SYMCEL

BEST PRACTICES IN EXPERIMENTAL DESIGN

This guide aims to bring insights into different aspects of experimental design for biocalorimetry. There are many aspects to consider when designing a calorimetric experiment. This document hopes to guide the user through the design process for isothermal microcalorimetry (IMC) experiments.

Aspects of experimental design

1. Thermodynamic references
2. Replicates
3. Volume and media type
4. Bacterial inoculum size and seeding density
5. Experimental time
6. Assay complexity
7. Vial and machine maintenance

1. THERMODYNAMIC REFERENCES

To increase sensitivity and increase the signal-to-noise ratio, the sample heat flow is measured against an inert reference, also referred to as a thermodynamic reference. For the calScreener, these references are located in rows A and F. For other instruments, the setup may vary.

Thermodynamic references should be loaded with a non-reacting material with the same heat capacity as the corresponding sample. This can be achieved, for example, by using the same volume of sterile culture media or other sterile solutions with similar heat capacity, for example, phosphate-buffered saline.

Alternatively, inert solid references corresponding to the heat capacity of specific solution volumes can be used. The slugs of solid references can be combined to achieve the required volume, for example, 100 µL + 200 µL for 300 µL. Solid references minimize sources of error, such as the risk of contamination and potential for leakage (Fig. 1).

Figure 1 Selection of solid references and combining them to achieve the required volumes.

Thermodynamic references should mimic the sample as much as possible, but this is not always possible. Depending on the experimental setup, there will be unknown components. Researchers often do not have the resources to determine specific heat capacities for their samples. In those cases, the heat capacity of the material could be used based on a literature search. Thermodynamic reference heat capacities within the 20% range of the sample heat capacities are acceptable.

2. REPLICATES

The number of replicates is highly dependent on the experimental system and is a trade-off between throughput and statistical power. More variable biological processes, for example, eukaryotic cells or attachment to a material, may need more replicates compared to more robust systems such as bacterial dilution series. This also depends on the variance you expect between your conditions. If the treatment effect between groups is low, you will need more replicates.

The consensus in the scientific community is that at least three biological replicates of each condition should be measured, which is also the minimal number

of replicates that would be required to rule out any outliers. As a best practice, three technical replicates should be used on the same run and three runs should contain different biological replicates. It is up to the user to determine if this can be done within the required timeframe and budget. If the number of replicates needs to be reduced it would be better to reduce the technical replicates on a plate.

Replicates should be connected to different thermodynamic references. In the calScreener, it means that the replicates should be placed side-by-side or diagonally, rather than in the same column (Fig. 2).

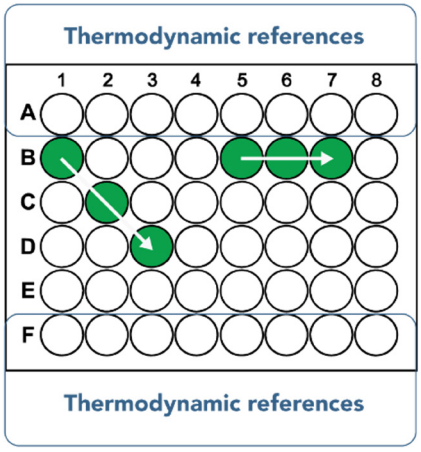

Figure 2 The recommended placement of replicates is indicated by the green circles.

Besides experimental samples, running media-only negative controls is a good practice. This will be helpful when trying to determine any low signals from the vials. Furthermore, this shows if there is any contamination of the media itself.

3. VOLUME AND MEDIA TYPE

Volume and media type can have a huge effect on metabolism. However, their effect differs between eukaryotic and prokaryotic samples.

As with all other aspects of calorimetric experiments, deciding the right media requires careful consideration and knowledge of your model organism. Calorimetric vials are hermetically sealed and contain only a defined amount of oxygen. Often, increasing the media volume increases the prokaryotic signal. On the other hand, the head space above the sample can be reduced, thereby reducing the total amount of oxygen contained in the vial (Fig. 3A). For obligate aerobes, signal intensity is usually not a problem and running at lower volumes with more headspace is more suitable. On the other hand, anaerobic bacteria with low metabolism would benefit from running at maximal volume. The appropriate volume should be determined at the beginning of the process.

For mammalian cells, increased media volume does not improve the signal output, as attached cells tend to suffocate with excess media volume above them. The optimal ratio of sufficient nutrients to oxygen diffusion for each cell type should be determined. The diffusion coefficient of oxygen in water is extremely low, leading to a limited depth of penetration into culture media. In a static culture, such as that within a calorimetric vial, oxygen diffusion into the medium happens solely at the liquid's surface where it is exposed to the atmosphere, and this can consequently lead to oxygen deprivation despite sufficient headspace. Volumes of 100–200µL have previously produced good results. It is important to use buffered media (HEPES), as the pH of media reduces with the release of carbon dioxide.

As discussed above, there is always a trade-off between oxygen availability and the amount of nutrients in the vial. But this is taken to the next level when bacteria are grown on solid agar. It is possible to cast solid agar in the plastic inserts. Despite the limitation of nutrients under a growing colony, excess air boosts metabolism (Fig. 3B). This method is particularly suitable for some fungi and Mycobacteria. Additionally, solid or semi-solid agar may be suitable when bacteria need to be immobilized, for example, when investigating the antimicrobial properties of materials.

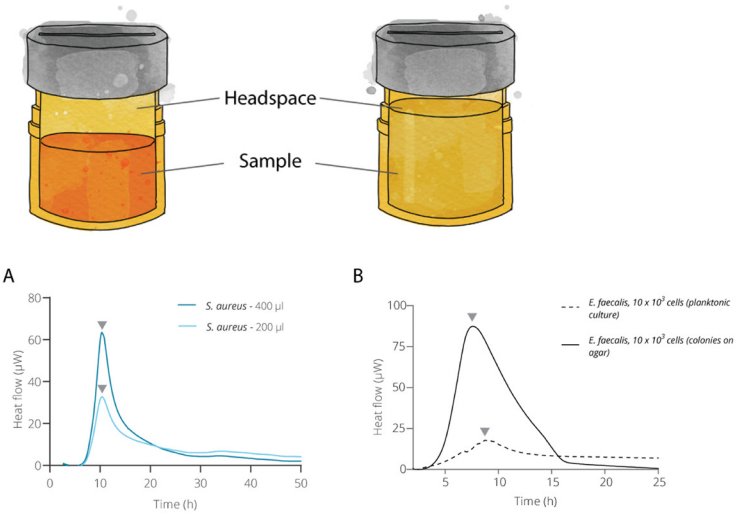

Figure 3 The effect of media volume, headspace, and type on thermograms.

Reducing agents in the media could cause background signals if they react with available oxygen. More defined media usually provides more nuanced thermograms compared to rich media. On the other hand, the biggest strengths of calorimetry are displayed when complex in vivo-like media is used.

Defined media is well suited for microcalorimetry in cases of media optimization or basic metabolic investigation. Adding or removing media components can affect the shape and total output of the culture, giving powerful insights into each component's attribution to growth and metabolism. If removing carbon source A reduces the total energy by 90%, it might be a preferred energy source over source B.

But this might only be the case if trace metal C is present in a concentration that is high enough, etc. In some cases, it is possible to link specific peaks and features on the thermogram to specific media components. For example, when using glucose as a sole carbon source in minimal media, it can be observed when added glucose is consumed and secondary metabolism needs to kick in to support further metabolism (Fig. 4).

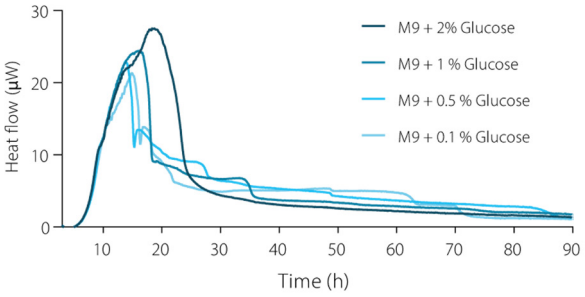

Figure 4 *E.coli* in minimal media with addition of glucose as carbon source.

One should be aware that batch-to-batch variation may produce varying thermograms when using off-the-shelf complex media such as BHI, LB, or TSB media. These media inherently have variations in their precise composition due to variations in ingredients such as yeast extract or animal infusion. Microbial cultures will metabolize these slightly differently, which can potentially show up as shifts in the thermogram.

4. BACTERIAL INOCULUM SIZE AND SEEDING DENSITY

IMC is much more sensitive than standard OD measurements, therefore lower inoculum sizes should be used to obtain the full thermogram. This paragraph focuses on the cases where the full bacterial growth spectrum is of interest. Determining a suitable inoculum size for each bacterium in a broth is good practice. It is easiest done by running a ten-fold dilution series (Fig. 5). As a rule of thumb, 10^4–10^5 CFU per vial for fast-growing bacteria is a good amount. This gives sufficient lag time for the system to equilibrate and does not extend the run-on period for too long. Very low CFU also gives rise to more variability due to random stochastic distribution in vials between replicates.

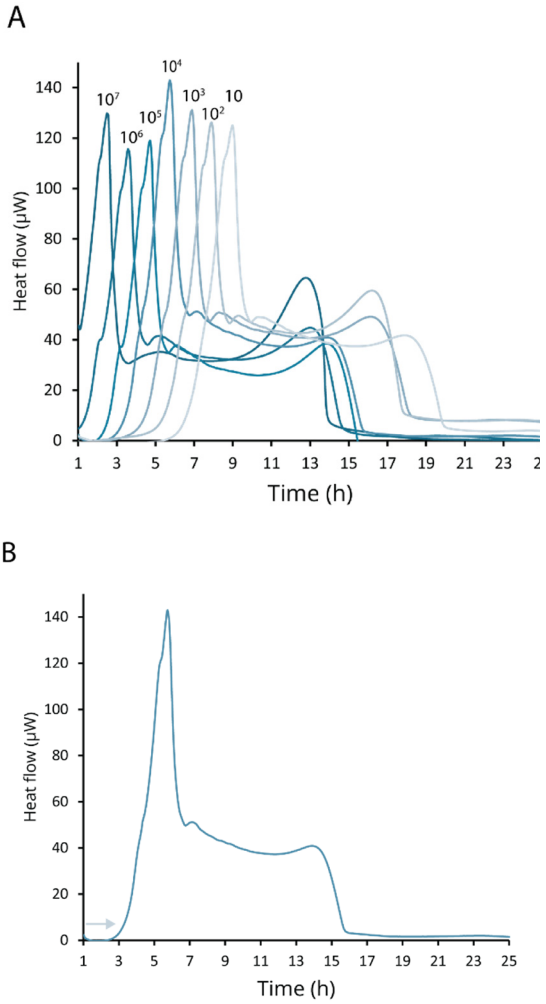

Figure 5 Dilution series of *E.coli* in MHB. A) Shows the full series with ten-fold dilutions, with CFU counts indicated above. B) Demonstrates the curve with 10^4 CFUs and good lag and run times.

Similarly, suitable seeding density for mammalian cells should be determined experimentally. The key is to find cell density that gives a good signal without overcrowding and killing the cells. The surface area of calVial inserts is about half that of the normal 48-well.

5. EXPERIMENTAL TIME

In IMC, experimental time is not limited by default. The experiment stops when the operator decides to do so. If possible, the experiments should be run until the signal returns to the baseline value. For most bacteria, this will take 24–48 hours, but it may take days for slow-growing organisms. Experimental time also depends on the research question. For example, when semi-quantifying bacterial inoculum is based on the time to peak, there is no need to wait afterwards for the whole thermogram to finish.

	Reason	Components			
		Synthetic wound fluid	Treatment	Gauze	Baceria
Control A	Chemical and physical reactions from the gauze or treatment	X	X	X	
Control B	Growth control of bacteria in wound fluid	X			X
Control C	Growth control of bacteria in presence of gauze	X		X	X
Sample	Treatment effect of gauze on bacterial growth	X	Several different treatment concentrations/ times	X	X

Table 1 Example setup for wound model experiments, including controls.

6. ASSAY COMPLEXITY

Complex model systems are very suited for microcalorimetry as the measurement of the heat flow disregards complex matrices or inert abiotic materials. For example, microcalorimetry is ideal to study the dressing's impact on bacteria using a wound model that includes bacteria, synthetic wound fluid, and a treated gauze product. However, to interpret the data from the model correctly, the assay has to be planned thoroughly and must rely heavily on controls. Suggested controls for such experiments are listed in Table 1. This way, it will be possible to elute specifically how each component affects the thermogram. More data could be produced by increasing or decreasing the dressing concentration and revealing the concentration-dependent treatment kinetics.

Other complex model systems, such as soil, fecal matter, tissue, etc., have been investigated successfully using microcalorimetry. All rely on a well-planned set of controls to isolate the effect of a certain intervention. Any unexpected results such as changes to thermograms should be subjected to further analysis, may it be plating on agar plates to determine colony morphology or investigating under a microscope for mammalian cell survival.

7. VIAL AND MACHINE MAINTENANCE

The machine does not require much maintenance. It is recommended an annual service of the equipment is carried out by authorized personnel. The machine can be cleaned externally using a soft, moist cloth with 70% ethanol as a disinfectant solution. However, it is important to avoid excess liquid coming into contact with connectors. It is therefore recommended that the Temperature Control Unit is cleaned using a dry cloth only. After the run is done, shut down the software. Restart the computer once every second month to allow for Windows updates to occur.

Avoid using excessive force when handling the calPlate, calVials, and lids with o-rings. Structural damage may cause the plate to get stuck in the calScreener and potentially damage the sensors. All of the above can be sterilized by autoclaving or using dry heat (overnight at 120˚ C or higher). When anomalies are seen in the data, inspect the vial's rim closely for any nicks or other damage that might prevent tight closure of the lid. If such damage is present, replace the vial and/or lid. If no plastic inserts are used during the experiment, clean the vials well before sterilization. If necessary, lids can be cleaned with ethanol and cotton swabs, but it is important to avoid excessive use of liquid.

Tweezers can be sterilized by autoclaving or using dry heat (overnight at 120˚ C or higher). If tweezers have been in contact with liquid broth, wipe them with ethanol before sterilization to prevent the build-up of residues on the surface. Additional cleaning by ultrasonication can be performed on the tweezers.

If needed, the loading station and torque wrench can be cleaned by wiping with ethanol. The torque wrench should also be calibrated as part of the annual service. Remember that everything needs to be completely dry before starting a new experiment.

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