MOLECULAR DEVICES

TECHNICAL NOTE

Temperature controlled kinetics with SpectraMax microplate readers

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Introduction

Temperature controlled kinetic measurements are often used for enzymatic reactions as well as cell-based assays. Here, we provide technical background on the heating element design of the Molecular Devices microplate readers and general guidelines for working with temperature-controlled kinetic assays.

Heating element design

The Molecular Devices microplate reader portfolio comprises various instruments, which differ regarding the design of the heating elements, as well as temperature control, and are therefore divided into three categories as shown in Table 1.

Category	Heating elements	Gradient heating	Maximum temperature	Microplate reader models
1	One top and one bottom chamber heating foil	Yes	45	SpectraMax® i3/i3x, Paradigm, ABS/ABS Plus, Mini, and FilterMax™ F5
			66	SpectraMax® iD3/iD5
2	Three heating elements in top and bottom locations	Yes	45	SpectraMax® M2/M2e
			60	SpectraMax® M3/M4/M5/M5e, Gemini™ EM/XPS, FlexStation® 3
3	Four heating elements in top and bottom locations	Yes	45	SpectraMax® Plus384, 340PC384, 190, and VersaMax™

Table 1. Categories of heating element design. Within the three main categories, microplate readers may differ in the maximum temperature enabled.

The heating element design of the category 1 microplate readers, such as the SpectraMax i3x Multi-Mode Microplate Reader, applies a dual area sensor for temperature control. One sensor measures the temperature of the top chamber heating foil, and another is used to measure the bottom chamber heating foil. Each heating element is independently regulated by a proportional-integral-derivative (PID) controller to guarantee accurate temperature adjustment. Furthermore, a gradient heating design (higher temperature for the top heating foil than for the bottom) is applied as shown in Figure 1. This reduces condensation for microplates covered with adhesive seals or plastic lids.

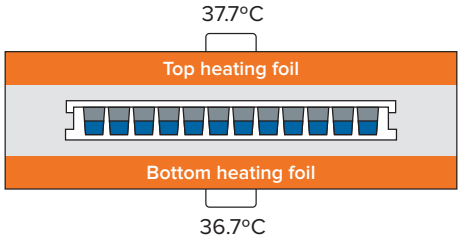

Figure 1. Plate chamber design of the SpectraMax category 1 microplate readers. The drawing shows a standard microplate of 15 mm height covered with a lid. When the user applies a set temperature of 37°C, the top is heated to 37.7°C and the bottom to 36.7°C (gradient heating design).

Category 2 and 3 microplate readers use gradient heating as well. In contrast to category 1, multiple heating elements are located in the plate chamber. The plate chamber of category 3 microplate readers has four heaters located in various positions above and below the plate carriage (as well as one additional heater for the cuvette), whereas category 2 has three heaters (plus one for the cuvette). A firmware change was applied in 2006 for category 2 and 3 microplate readers to support improved heat distribution in the plate chamber. To ensure that you have the latest firmware, contact the Molecular Devices technical support team.

Lidded microplates used with microplate readers that do not apply gradient heating can experience condensation on the underside of the lid, resulting in stray light that interferes with the measurement. This is extremely critical for fluorescence top measurements, as well as absorbance, where light must travel through the lid before reaching the sample. Other issues observed with temperature-controlled kinetics are evaporation, volume effects due to evaporation, and non-uniformity caused by temperature gradients. In the following section, general recommendations for temperature-sensitive kinetic measurements are provided.

General recommendations for temperature-controlled kinetics

To stabilize the temperature in the microplate chamber of the reader, it is recommended that you turn on the temperature control before you start the experiment. The microplate chamber requires at least 15 minutes to go from room temperature to a set temperature of between 37°C and 45°C.

During temperature equilibration, and while maintaining the temperature of the assay microplate, the so-called 'edge effect' may appear in your data, making interpretation of results difficult. Edge effect refers to the impact that evaporation, which occurs in wells of the microplate around the perimeter, can have on assay results. If you suspect you are seeing the edge effect in your data, you will need to test your experimental setup. To test the uniformity with your setup, use control wells in each corner, as well as in the middle of the microplate. Loading the entire microplate with control samples will give you an even better view of potential gradient effects across the microplate.

As a general guideline, consider that it takes approximately 30 minutes to warm 100 µL of reagent in a 96-well microplate from 21°C to 37°C. As the microplate warms from the outer edges to the middle it may take an additional 15 to 30 minutes to achieve a stable temperature across the entire 96-well microplate. To reduce the equilibration time to the desired temperature, consider prewarming the microplate and reagents. To prewarm a microplate, place it on a heat block at the desired temperature and add prewarmed reagents, then transfer the filled microplate to the prewarmed microplate reader.

Alternatively, you can prewarm the microplate containing most of the reagents in the microplate reader, and add the prewarmed final reagent to start the reaction just before beginning to read the microplate. If the final reagent cannot be prewarmed, use a maximum of 1/10 of the total reaction volume, and ensure proper mixing by shaking before reading the microplate.

Once the target temperature is reached, the outer wells require more energy to maintain temperature and therefore show increased thermal convection and evaporation. For this reason, cell-based assays often show an uneven distribution of cells in the outer wells, especially for microplates with low well volumes, such as higher density 384-well microplates. During the initial warming phase of the assay microplate, the increased evaporation rate of the outer wells decreases their temperature relative to the inner wells of the microplate. One way to avoid this edge effect is to fill the outer wells with water and only use the inner wells for the actual assay.

Alternatively, use microplates where the spaces between and around the wells can be filled with liquid, or where there is an outer moat that you can fill with liquid so that all 96 wells are available for your assay[1,2]. Microplates like this reduce the edge effect, as well as evaporation, and were developed for cell-based assays. Based on our experience using these microplates for temperature-sensitive enzymatic assays, it is best not to fill the inter-well space, since even when prewarmed water is used, the additional volume delays equilibration to the target temperature.

A microplate cover can be used to reduce evaporation, as well as contamination of adjacent wells. A plastic lid, sealant, or even mineral oil (if applicable) may be used as a cover. Transparent plastic lids with condensation rings reduce contamination between wells, while allowing gas exchange to occur during the experiment. Such a lid can be easily removed and replaced, so you can add reagents at any time during a kinetic experiment. If you need to minimize evaporation as much as possible, adhesive sealing sheets are a good alternative to lids. Sealants where adhesive is located in the area of the well are not recommended, since you cannot read through them. Non-transparent sealing sheets should only be used for data acquisition with fluorescence bottom-read mode.

For all other types of reads, such as absorbance and top-read fluorescence, it is recommended to use sealing sheets with optically clear windows with strong adhesive in the well edge areas, or optically clear heat seals typically used for qPCR. There are also gas-permeable, optically clear sealing sheets on the market, but typically they have a low gas transfer rate. For any transparent cover that you need to read through, keep in mind that the intensity of light in the optical path is decreased because of the reflective properties of the surface. If you need to work in the ultraviolet (UV) wavelength range, check for the UV-transparency of the well bottom and any cover materials you use[3,4]. If you read from the bottom, mineral oil is another option if it does not interfere with the assay components. If a higher gas exchange rate is required, non-transparent, gas-permeable sealing membranes may be used for bottom reads.

If you need to read through the cover, and condensate forms while incubating in the microplate reader, then apply a shake step before or between kinetic reads to allow droplets on the cover to come off (materials differ in properties to form condensate). For fluorescence reads, choose to read from the bottom if possible and use black-wall clear-bottom microplates. This avoids any stray light effects caused by condensation at the cover.

If condensate forms before adding the microplate to the reader, it is likely a handling issue. Minimize the time a prewarmed microplate spends at room temperature before it is placed in the prewarmed reader, as condensate can accumulate under the lid and distort results at the beginning of a kinetic reading. This also applies if the incubation temperature of the microplate outside of the microplate reader was higher than inside the microplate reader, i.e. the microplate was incubated in an incubator at 37°C and is then measured in the microplate reader at 30°C. This applies to absorbance and top-read fluorescence.

For kinetic absorbance assays, the assay volume-dependent pathlength affects the Vmax rate or slope determination, so be sure to use the same volume in all wells (see Figure 2).

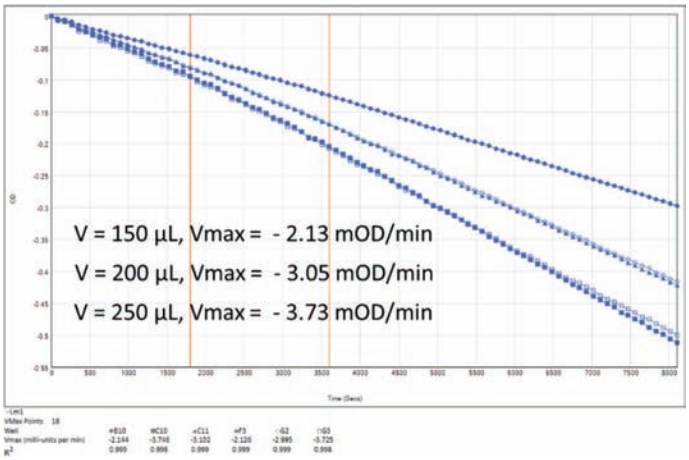

Figure 2. Effect of volume on the Vmax rate for a kinetic absorbance assay. The enzymatic assay was premixed as a bulk and then added at volumes in the range of 150 to 250 µL to a clear 96-well microplate. Vmax is calculated between 1800 and 3600 seconds (vertical orange lines). The slope is significantly different for different volumes used.

Although the information provided here will serve as guidance for assay setup, optimizing each assay on SpectraMax microplate readers will ensure that you get the best results possible. Following is a general checklist for reference:

1. Allow enough time to warm the microplate chamber of the microplate reader before use.
2. Use sample control wells to check for effects such as temperature gradient, evaporation and contamination.
3. To reduce edge effects
   - prewarm reagents and microplate
   - avoid using the outer wells, or change to a microplate with an outer moat
4. To minimize evaporation and contamination, use a microplate cover.
5. To avoid condensation for lidded microplates
   - minimize temperature changes while handling the microplate
   - avoid transfer of microplate from higher to lower temperature
6. Read fluorescence assays from the bottom, if applicable.
7. Use the same volume in all wells.

References

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3. UV Absorbance Measurements of DNA in Microplates, Evelyn L. McGown, *BioTechniques* 28:60-64 (January 2000)
4. UV/VIS Spectroscopy in Microplates, Application Note 073 041 April 2008, Greiner Bio-One GmbH, Germany

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11/22 2288B
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