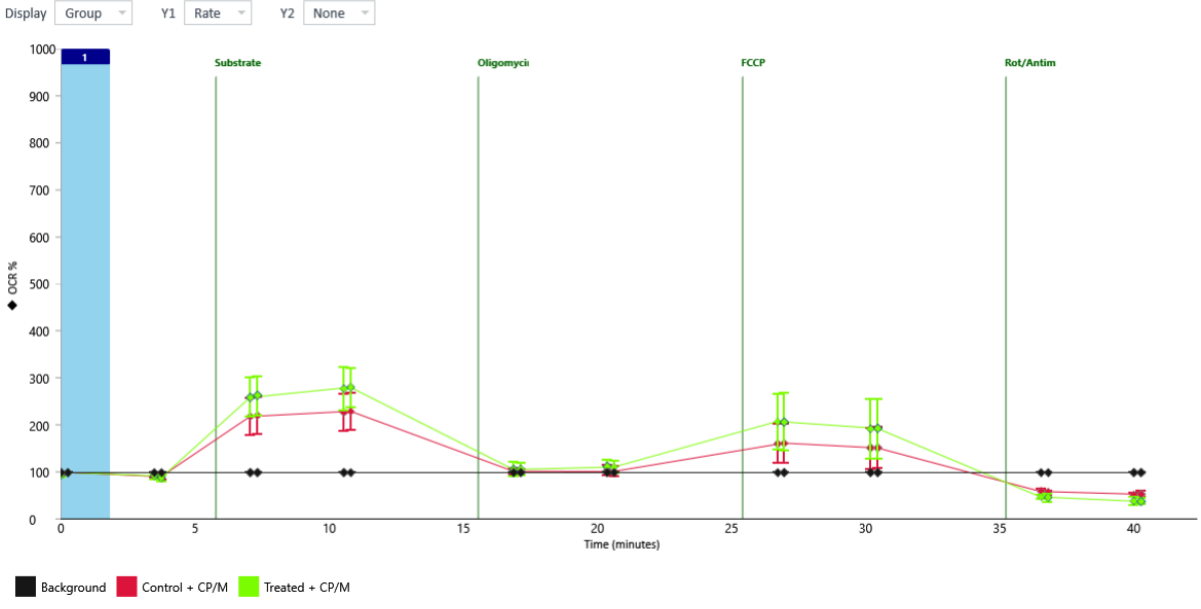


OCR %

	1	2	3	4	5	6	7	8	9	10	11	12
A	-87.18	112.37	105.40	111.01	118.14	106.92	130.64	175.98	137.39	89.43	100.71	-691.39
B	189.04	112.55	107.01	104.71	108.32	103.09	121.91	110.99	112.19	125.79	109.41	190.72
C	-26.87	102.64	110.37	108.20	106.35	115.99	113.22	112.07	127.01	135.97	112.25	193.98
D	603.06	112.25	108.29	110.41	111.87	111.13	117.81	118.09	117.86	133.12	116.79	220.52
E	69.30	110.54	108.25	112.36	111.23	102.98	129.97	127.84	103.52	110.87	105.19	367.16
F	41.34	114.81	106.99	113.65	108.88	109.65	118.63	116.87	116.48	125.39	108.53	381.55
G	100.00	116.97	108.10	114.14	112.81	111.36	116.70	120.28	98.19	114.34	106.50	100.00
H	100.00	108.79	96.79	103.23	100.53	105.01	132.19	162.31	128.95	112.78	98.51	100.00

Background Control + CP/M Treated + CP/M

B



OCR %

	1	2	3	4	5	6	7	8	9	10	11	12
A	109.67	119.31	107.26	111.99	93.02	98.57	110.06	109.54	99.76	117.41	105.78	10.15
B	314.07	116.12	113.05	109.64	112.97	104.69	106.47	114.40	108.94	108.62	104.11	87.78
C	581.68	**107.15**	**96.84**	**103.79**	**102.54**	**108.10**	110.71	113.72	108.50	104.54	117.42	619.83
D	2.35	**109.40**	**107.69**	**109.08**	**103.07**	**102.75**	107.01	112.61	105.21	106.26	113.28	-16.42
E	40.22	103.99	108.93	104.78	109.49	103.58	117.95	83.24	104.74	114.69	115.58	183.31
F	2504.15	111.78	106.71	109.53	107.84	101.02	110.68	107.23	105.18	110.30	112.12	48.84
G	100.00	110.05	110.76	110.39	104.88	109.35	111.68	109.41	106.10	102.60	117.53	100.00
H	100.00	98.46	99.45	107.54	103.04	107.85	107.21	97.60	97.86	107.93	119.92	100.00

Background Control + CP/M Treated + CP/M

Mitochondrial assay medium

	Stock (mM)	Volume from stock per liter (mL)	2x MAS (mM)	MAS (mM)
Sucrose	1000	140	140	70
Mannitol	1000	440	440	220
KH_2PO_4	1000	20	20	10
$MgCl_2$	200	50	10	5
HEPES	200	20	4	2
EGTA	200	10	2	1
ADP	200	20	4	2

Table 2

Command	Duration	Injected compound
Calibration	by default	
Equilibration	Yes	
Baseline		
2 Cycles		
Mix	30 s	
Wait	30 s	
Measure	2 min	
Inject Port A		**Substrates**
2 Cycles		
Mix	30 s	
Wait	30 s	
Measure	2 min	
Inject Port B		**Oligomycin**
2 Cycles		
Mix	30 s	
Wait	30 s	
Measure	2 min	
Inject Port C		**FCCP**
2 Cycles		
Mix	30 s	
Wait	30 s	
Measure	2 min	
Inject Port D		**Rotenone + Antimycin A**
2 Cycles		
Mix	30 s	
Wait	30 s	
Measure	2 min	

Table 3

			Volume in 5 mL			
Substrates	Stock conc.	Working conc.	**Stock**	**2x MAS**	**dH_2O**	Final conc.
Succinate/rotenone	1 M/20 mM	100 mM/10 µM	**500 µL/ 2.5 µL**	**2.5 mL**	**1997.5 µL**	10 mM/1 µM
Pyruvate/malate	1 M/100 mM	100 mM /10 mM	**500 µL/500 µL**	**2.5 mL**	**1500 µL**	10 mM /1 mM
Glutamate/malate	1 M/100 mM	100 mM /10 mM	**500 µL/500 µL**	**2.5 mL**	**1500 µL**	10 mM /1 mM
Palmitoyl carnitine/malate	10 mM/100 mM	400 µM /10 mM	**200 µL/500 µL**	**2.5 mL**	**1800 µL**	40 µM /1 mM
Inhibitors						
Oligomycin	25 mM	15 µM	**3 µL**	**2.5 mL**	**2497 µL**	1.5 µM
FCCP	50 mM	40 µM	**4 µL**	**2.5 mL**	**2496 µL**	4 µM
Rotenone/antimycine A	20 mM/20 mM	10 µM/ 10 µM	**2.5 µL/2.5 µL**	**2.5 mL**	**2495 µL**	1 µM/ 1 µM

Moustafa Elkalaf, MBBCh,
Charles University
Faculty of Medicine in Hradec Králové
Third Faculty of Medicine
Czech Republic

June 30, 2021

Subject: Rebuttal letter
To:
Vidhya Iyer, Ph.D.
Review Editor
JoVE

Dear Dr. Iyer,

Thank you for forwarding us the editorial and reviewers' comments to our manuscript. We have modified the manuscript to address the concerns of the editors, reviewer 2 and reviewer 3. In this letter, we have included a point-by-point response to all comments and we hope that the manuscript is now ready for publication in JoVE.

Yours sincerely,

Moustafa Elkalaf

Please revise the manuscript to thoroughly address the reviewers' concerns and all the editorial comments. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Done

2. Please revise your title to “Measuring Mitochondrial Substrate Flux in Recombinant Perfrinolysin O-permeabilized cells.”

Done

3. Please provide an email address for each author.

The original manuscript had 6 emails for 6 authors.

4. Please delete the template instructions before you submit your revision.

Done

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example, Wave controller; MAS-BSA-XF PMP; Wave Desktop; Falcon tube etc

Although the Seahorse XFe96 analyzer is clearly crucial for your protocol, avoid mentioning the commercial name too many times and especially in the title.

Even in the summary, please delete” that is available commercially as Seahorse XF plasma membrane permeabilizer”.

Line 127: please replace “using XF PMP” with “rPFO”.

All these terms have been changed in the revised manuscript. The product XF PMP has been replaced by recombinant perfringolysin O (rPFO)

6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done

8. Please consider providing solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

The original submission included the components as Table 1 and 3. Both tables were referenced in the text.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We tried to explain the steps in the most simplified manner.

10. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
Done

11. Consider providing information in Figure 2 as a table (.xls) and not as a figure (pdf).
We have decided to delete this figure from the revised manuscript. All figures have been renamed to match the new order.

12. As we are a methods journal, please add any limitations of your technique and the significance with respect to existing methods to the Discussion.
The original manuscript included the limitations in the discussion section

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Done

14. Please sort the Materials Table alphabetically by the name of the material.
Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The protocol provides a guidline to use the commercially avaible permeabilizer (Recombinant perfringolysin O, rPFO) to selectively permeabilize plasma membrane without affecting mitochondrial integrity. After permeabilization, it allows testing the flux of several mitochondrial substrates using Seahorse XFe96 analyzer.

Major Concerns:

For me,

(1) I saw no evidence supporting that the permeabilizer only permeabilize plasma membrane without affecting mitochondrial integrity. How about other permeabilizer, such as digitonin, saponin ect? The authors should provide convencing evidence that demonstrating the permeabilization is working.

We don't believe adding the mechanism of action or the chemistry of permeabilization would improve by anyway the protocol discussed in a methodology journal. A paper that was cited in the manuscript have compared permeabilizing agents (reference 7). Also, references 8 and 9 discussed in more detail the mechanism of rPFO. Adding such explanation to the text would be trivial.

(2) The authors should highlight the necessarilatiy for performing flux assay of several mitochondrial substrates. Why do we need to compare different substract oxidizing pathways or capacities?

The importance of testing mitochondrial substrate flux is mentioned in the abstract and introduction section.

(3) Do the authors also include injection soultions into the empty wells, or not? Based on our experience, even the injection pores for the empty wells must be filled as well, otherwise injections could have some problems. The authors should provide this information.

Yes. It was explained in figure 4 in the original submission, now it is figure 3.

(4) Why the authors using so many empty well for background? Generally 4 wells are succifient.

Because not all wells are treated with the same compound in the first injection (Port A). This part specifically was discussed thoroughly in the text and figures (6-9) now (5-8).

(5) In all the assays, FCCP-induced maximal respiration was also impaired? How would the authors interpret this? Why the authors do not perform quantitive and statistic assays?

We see that FCCP induced respiration was not impaired in any condition, but rather the treated group had higher substrate induced respiration (coupled) and FCCP induced respiration (uncoupled).

For the statistics and the quantitative assays, it is not within the journal scope. We are discussing the methodology and how we do the method. I'd quote the comment of reviewer 3 as a possible answer "This protocol is mainly focused on the application of the XFe96 analyzer in combination with permeabilized cells to gain insights into mitochondrial function that would otherwise require arduous isolation of mitochondria".

Reviewer #2:

Manuscript Summary:

This is a new optimized protocol modified protocol to compare mitochondrial substrate flux of different cellular phenotypes or genotypes and can be customized to test various mitochondrial substrates or inhibitors.

The manuscript it very well written and the protocol will be useful for many labs who are assessing mitochondrial flux

Major Concerns:

9 figures are too much and I would suggest to reduce them the major 3-4 figures.

We believe that the figures are necessary to include to guide the reader through the protocol. Figure 2 has been removed and the new number of figures is 8.

Reviewer #3:

Manuscript Summary:

This current manuscript by Elkalaf et al. presents a modified protocol that was based on previously published protocols for measuring mitochondrial function using the Seahorse extracellular flux analyzer XFe96. This protocol is mainly focused on the application of the XFe96 analyzer in combination with permeabilized cells to gain insights into mitochondrial function that would otherwise require arduous isolation of mitochondria.

Overall, the protocol is well written and provide a consistent guideline on how to apply this method reproducibly. The figures and tables are informative and used effectively to guide readers through the practical setup of an experiment. Specific deviations from previous publications were addressed in the discussion and reasons for each change were explained well with adequate reasoning.

At this time, I think that I should be able to perform this protocol without difficulties, however this would be confirmed upon actually performing the experiment, which I have not done. I only have a few minor comments to be considered for revision.

Minor Concerns:

1. This manuscript seems to be a kind of companion for previously published protocols. The current protocol work well if the reader is familiar with these previous protocols. But if this is not the case, the reader may face various deviations from the standard protocols provided by the producer (Agilent). It may be helpful to add a section with a very brief comparison between the current protocol and the closest protocol from Agilent. Note that this would not necessarily improve any aspect of the protocol itself and would only be helpful for novice researchers or labs.

The original manuscript contained the differences and the main modifications that we have made in the discussion section.

2. Some additions in various mixes were only explained explicitly in the legends of figures (e.g., malate). Consequently, the effects of such components may not be obvious at first and could therefore be improved.

In this work we are trying to describe the protocol we are using and explaining the need of these addition mixes was described only as part of Figure 1 to highlight the pathway of each used substrate. We have explained only “malate” since it is a common addition to 3 different mixes. We agree that the description of these addition can be improved, but we don’t believe that it is required for the method.

3. The figures are very informative regarding the setup in general. However, it is clear what the authors wanted to achieve with figure 2, it seems somewhat overly simplified and a redesign to make the figure easier to use as a checklist for each day of the protocol would be recommended. Currently, the figure is quite large and the subsequent steps seem to be quite scattered over the page. Therefore, improving the visualization of the sequence of the steps may prevent researchers from missing one.

We have deleted figure 2 in the revised manuscript. We didn’t want to make it as a checklist since it would be a replica of the work of Salabei et al. Figure 2. This work is referenced in the manuscript as reference no. 7.

4. The manuscript still contains some small typos that should be corrected.

We have revised the text for proofreading