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TITLE:

Metabolic Profiling to Determine Bactericidal or Bacteriostatic Effects of New Natural Products using Isothermal Microcalorimetry

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KEYWORDS:

Isothermal microcalorimetry; antimicrobial resistance; natural products; microbiology; antibacterial; bacteriostatic; bactericidal; Myxobacteria

SUMMARY:

The elucidation of the mode-of-action of a novel antibiotic is a challenging task in the drug discovery process. The goal of the method described here is the application of isothermal microcalorimetry using calScreener in antibacterial profiling to provide additional insight into drug-microbe interactions.

ABSTRACT:

Due to the global threat of rising antimicrobial resistance, novel antibiotics are needed urgently. We investigate natural products from Myxobacteria as innovative source of such new compounds. One bottleneck in the process is typically the elucidation of their mode-of-action. We recently established isothermal microcalorimetry as part of a routine profiling pipeline. This technology allows for investigating the effect of antibiotic exposure on the total bacterial metabolic response, including processes that are decoupled from biomass formation. Importantly, bacteriostatic and bactericidal effects are easily distinguishable without any user intervention during the measurements. However, isothermal microcalorimetry is a rather new approach and applying this method to different bacterial species usually requires pre-evaluation of suitable measurement conditions. There are some reference thermograms available of certain bacteria, greatly facilitating interpretation of results. As the pool of reference data is steadily

growing, we expect the methodology to have increasing impact in the future and expect it to allow for in-depth fingerprint analyses enabling the differentiation of antibiotic classes.

INTRODUCTION:

The aim of this method is to apply isothermal microcalorimetry (IMC) as a medium throughput assay in mode-of-action (MoA) profiling of new antibacterial compounds. This method reveals data regarding the activity of compounds on bacterial species and provides information on the bactericidal or bacteriostatic nature of the compound itself.

The rise of emerging antimicrobial resistance (AMR) is a global problem and it leads to less effective treatments of common infections with known antibiotics[1]. However, the search for new compounds and drugs that can replace or work in combination with known antibiotics are ongoing and this is built on diverse approaches. Natural products are a key player in current drug discovery campaigns and particularly in anti-infective drug discovery[2]. However, identifying new lead structures for antibiotic development is a lengthy and financially demanding process[3]. Thus, the early steps of discovery are extremely important in order to filter on most promising scaffolds already at an early stage. Initial steps in natural product drug discovery include obtaining a compound's structure, determining the activity in vitro along with MoA and target identification. Most successful compounds being eligible for further development should display a favorable spectrum of activity (i.e., broad-spectrum activity in the case of antibacterials) and a novel MoA by which pre-existing AMR can be overcome. Promising scaffolds are then typically screened in secondary assays, which include in vivo bioavailability, toxicity, and metabolism[4]. Besides the financial concerns, natural product drug discovery faces further challenges concerning the costs and technical difficulties related to compound isolation and purification, which, in turn, can make it difficult to obtain multi-milligram or even gram amounts in the early stages of the discovery process[5,6]. Therefore, it is of utmost importance in natural product research to be able to perform state-of-the-art primary screening with minimal compound amounts in order to take a well-informed decision about further investments to make a novel natural product accessible for pre-clinical development. With the use of IMC for antibacterial profiling, the amount of compound needed is significantly reduced in comparison to standard methods. The technique also provides more in-depth information regarding the interaction of new drugs with the microbial community[7].

IMC is a well-established method for measuring total energy as a result of all biological, physical and biochemical processes and reactions in a biological system. Bacterial energy release is proportional to the total metabolic reactions[8]. Within a closed system, such as the used microcalorimeter, the heat levels can be measured in the microwatt range to study the metabolic kinetics of bacteria [9-12]. The heat (energy) that is released by bacteria is linked to cellular functions that underlie their metabolism and that are not necessarily proportional to the cellular biomass.

Initially, the applicability of isothermal calorimetry for microbiological assays has been limited due to its low throughput and high testing volumes. However, the used microcalorimeter is unique as it combines the advantages of isothermal calorimetry with increased throughput and lower compound requirements, which makes it a valuable tool for drug discovery applications[10].

Furthermore, the instrument provides further advantages over alternative methods for measuring bacterial growth kinetics, such as the standard turbidity method, which is based on the measurement of optical density at 600 nm (OD_{600}). Measuring OD_{600} is based on the assumption that increased optical density is equal to microbial growth, thereby neglecting the presence of non-viable cells. This method has also been criticized as it excludes small colony variants and persister cells[11]. In contrast, IMC allows the real-time observation of any type of viable cells. If cells are dormant, they still exhibit metabolic activity and they can thus be detected by IMC, whereas such phenomena are not detectable by the standard turbidity method[11]. Other advantages of IMC include a shorter antimicrobial susceptibility testing time, measuring drug interactions in a complex community and standard analysis methods without destroying the sample[7].

The IMC technology has been implemented in a wide range of studies, ranging from microbiology to thermogenesis and cancer biology[13-17]. The microbial applications include the determination of minimum inhibitory concentrations (MIC) of compounds against various bacterial strains. Several studies have been done and it has been concluded that MIC data from isothermal calorimetry for the majority of bacterial species can be obtained faster and results are similar compared to other (standard) methods for MIC determination[12,18,19]. Further applications of IMC include observing the interaction of drugs and combination of drug treatments with complex bacterial communities such as biofilms[11]. A study focusing on MoA profiling showed that the microcalorimeter can detect a difference in first- and second-generation cephalosporins, while different antibiotics with the same MoA exhibit a similar heat flow curve compared to each other[18].

Here, we describe the use of IMC for MoA profiling of new natural products using the new isothermal microcalorimetry instrument. The method is used to determine effective antibiotic concentrations and to describe characteristics of antibiotics in terms of bactericidal or bacteriostatic mechanisms. The method can be broadly implemented in MoA profiling of compounds and it might replace or at least complement standard microbiological methods. Future studies will include in-depth fingerprint analyses that will enable the differentiation of antibiotic classes based on target mechanisms.

PROTOCOL:

NOTE: The instrument temperature must be set according to the bacterium used at least a day in advance to ensure stability of the system. Here, *Acinetobacter baumannii* DSM-30008 samples are run at 30 °C.

1. Culture preparation

1.1. Streak out the strain under investigation (here: *A. baumannii*) on a CASO agar plate and incubate overnight in a static incubator at 30 °C.

1.2. Prepare an overnight culture by inoculating a single colony in MHB (Mueller-Hinton broth)

and incubate on a shaking incubator at 180 rpm at 30 °C.

2. Sample preparation

2.1. Use 1.5 mL tubes to prepare the concentration range of selected drug or compound (e.g., by adding 1.5 µL of 100x stock solutions in DMSO).

2.2. Dilute the overnight culture in MHB medium.

2.2.1. Measure the optical density of the overnight culture using a spectrophotometer at a wavelength of 600 nm.

2.2.2. Dilute the culture to obtain 5 x 10^5 colony-forming units (CFU)/mL in fresh MHB medium. An OD_{600} of one equals approximately 5 x 10^8 CFU/mL (e.g., *Escherichia coli*, *Staphylococcus aureus*, *A. baumannii*).

NOTE: It is important to calibrate the conversion factor OD_{600} to CFU/mL for each individual bacterial strain under the applied culturing conditions.

2.3. Add 150 µL of the cells to the test tubes prepared in step 2.1, ensuring correct final concentration of tested drug or compound and correct final cell concentration.

2.4. Mix the compound with the cells by vortexing.

NOTE: The sample plate (see **Table of Materials**) has six rows, each containing eight wells, a total of 48-wells; Row A and Row F are the thermodynamic reference. Therefore, no samples can be loaded into those wells, corresponding media is loaded into those wells. 32 test samples can be measured per run, using wells in rows B-E. Individual test sample should be run at minimum in duplicate (here: all samples were run as triplicates). Growth controls should be included.

3. Insert preparation

3.1. Transfer 120 µL from the mixture prepared in step 2.4 to the plastic inserts.

NOTE: Use reverse pipetting in step 3.1 to prevent liquid from spraying on the sides of the plastic inserts, which can lead to interference with correct signal readouts.

3.2. Place all titanium vials into the holders (see **Table of Materials**) with tweezers.

3.3. Gently transfer inserts into the titanium vials in the holder plate.

3.4. Loosely place titanium lids on all titanium vials.

4. Insert loading

4.1. Transfer the –holder with titanium cups onto the sample station and place on designated area.

4.2. Use the torque wrench, set to 40 cNm force, to tighten all the lids.

5. Running of samples

5.1. In calView software, start a new experiment (**Supplemental Figure 1**).

5.2. Retract the sample insertion arm from the instrument.

5.3. Place the cup holder on the "bridge", column 8 facing the sample insertion opening and gently push the cup holder into the instrument at the designated "Position 1". Wait 10 minutes for the system to stabilize. Label the experimental wells.

5.4. Push the sample insertion arm until the cup holder is at the designated "Position 2". Wait 20 minutes for the system to stabilize.

5.5. Push the sample insertion arm into "Position 3" and retract the sample insertion arm until it is at the "Running position". Highlight all the wells in the software and select **Reaction Start** (**Supplemental Figure 4**).

5.6. Run the experiment until the heat emission reads are stably back at zero.

NOTE: Be sure that all wells behave similar within these steps. **Supplemental Figure 5** illustrates what should be observed if the wells are loaded correctly. If loaded incorrectly, repeat steps 5.2-5.4.

6. Remove the cup holder

6.1. In the software, select **Stop** (**Supplemental Figure 6**). The software will then ask if "you are sure", select **Yes** (**Supplemental Figure 7**) and save the experiment on a drive or desktop for data analysis (**Supplemental Figure 8**).

6.2. Insert the sample insertion arm completely into the instrument and engage the magnets to retrieve the cup holder.

6.3. Loosen the lids, remove the inserts and place vials and lids into glass holders and place for 4 hours at 180 °C followed by placing the vials and lids into a desiccator to ensure lids and vials are dry.

7. Analyzing data

7.1. Open the software (**Supplemental Figure 9)**, select **Open experiment** in the left upper corner (**Supplemental Figure 10**). In the popup window select the experiment of interest and press **Open** (**Supplemental Figure 11**). The application will open the experiment in default wells view (**Supplemental Figure 12**).

7.2. Press **Select all** or **Ctrl+A** (**Supplemental Figure 13**).

7.3. Press **Define Baseline**, this parameter normalizes the data in each position (**Supplemental Figure 14**). In the popup window select a time period of >30 min located in the lag phase (the heat flow has to be low, between zero and ten µW; **Supplemental Figure 15**). After selection of base line time period the chosen baseline will appear in green in the thermogram. Close the **Define Baseline Section** window.

7.4. Press **Save** or **Ctrl+S** and close the software (**Supplemental Figure 16**).

7.5. Open web-based Symcel Calorimetry analysis application (https://symcel.shinyapps.io/symcel_calorimetricgrowth/).

7.6. To upload the file to the Calorimetry analysis application, press **Browse** (**Supplemental Figure 17**), select the experiment and press **Open** (**Supplemental Figure 18**). The metabolic parameters will be calculated automatically for the 32 samples in the web application (**Supplemental Figure 19**).

7.7. In order to fit the heat Flow data to Gompertz and/or Richard´s growth models click **Growth Function**. Growth models fit will be displayed in the section "Cumulative", also compared to the raw data in the section "Flow" (**Supplemental Figure 20**).

7.8. To download all calculated parameters, press **Download Measures**. Select the file location and press **Save** (**Supplemental Figure 21**). The file will be exported to a spreadsheet for further calculations.

REPRESENTATIVE RESULTS:

A sufficient number of bacteria producing heat is needed for the instrument to record a heat signal. If there is a delay in time until a heat flow is detectable it means that the bacteria do not yet produce a heat signal above the detection limit. The detection of released heat from the bacterial sample is therefore directly related to increasing bacterial activity, including bacterial growth. Bacterial growth and other metabolic activities are known to be strongly influenced by the addition of an antibacterial drug. To determine whether a new natural product under investigation exerts bactericidal or bacteriostatic effects or a combination of both MoAs, we have chosen a small set of reference drugs and we recorded thermograms to which we compared data from experiments with the natural product. Based on the potency of selected antibiotics a range of concentrations was selected being close the minimum inhibitory concentration (MIC) as determined by microbroth dilution.

Ciprofloxacin targets bacterial DNA gyrase and topoisomerase IV and consequently, it exhibits a bactericidal MoA. However, bacterial killing induced by ciprofloxacin is concentration-dependent and when it is dosed at insufficient concentrations it can also have a bacteriostatic effect[21]. Tetracycline and chloramphenicol target the ribosome at the 30S and 50S subunit, respectively, and they act bacteriostatic due to inhibition of protein synthesis[22,23]. Rifampicin acts by inhibiting DNA-dependent RNA polymerase and it can have both, bactericidal and bacteriostatic effects, depending on the dose used[24]. The natural product that we are investigating here was isolated from Myxobacteria and it showed potent activity against Gram-negative and Gram-positive bacterial pathogens. While investigating its MoA and molecular target, we were interested in determining whether the new natural product exerts bactericidal and/or bacteriostatic effects and whether the heat profiles of treated *Acinetobacter baumannii* are similar to thermograms from bacteria that were treated with the reference drugs mentioned above.

The thermograms obtained by exposing *A. baumannii* DSM-30008 to ciprofloxacin in serial dilution are displayed in **Figure 1A**. Concentrations between 0.005 µM and 0.1 µM have a minimal effect on the growth and metabolism of *A. baumannii.* However, treating the cells with 0.5 µM ciprofloxacin leads to a significant shift in lag phase duration and lower maximum heat flow. These two changes together affect the time to peak (**Figure 1C**), which is increased by approximately 6 hours. In **Figure 1B**, the cumulative released heat is plotted against time. Here, we see the effect of concentrations, which is reflected by an incline of slope. Quantification of the thermogram's incline gives us the maximum metabolic rate of *A. baumannii* in presence of ciprofloxacin as displayed in **Figure 1D**, where we can observe a concomitant decrease of metabolic rate of cells treated with 0.5 µM ciprofloxacin. Changes of metabolic rate of cells treated with lower concentrations are minimal. This experiment could be further improved by the addition of intermediate concentrations covering the 0.1-1 µM range to observe a more pronounced gradual change between the concentrations that have no effect and a concentration resulting in a significant delay of lag phase and metabolic rate. However, the trends of change support a bactericidal MoA of ciprofloxacin.

[Place **Figure 1** here]

In the case of tetracycline, we did not observe significant changes in the thermograms for all concentrations tested until late exponential phase starting after 8 hours (see **Figure 2A**). Nevertheless, major changes are observed in stationary phase heat emission, where the second peak of heat flow is significantly lowered for *A. baumannii* treated with 5 µM and 10 µM tetracycline. Lower concentrations had an effect as well, which was however less pronounced. This effect also causes a prolongation in time to peak as displayed in **Figure 2C**. The cumulative released heat curves (**Figure 2B**) show that non-treated and treated cells do not display significant differences in the overall curve shape but by tendency, the slope of the curves declines at higher concentrations of tetracycline. This also translates into quantified metabolic rates displayed in **Figure 2D**, where a concentration-dependent effect, (i.e., decrease of metabolic rate at increasing antibiotic concentrations) is observed. These findings support the fact that tetracycline has a bacteriostatic effect.

[Place **Figure 2** here]

Even more pronounced effects can be observed when treating *A. baumannii* with the protein synthesis inhibitor chloramphenicol that targets the 50S ribosomal subunit. Exposure to increasing concentrations of chloramphenicol leads to prolongation of the lag phase and significant changes of the metabolic activity in the stationary phase (**Figure 3A** and **Figure 3B**). No change in metabolic rate is observed for the lowest tested concentration and the highest test concentration chosen (50 µM) prevents most energy release of the sample. Looking at the intermediate test concentrations (5 µM and 10 µM), the time to peak is significantly increased by approximately 8-9 hours (**Figure 3C**). Simultaneously, the metabolic rate of treated cells is significantly reduced, with 50 µM being lethal (**Figure 3D**). Overall, the changes observed at concentrations up to 10 µM chloramphenicol are consistent with a bacteriostatic effect of this antibiotic class.

[Place **Figure 3** here]

Rifampicin treatment in the selected concentration range has a dramatic effect on the thermograms of *A. baumannii* DSM-30008 related to the lag phase duration and effects on growth until the late stationary phase (**Figure 4A**). A significant reduction of heat emission can be seen in **Figure 4B** that goes along with a decrease in metabolic activity. **Figure 4C** and **Figure 4D** illustrate the influence of the prolongation of the lag phase and changes of the metabolic activity in the stationary phase. **Figure 4C** shows a definite increase in time to peak for all concentrations used. **Figure 4D** illustrates the decrease in the metabolic rate caused by the decrease in slope for all concentrations that is usually ascribed to a bactericidal effect. Due to antibiotic-induced killing of bacterial cells, the metabolic activity is expected to be lower due to a smaller number of active bacteria present. The data collected is in agreement with the fact that rifampicin can act bactericidal and bacteriostatic, and the data presented here support mainly bactericidal effects of the chosen concentrations.

[Place **Figure 4** here]

The lowest selected test concentration of the natural product antibiotic (0.25 µM) has no or only marginal effects on the thermogram of *A. baumannii* DSM-30008. However, other tested concentrations exhibit some effect on lag phase duration, and affect the growth until the late stationary phase (**Figure 5A**). The most obvious effect is the significant reduction of heat emission in the stationary phase. Data displayed in **Figure 5B** clearly show that released energy is significantly decreased for all effective concentrations and the slope is decreased as well. These effects translate into a decrease of time to peak (**Figure 5C**) and more importantly, a significant and clearly dose-dependent decrease in metabolic rate is observed (**Figure 5D**). The investigation of the new myxobacterial natural product revealed a combined bacteriostatic and bactericidal effect.

[Place **Figure 5** here]

Figure 1: Effect of ciprofloxacin on *A. baumannii* DSM-30008 growth and metabolism. (**A**) Thermograms shown as heat flow (µW) vs. time (h) for wild type (WT) *A. baumannii* DSM-30008, non-treated and exposed to ciprofloxacin. (**B**) Cumulative heat (mJ) vs. time (h). (**C**) Time to peak (h) with error bars (standard deviation). (**D**) Metabolic rate (µW) with error bars (standard deviation).

Figure 2: Effect of tetracycline on *A. baumannii* DSM-30008 growth and metabolism. (**A**) Thermograms shown as heat flow (µW) vs. time (h) for wild type (WT) *A. baumannii* DSM-30008, non-treated and exposed to tetracycline. (**B**) Cumulative heat (mJ) vs. time (h). (**C**) Time to peak (h) with error bars (standard deviation). (**D**) Metabolic rate (µW) with error bars (standard deviation).

Figure 3: Effect of chloramphenicol on *A. baumannii* DSM-30008 growth and metabolism. (**A**) Thermograms shown as heat flow (µW) vs. time (h) for wild type (WT) *A. baumannii* DSM-30008, non-treated and exposed to chloramphenicol. (**B**) Cumulative heat (mJ) vs. time (h). (**C**) Time to peak (h) with error bars (standard deviation). (**D**) Metabolic rate (µW) with error bars (standard deviation).

Figure 4: Effect of rifampicin on *A. baumannii* DSM-30008 growth and metabolism. (**A**) Thermograms shown as heat flow (µW) vs. time (h) for wild type (WT) *A. baumannii* DSM-30008, non-treated and exposed to rifampicin. (**B**) Cumulative heat (mJ) vs. time (h). (**C**) Time to peak (h) with error bars (standard deviation). (**D**) Metabolic rate (µW) with error bars (standard deviation).

Figure 5: Effect of a new antibacterial natural product on *A. baumannii* DSM-30008 growth and metabolism. (**A**) Thermograms shown as heat flow (µW) vs. time (h) for wild type (WT) *A. baumannii* DSM-30008, non-treated and exposed to the natural product. (**B**) Cumulative heat (mJ) vs. time (h). (**C**) Time to peak (h) with error bars (standard deviation). (**D**) Metabolic rate (µW) with error bars (standard deviation).

Supplemental Figure 1: Selecting a new experiment in the software interface. In a red square the step to select a new experiment is depicted.

Supplemental Figure 2: Naming a new experiment in the software interface. In a red square the step to name and confirm the new experiment is depicted.

Supplemental Figure 3: Starting a new experiment in the software interface. In a red square the step to start a new experiment is depicted.

Supplemental Figure 4: Well selection and reaction start in software interface. All reaction wells are selected (wells colored in deep blue color, button **Select all** depicted in a red square) and reaction start is selected (depicted in a red square).

Supplemental Figure 5: Correct loading of cup holder. Reference wells are selected (deep blue

color, red square) and thermograms are displayed in a popup window. When the loading is performed correctly, we observe a steep decline in heat emission signal detected that must reach a plateau phase and remain in this phase for approximately 2-3 min, afterwards the signal returns to the starting point. When this is observed the loading of the cup holder is correct.

Supplemental Figure 6: End of experiment. In red, the **Stop** button in the experiment is depicted.

Supplemental Figure 7: Confirmation of the end of experiment. In red, the **Yes** button to confirm the end of running experiment is depicted.

Supplemental Figure 8: Saving the experiment file. A popup window with save file options is shown and in red the button **Save** is depicted.

Supplemental Figure 9: Software interface.

Supplemental Figure 10: Accessing saved experiments. In red the button **Open experiment** to access saved experiments is depicted.

Supplemental Figure 11: Opening of selected experiment file. In red the button **Open** is depicted.

Supplemental Figure 12: Default wells view. All experimental well and corresponding thermograms are visible.

Supplemental Figure 13: Selecting wells for analysis. In red the button **Select all** is depicted.

Supplemental Figure 14: Defining the baseline. In red the button **Define baseline** is depicted.

Supplemental Figure 15: Selecting the baseline signal. Minimum of 30 min of signal in the lag phase is selected (red box).

Supplemental Figure 16: Saving changes to the experimental file. In red the button **Save** is depicted.

Supplemental Figure 17: Web based Calorimetry analysis application. The online software interface and file upload pathway is shown.

Supplemental Figure 18: Upload of selected experimental file. In red the button **Open** is depicted.

Supplemental Figure 19: Analysis of the thermograms. Metabolic parameters calculated for each experimental well are depicted in red.

Supplemental Figure 20: Fitting the experimental data to theoretical growth models. Heat flow

data is fitted either to a Gompertz or a Richard´s growth model.

Supplemental Figure 21: Exporting the measurements. In red the buttons **Download Measures** and **Save** are depicted.

DISCUSSION:

Isothermal microcalorimetry measures energy emitted from biological system over time and this energy release is a result of all biological, physical and biochemical processes. The measured heat flow can be exploited to evaluate or determine antibacterial effects of substances as it enables continuous real-time monitoring of metabolic activity.

In order to obtain reliable data for analysis, correct starting colony forming units (CFU) need to be determined individually for each species or strain used. In case the CFU count is too low, this leads to a prolonged lag phase as it takes longer for the system to reach a critical amount of biomass producing enough heat to be detected. In case the CFU count is too high the lag phase will be very short, and the amount of heat produced can cause heat transfer to neighboring sensor cells (reference and experimental wells) and cause distortions of the thermograms. High numbers of CFU will also lead to a faster oxygen depletion and switch to anaerobic conditions. It also has to be taken into account that during the first 30 minutes of the experiment, when the system is equilibrating, data collection is not possible and the actual recording of effects is delayed. In addition, incorrect CFU determination leads to false MIC determination, ultimately affecting the experiment and the changes observed[25]. Another critical point is the correct determination of the baseline. Usually, the baseline signal is selected during the lag phase when the heat flow signal is zero and ideally, the time range for baseline definition is >30 min. However, this is not always possible as the time that the bacteria requires to reach the heat signal detection limit differs between strains and species. Some species or strains require more than 30 minutes to reach the heat signal detection limit while other strains or species reach it within the first 30 minutes. In that case, it is possible to select the baseline signal at the end of the experiment when all the heat signals have dropped back to zero and remain stable. Alternatively, baselines from other experiments with the same bacterial strain can be used, which is however not recommended.

The system allows for some flexibility in terms of design and optimization of experiments and troubleshooting. Volumes used are in the range of 100-300 µL when using plastic inserts and 100-600 µL when using titanium cups without the plastic inserts. The recommended working volume by the manufacturer is 120 µL. The use of different volumes in setting up a new experimental series, and while finding optimal conditions for measurements, has an impact mainly on two parameters. By using lower volumes, the amount of required test compound can be decreased, which is particularly important for compounds, which are available only in small amounts. In addition, the used volume directly impacts oxygen availability during the measurement with lower volumes increasing the amount of available oxygen required for bacterial growth. Oxygen depletion is one of the main factors contributing to the maximum possible duration of the experiment. Importantly, it is possible to use solid media, not only liquid media. This is especially important for slow-growing microorganisms as growth on the interface between solid and gas

phase enables better oxygen access[9].

IMC is a useful analytic tool to discover unknown processes with applications in physics, chemistry and biology. The method measures the heat exchange within a closed system and analysis of the recorded heat exchange provides additional information that cannot always be obtained with standard methods. In microbiology and antibiotics research, one of the biggest advantages of IMC is its ability to distinguish between live, dead and persister or dormant cells, which is not possible using standard turbidity methods[11]. In addition, IMC is highly sensitive and it can detect heat emission from as few as 10^4-10^5 cells[9]. Another advantage is that the experimental setup is fast and easy, and it allows for continuous, real-time tracking with minimal to no user interference. Further, IMC is non-destructive, which enables further analysis of samples. Data analysis allows for decoupling of biomass formation until late exponential or early stationary phase and metabolic activity in stationary phase.

Besides the novel and exciting application features mentioned above, there are also drawbacks to this method. The major limitation is that IMC instruments measure the total heat produced and released within a specific system, which also include non-specific signals. This highlights the importance of experimental planning with appropriate controls to be able to assess the heat signal changes that are measured by recording heat flow[9, 20].

In our hands, IMC is an important tool to study antibacterial effects of new natural products and to determine effective concentration ranges. Apart from differentiating bacteriostatic and bactericidal effects, it could be used in future as part of target identification studies and MoA determination. This can be done by comparing thermograms of different antibiotic classes to thermograms of new active compounds as displayed here. However, it still has to be investigated whether certain quantifiable parameters that can be extracted from the measured data are sufficient for such comparisons or if it will be necessary to work on algorithms giving fingerprints based on full thermograms. Another possible application in the field of antibiotic research is the comparison of wildtype to resistant clones, coupled with whole genome sequencing, which can help elucidating the mode-of-resistance (MoR) of new antibacterials. Because of the static nature of the method, investigation of efficacy of new agents on either biofilm formation or on already established biofilms could lead to better understanding of the biological process and the effect of selected agents on microbes in different stages of dormancy. IMC records total energy released in the form of heat making it a suitable method to investigate also sub-inhibitory effects of active substances that might be coupled to transcriptomics. This method can also be used in clinical settings to detect contamination of samples or for the determination of antibiograms helping to rapidly decide on definite treatment of the patients[11].

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DISCLOSURES:

The project was performed in collaboration with Symcel, developer of the calScreener technology. The authors have nothing to disclose.

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Figure 1A

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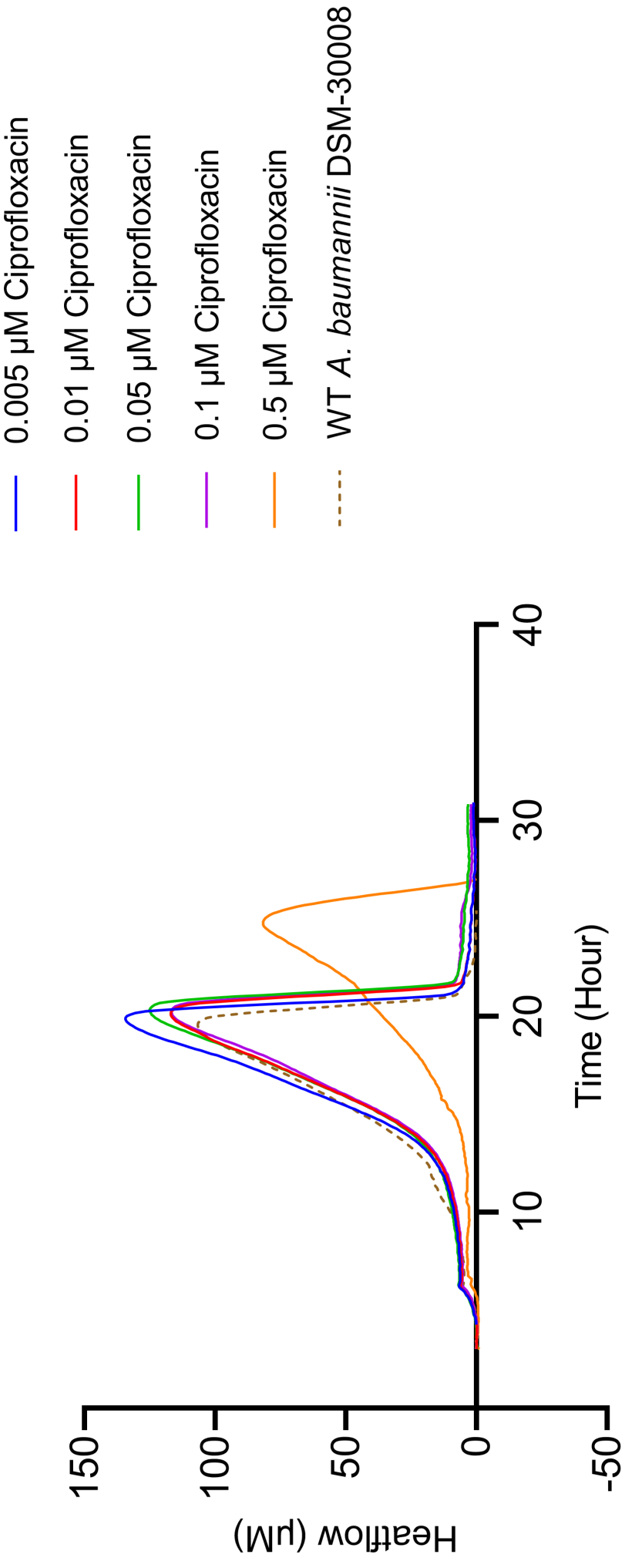

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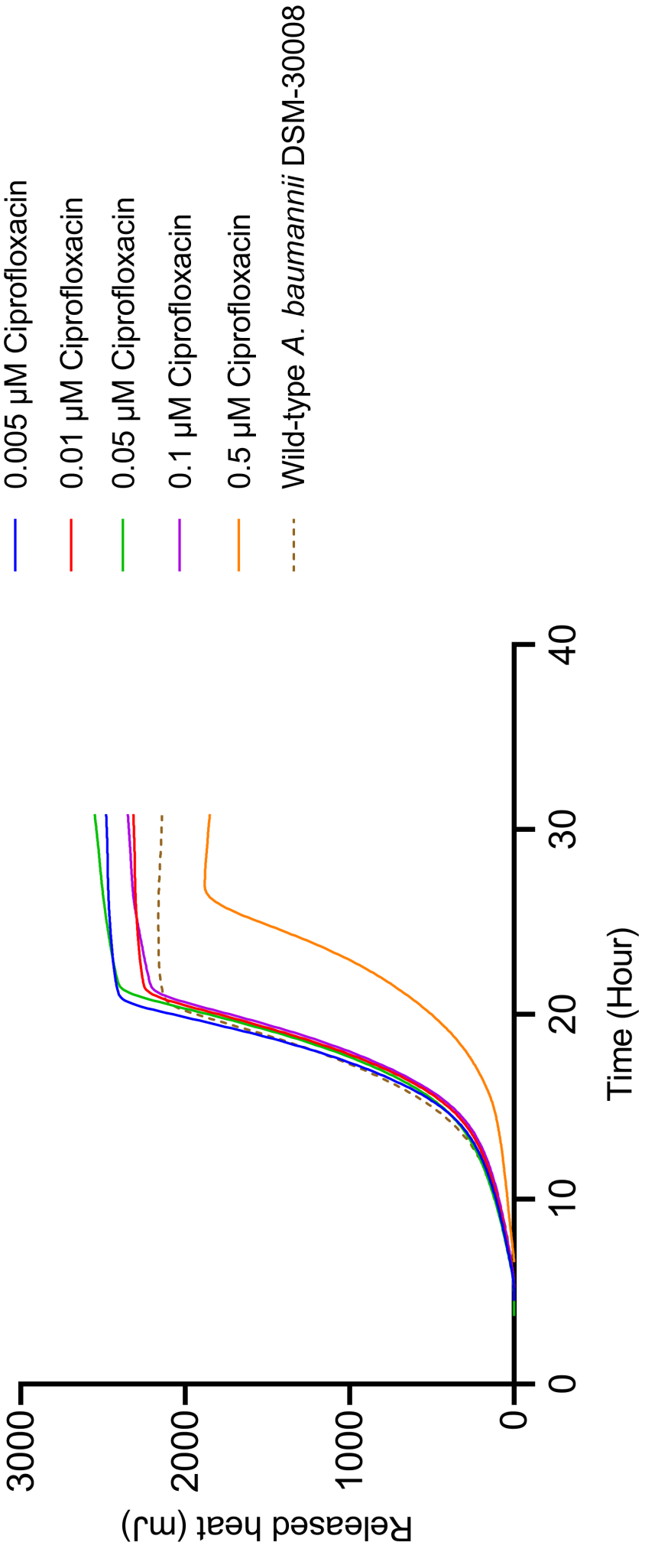

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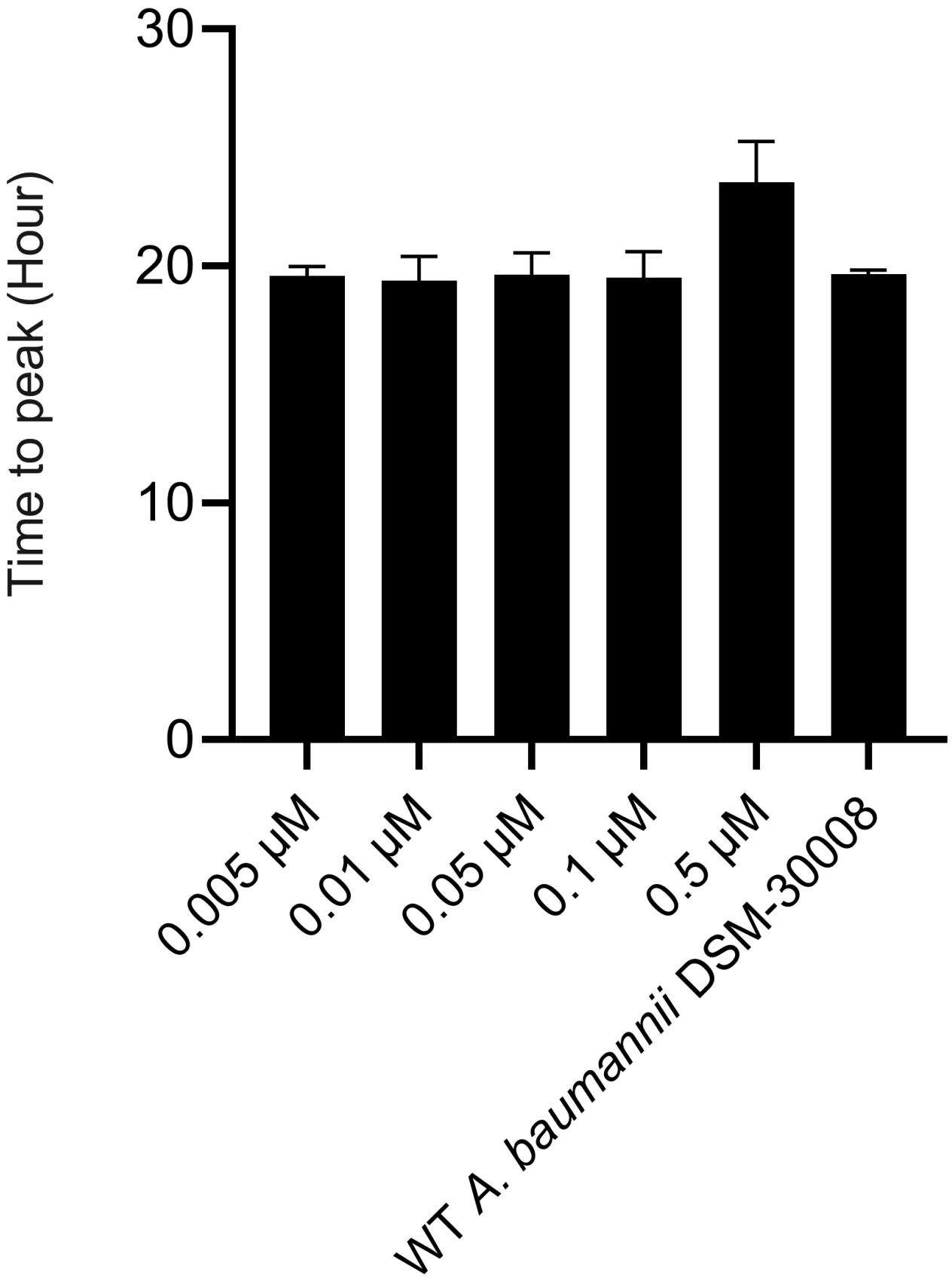

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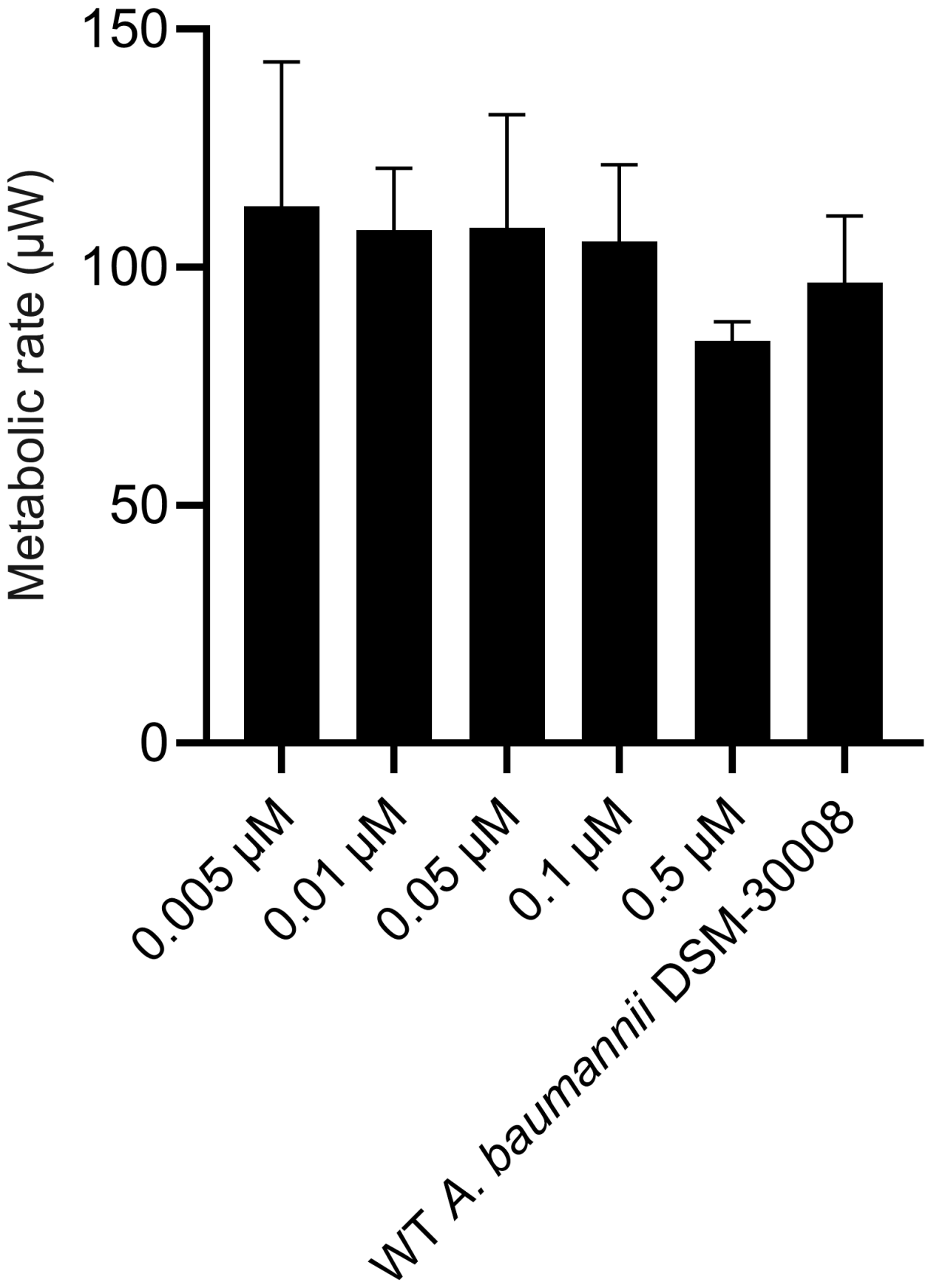

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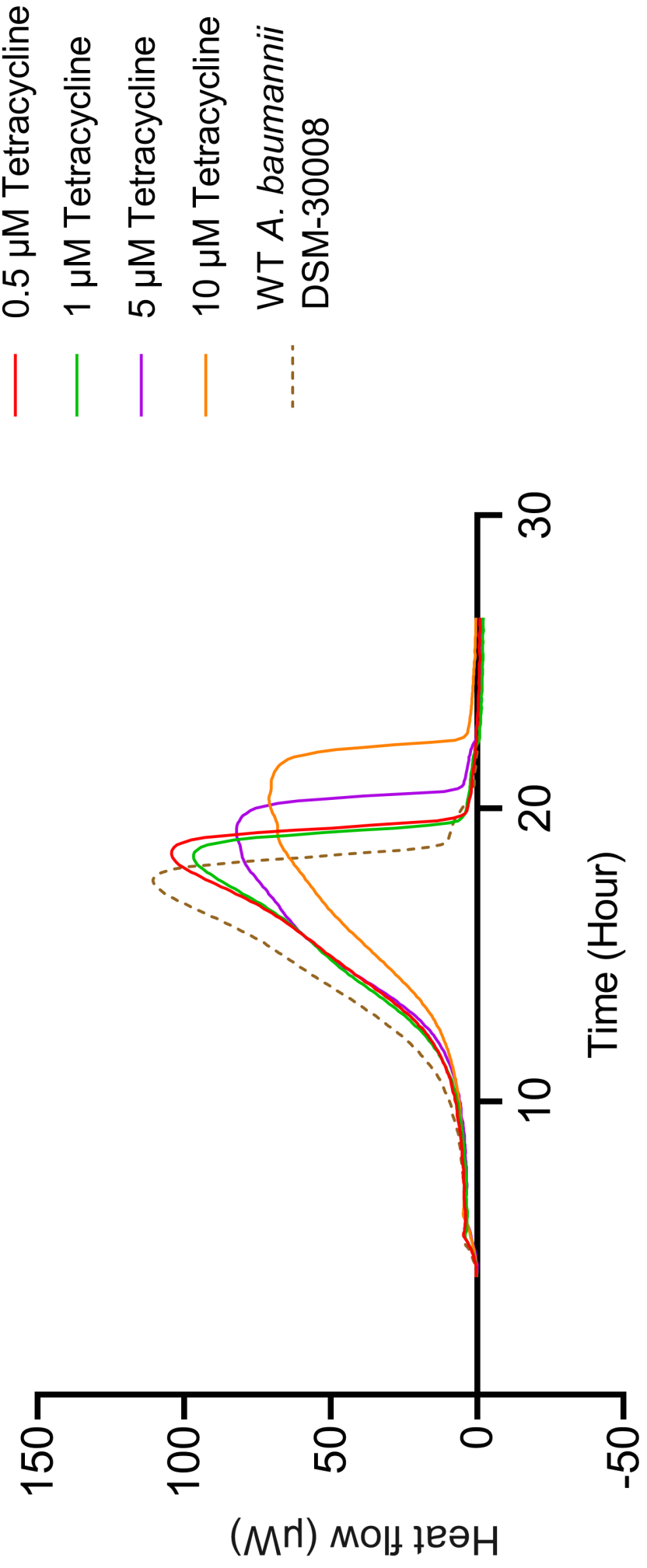

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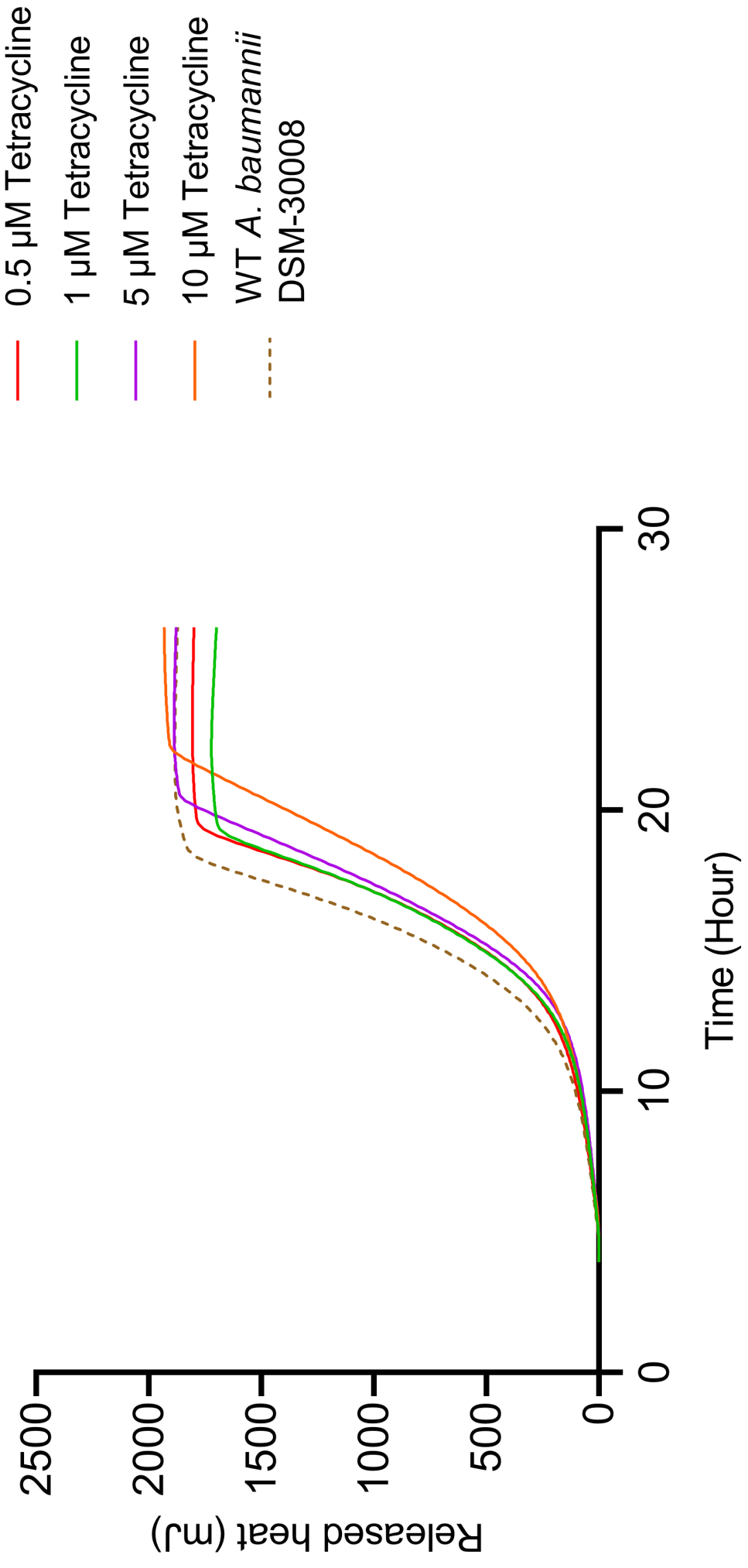

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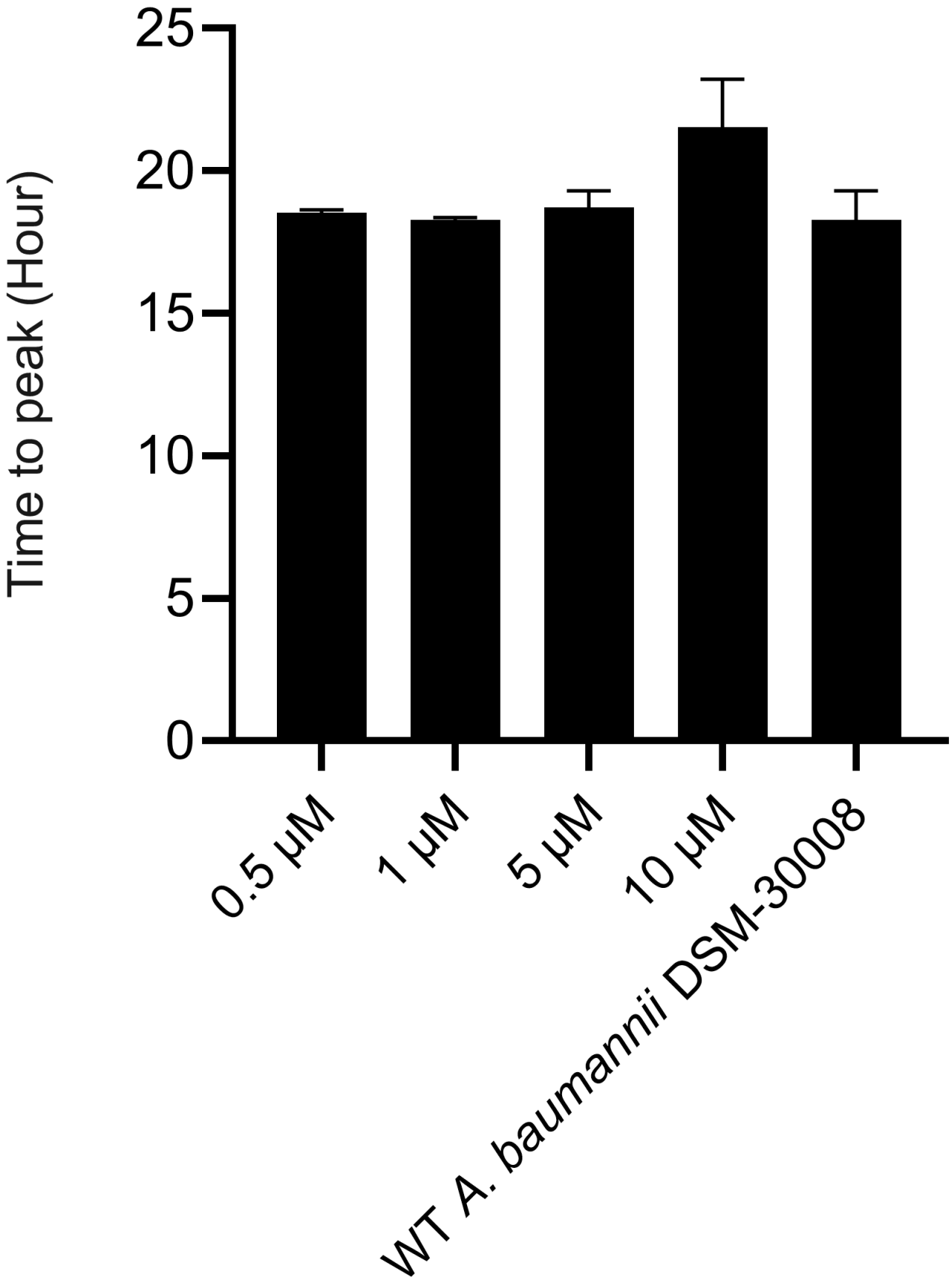

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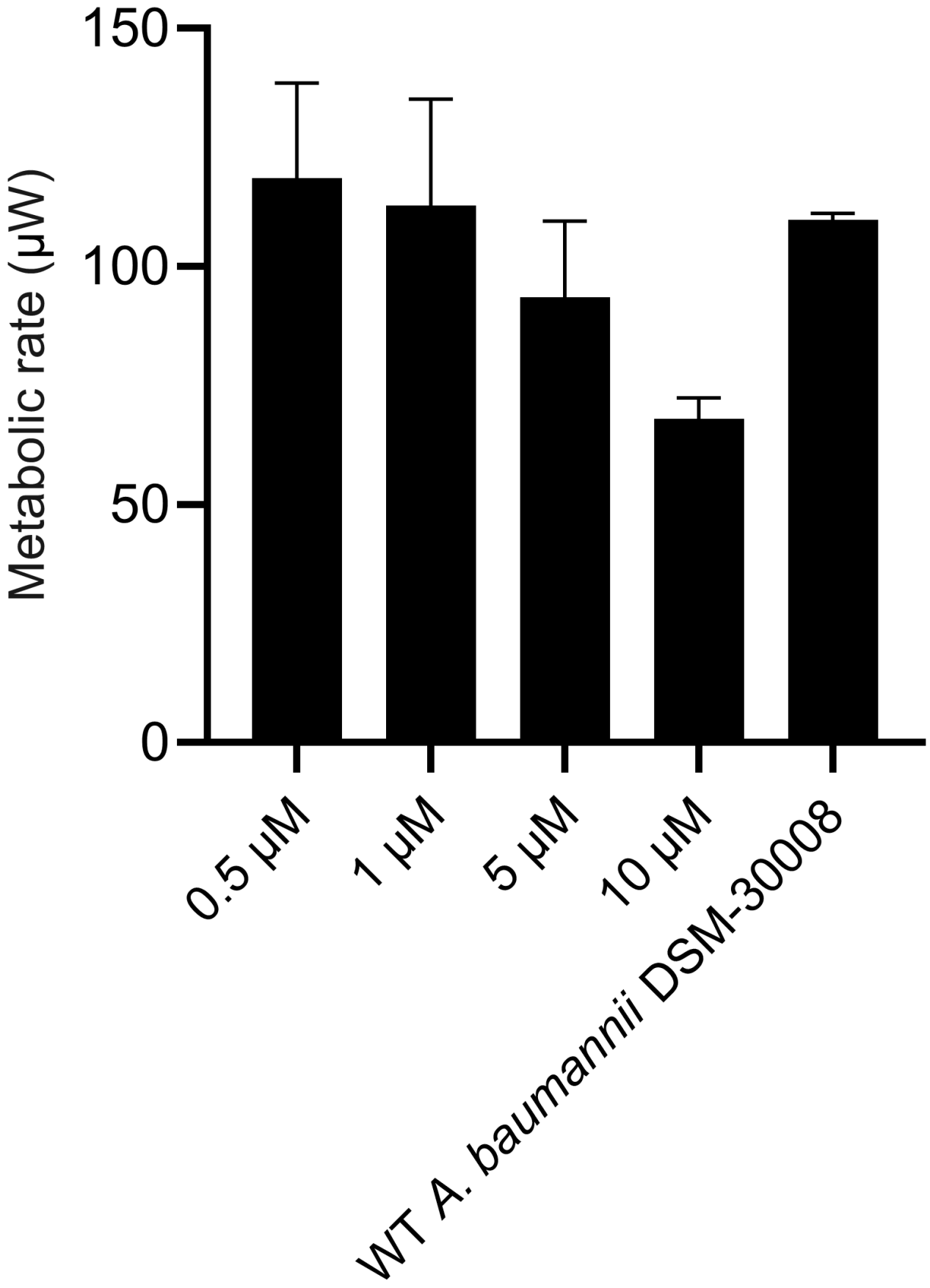

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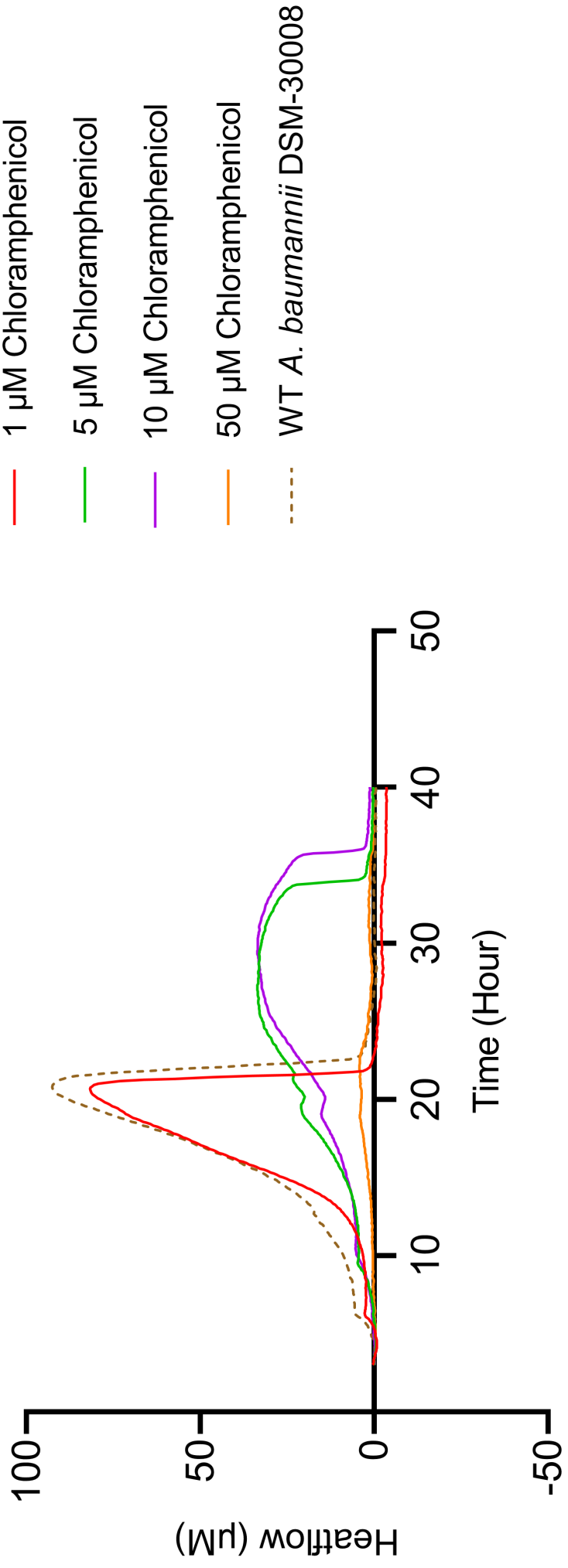

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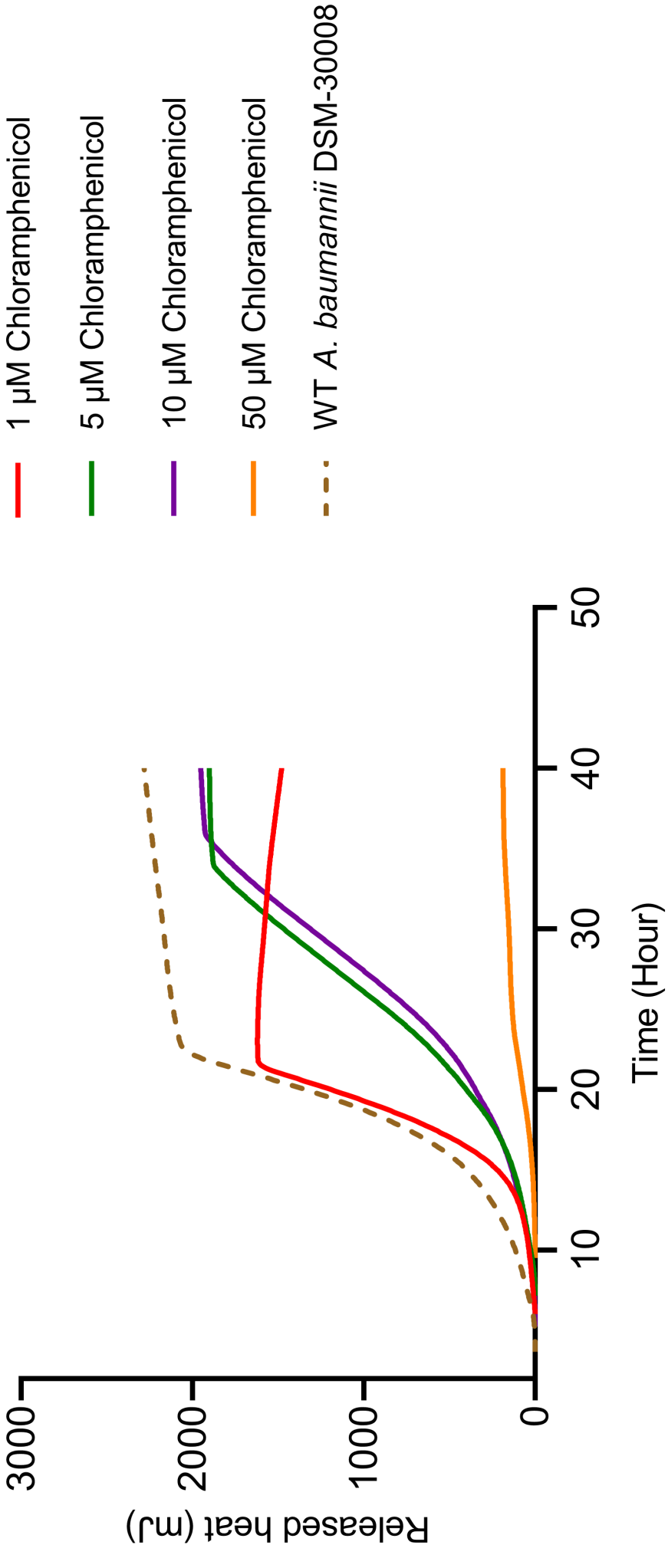

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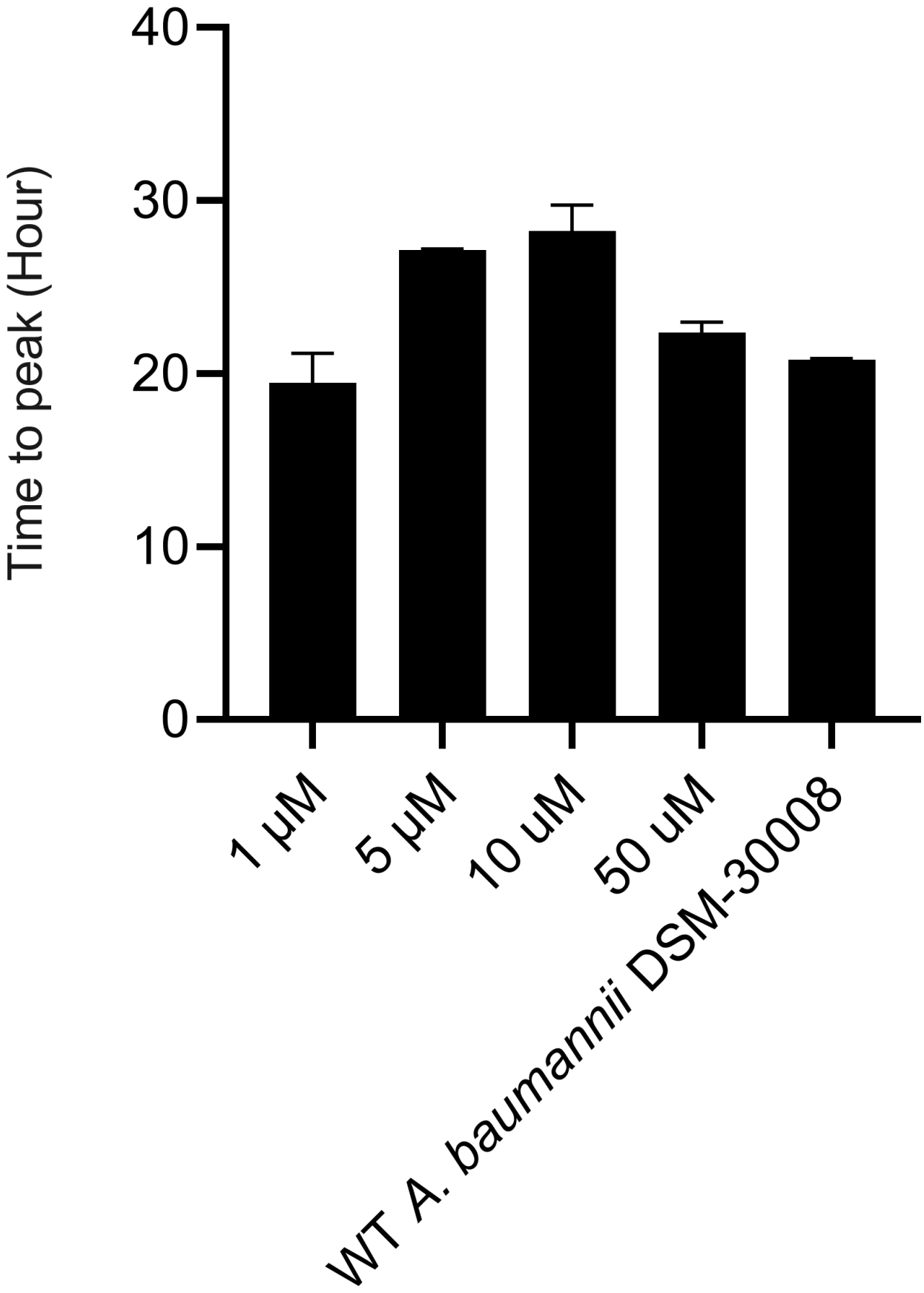

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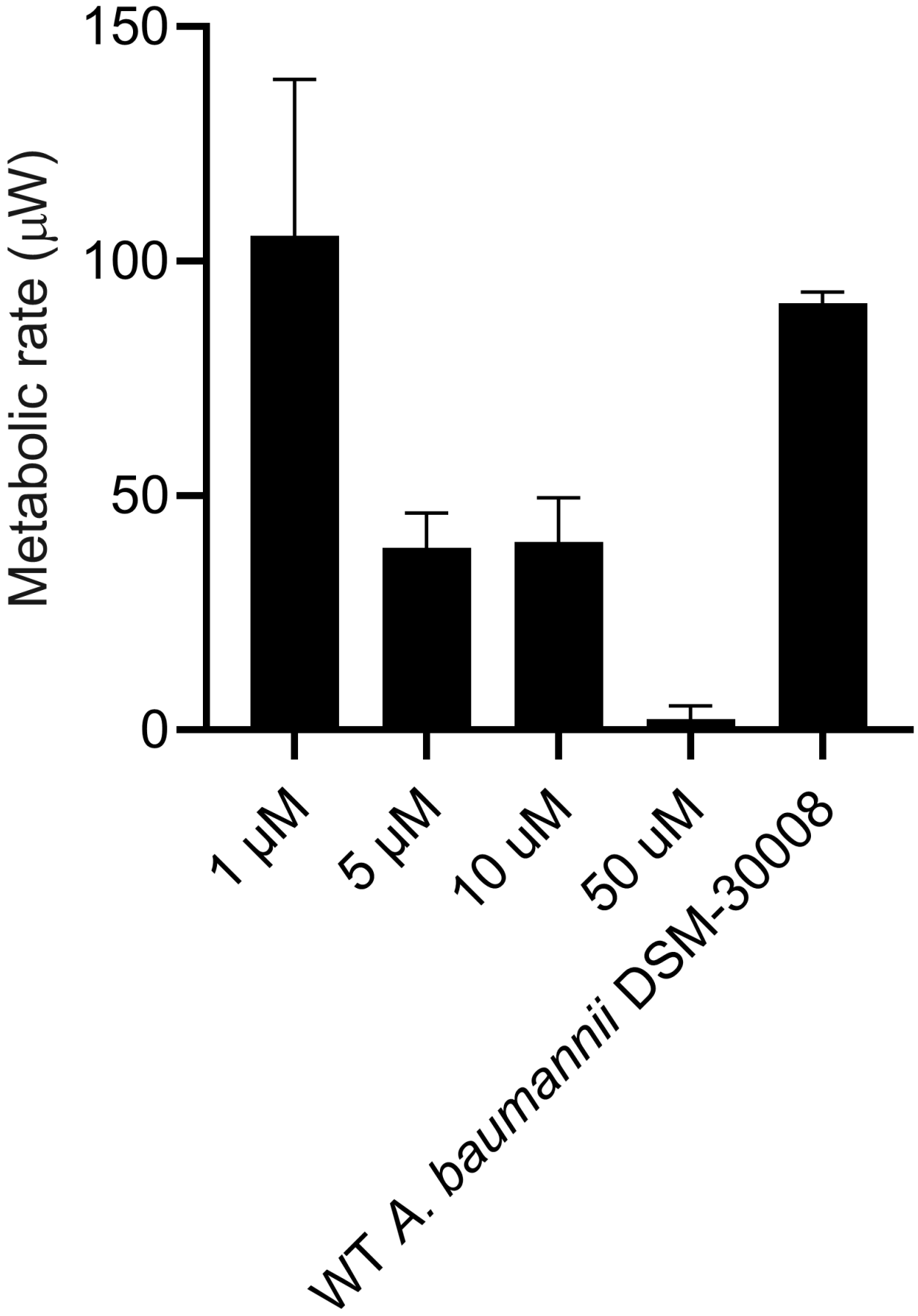

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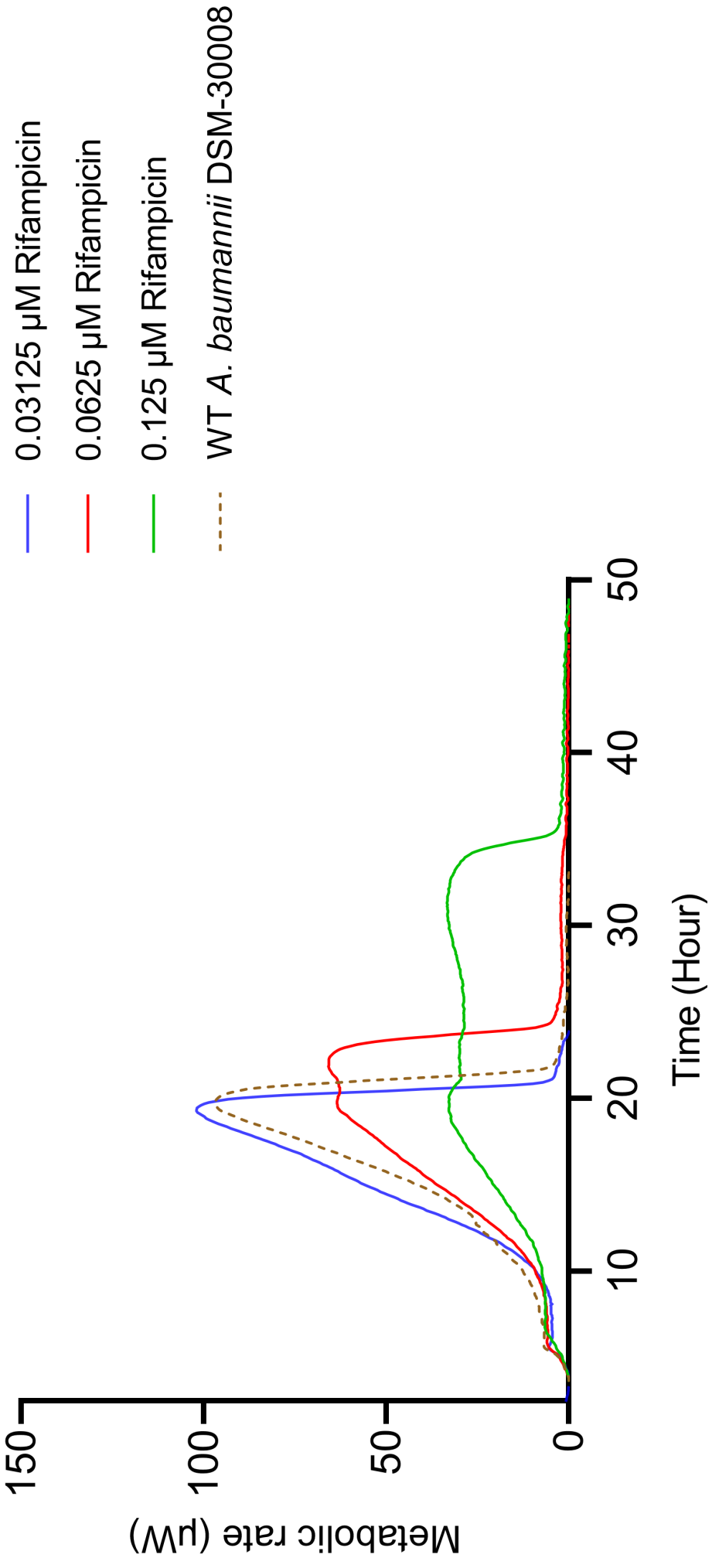

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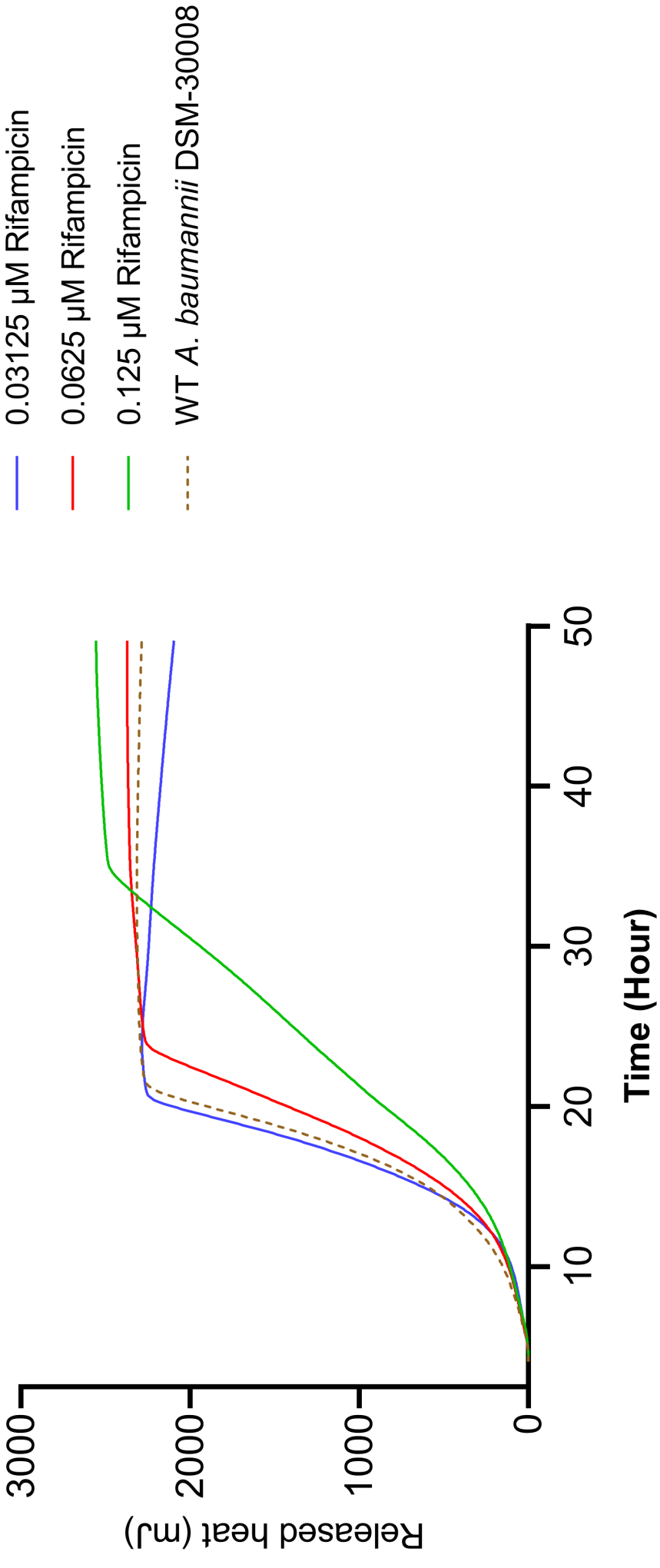

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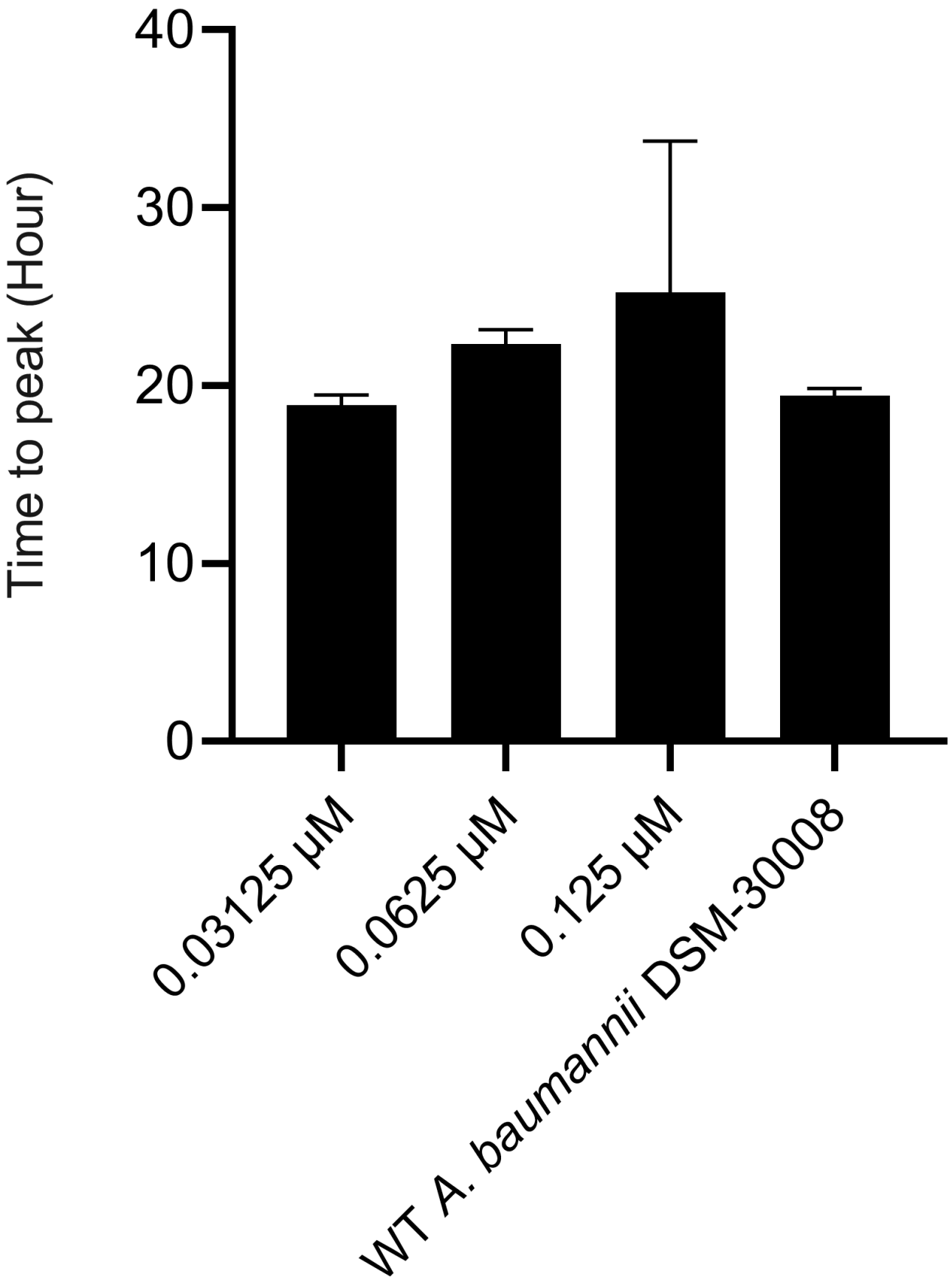

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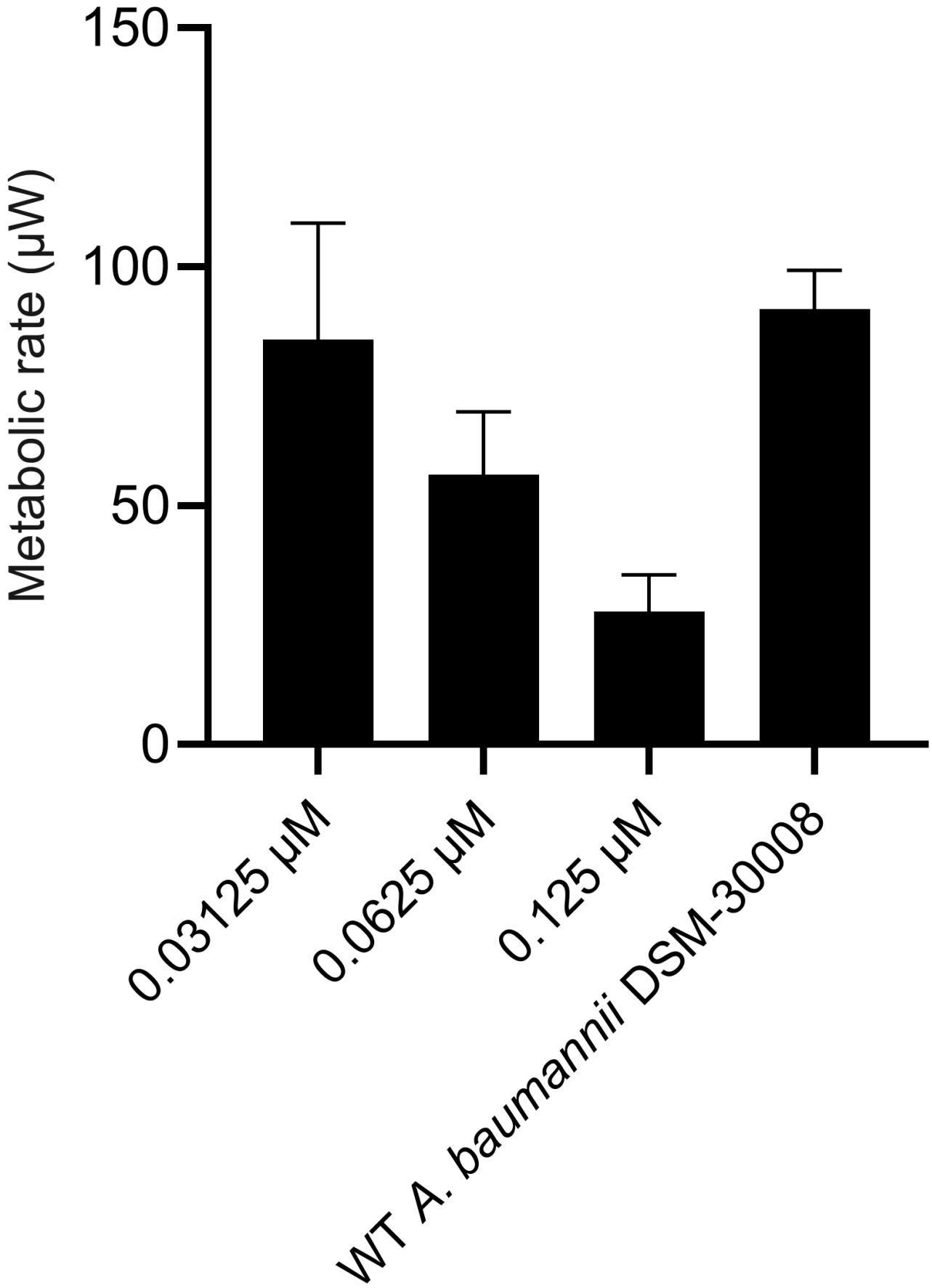

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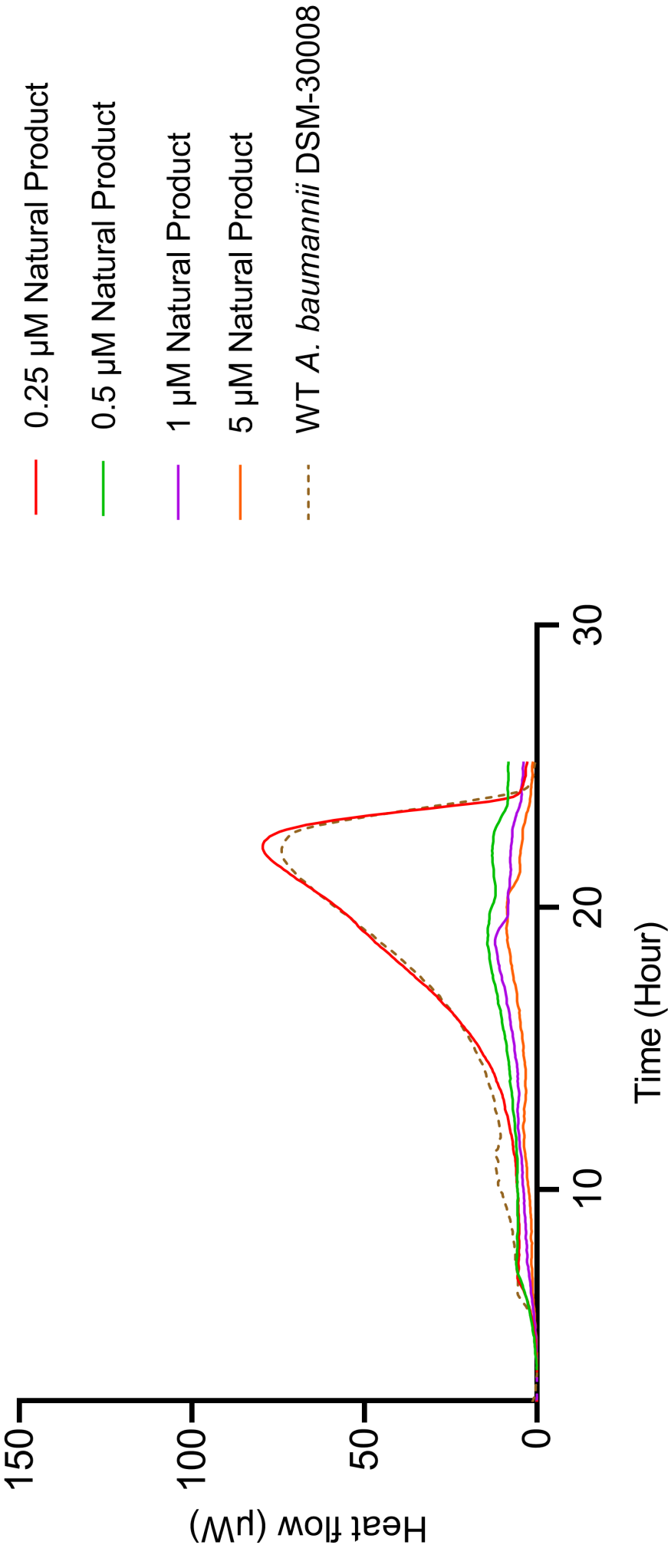

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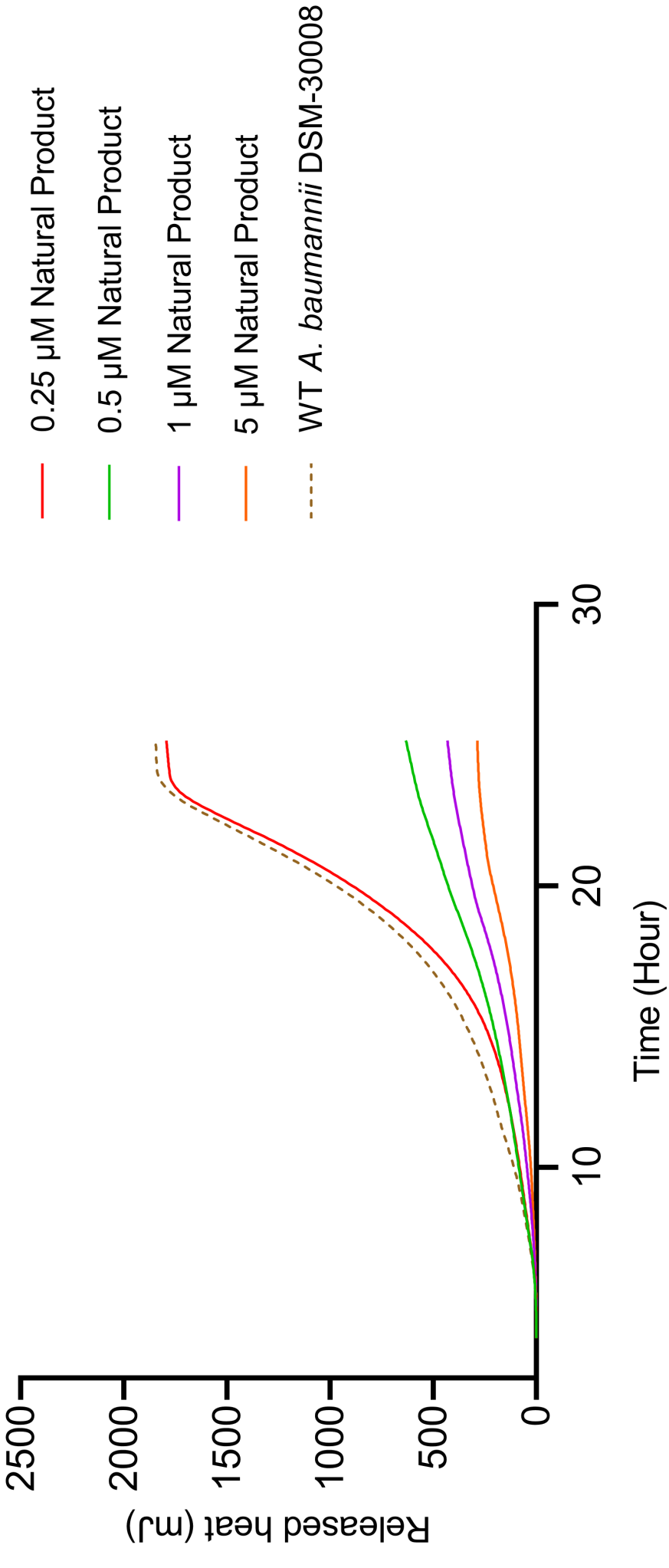

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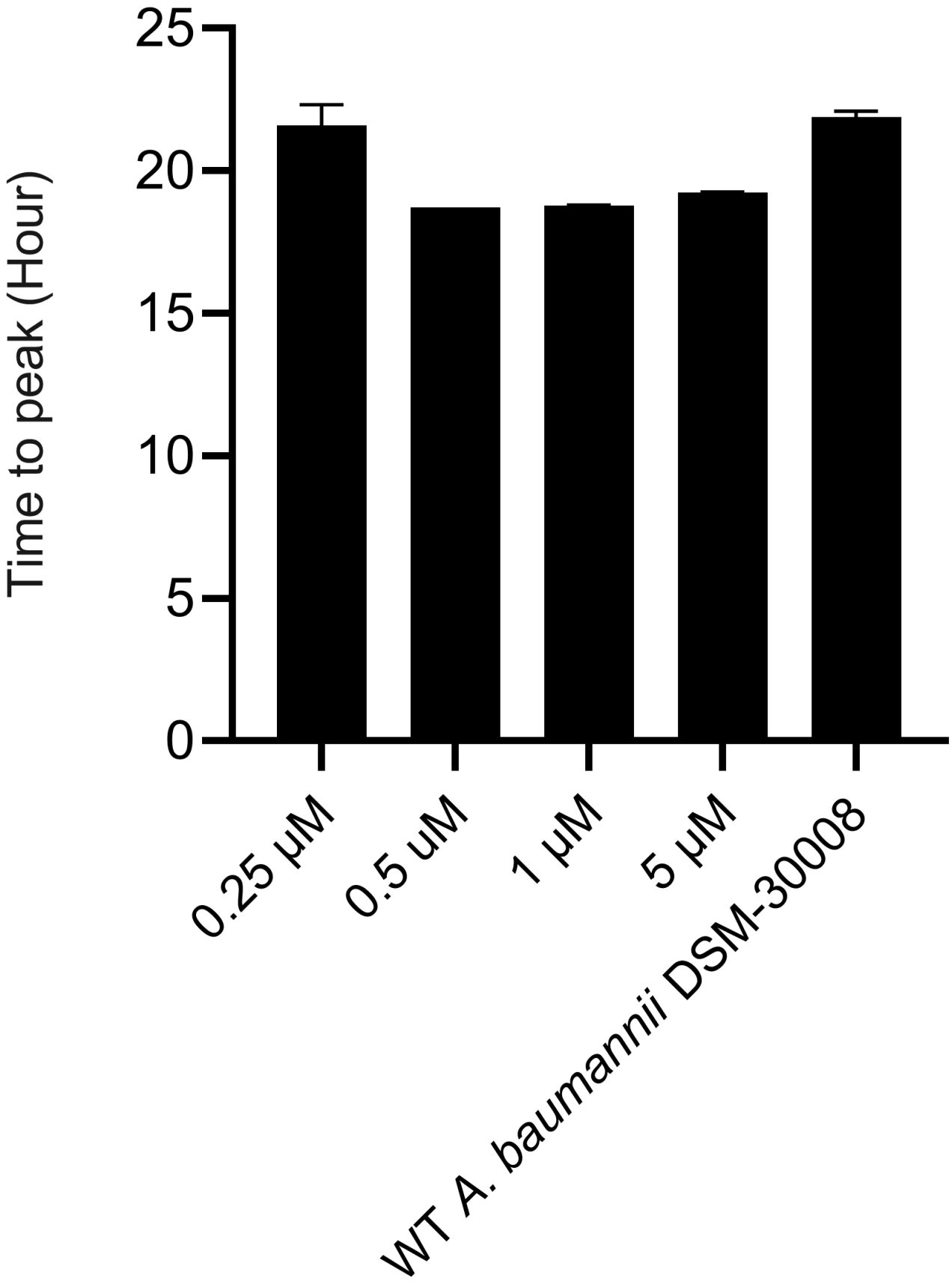

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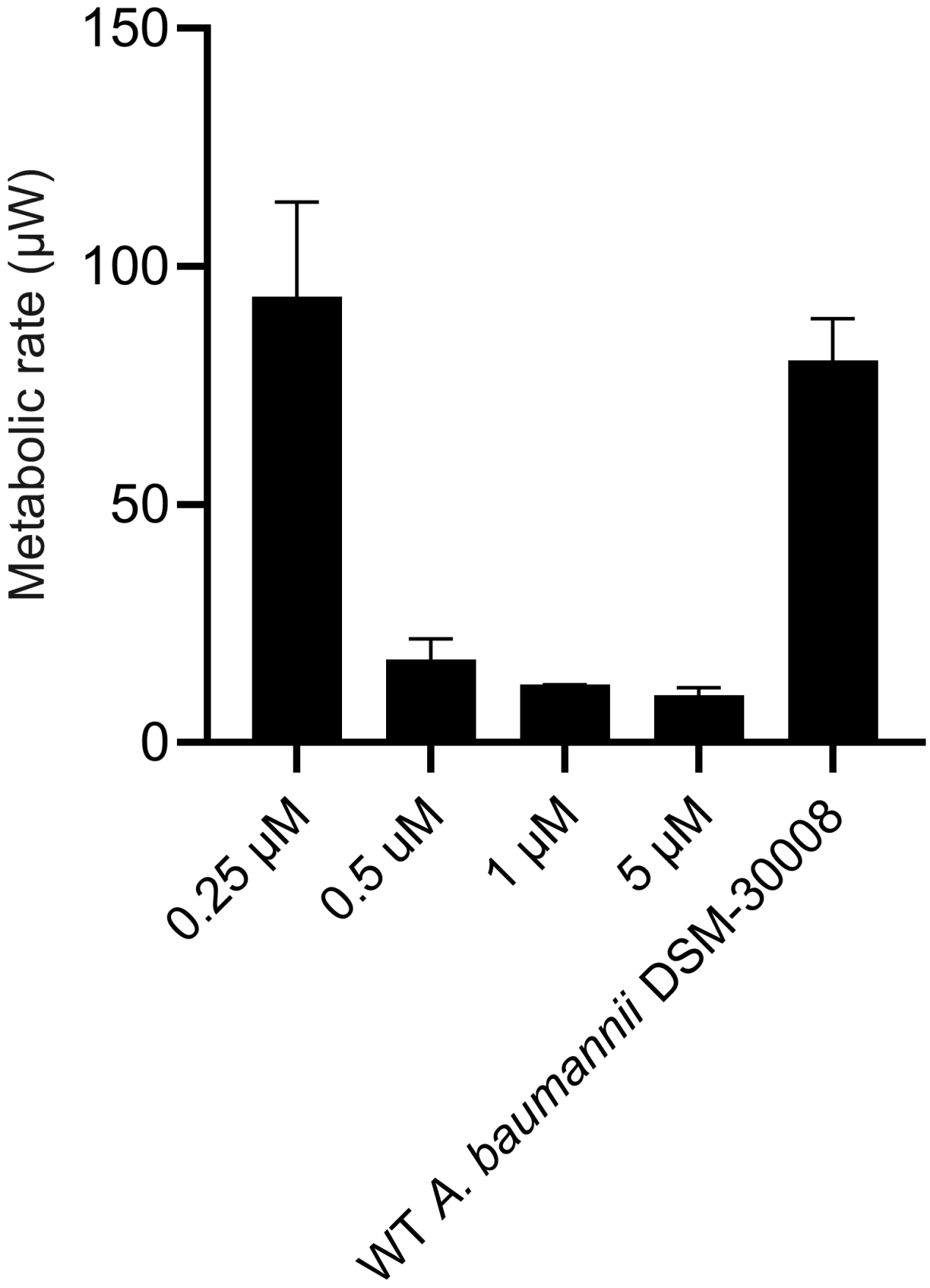

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acinetobacter baumannii	DSMZ	DSM-30008	reference strain used in this study
calPlate	Symcel	1220093	48-well plate for titanium cups to be inserted
calScreener	Symcel	1200001	isothermal microcalorimetry instrument
calView software	Symcel		collection and analysis software
calWell	Symcel	1901004	micro-bio grade (non-active) 48-well plate with plastic inserts which are inserted into the
CASO agar	Carl Roth	X937.1	isolation and cultivation of microorganisms
Chloroamphenicol	Sigma-Aldrich	C0378	antibiotic
Ciprofloxacin	Sigma-Aldrich	17850	antibiotic
Cuvettes	Brand	759015	1.5 mL cuvettes
Disposable inoculation loops	Sarsted	86.1562.050	10 µL inoculation loops
Dimethylsulfoxid (DMSO)	Thermo Fisher Scientific	85190	
Eppendorf tubes	Eppendorf	30120086	1.5 mL eppendorf safe-lock tubes
Ethanol	Thermo Fisher Scientific	10428671	
Falcon Tubes	Sarsted	62.554.502	15 mL falcon tubes
Hydrochloride (HCL)	Thermo Fisher Scientific	10316380	
Methanol	Thermo Fisher Scientific	A412-500	
Mueller Hinton Broth	Sigma-Aldrich	70192	liquid medium for antibiotic susceptibility studies
Mueller Hinton Broth II cation adjusted media	Sigma-Aldrich	90922	Mueller Hinton Broth cation-adjusted to 1.25 mM $CaCl_2$, 0.8 mM $MgCl_2$
Petri dishes	LABSOLUTE	7696404	
Pipette 100 - 1000 µL	Brand	705880	
Pipette 2 - 20 µL	Brand	705872	
Pipette 20 - 200 µL	Brand	705878	
Pipette tips 100 - 1000 L	Brand	732032	
Pipette tips 2 - 200 µL	Brand	732028	
Polymyxin B sulfate	Sigma-Aldrich	P0972	antibiotic
Rifampicin	Sigma-Aldrich	R3501	antibiotic
Serological pipette	Thermo Fisher Scientific	170356N	10 mL Nunc serological pipette
Spectrophotometer	Eppendorf AG	6135 000.017	
Sterile filters	Minisart	16534----------K	0.2 µm pore size sterile filters
Syringe 50 mL	NORM-JECT	22778	
Tetracycline	Sigma-Aldrich	87128	antibiotic
Titanium cups	Symcel	1220089	inserted in 48-well titanium calPlate
Titanium lids	Symcel	1220091	screwed and tightend to the titanium cups
Trimethroprim	Sigma-Aldrich	T7883	antibiotic
Tweezers	Symcel	1900602	

Letter to editors (editorial comments) Click here to access/download;Rebuttal Letter;Letter_to_editors.pdf

Dear editors,

Please find our point-to-point response to the editorial comments below in the text.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

After careful proofreading, we did not include major corrections in the text.

• **Protocol Detail:** 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 that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) Line 132: how is the temperature selected?

We carefully checked all steps in the protocol. We added information on the medium used for growing *A. baumannii* and the used temperature of 30 °C (optimal growth condition for *Ab*) was added as well.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

After our revision, the protocol is within the 10-page limit and we highlighted a section not exceeding the 2.5-page limit.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

This is included in our manuscript.

Figure/Table:
1) Define all error bars.

This has been done (Figures 3-5).

2) Place all legends after the representative results section. Placeholders can remain as they are.

This has been done.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are calScreener (Symcel AB), Eppendorf, calWell, calPlate, Symcel Calorimetry,

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We replaced all commercial sounding language in the manuscript and referred to the table of materials where appropriate. As the calScreener instrument is unique (it is the only microcalorimeter suitable for biological evaluations), we left its name at its first appearance in the text (Summary) but removed the brand name in the rest of the manuscript. We also left specific names upon first appearance in the text (Software tools) as these are unique and cannot be replaced by any other software program.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

n/a

With best regards,

Jennifer Herrmann, on behalf of the authors

Rebuttal Letter
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Dear reviewers,

Many thanks for the careful revision of our manuscript and the overall positive evaluation. We acknowledge and appreciate your comments that helped us to improve the manuscript. Please find a point-to-point response to your comments below.

Reviewer #1:

Manuscript Summary:

Metabolic profiling to determine bactericidal or bacteriostatic effects of new natural products using isothermal microcalorimetry

The authors describe a new method to be used for determination of mode of action of antimicrobial compounds. The method uses isothermal microcalorimetry with calScreener allowing a high-throughput analysis with low volumes. The paper is well organized and clearly written. My recommendation is accept with minor modifications.

Overall comments:

Define better which mode of action you want to measure. Mode of action could also be interpreted as activity against e.g. cell wall synthesis or protein synthesis. It should be mentioned which media and conditions were used to create the representative results.

The presented method can be used to get first insights into the mode-of-action (MoA) of new antibiotics regarding major pathways being inhibited. This application is however still under investigation. Here, we only look at mechanisms by differentiating between cidal and static effects. This is highlighted in the title and abstract. We would like to keep some general comments on the possibility to use IMC for more in-depth MoA analyses but this was not part of the presented study. Media and conditions used to generate the representative results have been added to the protocol section.

Find below my comments to the sections:

Introduction
Line 99-102: may also comment on the production of EPS that can lead to higher OD values than for the actual cell count. Also, IMC detects growth earlier than OD measurements.

Thanks for this comment. In a separate study, we are currently evaluating the formation of biofilms of pathogens that heavily interact with surfaces and the effects of antibiotics on pre-formed biofilms. However, these experiments have not yet been elaborated and in the presented study, we focussed on antibacterial effects assuming that the initial *A. baumannii* population mostly consists of planktonic cells in the setup we used. We currently lack data for a concise evaluation of the dependency of e.g. OD vs. EPS formation vs. heat release reads. Since we did not present data on the correlation of OD vs. IMC, in terms of detecting bacterial growth, we did not further comment on this in the manuscript.

Protocol

Line 148: Why you need 2 passages after streaking out on the plate? Is there a difference in the measurements if used less or more passages? In phenotypic measurements, the amount of passages can lead to a slightly different profile.

In the first culturing step, we streak out the cryopreserved culture on CASO agar plates to obtain single colonies. For the actual experiment, we obtain most reproducible results starting from an overnight culture in liquid medium and then diluting this culture to the desired target concentration (CFU/mL). With a liquid overnight culture, it is easier for us to ensure that we have the same starting number of bacterial cells between experiments and thus, low plate-to-plate variations. Experiments can be set up as well by inoculating the assay culture directly from single colonies on solid media, however, in our hands, the CFU count variability was slightly higher compared to the presented protocol. The major difference that we observe when having minor variations in the starting bacterial cell number is the lag phase duration.

Line 153-155: Although OD is an easy way to adjust the density, is it valuable for any bacteria? See my comment above for EPS production. However, it can be valid if a basic calibration is done (e.g. one time measurement OD and cfu). On the other hand, if you use OD for determination of the inoculum, skip the correlation with cfu.

This is true. We typically perform concise studies on the correlation of OD to CFU/mL for all microbes that we use. This has been also done for the *A. baumannii* strain by measuring OD (following the culturing protocol as described in the manuscript), plating of serial dilutions, and colony counting. E.g. starting from an OD_{600} of 1, which equals in this case ~ $5x10^8$ CFU/mL we stepwise dilute the culture for starting the experiment by factor 1000 to finally achieve $5x10^5$ CFU/mL, which is recommend for IMC. We added a note to the manuscript.

Line 162-165: Are no growth controls used? Meaning wells without antimicrobial compound?

Thanks for this comment. Indeed, growth controls are used and we added this information to the protocol section.

Line 262: May define what low heat flow means

Low heat flow in the beginning stages of the experimental run means heat emission is equal or above zero, but not higher than 10 µW. This has been added to the protocol section.

Line 260-267: May rewrite it since it seems that the description of baseline definition is repeated

We carefully checked the manuscript for repetitions and we could not identify sections that might be skipped in the protocol.

Line 273-274: You may want to mention that it is a web-based application

The analysis of data is performed with the web-based application mentioned in step 7.6 (given along with the link). The initiation and stopping of experiment is done on software installed on the computer connected to the instrument.

Line 276-285: Modify it as above to avoid repetitions (mention the figures only once)

We are not sure how to implement this. Each figure panel is referred only once in the text.

Representative results

Why did you not test directly at the MIC and may one level above?

To ensure the correct determination of bacteriocidal and bacteriostatic effects (and possible dual MoAs), we typically assess a broader range of concentrations. However, MIC data is generated in parallel.

Line 309-311: Why was the MIC not already determined using calScreener and directly?

In our standard workflow, we determine MIC using protocols recommended by EUCAST as this is already widely accepted. However, we do compare microbroth dilution to IMC-based MIC and data correlate well. We did not comment on this in the manuscript as the focus of the presented method was the differentiation of cidal vs. static MoA.

Line 336-340: Why did you not do this experiment?

The displayed results supported the published cidal mechanism of ciprofloxacin. Indeed, we are refining (and extending) our data sets with reference antibiotics. Here, we think it is of interest to also display an experiment that should undergo further optimization (adapting the assay concentration range) to further improve the experimental outcome.

Line 341-345: May add how many replicates you did

All tests were done in triplicate and we added this info to the manuscript.

Line 398-407: In the same manner as rifampicin or with a lower effect?

We did not do a direct comparison of individual data sets but we analyzed each antibiotic separately to conclude in cidal/static effects. However, in the presented case, the effect was similar to rifampicin but more pronounced.

Discussion
Line 423-425: It may also increases the MIC

Yes. We added a comment and a reference.

Line 449-450: Rewrite the sentence on solid media

This has been done.

Major Concerns:

none

Minor Concerns:

The protocol might need to be adapted for other microorganisms than those used.

This point is well taken and we agree. Indeed, we test different microorganisms in different media using different concentrations of antimicrobial compounds to achieve fully informative results.

Reviewer #2:

Manuscript Summary:

This manuscript introduces the isothermal microcalorimetry for primary screening of candidates with potential bactericidal or bacteriostatic activity on target cells. This is an interesting work, and the description of the protocol is complete. However, because the assayed samples are some purified compounds, the applications of this protocol will be limited. Based on the policy of this journal, I recommend acceptance of this manuscript for the publication.

Major Concerns:

The authors establish this protocol based on that if the compounds affected the growth of target cells, the IMC value will change, which could be easily detected by a isothermal microcalorimeter. My major concern is that, if the assayed samples are not purified in advance, i.e. assaying on a mixture, then how about the rate for false positives?

The described method is applicable to purified compounds. We do work with microbial crude extracts as well but so far, we did not apply IMC to determine antimicrobial activities of such samples. We fully agree that this application might be challenging as crude extracts are very complex and they typically contain multiple natural product classes, which might lead to results that are not easy to interprete if the assay system and/or readout is complex. We routinely assess crude mixtures in microbroth dilution assays. Only if actives are purified and structurally characterized, we continue with in-depth profiling, thereby also applying IMC. It will be interesting to see which additional data can be retrieved for extracts when switching from typical microbroth assays to IMC. However, we believe that only well characterized (i.e. fully dereplicated) extracts should be used in such a pilot study.

With kind regards,

Jennifer Herrmann (on behalf of the authors)

SI 1

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SI 3

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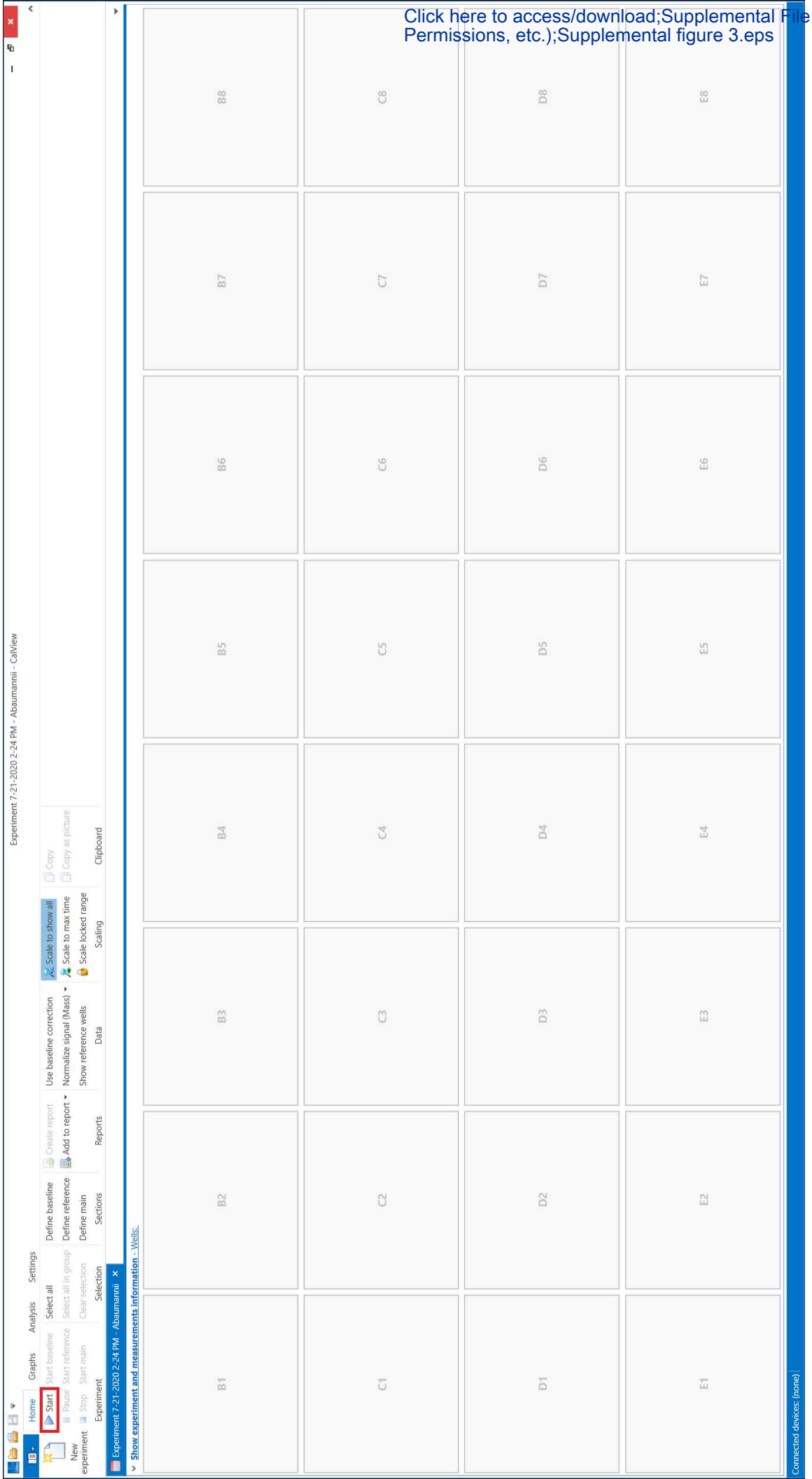

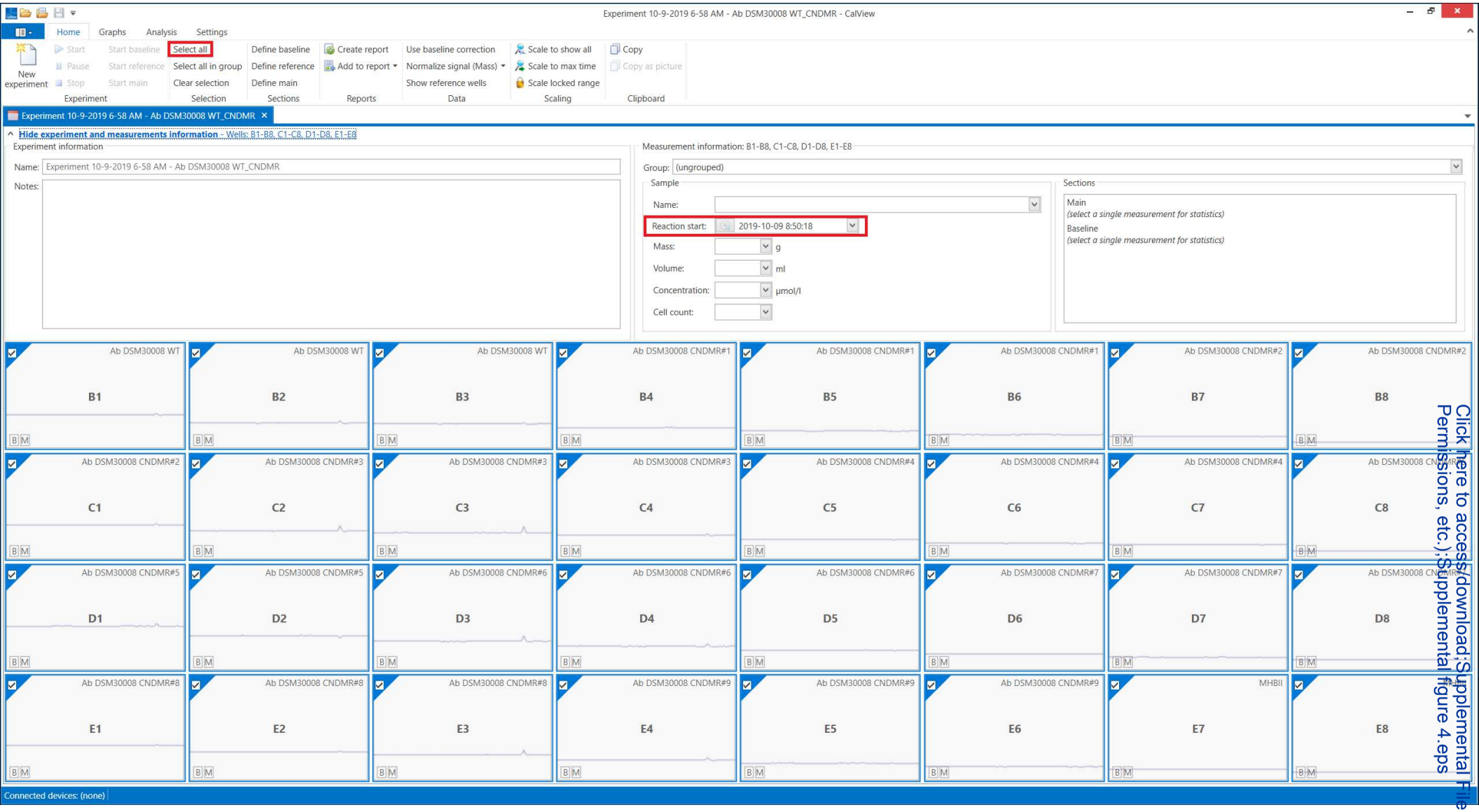

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SI 5

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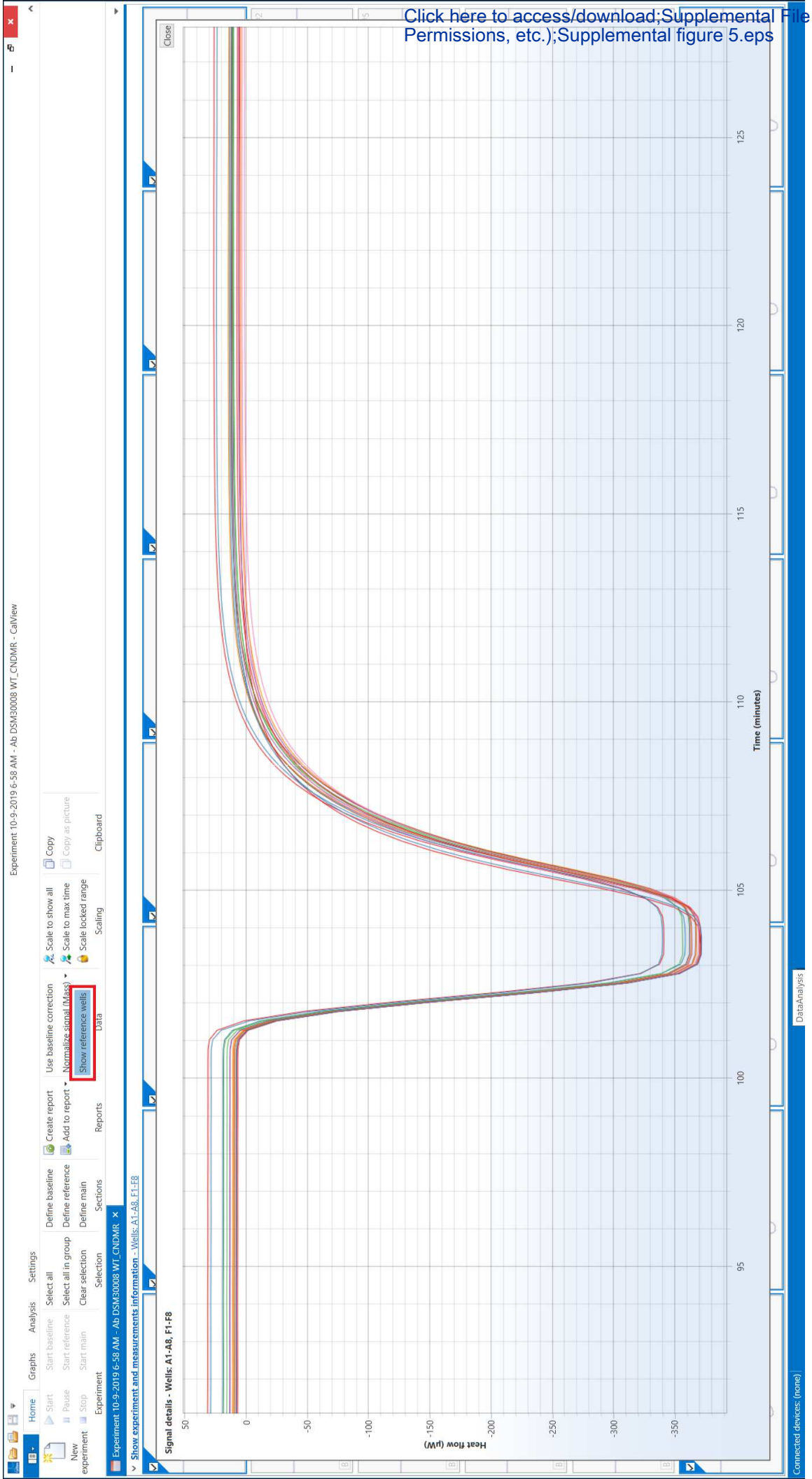

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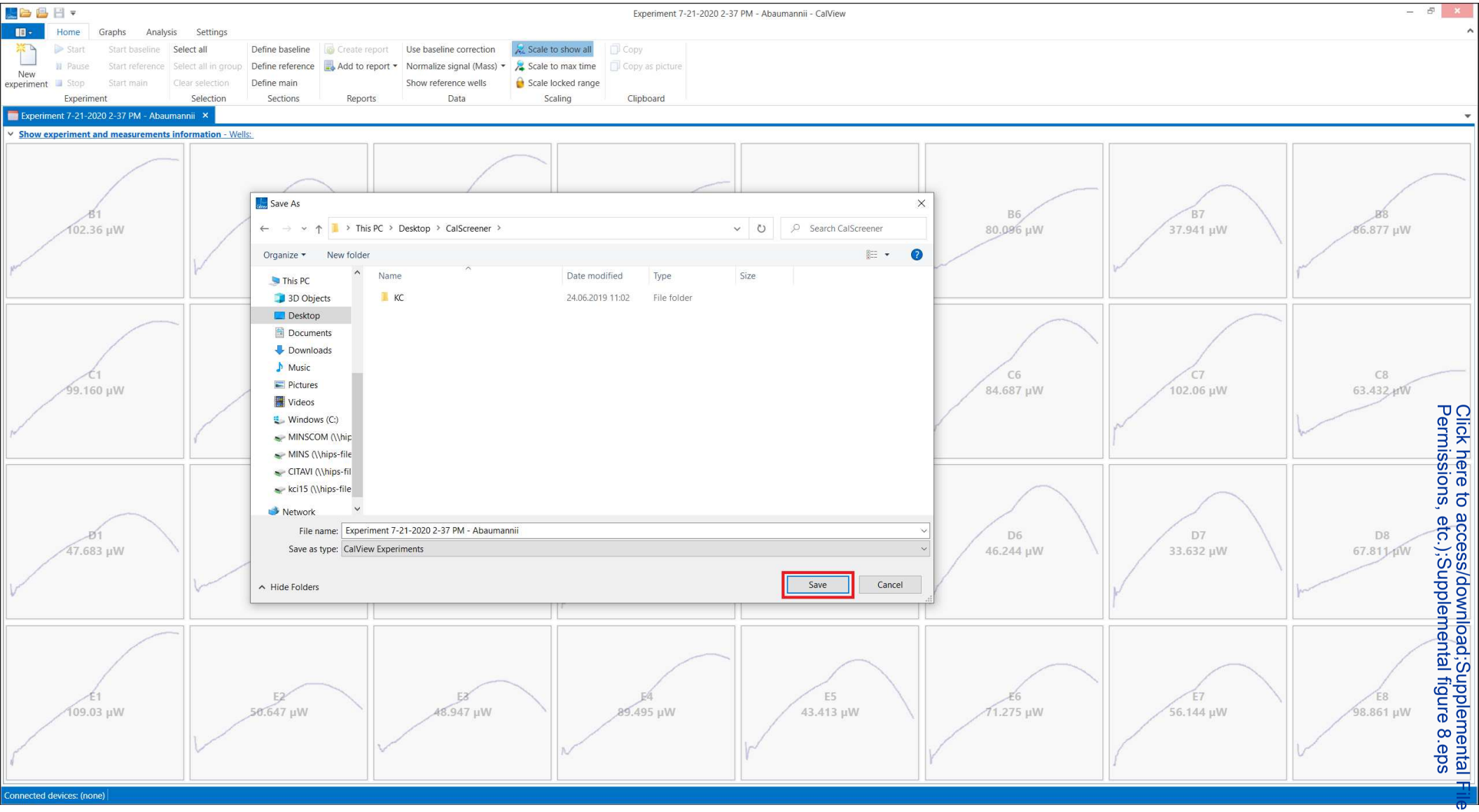

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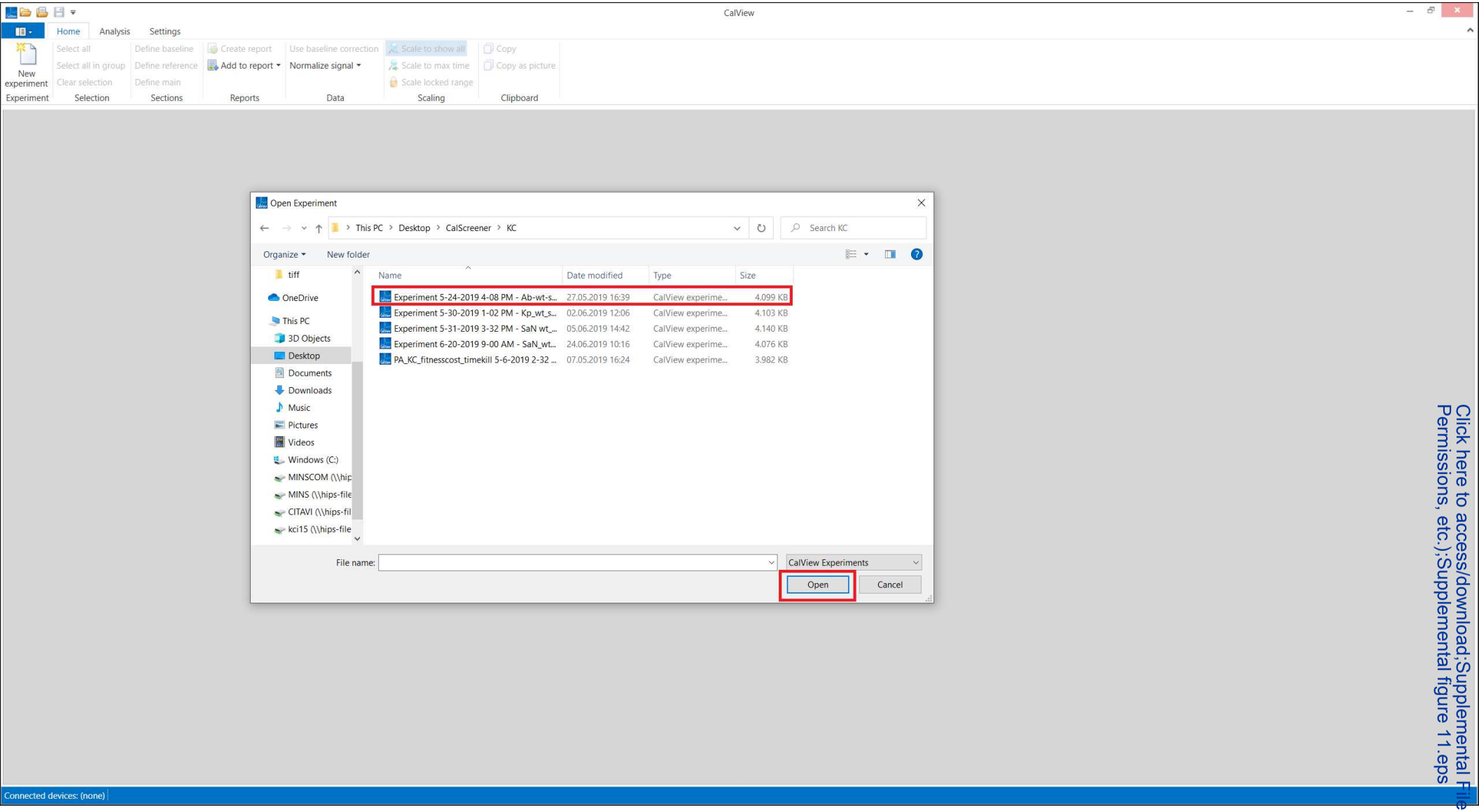

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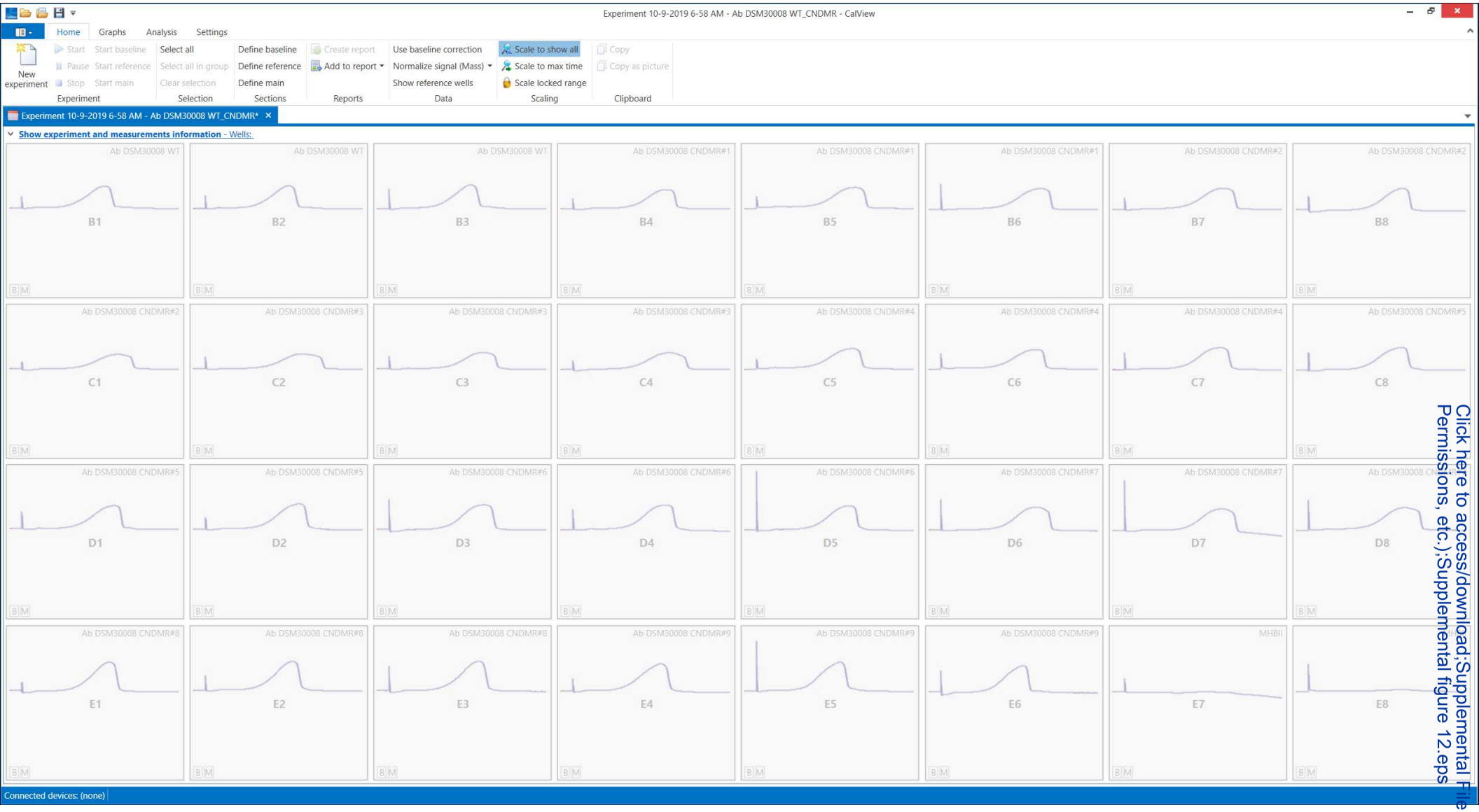

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SI 13

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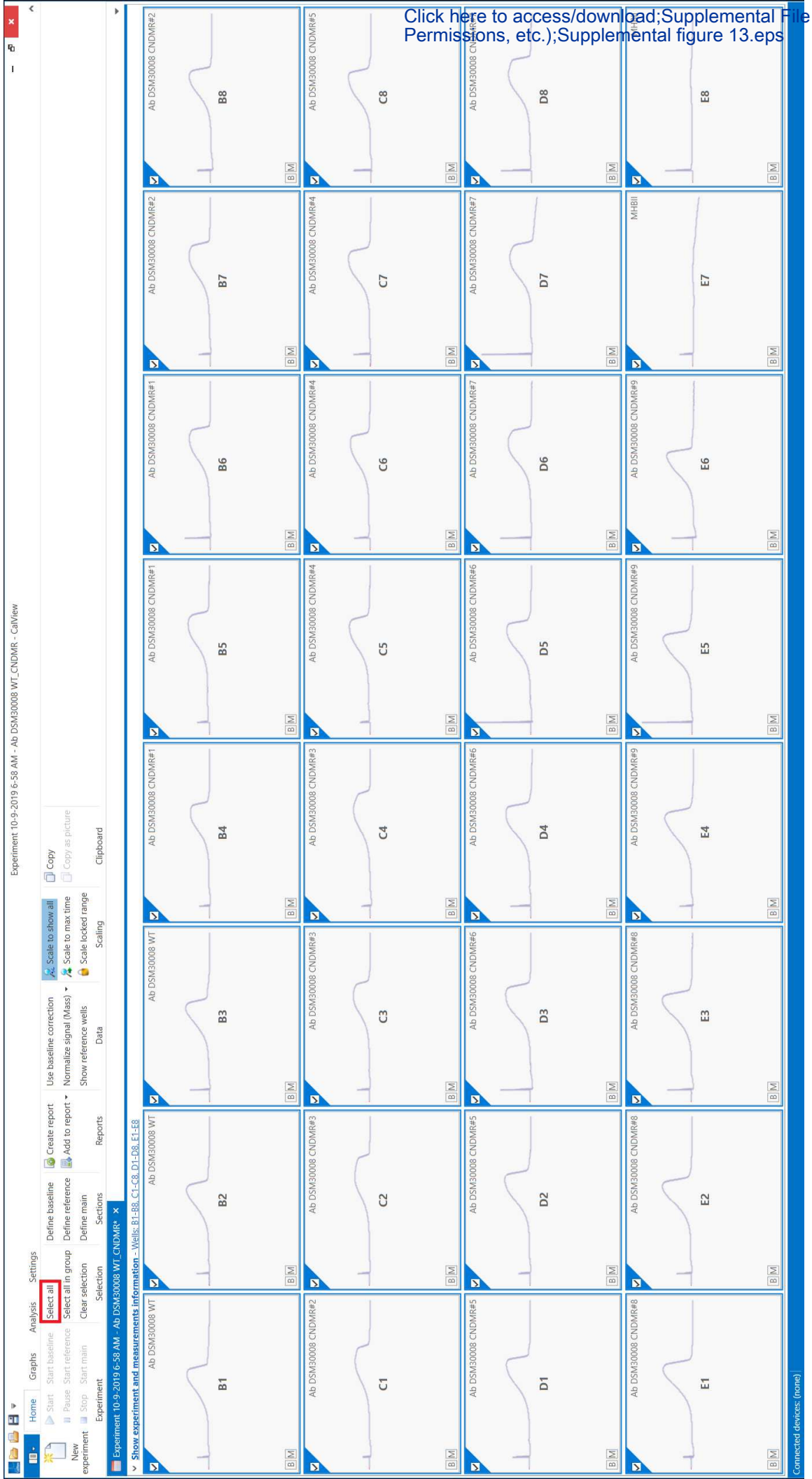

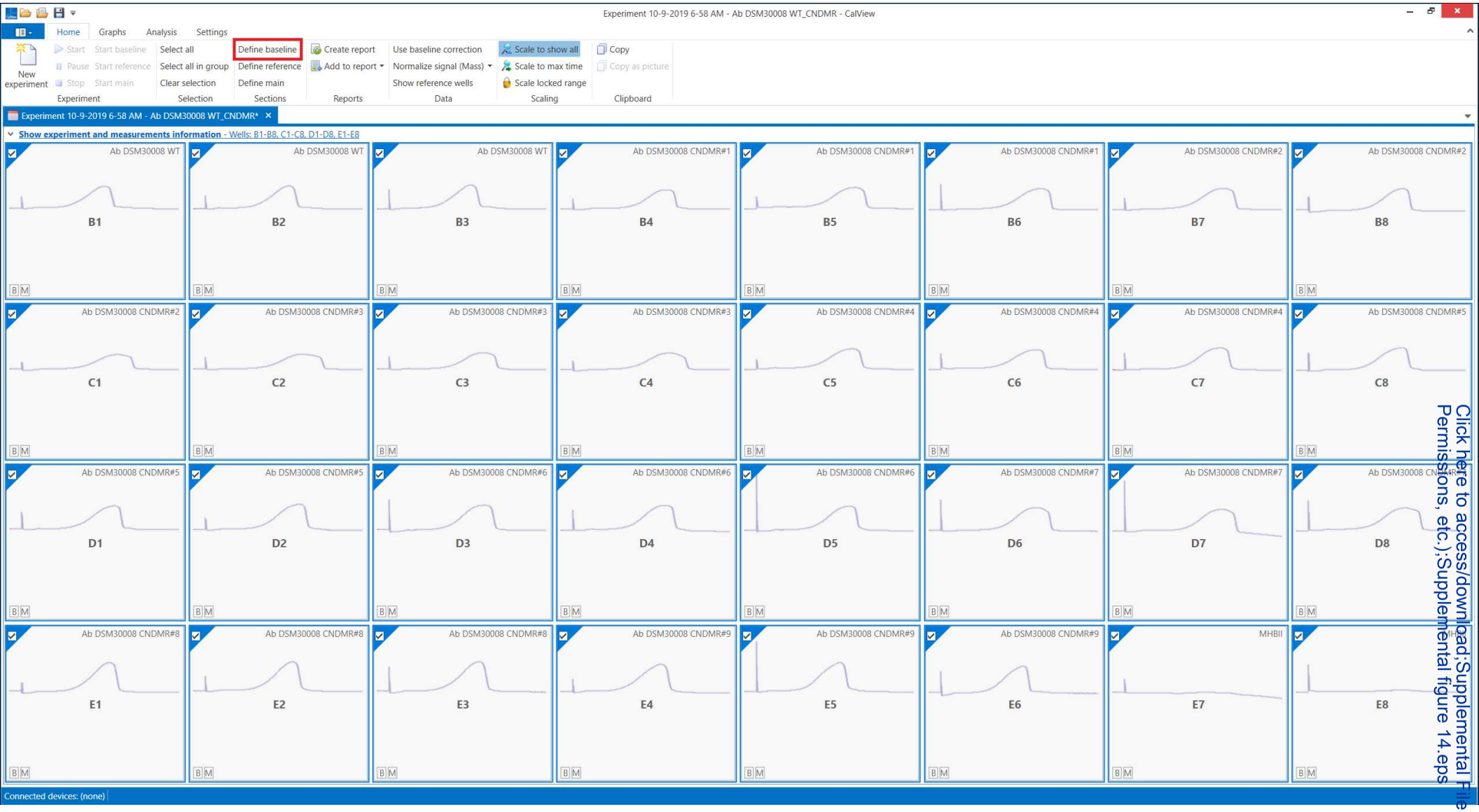

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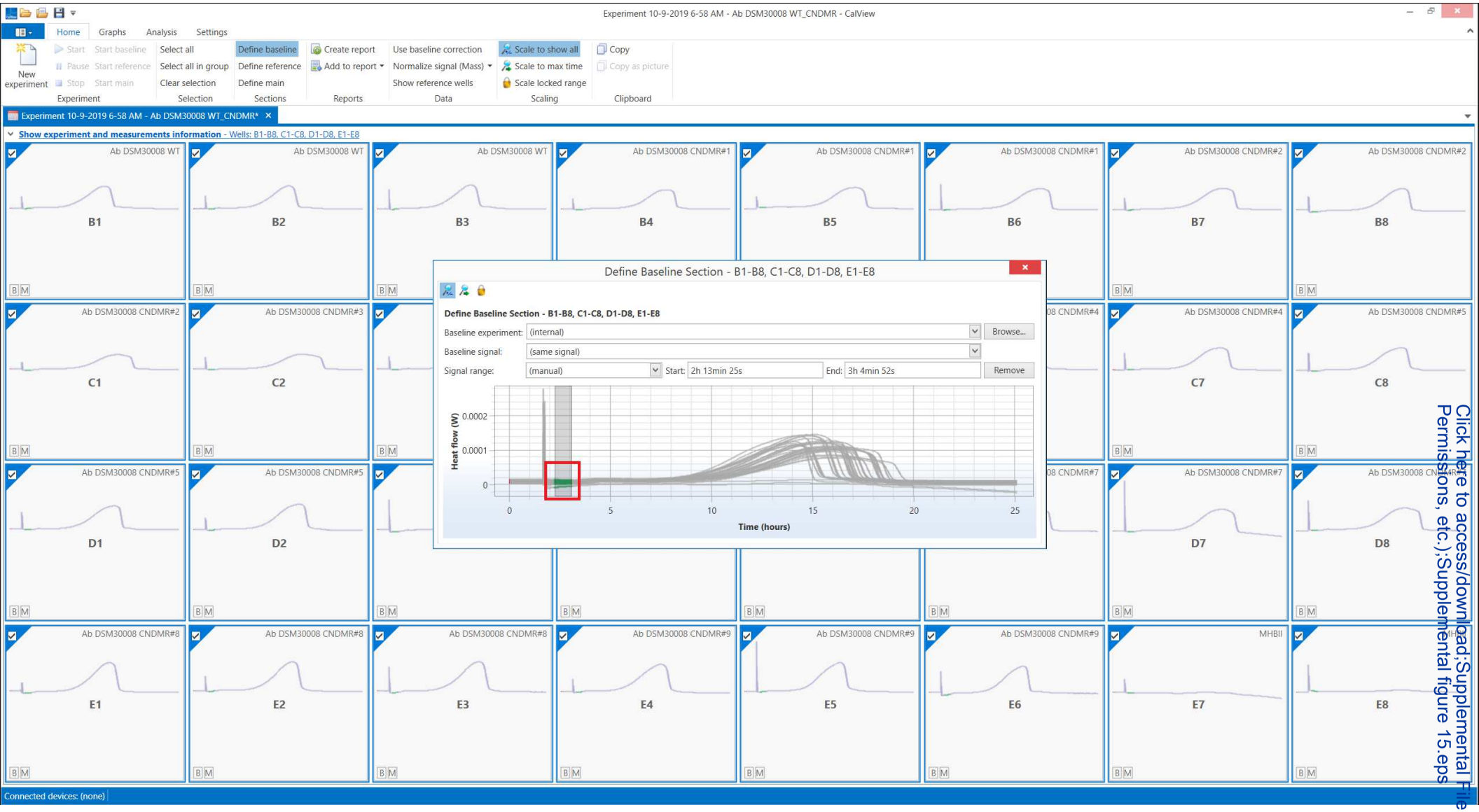

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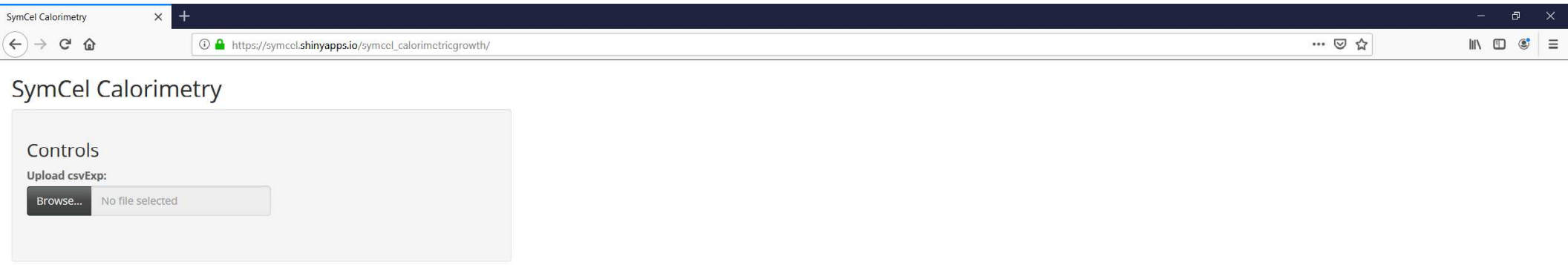

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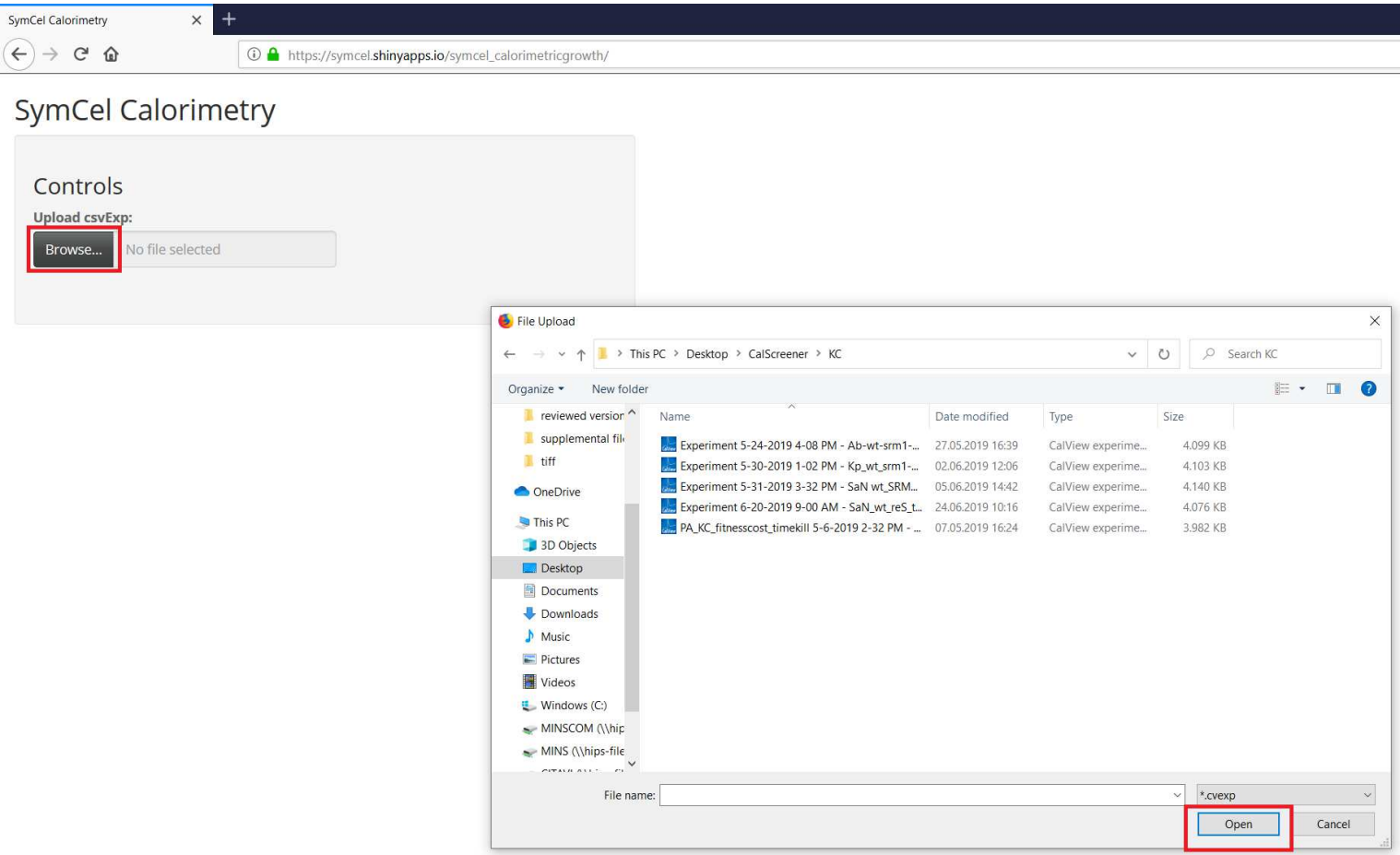

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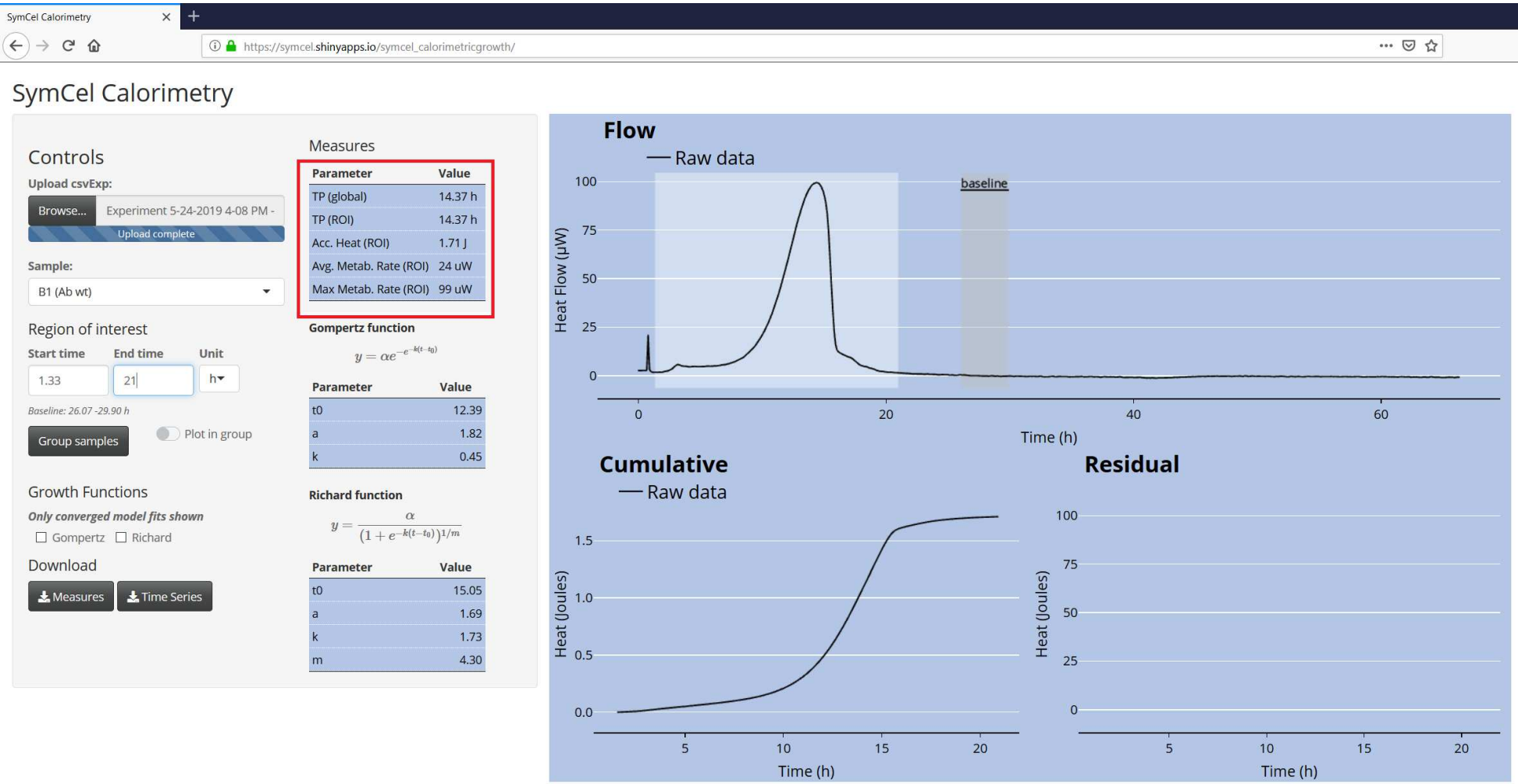

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SymCel Calorimetry

https://symcel.shinyapps.io/symcel_calorimetricgrowth/

SymCel Calorimetry

Controls

Upload csvExp:

Browse... Experiment 5-24-2019 4-08 PM -

Upload complete

Sample:

B1 (Ab wt)

Region of interest

Start time	End time	Unit
1.33	21	h

Baseline: 26.07 -29.90 h

Group samples Plot in group

Growth Functions

Only converged model fits shown

☑ Gompertz ☑ Richard

Download

Measures Time Series

Measures

Parameter	Value
TP (global)	14.37 h
TP (ROI)	14.37 h
Acc. Heat (ROI)	1.71 J
Avg. Metab. Rate (ROI)	24 uW
Max Metab. Rate (ROI)	99 uW

Gompertz function

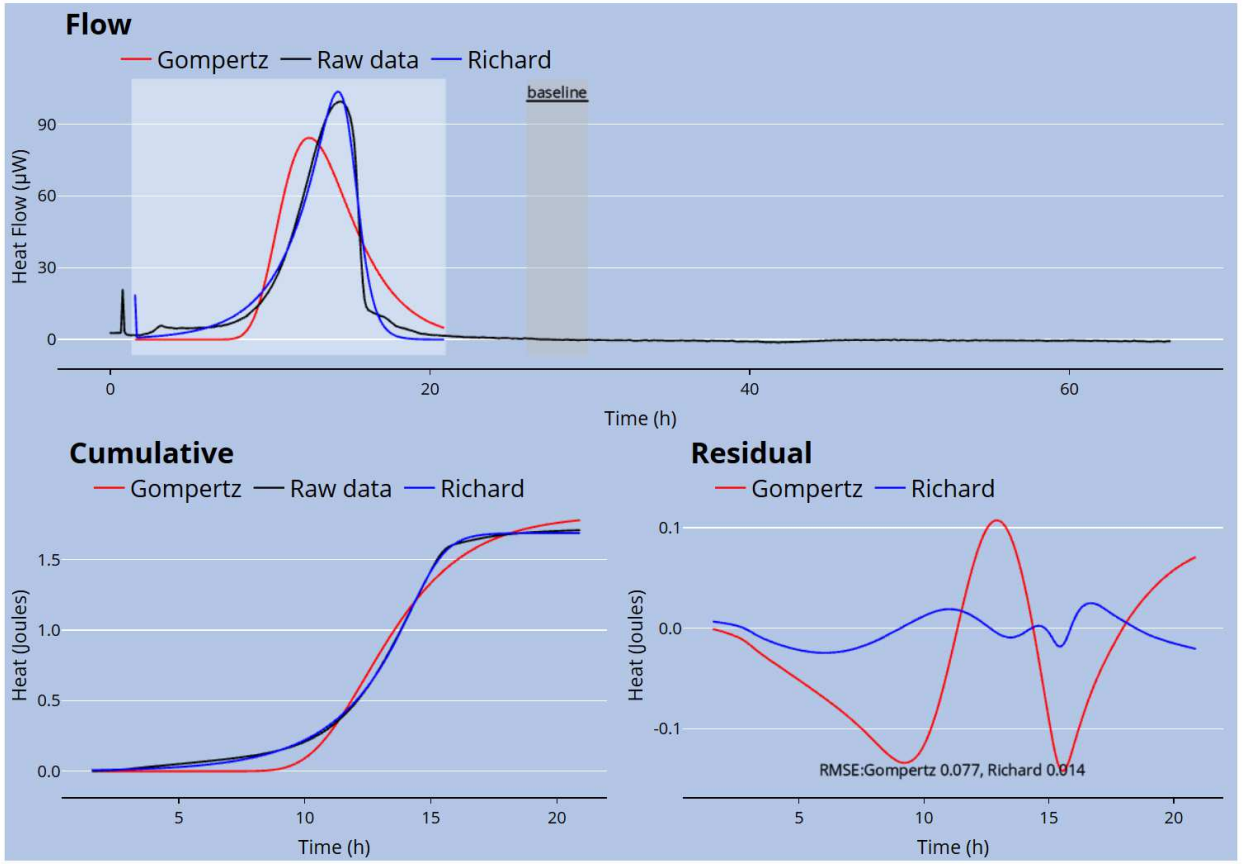
$$y = \alpha e^{-e^{-k(t-t_0)}}$$

Parameter	Value
t0	12.39
a	1.82
k	0.45

Richard function

$$y = \frac{\alpha}{(1+e^{-k(t-t_0)})^{1/m}}$$

Parameter	Value
t0	15.05
a	1.69
k	1.73
m	4.30



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SymCel Calorimetry

https://symcel.shinyapps.io/symcel_calorimetricgrowth/

SymCel Calorimetry

Controls

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Browse... Experiment 5-24-2019 4-08 PM -

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