# Assay of Oxyrase Activity

Measurement of Oxyrase Activity starts with the definition of an Oxyrase Unit. An Oxyrase Unit is that amount of Oxyrase that, under defined conditions, reduces dissolved oxygen at the rate of 1% per second.

The procedure presented here is not intended as specific instructions for the assay, but an outline of the method used and precautions taken. Temperature, pH, aeration of the buffer, sample volume, and cleanliness of equipment and supplies have all been found to be critical points of control. To minimize variation of measurements, these parameters have been defined and must be strictly adhered to when performing this assay.

The Gilson Oxygraph was used to create the assay of Oxyrase activity; however, the Oxygraph is no longer being manufactured, so this assay will need to be adapted to an equivalent instrument.

**Preparation:**

First, prepare water bath(s) that will maintain temperatures between 36.0 - 38.0°C . Prepare 100 ml of buffered substrate(s), and place the buffered substrate into the 37.0°C water bath. Aerate the substrate(s) for a minimum of 30 minutes by bubbling air through the buffer. If used over a period of time, the buffer should be re-aerated every 3-4 hours for a minimum of 10 minutes. Continuously monitor the temperature throughout the testing process to ensure the temperature stays in range. For daily testing, prepare fresh buffer and discard at days end.

**Testing Process:**

Standardize with a known value of Oxyrase activity prior to running test or unknown samples. To make a standard, measure a known activity level three times by three different operators. The average of each determination is combined into a grand average. The grand average becomes the value of the standard (the grand average should lie within $\pm$ 5% of each average of each operator). The initial volume of Oxyrase, may now be aliquoted into small vials and stored at a constant -20.0°C or colder temperature until used. Oxyrase activity is very stable and decays at the rate of about 5% per year under these conditions.

To standardize the instrument, activity for a standard aliquot should be measured a minimum of 3 times and until the values (units/mL) for three sequential determinations agree within a range of $\pm$ 5% of each other, and their average $\pm$ 5% of the standard's activity. This requirement ensures the stability of the enzyme standard and the instrument.

All samples should be measured in triplicate with all measurements (units/mL) being within $\pm$ 5% of each other. Repeat testing until 3 sequential readings are within $\pm$ 5%. All samples should be stored in an ice bath to ensure stability of enzyme activity during testing.

# Qualitative Methylene Blue Assay of Oxyrase Activity

Many times an Oxyrase user is interested in knowing, "Does the Oxyrase product have activity?" without resorting to a quantitative assay. The following method quickly and inexpensively reveals if the Oxyrase is still active. This qualitative assay is instructive as to how Oxyrase works to remove oxygen again and again.

**Reagent:**

Make up 100 ml of 40 mM Phosphate Buffer at pH 8.4 with 20 mM of dl-lactate and 1 ug/ml of methylene blue.

**Procedure:**

1. Put 5.0 ml of the Reagent into each of two screw-cap tubes (fill each tube to about ½ total volume), and warm to 37°C in a water bath.

2. Add the recommended amount of Oxyrase product (for example, 1:100 of EC-Oxyrase or 1:50 of Oxyrase for Broth) to one of the tubes. Do not mix. Affix the screw cap. Incubate at 37°C.

3. Observe with time. Within 10 to 15 minutes, one will see a clearing of the methylene blue at the bottom of the tube containing the Oxyrase product. With time, the clearing will progress up the tube to the top until a light blue line will remain at the meniscus. This blue line represents oxygen dissolving into the buffer from the head space, after which it is quickly reduced by the Oxyrase.

4. Clearing of the methylene blue may be better visualized by placing a white card behind the tubes.

5. After clearing has reached the top, invert the tube one time. The buffer will turn blue because the oxygen in the head space has dissolved back into the buffer. Within approximately the same time, the Oxyrase product will turn the solution clear once more.

*Warning: Constant / rapid mixing of the tube may cause the enzyme to denature, greatly effecting both, the activity and the results generated by the Methylene Blue Assay.*