# the 1:1 sample to lysate ratio: its importance and the verification process on the Sievers Eclipse* BET platform

## purpose

With the introduction of innovative technology for bacterial endotoxins testing (BET), it is crucial to show equivalency across platforms. The Sievers* Eclipse Ratio Verification Test was designed to verify that the 1:1 ratio of sample to lysate routinely used in a 96-well microplate is always present using the Eclipse microplate. Delivering a 1:1 ratio every time maintains a consistent biochemical interaction between samples and Limulus Amoebocyte Lysate (LAL). Even with a reduction in volume when using the Sievers Eclipse platform, the ratio remains the same and end users can be confident that endotoxin values for the samples and standards are equivalent and valid.

## background and importance

The kinetic chromogenic BET technique with a 96-well microplate involves mixing of standards and samples with reagent in each well and observing the color change over time. Precisely controlling the amount of sample versus the amount of lysate is an essential factor for success. USP <85> states to follow the lysate manufacturer's instructions for use (IFU) with respect to certain test parameters such as volume ratios, incubation time, or pH.

The manufacturer's IFUs indicate how to arrive at the correct volume ratio of sample to reagent in two ways: (1) directly stating that the ratio of sample to reagent should be 1:1 or (2) instructing end users to add 100 µL of LAL reagent water blanks, endotoxin standards, product samples, and positive product controls followed by adding 100 µL of LAL to all utilized wells.

In directing users that the ratio of sample to reagent should be 1:1, one IFU recommends a total well volume of 100 µL, instead of the typical 200 µL, stating that it is optimal for the greatest sensitivity. This IFU demonstrates that the 1:1 ratio is the significant component to this assay, not the total well volume. As long as there is a 1:1 ratio of sample to lysate in each well, the reaction will proceed accurately, and the results produced will be equivalent.

If the ratio is disproportionate, meaning the precise amount of sample to reagent is no longer correct, it could have a significant impact on reaction kinetics and the overall assay results. Despite the overall well volume, the reaction could occur faster, producing a shorter onset time and subsequently higher endotoxin concentrations. Or the opposite could occur, resulting in a slower onset time and lower endotoxin concentrations.

Having an inconsistent ratio could also impact the natural buffering capacity that the lysate provides when mixed with the sample. Without a precise 1:1 ratio, the pH of the reaction mixture can be altered outside the recommended range of 6-8 and influence the overall kinetics of the assay.

## 1:1 Ratio Verification Test

The 1:1 Ratio Verification Test was developed to demonstrate that the precise ratio of lysate and sample is achieved on the Sievers Eclipse microplate in each of the 104 wells. To complete the test, the user will need a new Sievers Eclipse microplate and the Sievers Eclipse 1:1 Ratio Verification Kit (STD 85000-01), which consists of a water vial and a dye vial. The Eclipse software leads the user through the setup and injection of water and dye into the corresponding

segments and the LAL port before analysis starts. The Eclipse microplate then proceeds through the same protocol as normal analysis, but since the 1:1 Ratio Verification Test does not rely on a kinetic enzymatic reaction, the overall testing time is reduced.

## report details for the 1:1 Ratio Verification Test

When analysis is complete, a report is generated in the 1:1 Ratio Results tab of the Eclipse software. In addition to the general information entered prior to running the test (e.g., analyzer serial number, Eclipse microplate information, and 1:1 Ratio Verification Sample information), the report shows the average optical density for each of the 104 optical wells.

Under the results section of the report, there is an overall average optical density for the dye portion of the plate and an overall average optical density for the water portion of the plate. Dye is mixed with dye on half of the plate, and dye is mixed with water on the other half of the plate producing these two averages. **Equation 1** below is used to obtain an overall ratio value indicating how successful the mixing was. This is indicative of how the sample and LAL will be mixed during normal analysis.

**Equation 1.**

$$\frac{Dye\ Average}{Water\ Average} = Ratio\ Value$$

In the wells where dye is mixed with dye, a perfect ratio would be 1 (Dye Average). In the wells where dye is mixed with water, the dye is diluted to half of its original concentration making the perfect ratio 0.5 (Water Average). When these averages are divided, this leads to the ideal Ratio Value of 2, indicating the exact same amounts of both dye and water reached the optical wells. A ratio between 1.90-2.10 is considered valid for this test and will not affect the overall kinetic curve and recovery of endotoxin.

## conclusion

The Sievers Eclipse microplate is a precision-crafted microfluidic liquid handling device that leverages the use of metering chambers, along with consistent channel geometry and motion, to deliver the exact same amount of sample and lysate to the optical wells simultaneously. It is this precise liquid handling with the Eclipse microplate that ensures the critical 1:1 sample to lysate ratio is achieved in all 104 optical wells for every assay.

While lysate manufacturer's IFUs either directly state to have a 1:1 ratio, or to add a certain amount of sample and reagent, it is not always 200 µL. The volumes recommended are typically set to help improve precision due to a longer path length or to allow for more accurate pipetting by using a larger volume. They are also within the typical well working volume of 75-200 µL that is recommended for the size of the wells on a standard 96-well microplate. "In order to have an efficient and realistic measurement, the lowest volume recommended for a microplate well is generally >1/3 of the maximum volume of the well".[1] This leads to the important factor being a 1:1 ratio of sample to lysate in every well, every time, not the total well volume.

The 1:1 Ratio Verification Test on the Sievers Eclipse proves that even with a significantly smaller total well volume, the crucial 1:1 ratio is achieved in every optical well, every time. With the precise ratio always present, the end user can be certain the biochemistry is equivalent. It is recommended that this verification test be completed annually by a SUEZ Certified Field Service Engineer or representative.

References

1. Pusterla, Tobias, PhD. "Which is the best microplate for my assay?" BMG Labtech, 2018 May 30. https://www.bmglabtech.com/which-is-the-best-microplate-for-my-assay/

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