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Bio-energetics Investigation of Candida albicans Using Real-time Extracellular Flux Analysis

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Nandita Singh, Ph.D.
Senior Science Editor
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November 11, 2018

Dear Dr. Singh,

Please see attached our revised manuscript entitled “Bio-energetics investigation of Candida albicans by using real-time extracellular flux analysis”. My apologies for the delay in submitting our revised manuscript. I would like to thank the reviewers for their insights and thoughtful comments. We have made every possible effort to address all of reviewers’ comments. Hence, we believe that the revised manuscript is significantly improved with respect to focus and depth.

Thank you,

Sincerely,

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TITLE:

Bio-energetics Investigation of *Candida albicans* Using Real-time Extracellular Flux Analysis

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KEYWORDS:

Candida albicans, bioenergetics, metabolism, mitochondria, respiration, oxygen consumption, extracellular acidification, glycolysis

SUMMARY:

Here, we present a stepwise protocol to investigate the mitochondrial respiration and glycolytic function in *Candida Albicans* using an extra flux analyzer.

ABSTRACT:

Mitochondria are essential organelles for the cellular metabolism and survival. A variety of key events take place in mitochondria, such as cellular respiration, oxidative metabolism, signal transduction, and apoptosis. Consequently, mitochondrial dysfunction is reported to play an important role in the antifungal drug tolerance and virulence of pathogenic fungi. Recent data have also led to the recognition of the importance of the mitochondria as an important contributor to fungal pathogenesis. Despite the importance of the mitochondria in fungal biology, standardized methods to understand its function are poorly developed. Here, we present a procedure to study the basal oxygen consumption rate (OCR), a measure of mitochondrial respiration, and extracellular acidification rates (ECAR), a measure of glycolytic function in *C. albicans* strains. The method described herein can be applied to any *Candida spp.* strains without the need to purify mitochondria from the intact fungal cells. Furthermore, this protocol can also be customized to screen for inhibitors of mitochondrial function in *C. albicans* strains.

INTRODUCTION:

Invasive fungal infections kill over 1.5 million people a year worldwide. This number is on the rise due to an increase in the numbers of people living with compromised immunity, including the elderly, premature infants, transplant recipients, and cancer patients[1]. *C. albicans* is an opportunistic human fungal pathogen that is a part of the human microflora. It also inhabits mucosal surfaces and the gastrointestinal tract as a commensal organism. *C. albicans* produces serious systemic disease in people who have immune deficiencies, who have undergone surgery, or who have been treated with long courses of antibiotics. The *Candida* species rank among the top three to four causes of nosocomial infectious diseases (NID) in humans[2-7]. The annual global number of *Candida* bloodstream infections is estimated to be ~400,000 cases, with associated mortalities of 46-75%[1]. The annual mortality because of candidiasis is roughly 10,000 in the United States alone. The extent of NID caused by fungi is also reflected in astronomical patient expenses[5]. In the United States, the yearly expense for the treatment of invasive fungal infections surpasses $2 billion, adding a huge strain to already overburdened health care system. Currently, available standard antifungal therapies are limited because of toxicity, increasingly prevalent drug resistance, and drug-drug interactions. Therefore, there is an urgent need to identify new antifungal drug targets that will result in better treatment options for high-risk patients. However, the discovery of new drugs acting on fungal targets is complicated because fungi are eukaryotes. This greatly limits the number of fungal-specific drug targets.

Recent studies have indicated that mitochondria are a critical contributor to the fungal virulence and tolerance to antifungal drugs since mitochondria are important for cellular respiration, oxidative metabolism, signal transduction, and apoptosis[8-11]. Both glycolytic and non-glycolytic metabolism are essential for the survival of *C. albicans* in the mammalian host[12-16]. Furthermore, several *C. albicans* mutants lacking mitochondrial proteins, such as Goa1, Srr1, Gem1, Sam37 etc. have been shown to be defective in filamentation, an important virulence factor of *C. albicans*[17-22]. In addition, these mutants were also shown to be attenuated for virulence in a mouse model of disseminated candidiasis[17-22]. Thus, fungal mitochondria represent an attractive target for drug discovery. However, the study of mitochondrial function in *C. albicans* is challenging because *C. albicans* is petite negative[23], which means that it cannot survive without the mitochondrial genome.

Here, we describe a protocol that can be used to investigate mitochondrial and glycolytic function in *C. albicans* without the need to purify mitochondria. This method can also be optimized to investigate the effect of the genetic manipulation or chemical modulators on mitochondrial and glycolytic pathways in *C. albicans*.

PROTOCOL:

NOTE: The detailed stepwise protocol of the assay is described below, and the schematic protocol is shown in **Figure 1.**

1. *C. albicans* strains and growth conditions

1.1. Grow the *C. albicans* strains in liquid Yeast Extract-Peptone-Dextrose (YPD) medium at 30 °C in an incubator shaker overnight.

NOTE: Maintain *Candida* strains as frozen stocks and grow on YPD agar (1 % yeast extract, 2% peptone, 2% dextrose, and 2% agar).

2. Preparation of reagents

2.1. Prepare the assay medium as follows:

2.1.1. For mitochondrial function assay, dissolve 1.04 g Roswell Park Memorial Institute (RPMI) 1640 powder and 2 g glucose (2%) in 90 mL sterile water and warm the media to 37 °C. Adjust the pH to 7.4 using 5 M NaOH and make up the volume to 100 mL with sterile water.

2.1.2. For glycolytic stress assay, dissolve 1.04 g RPMI 1640 powder alone in 90 mL sterile water, warm the media to 37 °C and adjust the pH to 7.4. Make up the volume to 100 mL with sterile water. RPMI 1640 powder has no bicarbonate, which is critical to monitor the pH change as a measure of glycolysis during the assay.

2.2. Injection compounds.

2.2.1. Prepare 1 M glucose stock in sterile water and store at -20 °C.

2.2.2. Prepare 100 mM oligomycin stock in dimethyl sulfoxide (DMSO), aliquot in small volumes and store at -20 °C.

2.2.3. Prepare 100 mM antimycin A stock in DMSO, aliquot in small volumes and store at -20 °C.

2.2.4. Prepare 100 mM SHAM (salicylhydroxamic acid) stock in ethanol on the day of assay.

2.2.5. Prepare 1 M KCN in the sterile water on the day of the assay.

3. Coating of the assay plate with Poly-D-Lysine (PDL)

NOTE: Perform all the below steps in a laminar hood.

3.1. Dissolve Poly-D Lysine in tissue culture grade water to make 50 µg/mL final concentration. Mix it well and aliquot into a 1.5 mL microcentrifuge tubes and store at -20 °C for the long term.

NOTE: 50 µL per well is needed, and for 24 wells, 1.2 mL is required. Therefore, aliquot at least 1.3 mL per microcentrifuge tube.

3.2. Add 50 µL per well and incubate at room temperature with the lid covered for 1-2 h.

3.3. Aspirate the solution and rinse one time with 500 µL sterile tissue culture grade water.

3.4. Open the lid and allow the wells to air dry. Use the plate on the same day or store at 4 °C for a maximum of 2-3 days.

4. Hydration of sensor cartridge

NOTE: Perform this step one day before the experiment.

4.1. Open the extra flux Assay Kit and remove the contents. Place the sensor cartridge upside down next to the utility plate (**Figure 2**).

4.2. Fill each well of the utility plate with 1 mL of calibrant and place the sensor cartridge back. Make sure the sensors which contain fluorophores (to measure the oxygen and pH) are submerged in the calibrant.

4.3. Incubate the sensor cartridge overnight in a non-CO_2 incubator at 37 °C.

5. Growing and seeding cells in the PDL-coated plates

5.1. Inoculate *C. albicans* in the YPD broth and grow overnight at 30 °C in a shaker at 200 rpm.

NOTE: Based on the assay design and the interest, *C. albicans* can also be grown in YPG or minimal medium.

5.2. On the day of assay, dilute an appropriate number of cells in the assay medium to yield a final concentration of 100,000 cells per 100 µL.

5.3. Add 100 µL of the diluted cells into each well of the assay plate except wells A1, B4, C3, and D6, in which add only 100 µL of the assay medium for background correction (**Figure 3**).

5.4. Transfer the plate to a non-CO_2 incubator at 37 °C and incubate for 60 min, which will let the cells adhere to the plate surface.

6. Assay Protocol

NOTE: The protocol outlined here is for the 24-well format of the instrument. Volumes will need to be adjusted if another format is used.

6.1. Mitochondrial function assay

6.1.1. Preparation of compounds

6.1.1.1. Prepare compounds at 10x concentration for mitochondrial function assay: Prepare 20 mM SHAM, 100 µM Oligomycin, 100 mM KCN, and 20 µM Antimycin A in the corresponding assay medium.

6.1.1.2. Add 50 µL SHAM into the port A, 55 µL Oligomycin into port B, 62 µL KCN into port C and 68 µL Antimycin A into port D (**Figure 4**).

6. 2. Glycolytic stress assay

6.2.1. Preparation of compounds

6.2.1.1. Prepare compounds at 10x concentration for glycolytic stress assay. Prepare 100 mM glucose, 100 µM Oligomycin, 500 mM 2-Deoxy Glucose (2DG) and 20 µM Antimycin A in the corresponding assay medium.

6.2.1.2. Add 50 µL glucose into port A, 55 µL Oligomycin into port B, 62 µL 2-DG into port C and 68 µL Antimycin A into port D.

6.3. Employ extra flux analyzer, which measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells in a 24-well plate format. Set up the assay protocol in advance.

6.4. Open the extra flux analyzer and set up the assay template by using the assay wizard tab and follow step by step instruction to fill out all the information that pops out during the setup. Generate the group layout as similar to shown in **Figure 5**. Set up the protocol as shown in **Table 1**. Set up these layouts well ahead before assay and save in the computer. At the time of assay, restore the saved protocol by opening the corresponding file in the **open file** option in the assay wizard tab (**Figure 5**).

6.5. Load the 10x compounds in the respective ports of the hydrated sensor cartridge containing the calibrant and load in the carrier tray of the extra flux analyzer. Start the calibration by pressing the **Start** button on the screen.

6.6. Add 350 µL of the assay medium gently to the cell plate along the side of the wells to minimize cell disturbance to bring the final volume to 450 µL.

6.7. Replace the utility plate containing the calibrant with the assay plate and continue.

6.8. Remove the sensor cartridge and the plate once the assay is completed. Save the file in the appropriate destination folder.

7. Data Analysis

7.2. Use the area under the curve- analysis of variance (AUC-ANOVA) analysis tab in the software to calculate the significant difference between the groups by selecting the respective parameters (OCR or ECAR) as shown in **Figure 6**.

7.3. Select the groups that need to be compared.

7.4. Add to the analysis groups for ANOVA and click **Ok**.

NOTE: This AUC ANOVA analysis will add a new sheet to the file in which the AUC is calculated for each group and compared between them by ANOVA. This will give a table of p values to show the significance.

REPRESENTATIVE RESULTS:

The focus of this protocol is to determine the bioenergetic functions of *C. albicans* assessed by extra flux analyzer. A *C. albicans* mutant lacking mitochondrial protein Mam33 is also included along with its complement strain, *mam33Δ/Δ::MAM33* to study the effects of the deletion of a mitochondrial protein on OCR and ECAR. *MAM33* encodes for a putative mitochondrial acidic matrix protein and its function in *Candida* is not known.

Cell numbers for extra flux assay

We employed 1x 10^5 *C. albicans* wild type cells per well in the extra flux assay, which showed an OCR of 145 pmol/min (**Figure 7**), which is within the optimal range of 100-300 pmol/min for any extracellular flux assay. It is also important to titrate the cell number to obtain an optimal OCR before the assay or when there is a change in growth medium composition between different assays.

Analysis of data

The area under the curve- analysis of variance (AUC-ANOVA) analysis tab in the software was used to calculate the significant difference between the groups by selecting the respective parameters (OCR or ECAR) as shown in **Figure 7**. This analysis added a new sheet to the file in which the AUC was calculated for each group and compared between them by ANOVA automatically, which gave a table of p values.

Mitochondrial function assay

In the mitochondrial stress test, the assay starts with measuring the basal oxygen consumption rate (OCR) followed by injection of inhibitors of an alternate oxidase (AOX), ATP synthase, and electron transport chain complexes, IV and III. In addition to the classical respiratory chain, *C. albicans* also utilizes the AOX pathway for energy generation[24]. Therefore, we investigated the effect of AOX system on OCR by inhibiting its activity by using salicylhydroxamic acid (SHAM)[25]. The mitochondrial function, which was measured as an oxygen consumption rate (OCR) in pmol/min at basal and after injecting respective inhibitors is shown in **Figure 7**. The basal OCR of wild type, *mam33Δ/Δ,* and *mam33Δ/Δ::MAM33* strains showed no differences (**Figure 7A and B**). Similarly, no significant difference in the basal ECAR was observed between these strains (**Figure 7C and D**). However, by inhibiting complex IV by KCN, wild type and *mam33Δ/Δ::MAM33*

showed a substantial shift toward the glycolysis in contrast to *mam33Δ/Δ*, which significantly failed to show a compensatory glycolytic shift (**Figure 7E**), suggesting that C-IV dependent glycolytic pathway is impaired in the *mam33Δ/Δ* mutant compared to wild type and *mam33Δ/Δ::MAM33* strains.

Glycolytic stress assay

In the glycolytic stress test, cells were starved for glucose for 1 h and the basal OCR and ECAR was measured. Upon starvation, *mam33Δ/Δ* showed significantly lower OCR (**Figure 8A and B**) and ECAR (**Figure 8C and D**) compared to the wild type and *mam33Δ/Δ::MAM33* strains suggesting an impaired utilization of glucose for both respiration and glycolysis when cells are forced to the starvation condition. After injecting glucose, all strains showed stimulation of both OCR and ECAR. Although oligomycin has less effect on both OCR and ECAR, 2-DG, which is a competitive inhibitor of glycolysis, inhibits both OCR and ECAR. Specifically, 2-DG partially inhibits OCR, which is further inhibited by Antimycin A. In contrast, 2-DG almost completely inhibits ECAR.

FIGURE AND TABLE LEGENDS:

Table 1. Protocol commands for the assay

Figure 1. Schematic representation of the experiment

Figure 2. Sensor cartridge (green) placed upside down near the utility plate before adding the calibrant. Fluorophores are indicated by arrows.

Figure 3. **Cell culture plate showing background wells A1, B4, C3, and D6.** The blue mark shows the notch, oriented to the lower left.

Figure 4. **The top portion of the sensor cartridge showing ports A, B, C and D.** The arrow mark shows the notch, oriented to the lower left.

Figure 5. The layout of wells and group assignment as visualized in the software. WT- wild type; MT- *mam33Δ/Δ* mutant*;* COMP- *mam33Δ/Δ::MAM33* complement strain. Arrow mark shows the open file tab.

Figure 6. The area under the curve (AUC) ANOVA analysis tab to calculate the significance between the groups. Select the groups that need to be compared and add to the analysis groups for ANOVA and click ok. This AUC ANOVA analysis will add a new sheet to the file in which the AUC is calculated for each group and compared between them by ANOVA. This will give a table of p values to show the significance.

Figure 7. Mitochondrial function assay in *C. albicans*. Oxygen consumption and extracellular acidification rates were measured in wild type, *mam33 Δ/Δ,* and *mam33 Δ/Δ::MAM33* strains of *C. albicans*. A) A real-time graph of oxygen consumption rate (OCR) at basal respiration followed

by sequential injection of SHAM (2 mM), Oligomycin (10 µM), KCN (10 mM), and Antimycin A (2 µM). B) Comparison of area under the curve (AUC) basal OCR between wild type, *mam33Δ/Δ,* and *mam33Δ/Δ::MAM33* strains of *C. albicans* show no significant difference. C) A real-time graph of extracellular acidification rate (ECAR) at basal glycolysis followed by sequential injection of SHAM (2 mM), Oligomycin (10 µM), KCN (10 mM), and Antimycin A (2 µM). D) Comparison of area under the curve (AUC) basal ECAR between wild type, *mam33Δ/Δ,* and *mam33Δ/Δ::MAM33* strains of *C. albicans* show no significant difference. E) Comparison of area under curve (AUC) ECAR between wild type, *mam33Δ/Δ* and *mam33 Δ/Δ::MAM33* strains of *C. albicans* after the injection of C-IV inhibitor KCN show significant decrease in glycolytic shift in *mam33Δ/Δ* mutant compared to wild type and complementary strains Values are expressed as average ± standard error mean. * $p<0.05$, ** $p<0.001$ is considered as significant by one-way ANOVA.

Figure 8. Glycolytic stress assay in *C. albicans*. Oxygen consumption and extracellular acidification were measured in wild type, *mam33Δ/Δ* mutant after they were starved for glucose followed by acute injection of saturated glucose concentration. A) A real-time graph of oxygen consumption rate after cells were subjected to basal ECAR followed by sequential injection of glucose (10 mM), Oligomycin (1 µM), 2-DG (40 mM), and Antimycin A (2 µM). B) Bar graph showing the area under the curve ECAR of basal glycolysis between wild type, *mam33Δ/Δ,* and *mam33Δ/Δ::MAM33* strains of *C. albicans*. C) A real-time graph of the glycolytic function after cells were subjected to basal ECAR followed by sequential injection of glucose (10 mM), Oligomycin (1 µM), 2-DG (40mM), and Antimycin A (2 µM). D) Bar graph showing the area under the curve ECAR of basal glycolysis between wild type, *mam33Δ/Δ,* and *mam33Δ/Δ::MAM33* strains of *C. albicans* Values are expressed as average ± standard error mean. * $p<0.05$, *** $p<0.0005$, **** $p<0.0001$ is considered as significant by one-way ANOVA.

DISCUSSION:

The bioenergetics extra flux assay serves as an excellent tool to read out the mitochondrial function by measuring oxidative phosphorylation (OXPHOS)-dependent oxygen consumption in real-time. In addition, a glycolytic function which is measured as an extracellular acidification rate (change in extracellular pH) can also be investigated at the same time in real-time analysis.

Successful plating of *C. albicans* in the assay plate is one of the critical steps in the assay because the incubation of the cells in the PDL coated plates allow the cells to adhere to the assay plate. Visualization of adhering *C. albicans* cells is accomplished by light microscopy. Improper cell adherence may lead to cells floating in the wells, which will affect the assay outcome with inconsistent readings between replicates. Similarly, it is important to perform the cell number titration as the first step in this assay to obtain an OCR between 100-300 pmol/min, which is an optimal range. Also, titrating the compounds to obtain the optimal concentration is also critical to achieving the optimal response. This may be performed whenever there is a change in the basal medium composition.

DMEM is the most commonly used basal medium in the extra flux assay for mammalian cells. However, in our hands, RPMI 1640 medium gave the optimal results with *C. albicans*. This was

primarily due to the efficacy of mitochondrial electron transport chain complex inhibitors that worked better in RPMI 1640 medium compared to DMEM.

In this assay, wild type, *mam33Δ/Δ,* and *mam33Δ/Δ::MAM33* strains of *C. albicans* were used to assess the mitochondrial respiration and glycolytic efficiency. The results clearly show that the deletion of *MAM33* gene reduced the ability of the *C. albicans* to shift to glycolysis under stress conditions such as inhibition of C-IV (**Figure 7C and 7E**). Similarly, when cells were subjected to stress condition like glucose starvation, both mitochondrial respiration and glycolytic efficiency is significantly compromised in the *mam33Δ/Δ* mutant but not in the wild type and complement strains (**Figure 8**). Therefore, depending on experiments, carbon sources in the RPMI 1640 may also be modified to glycerol or galactose, which forces the cells to use OXPHOS rather than glycolysis.

The significance of using the extra flux assay is that by using various substrates and compounds that perturb mitochondrial function, the cells ability to utilize OXPHOS versus glycolysis can be inferred. This may be used as an informative tool to understand the effect of any treatments or gene on the mitochondrial function and glycolysis in *Candida albicans* without isolating mitochondria. The assay measures basal oxygen consumption rate (OCR), which is a measure of mitochondrial respiration, and extracellular acidification rate (ECAR), which is a measure of glycolytic function in both the presence of glucose and after glucose starvation. The extra flux cellular assay is well employed in a variety of mammalian models[26]. Despite the medical importance of *C. albicans*, research into fungal mitochondria has been somewhat hampered due to the lack of standardized experimental procedures to study mitochondrial function and glycolysis in real time.

This method provides an easy and convenient way of investigating the mitochondrial respiration and glycolytic efficiency by using real-time extracellular flux assay in *C. albicans.* In future, this assay may also be applied to study the drug-efficacy testing using commonly employed antifungals such as fluconazole. Additionally, another potential future application of this method is in drug discovery, especially for screening compounds targeting mitochondrial function of *C. albicans*.

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DISCLOSURES:

The authors have nothing to disclose

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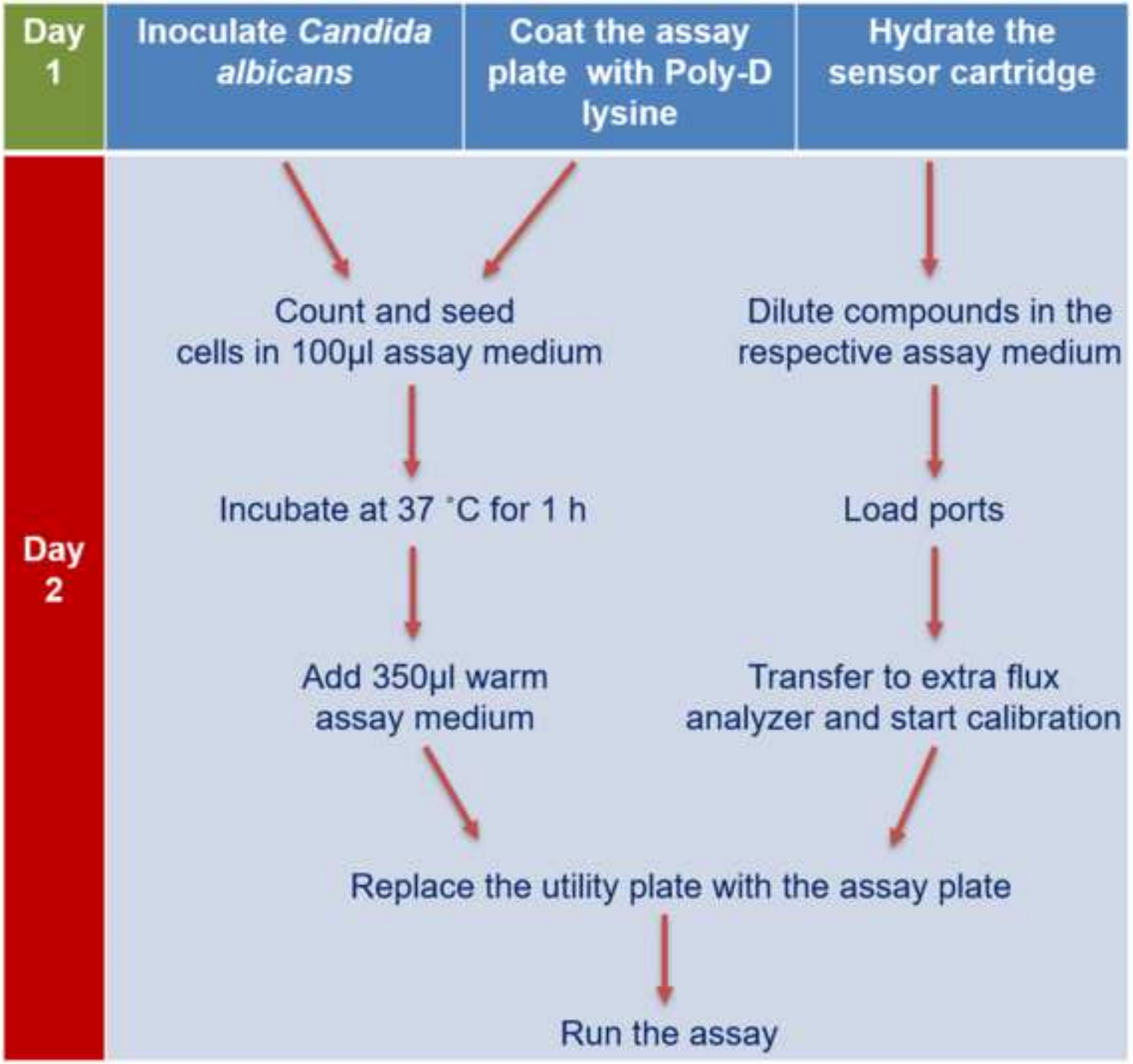

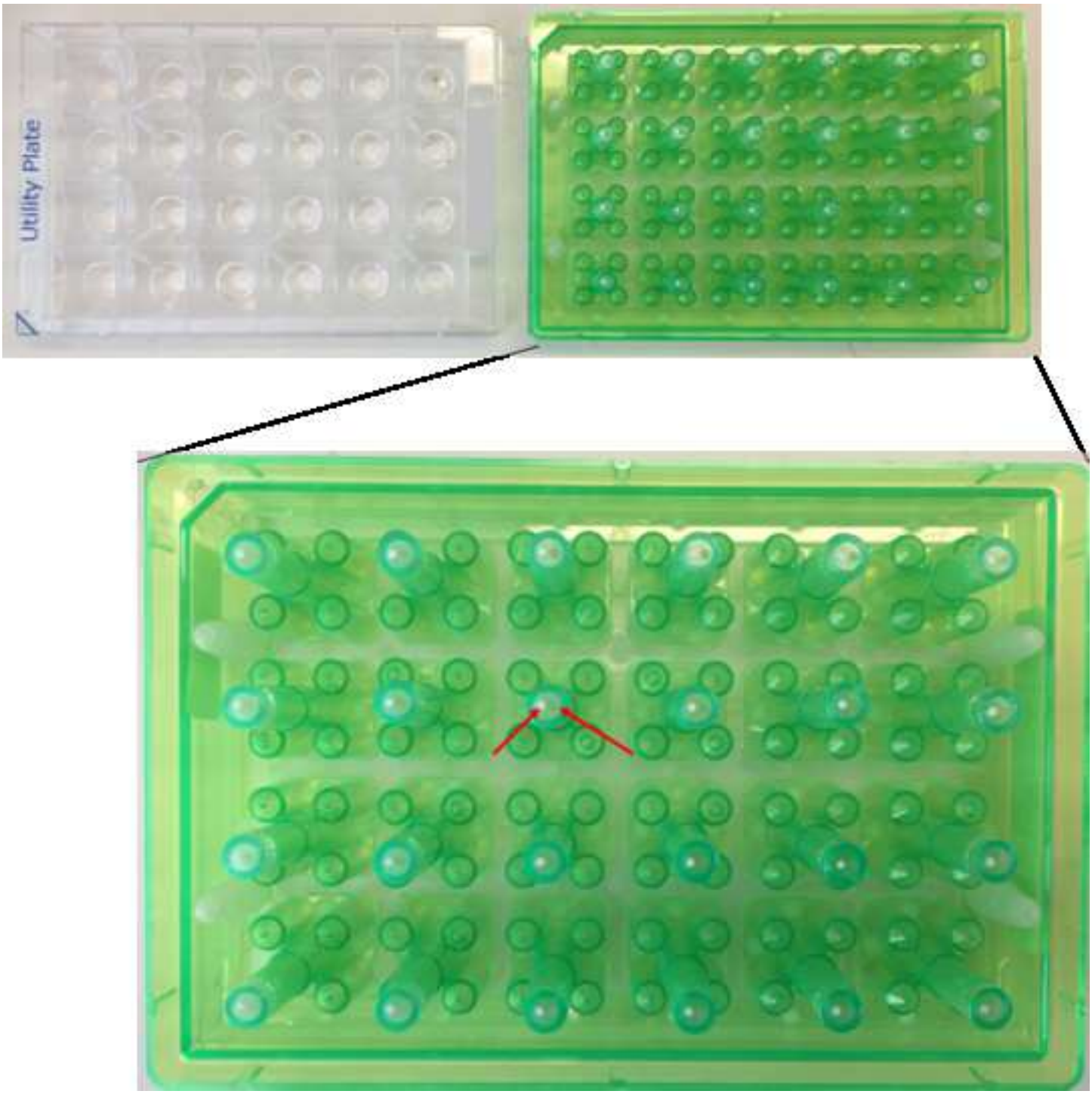

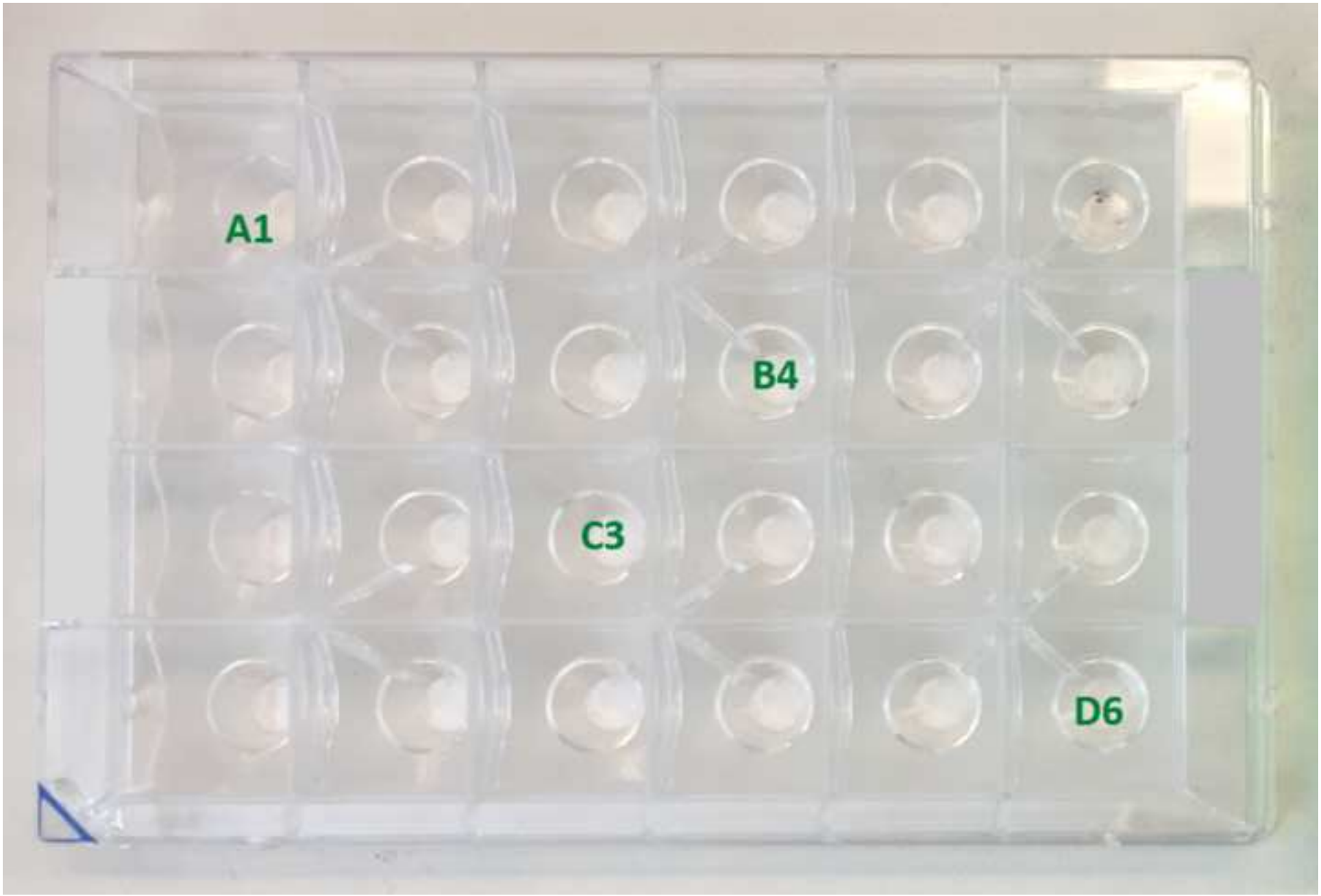

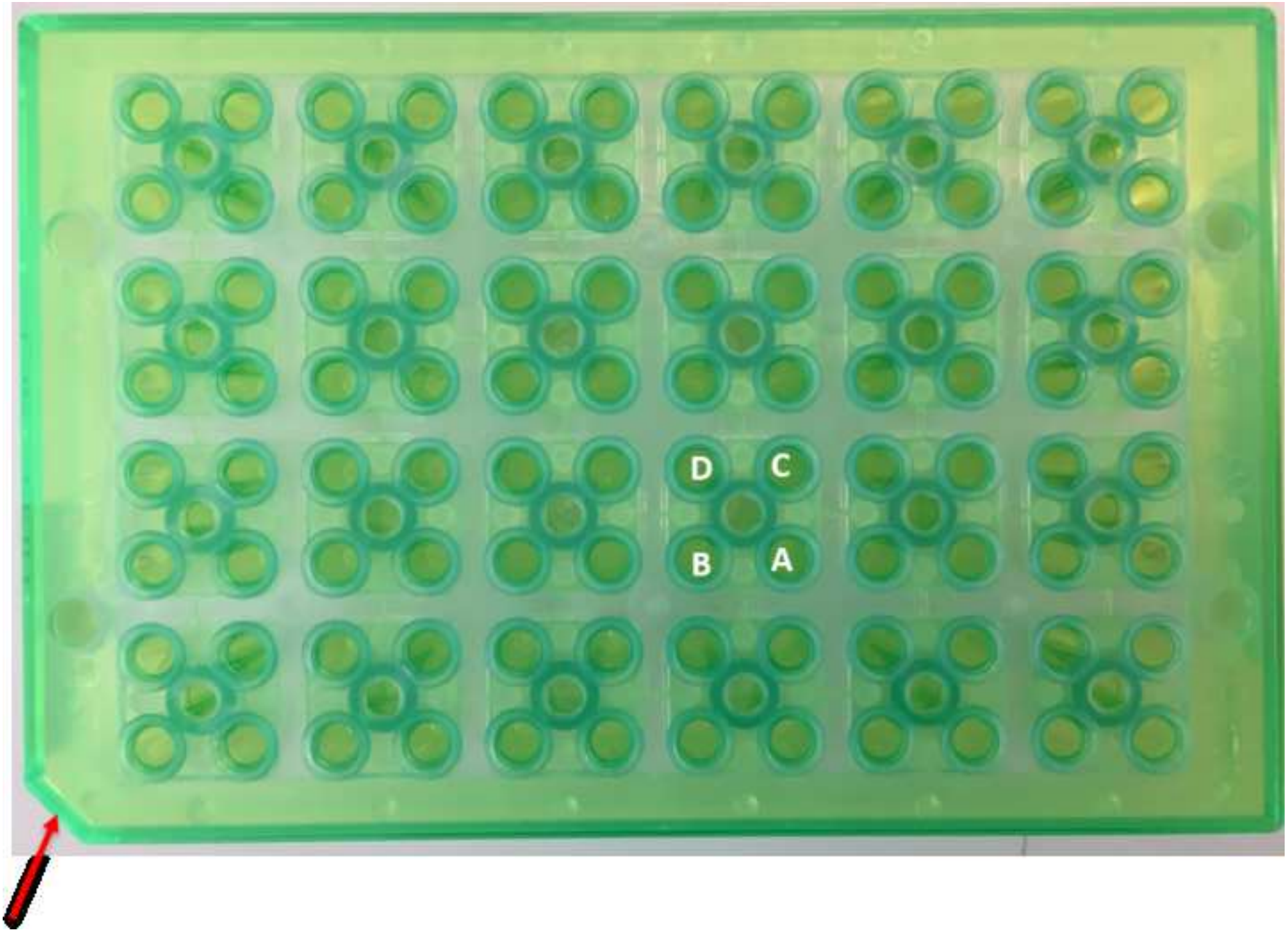

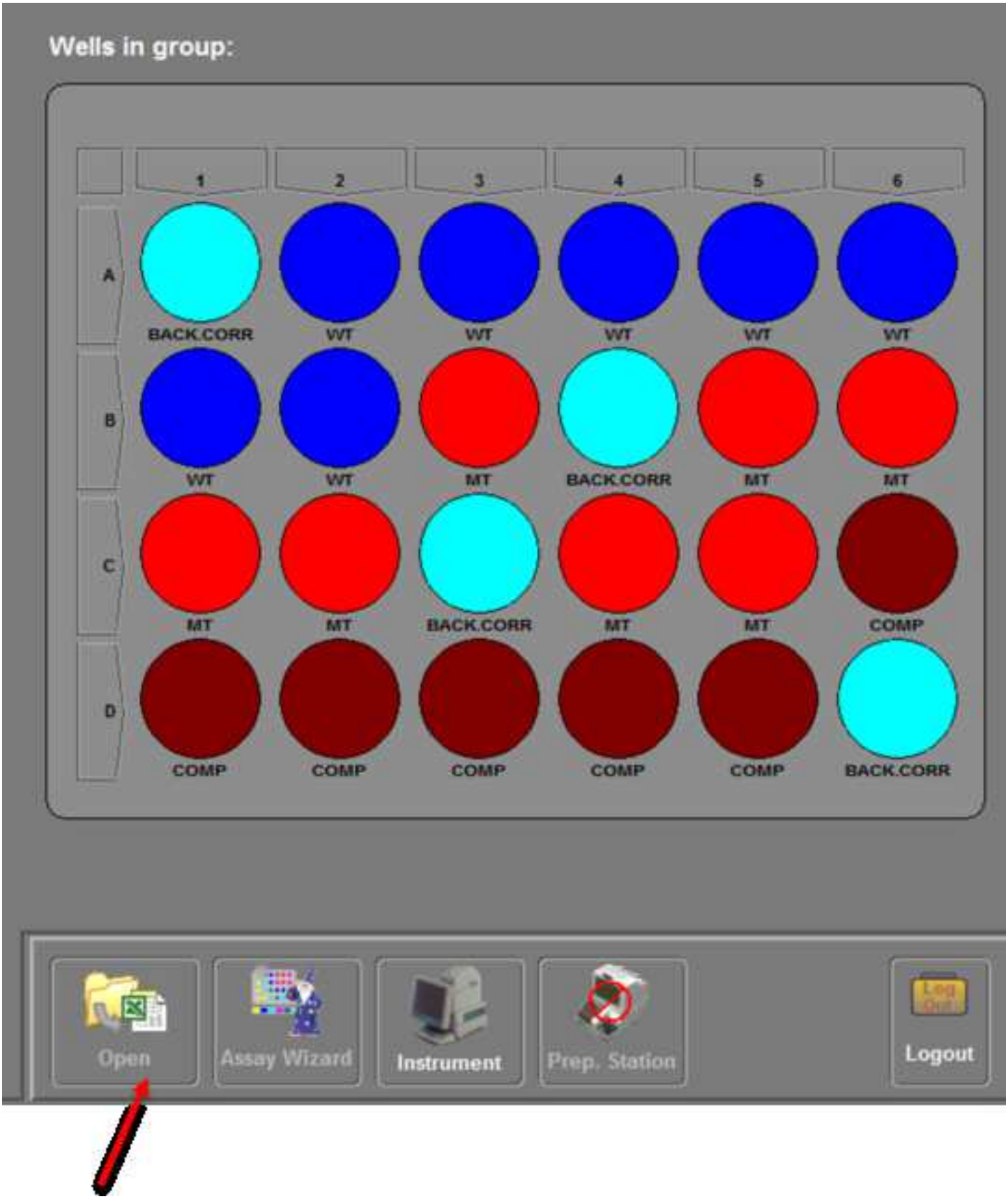

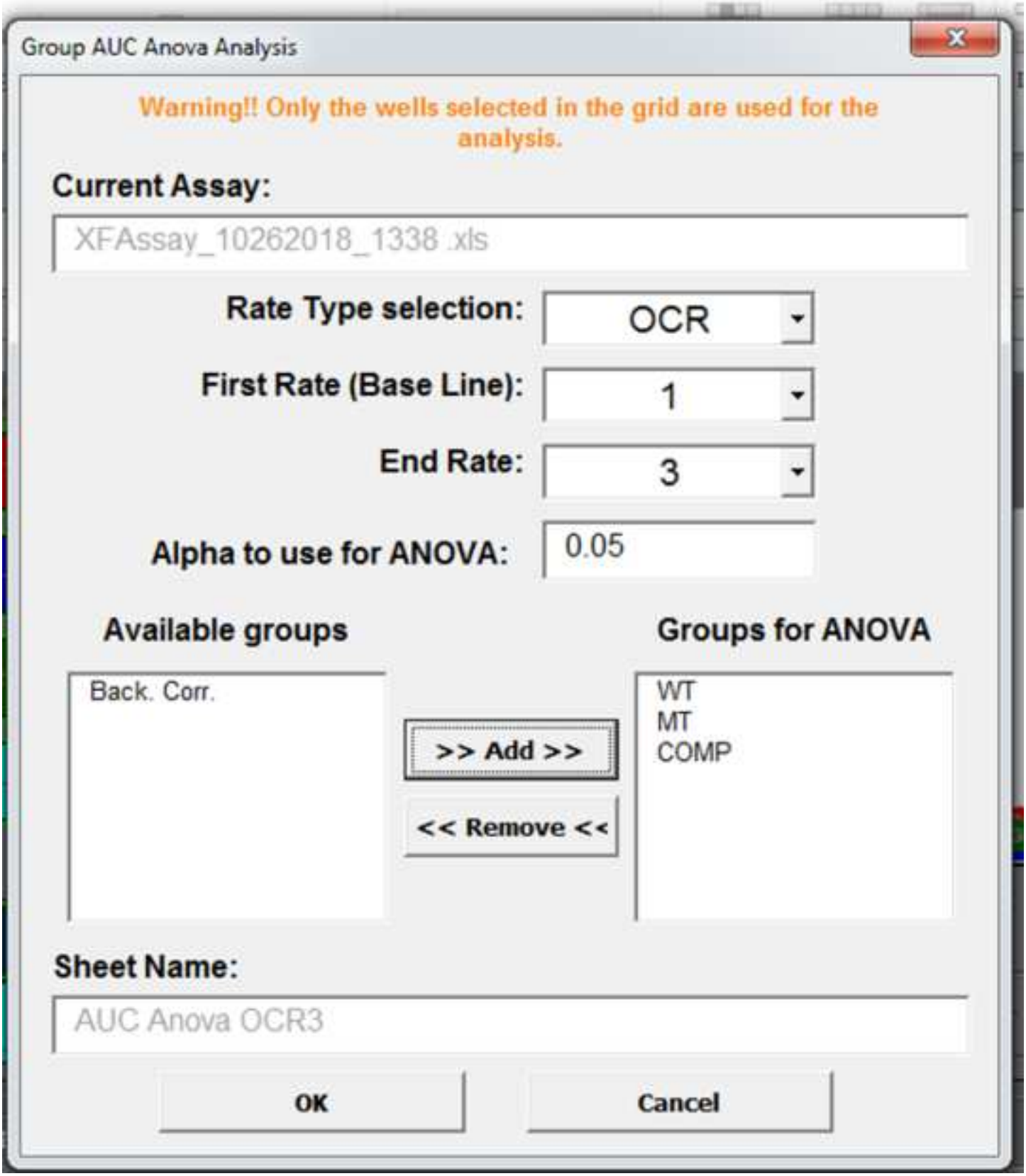

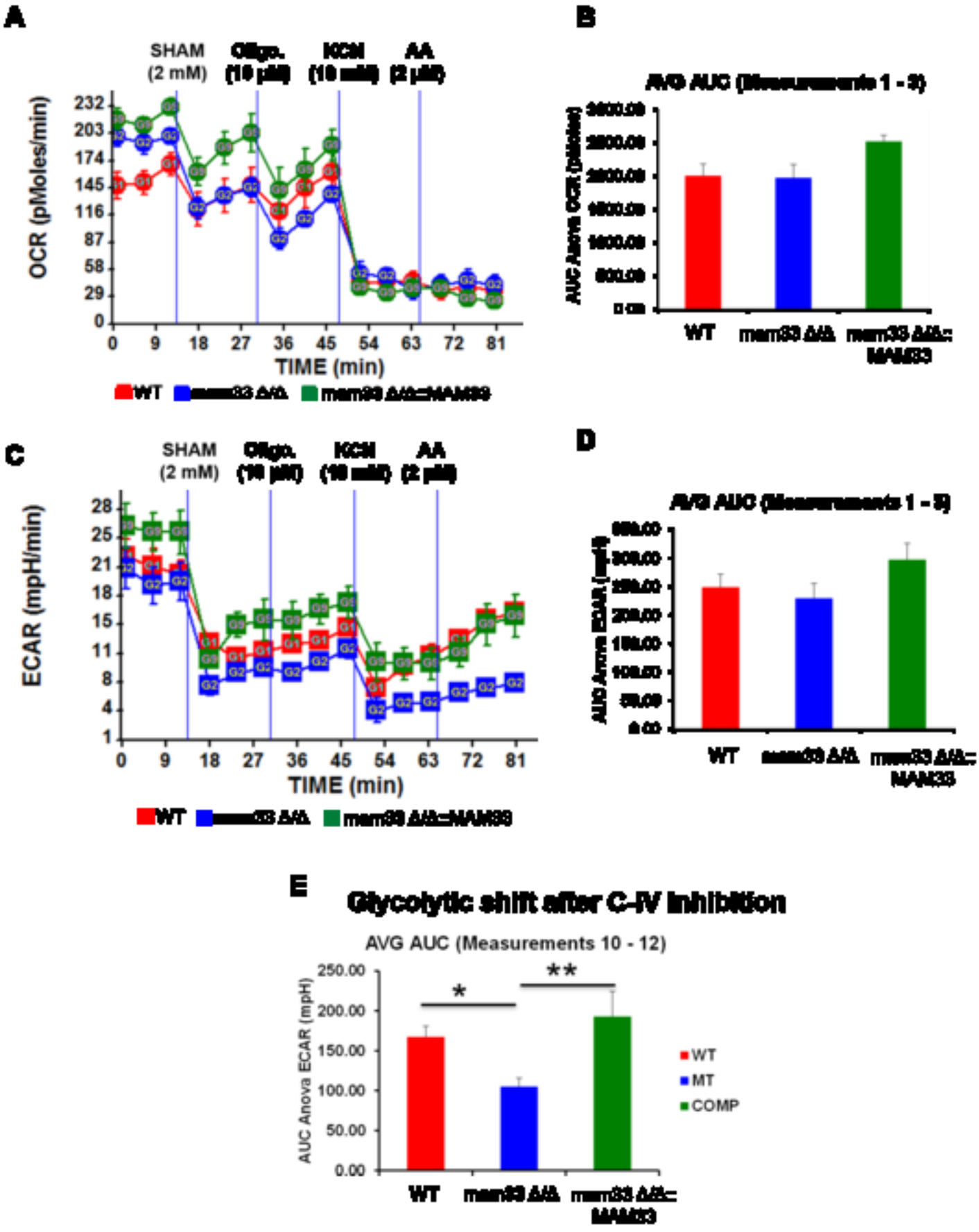

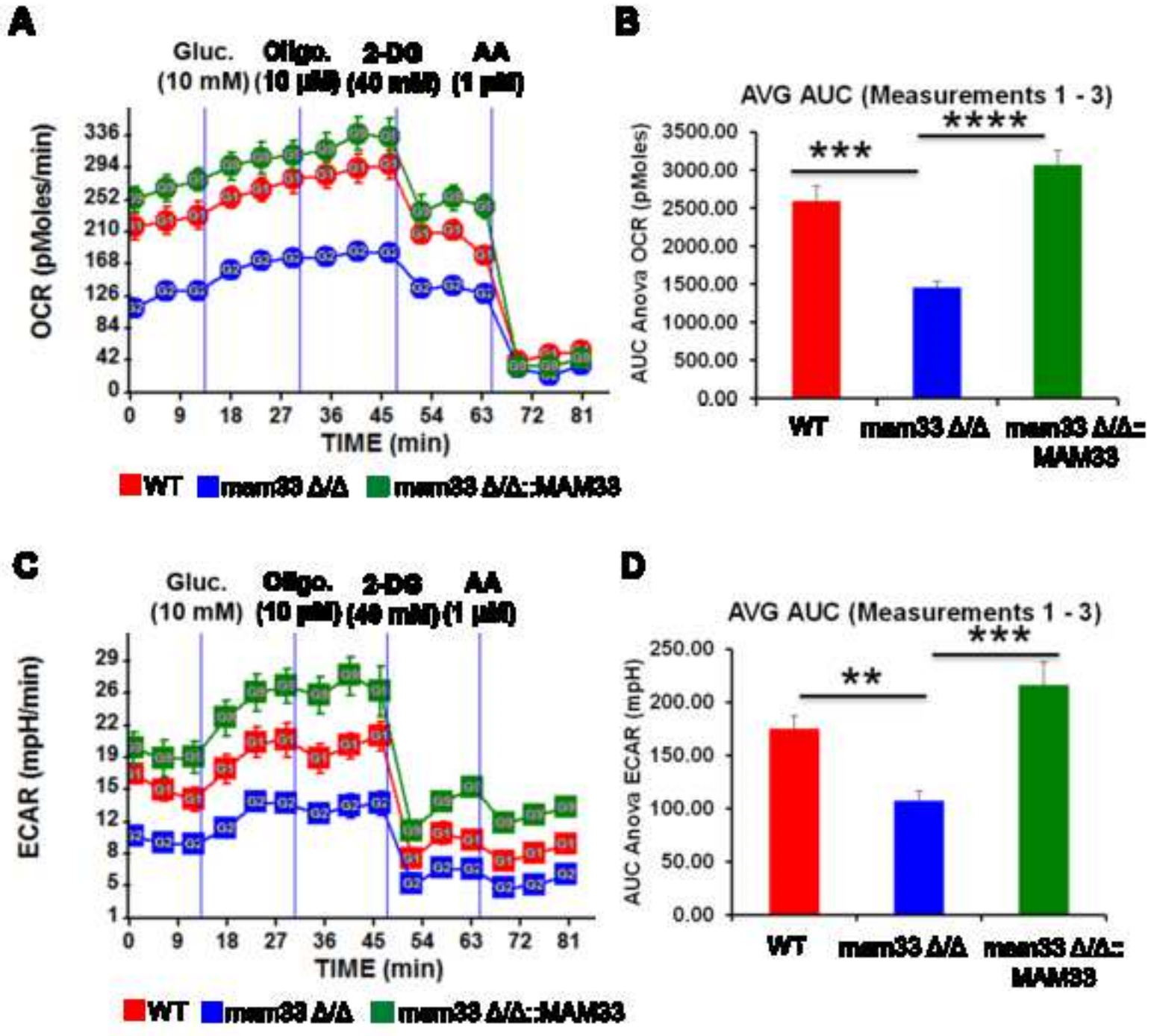

Table 1

Start Protocol		
Command	**Time (min)**	**Times (n)**
Calibrate	15	1
Equilibrate	20	1
Basal		
Loop Start		3
Mix	1	
Wait	2	
Measure	2	
Loop End		
Inject Port A		
Loop Start		3
Mix	1	
Wait	2	
Measure	2	
Loop End		
Inject Port B		
Loop Start		3
Mix	1	
Wait	2	
Measure	2	
Loop End		
Inject Port C		
Loop Start		3
Mix	1	
Wait	2	
Measure	2	
Loop End		
Inject Port D		
Loop Start		3
Mix	1	
Wait	2	
Measure	2	
Loop End		

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments
RPMI 1640	Corning	MT50020PB	
Antimycin A	Sigma	A8674	
KCN			
Mito stress kit	Agilent	103015-100	
Oligomycin	Calbiochem	495455	
pH meter	Accumet	AR20	
Phenol red	Sigma	P5530	
Poly-D lysine	Sigma	P6407	
Rotenone	Santa cruz	203242	
Seahorse XF24 FluxPak	Agilent	100850-001	
SHAM			
Sodium Chloride	Amresco	241	
Sodium hydroxie pellets	J.T Baker	3722	
Tissue culture grade water	Gibco	1523-0147	
XF assay calibrant solution	Agilent	100840-000	
Yeast extract Peptone Dextrose	Fisher scienti	BP2469	
Yeast extract Peptone Dextrose Agar	Sigma	A1296	
Yeast extract Peptone Glycerol	Sigma	G2025	

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Author(s):	Sundarajan Venkatesh, Manju Chauhan, Carolyn Suzuki, Neeraj Chauhan

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Title:	Bio-energetics investigation of Candida albicans by using real-time extracellular flux analysis

Signature:	Neeraj Chauhan	Date:	08/06/18

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We would like to thank the reviewers for their insights and thoughtful comments. We have made every possible effort to address all of reviewers' comments, as outlined in detail below. Hence, we believe that the revised manuscript is significantly improved with respect to focus and depth. Below is my response to their comments.

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript is thoroughly revised and checked for spelling and grammatical errors.

2. Please revise lines 60-63 and 98-101 to avoid previously published text.

Revised

3. Affiliations: Please note that numbering follows the order of authors. First author gets 1, next author with different affiliation gets 2, etc., following from first to last.

Revised

4. Abstract: Please do not include references here.

References are now removed from the abstract

5. Please spell out each abbreviation the first time it is used.

Spelled out on the first time

6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Revised

7. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Revised

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Revised

9. Please revise the protocol to contain only action items that direct the reader to do something (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." Please include all safety

procedures and use of hoods, etc. However, notes should be used sparingly, and actions should be described in the imperative tense wherever possible.

Revised

10. Lines 86-95: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the discussion about the protocol to the Discussion.

Revised as per the suggestion

12. Line 162: Please reference Figure 2 in this step so the readers know what wells A1, B4, C3 and D6 refer to.

New figure showing wells A1, B4, C3 and D6 is incorporated

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

Single-line spaces are included now

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Highlighted in yellow

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Highlighted in yellow

17. Figure 1: Please use SI abbreviations for all units (µL, h) instead of µl and hr. Please include a space between all numerical values and their corresponding units (4 °C, 30 °C, 100 µL).

Corrected

18. Figure 2: Please define the acronyms (WT, MT, COMP, etc.).

Defined in the figure legend

19. Figures 3 and 4: Please define error bars in the figure legend. Please include space between all numerical values and their corresponding units (10 mM, 1 µM).

Corrected

20. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Discussion is revised to include all critical steps in the protocol

21. Please combine the future application paragraph with Discussion section.

Combined.

22. References: Please do not abbreviate journal titles.

JoVE endnote style has been used

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript reports the use of "Seahorse bioenergetics assay" for studying perturbations in mitochondrial functions in C. albicans. The authors make use of this assay to flesh out information on the respiratory and glycolytic ability of a mitochondrial mutant.

1. **While the assay looks promising, the authors need to perform a few more control experiments to prove the efficacy of their protocol.**
   We have performed additional experiments and a different assay medium to confirm the efficiency of the protocol. By using RPM1 1640 medium as suggested by the reviewer, we found better responses to the mitochondrial inhibitors.

2. **The authors do not mention which mitochondrial mutant they have used in this study. They should mention which gene has been mutated and what functions are affected in this null mutant. At least two more mutants should be included in their study to prove the statistical significance of their protocol.**
   We have included the mutant name (*mam33Δ/Δ*) in this manuscript and provided its description. Since this is a method paper, analyzing more mutants is beyond the scope of this paper.

3. **Additionally, the authors should keep the naming of the strains consistent in the study. They write "mutant" and then also use "MT" as another abbreviation for the mutant in the same figure 3. Same goes for the complementary strain too**.

   In the revised paper, the mutant and the complementary strain names are kept consistently throughout.

4. **Adherence assays in Candida also make use of RPMI 1640 routinely. The authors should check the efficiency of their protocol with this medium also.**

   Thanks for the pointing out about RPMI 1640. When we used RPMI, we found RPMI 1640 worked better in terms of the efficiency of oxphos inhibitors. In the revised submission, we employed RPMI 1640 instead of DMEM.

5. **The inhibitors of mitochondrial function did not work in their assay. The authors should give some more explanation to this. If the routinely used inhibitors do not work in this assay, the assay does not have any therapeutic potential.**

   In the revised manuscript, we employed RPMI and found the inhibitors of mitochondrial function worked efficiently.

6. **Any other drug/compound that has been shown to affect mitochondrial function can also be included in the study.**

   We have now also investigated and included SHAM-an inhibitor of alternate oxidase (AOX) and KCN- an inhibitor of complex IV in the revised manuscript.

7. **Authors should mention the troubleshooting attempted while the assay was performed.**

We mentioned the inefficiency of DMEM for mitochondrial inhibitors in the revised manuscript.

8. **Line no 89 "the ability to" should be "the ability of".**

   Corrected

9. **Line no 108 "filter add store" should be "filter and store"**

   Corrected

10. **Line 216 : change 'are shown' to 'showed'**

    Deleted

11. **Line 218 the sentence should ne phrased as " to obtain and optimal OCR, titration of**

    Changed to “To obtain optimal OCR

12. the WT …..was performed with cell numbers ranging from….per well"

Corrected

13. Line 269 : instead of 'peturbate', 'perturbs' should be used.

Corrected

14. Line 288 : use word 'analyse/assess' instead of 'interrogate'

Corrected

15. Line 293: meaning of the statement is not clear.

Removed

16. Kindly rephrase the statement written in "future application".

Revised

17. Figure 1: Analysis should be Analyze

Corrected

Reviewer #2:

Manuscript Summary:
This manuscript uses real-time extracellular flux assay to investigate mitochondrial respiration and glycolytic function in C. albicans. The authors present a simple protocol that may be used to any Candida spp. strain. The method is interesting and can be useful to study new antifungals or gene manipulation.

I have a few comments:

1. **line 69: Authors report "Recent studies have indicated...drugs". No studies were cited. Please add references.**
   Now included.

2. **Please, define all abbreviation (e.g., WT, MT) in the manuscript.**

   Abbreviations are defined now.

3. **Both "g" and "RPM" are units used for centrifugation steps. It is better to use g, which refers to the acceleration applied to your samples. RPM is not as useful a unit, because the force varies with the radius of your machine (the bigger the radius,**

the more acceleration is applied to your samples for the same RPM). In this way, please, change rpm to g in the manuscript.

RPM is most commonly used in the shakers to grow microbial cultures

4. It seemed to me that there is an appropriate equipment for this assay. If this is true, please, clarify in the manuscript.

Instrument is clarified in the revised paper

5. **Also, improve the legends of figure 2 and table 1. For example: Fig. 2: Layout of wells and group assignment "as visualized from Seahorse X24 software" ???. Also, define abbreviations.**

Corrected to "Fig. 2. Layout of wells and group assignment as visualized in the Seahorse XF24 software" "Table 1. Protocol commands for the assay.

6**. In the last page, there are three lines highlighted in yellow. I am not sure what they mean**.

This was an inadvertent error. We have corrected it.

Reviewer #3:

Manuscript Summary:

1. **The manuscript by Venkatesh, Chauhan, Suzuki, and Chauhan details a method for utilizing the Seahorse platform to assess OCR and ECAR readouts for mitochondrial respiration and glycolytic function in C. albicans strains. While the goal is aimed at detailing this system, the lack of strain details provides only tangential conclusions to be made by the reader. This system, however, presents the opportunity to rapidly and reproducible assess the responsibility of specific genetic mutations to affect overall respiration within multiple strain backgrounds.**

The details of the mutant and complementary strains are detailed in the revised manuscript

2. **The abstract describes how antifungal tolerance is impacted by mitochondrial function but there is little to no description of this in the Introduction. A paragraph should be added to include how mitochondrial function is tied to antifungal tolerance or resistance.**

Importance of mitochondria on antifungal tolerance is highlighted in the introduction (lines 78-80). A more in-depth description on this topic is beyond the scope of this paper.

3. **Another major question is why this protocol is needed. There are a number of yeast-specific protocols for assaying mitochondrial function.**

Standardized methods to understand mitochondrial function in pathogenic fungi are poorly developed. The method presented herein is an effort to provide a protocol to measure the basal oxygen consumption rate (OCR), a measure of mitochondrial respiration, and extracellular acidification rates (ECAR), a measure of glycolytic function in *C. albicans* strains. The method described herein can be applied to any *Candida spp.* strains without the need to purify mitochondria from the intact fungal cells. Furthermore, this protocol can also be customized to screen for inhibitors of mitochondrial function in *C. albicans* strains.

4. **Line 89-90: This sentence is floating. What does this adaptation measure? Is it OCR, ECAR, or something else?**

Corrected

5**. Lines 86-95: The starting cell number for the assay should be known for this protocol. Is it known that cell density has no impact on respiration rates? I would expect the opposite to be true based on previous reports:**
https://www.sciencedirect.com/science/article/pii/S1550413112002380.

The starting cell number is critical for the assay. In fact, we titrated the optimal C. albicans cell number for this assay. The importance of starting cell number is described line 248-252.

6. **The first two sentences of the first paragraph are describing methods written in the protocol. I would suggest omitting these sentences and describing the purpose of the experiment in the context in which it was performed.**

Corrected

7. **This first paragraph also fails to state that the experiment was performed before moving into analysis of results. There's a major disconnect there.**

Corrected

8. **The mutant used in this study is not named and must in order for the experiments described here to be reported on any way. This is particular important for a methods paper.**

Mutant name is now included and detailed in the revised paper

9**. From Figure 3 it is not clear what part of the curve is used for area under the curve (AUC) measurements in 3A or 3B and why. My assumption would be that after the final injection, AUC would be calculated but this does not represent the given data. More clarification is needed.**

New figures are included, which is much more clear and detailed in the data analysis part.

10. Additionally, the complemented strain appears significantly different across the curve from the WT in 3A, which it should phenocopy. No statement is given on why this difference exists and if there's a significant different between the two.

New data more focused on the protocol and the method

11. The complemented strain is more similar to WT in 3B but is still significantly different across most of the assay. There's no comment on this and no difference is suggested from the bar graph. This requires much more explanation.

In our revised manuscript, we have addressed these concerns by optimizing the experiments and providing new data

12. Finally, the text states that the experiment is flawed based on the optimal OCR range. This should be redone so the data lies within an acceptable range of detection.

We have redone the experiments and found the optimum OCR range and cell number, corresponding changes are done

13. **The protocol numbering system is not consistent with the JoVe format.**

Corrected

14. **All steps requiring use of the machine would benefit from images as the location of these ports and method used for injection are not obvious from reading.**

Corresponding figures are now incorporated in the revised manuscript

15. **An additional section, Step 9, should be included for data analysis.**

The revised manuscript contains data analysis section.

16. **There's a lot of concerns regarding grammar across the manuscript.**

The manuscript is thoroughly revised and checked for spelling and grammatical errors.

17. **There are too many places where this is problematic to list but a selection are below. These grammatical issues are present in Figure 1 as well.**

Figure 1 is corrected

18**. Step 2. Part 1.1 is a massive run-on sentence and needs to be rewritten in its entirety. Please clarify that all of Step 3 Parts 1.2-1,4 should be performed in the hood as only 1.2 is currently noted as such.**

All the step 3, 1.2-1.4 should be performed in the hood. Corresponding changes have been made in the revised manuscript as below

NOTE: Perform all the below steps in a laminar hood.

1.1. Dissolve Poly-D Lysine in tissue culture grade water to make 50 µg/mL final concentration. Mix it well and aliquot into a 1.5 mL microcentrifuge tubes and store at -20 °C for the long term. You need 50 µL per well, and for 24 wells, it requires 1.2 mL. Therefore, aliquot at least 1.3 mL per microcentrifuge tube.
1.2. Add 50 µL per well and incubate at room temperature with the lid covered for 1-2 h.
1.3. Aspirate the solution and rinse one time with 500 µL sterile tissue culture grade water.
1.4. Open the lid and allow the wells to air dry. Use the plate on the same day or store at 4 °C for a maximum of 2-3 days.

19. **Step 4 Part 1.1 needs clarification. Turn what "upside down". What is it "it" in the next phrase, etc.,**

The sentence is now re-phrased.

18. **Line 29: This sentence is a little awkward. I'd suggest changing "..is gaining more importance in studying changes.." to "..is gaining more importance to the study of changes.."**

Corrected

21. **References in the abstract is unusual and should be saved for the body of the paper**.

References from the abstract are removed

22. **Line 63: "drug to drug" should be either "drug-drug" or "interactions between drugs".**

Corrected

23. **Line 66: stating conservation of targets for antifungals between fungi and humans could be stated more explicitly here.**

Line 75 explicitly states challenges in discovery of new targets due to the fact that fungi, like humans, are eukaryotes.

24. Line 71: There are a number of more recent references that can be used here that are more broad in scope as well. I'd include this review (PMID 25088819) and this recent paper (PMID 29719235).

Thank you for the suggestion. These references are now added into the text.

25. **Line 86: "a" should be inserted between "in" and "Seahorse"**

Corrected

26. Line 89: "to" should be "for"

Corrected

27. **Step 6, Part 1.3 - grammar.**

    Corrected

28. **Step 8, Part 1.2: Wording is awkward, consecutive "by"s**
    The entire protocol is rewritten and is clear now

29. **Step 8, Part 1.3 "form" should be "from"**

    Modified the sentence

30. **The "O" in the temperature is a zero and not give as an "O"**

    Corrected

31. **The Experimental Materials table is missing information. Please provide the company name and catalog number**

    Provided