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Lepore, A orcid.org/0000-0002-2037-673X, Kaci, FN, Bubici, C et al. (1 more author) (2023) *An Integrated Methodology to Quantify the Glycolytic Stress in Plasma Cell Myeloma in Response to Cytotoxic Drugs*. In: Papa, S and Bubici, C, (eds.) *Metabolic Reprogramming: Methods and Protocols*. *Methods in Molecular Biology*, 2675 . Springer , pp. 285-296. ISBN 9781071632468

https://doi.org/10.1007/978-1-0716-3247-5_21

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An integrated methodology to quantify the glycolytic stress in plasma cell myeloma in response to cytotoxic drugs

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Running title: Extracellular acidification rate in myeloma cells

Keywords: cellular stress, multiple myeloma, aerobic glycolysis, cytotoxic drugs, metabolic reprogramming

Abstract

Multiple Myeloma (MM) is an incurable plasma cell malignancy primarily localized within the bone marrow (BM). Myeloma plasma cells, like many other cancer cells, change their metabolism in response to internal and external stimuli. The main metabolic alterations of MM cells include deregulated glycolysis (commonly associated with enhanced uptake and utilization of glucose), lipid metabolism dysregulation, as well as deregulated mitochondrial respiration (commonly associated with the deregulated formation of reactive oxygen species). Over the past decade, the discovery of novel methodologies and the commercialization of sophisticated instrumentation and reagents have facilitated the detection of real-time changes in cellular bioenergetics. Of those, the Seahorse™ extracellular flux (XF) analyser has been widely used to evaluate the glycolytic flux and mitochondrial respiration in many cell types. While adherent cell lines are easy to use with this technology, non-adherent suspension cells are more difficult to handle especially when their metabolic activities are being investigated in response to drug treatment. Here, we provide an integrated protocol that allows the detection of extracellular acidification rate (ECAR) of live myeloma plasma cells in response to chemotherapeutic drugs. Our optimised protocol consists of treating myeloma cells with cytotoxic drug of interest in a standard culture plate prior to the real-time analysis in the XF analyser. Furthermore, we provide results of experiments in which the metabolic activities of myeloma cells in response to cytotoxic treatment were compared between the manufacturer's basic procedure and our optimised protocol. Our observations suggest that our integrated protocol can be used to achieve consistent, well-standardized results and thus it may have broad applications in studies focusing on the characterization of metabolic events in non-adherent suspension cells

1. Introduction

In recent years, a growing body of evidence has demonstrated that aberrant cellular metabolism is an emerging hallmark of cancer, including multiple myeloma (MM) – an incurable plasma cell malignancy within the bone marrow (BM) [1,2]. It has been proposed that alterations in metabolism of cell nutrients such as glucose and glutamine sustain increased biosynthesis of macromolecules including nucleotides, lipids, and amino acids, required for rapid cell growth and proliferation [3-7]. Increased glycolysis also helps to maintain cellular redox state, therefore contributing to the intrinsic survival (anti-apoptotic) mechanisms of myeloma plasma cells [3-7]. Consequently, dysregulated cell metabolism has been linked to tumour aggressiveness and treatment resistance [4]. Recent studies show that upon stimulation with chemotherapeutic drugs myeloma plasma cells rewire their metabolism [8,9]. Extracellular flux (XF) analyses such as cellular oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) provide powerful tools to study the metabolic rewiring in response to drug treatment in both adherent and non-adherent suspension cells [10,11]. In an attempt to minimize technical variation, we describe here an optimized protocol for testing glycolytic stress of plasma cell myeloma in response to current anti-myeloma drugs using a Seahorse™ XF analyser (**Figure 1**). In this protocol, we pre-treat myeloma cells with either chemotherapeutic drugs or vehicle control in standard culture plates, followed by real-time measurement of metabolic changes using fluorescent sensors in specialized XF microplates. This has the great advantage of having minimal loss of cells during agitation, mixing and change of medium of non-adherent suspension cells, when testing for metabolic changes in response to cytotoxic treatments. Furthermore, our protocol does not require the use of tissue adhesive Cell-Tak (that is often used to immobilize poorly adherent cells), which has been shown to affect cell shape in a cell line-specific manner and may change cellular metabolism [12].

2. Materials

2.1 Reagents and materials

1. L363 Myeloma plasma cell line (RRID:CVCL_1357)
2. Cytotoxic test lead compounds (your drug of interest, e.g., proteasome inhibitor Bortezomib, Velcade™)
3. Tissue culture plates, 6-well
4. 96-well microplates
5. 70% ethanol
6. Sterile pipette basins
7. Sterile plastic material for tissue culture
8. Protein colorimetric assay dye
9. Complete culture medium: RPMI 1640 Medium (with phenol red and without L-glutamine and HEPES) supplemented with 10% Foetal Bovine Serum (FBS), 150 U/mL Penicillin, 200 U/mL streptomycin.
10. Seahorse™ XF RPMI Medium pH 7.4
11. Seahorse™ XF 200 mM glutamine solution
12. XF Assay Media: XF RPMI Medium pH 7.4 supplemented with 2 mM glutamine; use 1 mL of XF 200 mM glutamine solution in 99 mL XF RPMI medium pH 7.4
13. Seahorse™ XF 1.0 M Glucose Solution
14. Seahorse™ XFe96 FluxPak containing sensor cartridges, 20 XFe96 cell culture microplates, and 1 bottle of Seahorse™ XF Calibrant Solution 500mL
15. Seahorse™ XF Glycolysis Stress Test Kit containing 300 µmol Glucose, 72 nmol Oligomycin and 1500 µmol 2-deoxy-glucose (2-DG).
16. 1X Phosphate-Buffered Saline (1X PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄.

2.2 Equipment

1. Seahorse™ XFe96 Extracellular Flux Analyzer
2. Microplate spectrophotometer
3. CO₂ incubator
4. Non-CO₂ incubator
5. Single and multi-channel pipettes with repetitive functionality
6. Water bath
7. Vacuum pump
8. Microplates shaker
9. Cell counter
10. Glass haemocytometer

2.3 Software

1. Seahorse™ Wave Desktop software 2.6
2. Microsoft Excel
3. GraphPad Prism 9.0

3. Methods

3.1 Growing and maintenance of myeloma cells

1. To ensure the highest level of cell viability, initiate the culture of L363 myeloma cells two weeks before performing Seahorse™ XF Glycolysis Stress Test. Do not use cells that have been cultured for more than 40 days.
2. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
3. Remove the vial from the water bath as soon as the contents are thawed and decontaminate by spraying with 70% ethanol. All the operations from this point on

should be carried out under strict aseptic conditions.

4. Transfer the vial contents to a 50 mL conical tube
5. Add dropwise 9.0 mL pre-warmth complete culture medium and spin at approximately 130 x *g* for 7 minutes.
6. Resuspend the cell pellet with the complete medium and dispense into a 25 cm² culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition to the vial contents, the culture medium be prepared fresh and maintained warmth into the water bath for at least 15 minutes.
7. Incubate the culture at 37°C in a 5% CO₂ incubator
8. Maintain cell density between 5 x 10⁵ and 2 x 10⁶ cells/mL and renew culture medium every 3 days (**Figure 1**).

3.2 Cell seeding, Day 1

From an actively growing cell culture of L363 myeloma cells, seed 500,000 cells in 2 mL final volume in a 6-well plate. Incubate plate at 37°C, 5% CO₂ overnight (**Figure 1**).

3.3 Setting up the XFe96 Extracellular Flux analyser, Day 2

1. Turn on the Seahorse XFe96 Extracellular Flux analyser and warm it up overnight (see **Note 1**).
2. Launch the Wave 2.6 desktop software and open the assay template. Make sure the software is updated to the latest version.
3. Each assay template contains four elements in the function toolbar (**Figure 2**): *Group Definitions*, *Plate Map*, *Instrument Protocol*, *Run Assay*.
4. In the *Group Definitions* element there are four tabs: *Injection Strategies*, *Pre-treatments*, *Assay Media*, *Cell Type* (**Figure 2**).
5. In the *Injection Strategies* tab fill in: Port A, 10 mM Glucose; Port B, 1 µM Oligomycin;

Port C, 50 mM 2-DG (see **Note 2**).

6. In the *Pre-Treatments* tab, select '*Control*' and indicate your drug-vehicle (i.e. DMSO), then select '*Experimental*' and indicate your treatment conditions (i.e. 4 nM Bortezomib).
7. In the *Assay Media* tab: indicate XF RPMI medium.
8. Within the *Cell Type* tab, click Add to entry the name of cell types you intend to analyse in your assay. It is recommended to add the seeding density to group name.
9. Once finished naming the groups, click *Generate Groups* and the software will automatically create your unique assay groups. In our case one for control DMSO and one for 4 nM Bortezomib.
10. The next step is to assign groups to the plate map. Click *Plate Map* element to design the 96 microplates.
11. Click individual wells on the plate map to assign the control and the experimental groups to the plate map.
12. Click the *Instrument Protocol* element in the toolbar to view or edit the instrument protocol. We recommend to use the default instrument protocol.
13. Last, click the *Run Assay* element in the toolbar (**Figure 2**) to save the template file.

3.4 Preparing a sensor cartridge before running an XF Glycolysis Stress Test, Day 2

1. Aliquot at least 20 mL of XF Calibrant into a 50 mL conical tube. Place this in a non-CO₂ 37°C incubator overnight.
2. Open the XFe96 Extracellular Flux Assay Kit under the hood and remove the utility plate and green sensor cartridge, and place the green sensor cartridge upside down next to the utility plate.
3. Fill each well of the utility plate with 200 µL of XF Calibrant solution and place the cartridge back into the utility plate, submerging the sensors into the XF calibrant solution.
4. Incubate the cartridge at 37°C in a non-CO₂ incubator overnight. To prevent

evaporation of the XF calibrant, verify that the incubator is properly humidified.

3.5 Cytotoxic treatment, Day 2.

Treat L363 myeloma cells with the desired concentration of cytotoxic test lead compound in the 6-well plate. Incubate the plate at 37°C in a 5% CO₂ incubator for 8-16 hours, depending on the treatment conditions (see **Note 3**) (**Figure 1**).

3.6 Seeding pre-treated myeloma cells into XF96 cell culture microplate, Day 3

1. Warm an appropriate volume of XF Assay Media to 37°C in a sterile bottle. In general, 100 mL is sufficient for one plate.
2. Collect the pre-treated L363 myeloma cells from each well of the 6 well-plate (from section 3.5) into a 15 mL conical tube (**Figure 1**).
3. Save an aliquot and count cells using the manual cell counter and glass haemocytometer.
4. Centrifuge cells at 130 x *g* for 7 minutes to pellet cells.
5. Discard medium and wash twice with 1X PBS
6. Resuspend the cell pellet in XF Assay Media at a final concentration of 500,000-600,000 cells/mL
7. Pipette 175 µL of the cell suspension along the side of each well. Use at least five wells for each condition (**Figure 1**).
8. As background/control wells use the wells in the four corners of the plate. Hence, pipette 175 µL of XF Assay Media into the background/control wells.
9. Centrifuge the XF96 cell culture microplate at 170 x *g* for 1 minute, with soft/slow settings for acceleration and deceleration (braking) ramps.
10. Transfer the XF96 cell culture microplate to a 37°C non-CO₂ incubator for 25-30 minutes to ensure that the cells have completely adhered to the bottom of the well.
11. Visually confirm cell adherence by examining the XF96 cell culture microplate with an inverted microscope.

12. Return the XF96 cell culture microplate into at 37°C non-CO₂ incubator for additional 30 minutes.
13. After an incubation time of 1 hour from the centrifugation step, the XF96 cell culture microplate is ready for the assay (see section 3.XX)

3.7 The making of the Injection Solutions, Day 3

1. Remove one pouch from the XF Glycolysis Stress Test Kit box, and remove the compound tubes (Glucose, Oligomycin and 2-DG).
2. Using a pipette, resuspend the contents of each compound tube with XF Assay Media using the volumes described in **Table 1**.
3. Place a cap on the tube, and vortex for 1 minute to solubilize the compounds
4. Prepare 3 mL of each injection solution by combining the appropriate volumes of XF Assay Media and stock solutions as described in **Table 2**.

3.8 Injection of the solutions and running assay, Day 3

1. Remove the hydrated XF Assay Cartridge from the non-CO₂ incubator (see section 3.4).
2. Gently dispense 25 µL of the appropriate injection solution into the ports according to the plate layouts (see **Notes 4 and 5**).
3. Visually inspect the injection ports for even loading. The liquid should be in the port, make sure there are no residual drops on the top of the sensor cartridge.
4. Before running your assay, view your cells under a microscope to check seeding uniformity and purity (no contamination), making sure cells have stably adhered following centrifugation.
5. Ensure your background wells do not contain cells.

3.9 Run the XF Glycolysis Stress Test, Day 3

1. From the Wave 2.6 software browse your created/saved experimental file (section

- 3.3) and launch it.
2. After reviewing the group definitions, plate map layout and instrument protocol, click *Start Run*.
 3. After entering the save location for your result file (following completion of the assay), the tray door on the XFe Analyzer will open.
 4. Place the sensor cartridge (hydrated and loaded with compounds) and Utility Plate onto the tray and press *I'm ready* dialog. The calibration and equilibration take approximately 15-20 minutes.
 5. Once sensor cartridge calibration is complete, Wave Controller will display the *Load Cell Plate* dialog.
 6. Click *Open Tray* to remove the Utility Plate and load the Cell Plate on the tray. The sensor cartridge remains inside the XFe Analyzer for this step.
 7. Click *Load Cell Plate* to initiate equilibration. After completing equilibration, the assay will automatically begin acquiring the assay measurements.
 8. Once the final measurement command in the instrument protocol is completed, Wave Controller software will display the *Unload Sensor Cartridge* dialog.
 9. Click *Eject* when ready to eject the sensor cartridge and cell plate. Save the cell plate for later analysis (i.e. normalization) (see section 3.10).
 10. After removing the sensor cartridge and cell plate, the *Assay Complete* dialog will appear.
 11. Click *View Results* to open your results or click *Wave Home* to return to the *Templates* view and begin another XFe assay.
 12. Save and transfer the assay result file to a USB drive and open using Wave Desktop software on your computer for data analysis. In alternative, all the data can be exported in Microsoft Excel or GraphPad format (command Export on the Wave software 2.6).

3.10 Data analysis and normalization

In order to minimize inconsistency and obtain accurate results, resultant data must be normalised. Regularly, for those Seahorse™ XF analysers equipped with image cytometers, image cell counting is the standard for normalization. However, it requires that cells are evenly distributed and firmly attached to the plate to obtain reproducible and reliable data. This requirement, however, is barely obtainable with suspension cells, even if XF96 plates are pre-coated with adhesive agents [12]. Alternatives methods for normalization consist of standardising resultant data from suspension cells against the amount of the total protein in each well. Here, we use this methodology. However, to avoid loss of cells after treatment with cytotoxic test lead compounds (i.e. your drug of interest) we stimulate myeloma cells in a 6-well plate, after treatment with bortezomib, cells are counted and seeded back in XF96 plates to allow the Glycolysis Stress Test response of pre-stimulated cells. This has the great advantage of having minimal loss of cells in the change of medium, when testing for stress test (**Figure 3**).

1. Prepare 20 mL of Protein Assay Dye Reagent diluting the stock solution 1:4 in H₂O.
2. Centrifuge the XF96 cell culture microplate at 170 x g for 1 minute, with soft/slow settings for acceleration and deceleration (braking) ramps.
3. Aspirate the XF RPMI pH 7.4 medium in the XF96 plate by using vacuum. The use of thin tips and low pressure is recommended to avoid aspirating pelleted cells.
4. Add 200 µL of 1:4 diluted Protein Assay Dye Reagent.
5. Incubate the XF96 plate on a plate shaker for 20 minutes at room temperature, allowing the reagent to lyse and stain the cells.
6. In a new 96-well microplate prepare a BSA standard curve ranging from 0 µg/µL to 12 µg/µL.
7. After incubation, mix by pipetting up/down the assay dye reagent with the cells and transfer the content of each well of the XF96 plate to the 96-well microplate.
8. Read the absorbance at 595 nm using a spectrophotometer to determine the protein concentration.
9. Use the protein concentration obtained to normalise the average and the standard

deviation of five measurement replicates to obtain the normalised ECAR value for each time point in the form of ECAR (mpH/min/ μ g protein) (**Figure 3**) [6].

4. Notes

1. The Seahorse XFe96 instrument needs to warm up for at least 5 hours to stabilize the temperature at 37°C.
2. Concentration of oligomycin to be used should be tested for each cell line.
3. The manufacturer's basic procedures for non-adherent cells recommend that the drug stimulation in suspension cells should be performed the same day of the XF assay directly into a Cell-Tak coated XFe96 Cell Culture plate and drug of interest should be administered using the Port A-system of the Sensor Cartridge injector. However, in our optimised protocol we recommend performing drug stimulation in a standard cell culture plates (i.e., 6-well plate) for the appropriate time and then re-seed cells into an XFe96 plate supplemented with XF Assay Media at a concentration of 87,500 total cells in 175 μ L volume for each well (**Figure 1**) (see also **Figure 3** for notable differences on using basic versus our optimised procedures).
4. Position the pipette tips (filled with your compounds for injection) into the desired column in the loading guide and orient the tips at a very slight angle. Insert the tips as far as they will go without resistance into the holes and dispense the compound. Do not force the tips completely into the holes. Avoid creating air bubbles. Do not tap any portion of the cartridge in an attempt to alleviate air bubbles. This may cause solutions to leak from the injection port.
5. To improve the loading of the solution into the injection ports of the sensor cartridge use a repetitive single channel pipette, if possible.

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Acknowledgements

The authors acknowledge the research funding from Blood Cancer UK (17014 to CB and SP), the Key Kendall Leukaemia Fund (KKL1361 to CB and SP) and Leukaemia & Myeloma Research UK (ref. 122498 to SP). FNK is sponsored by the Republic of Turkey Ministry of National Education Scholarship (MEB1416). Figures were created modifying illustrations provided by Servier (<https://smart.servier.com>) under Creative Commons Attribution 3.0 Unported License.

Tables

Table 1: Resuspension volumes for the XF Glycolysis Stress Test Kit compounds

Compound	Volume of XF assay medium	Stock concentration
Glucose (Blue)	3000 μ L	100 mM
Oligomycin (Light Blue)	720 μ L	100 μ M
2-DG (Green)	3000 μ L	500 mM

Table 2: Instructions for preparation of the injection solution.

Port & Compound	Stock Volume	Volume of XF assay medium	Port concentration	Volume for each port	Final well concentration
Port A – Glucose	2400 μ L	600 μ L	80 μ M (8X)	25 μ L	10 μ M
Port B – Oligomycin	270 μ L	2730 μ L	0.018 μ M (9X)	25 μ L	0.001 μ M
Port C – 2-DG	3000 μ L	-	500 μ M (10X)	25 μ L	50 μ M

Figure Legends

Figure 1. Schematic overview of our integrated procedures.

Overview of our integrated methodology to assess glycolytic stress in plasma cells myeloma in response to chemotherapeutic drugs using an extracellular flux (XF) analyser. Of note, myeloma cells are treated with cytotoxic drugs in 6-well plate before being tested for metabolic changes in a XF96 testing plate for the measurement of the extracellular acidification rate (ECAR) using a Seahorse™ XF Glycolysis Stress Test Kit and Seahorse™ XF analyser.

Figure 2. Overview of a window of Seahorse™Wave desktop software.

Shown is a representative window of the Seahorse™ Wave 2.6 desktop software showing the assay template containing four elements in the function toolbar: Group Definitions, Plate Map, Instrument Protocol, Run Assay.

Figure 3. Compare and contrast measurements of extracellular acidification rate (ECAR) using a standard protocol versus our integrated protocol.

A) Typical measurements of ECAR obtained in L363 plasma cell myeloma using the “*manufacturer’s basic procedure*” for non-adherent cells (as for Seahorse™ XF Glycolysis Stress Test Kit manufacturer’s recommendations) whereby untreated L363 myeloma cells were seeded the day before (day 2) into a Cell-Tak coated XFe96 plate. On day 3, myeloma cells were treated with bortezomib (4 nM) into a XFe96 plate for 8 hours. Next, drug-containing medium was removed by gentle vacuum aspiration, and myeloma cells were gentle-washed with 1X PBS and supplemented with XF Assay Media and then subjected to sequential injection of glucose (initiates glycolysis), oligomycin (eliminates mitochondrial ATP

production) and 2-deoxyglucose (inhibits glycolysis) to detect the metabolic changes in response to bortezomib. Values indicate the mean \pm SEM of five technical replicates. **B)** Typical measurements of ECAR obtained in L363 plasma cell myeloma using “*our integrated protocol*” whereby L363 myeloma cells (pre-treated 8 hours with 4 nM bortezomib in a standard 6-well plate on the same day of the assay) (Figure 1) were collected, counted, washed with 1X PBS, and re-seeded in a XFe96 plate supplemented with XF Assay Media. Following 25-30 minutes incubation at 37°C in a non-CO₂ incubator, myeloma cells were subjected to sequential injection of glucose, oligomycin and 2-deoxyglucose to detect the metabolic changes in response to bortezomib. Values indicate the mean \pm SEM of five technical replicates.

Figure 1

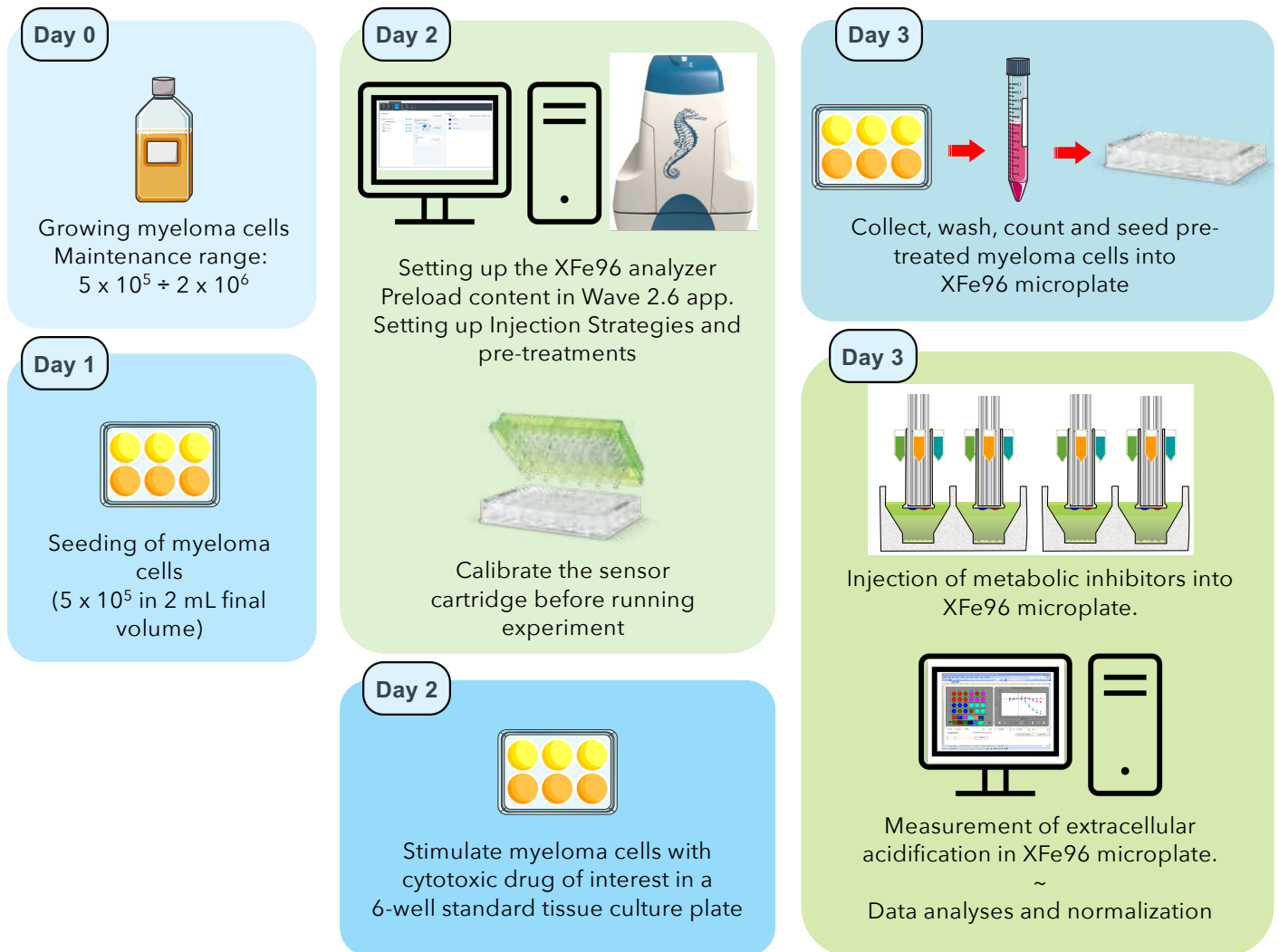


Figure 2

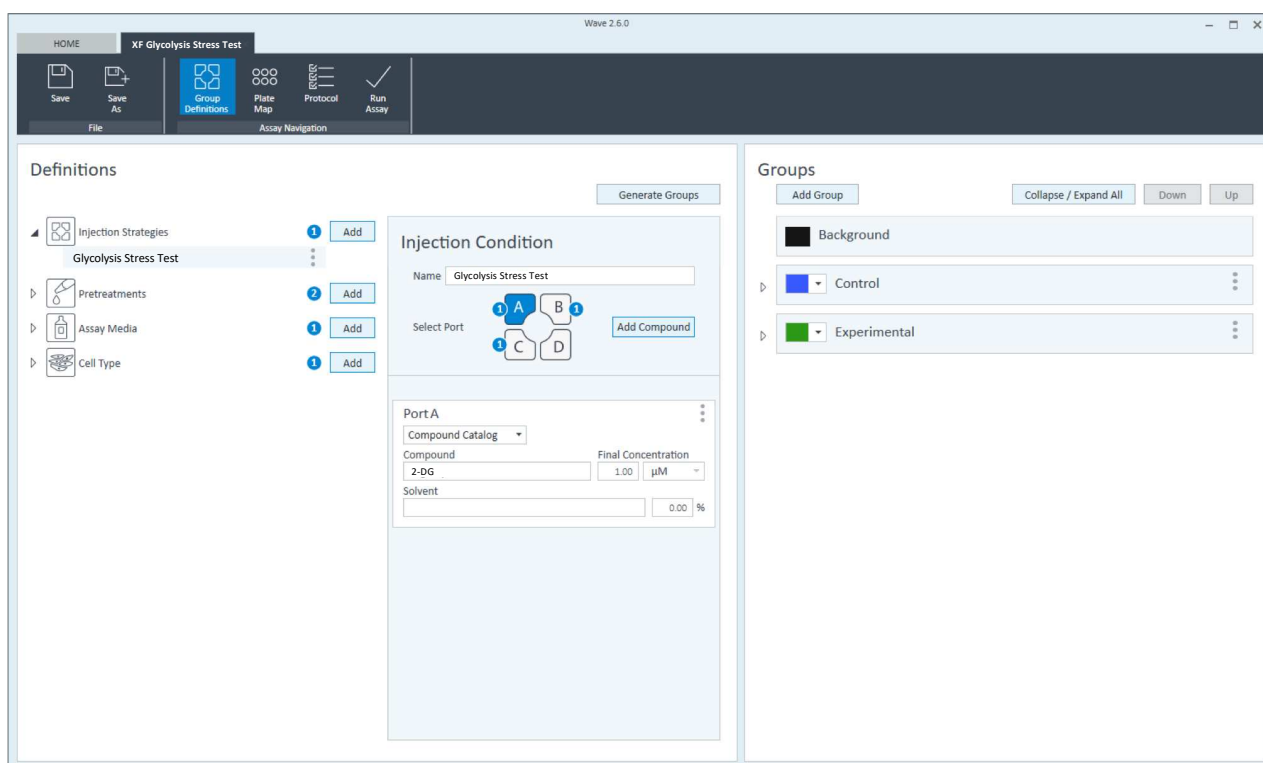
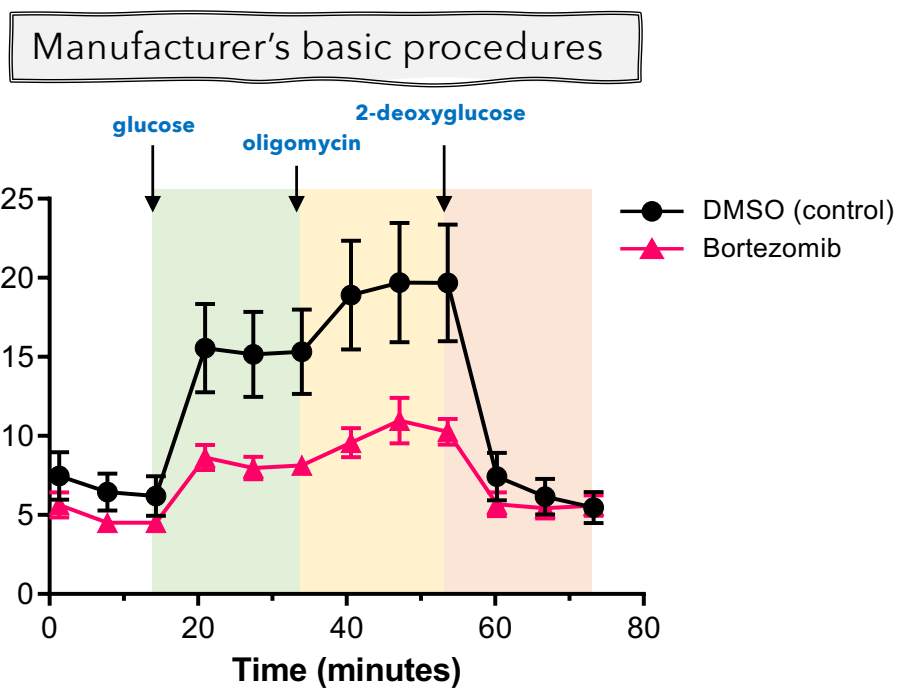


Figure 3

A



B

