

Technical Bulletin #448

BD™ Oxygen Biosensor System Data Collection and Normalization

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Introduction

The BD™ Oxygen Biosensor System (BD™ OBS) incorporates an oxygen-sensitive fluorophore into the wells of an automation-friendly BD Falcon™ Microplate. It is ideally suited for use in assays involving changes in the concentration or consumption of oxygen. Cell-based applications include cytotoxicity testing, antibiotics susceptibility/resistance testing, proliferation assays, oxidative burst and assessments of growth kinetics. Assays based upon oxidative enzyme reactions, such as mitochondrial oxidative phosphorylation or monoamine oxidase, are also possible.

The basis for the technology is the orange-colored fluorophore embedded in the hydrophobic silicone matrix at the bottom of each well. Because the dye's ability to fluoresce is reversibly quenched by oxygen, fluorescence varies inversely with oxygen concentration, in a predictable and reproducible fashion.

While the BD OBS provides a simple platform for conducting assays, the nature of the fluorescence signal differs from that of many other fluorescence probes in common use. Data collected using the BD OBS needs to be processed differently than does data from other fluorescence assays. Specifically, BD OBS fluorescence data needs to be normalized by division, not subtraction.

This Technical Bulletin addresses the collection and treatment of BD OBS data in more detail than the high-level *Guidelines for Use*. The information focuses on the basics that all users need to understand. The theory underlying the relationship between oxygen concentration and signal intensity and a protocol to convert signal to actual oxygen concentration are covered in a separate document (*Technical Bulletin #443*). This information can also be found on our website at: www.bdbiosciences.com/discovery_labware/products/drug_discovery/oxygen_biosensor_system

Signal Collection and Processing Basics

Collecting Fluorescence Data

The BD Oxygen Biosensor System should be read in a fluorescence plate reader, ideally from the bottom (to avoid issues associated with light scatter from well contents). The recommended excitation wavelength is 485 nm; the recommended emission wavelength is 590-630 nm. Gain should be manually set to a fixed value (i.e., any "auto gain" feature should be disabled), ideally one for which an empty BD OBS plate gives 1-10% of the full-scale signal. It is possible, but less optimal, to read from the top or at other wavelengths, if necessary. Such changes should be evaluated by the user prior to incorporation into an assay protocol. Contact our Technical Service Department at 800-343-2035 or e-mail at labware@bd.com if you have additional questions.

Baseline Signal vs. Noise

The signal observed in "pre-blanked" wells or negative control wells is not traditional "background". It is not simply noise to be subtracted out, but rather real "base-line" signal, corresponding to the ambient oxygen concentration (fluorescence is not 100% quenched at ambient conditions). The baseline signal should be divided into, not subtracted from, the signal intensities from the experimental wells.

Data Normalization

In accordance with the theory dictating the behavior of fluors (*Technical Bulletin #443*), the oxygen concentration at the test condition correlates to the ratio of fluorescence at the test condition to that at the reference condition. For this reason, fluorescence intensities should be normalized by division prior to subsequent analysis, and expressed in dimensionless "normalized relative fluorescence" units (NRFU). For practical simplicity, we recommend "ambient" oxygen concentration as the reference condition. NRFU thus represents the "fold" increase in signal relative to the ambient condition.

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Normalizing Against Negative Control Wells

The simplest approach for data normalization is to designate at least one negative control well on each plate as the reference. The observed raw fluorescence values for each well should be divided by the average of the negative control well(s) for that plate at that time point. This approach requires no pre-blank, and is thus high-throughput, but it explicitly lacks the ability to normalize out slight well-to-well differences in measured fluor content. It does, however, factor out any slight up-and-down variations between time points due to slight fluctuations in temperature or other machine drift at the time of reading. This approach is suitable for applications where large signals relative to baseline are anticipated, for which the small well-to-well variability in machine-perceived fluor concentration will be insignificant.

Optional Two-Step Normalization Protocol for Improved Quantifiability

To yield even better normalized fluorescence values, by accounting for this small well-to-well variability, take a "pre-blank" reading on each plate to be used in an assay, and first normalize each well's fluorescence by the value in this pre-blank reading, and then against the negative control(s) for the corresponding time point (as described herein). The easiest way to do this is to read each BD™ OBS plate empty prior to the start of the assay.

These reads do not need to be done at the same temperature conditions at which subsequent readings will be taken, since the ratio of the signal in a "dry" well at room temperature will differ from the signal in a "wet" well at assay temperature by a constant ratio, all else equal. This works because fluorescence intensity varies essentially linearly with fluor concentration.

Employing this two-step normalization protocol will yield results much tighter than the raw values. For instance, CV's on raw fluorescence numbers for repli-cate measurements may be reduced from 9% to less than 2%. This tightening of the assay data can be particularly valuable when looking for small rates or small differences in rates of oxygen consumption.

It should be evident that pre-blanking will not normalize out unintentional differences in the assay content of each well. That is, if the actual number of cells (for instance) varies well-to-well, there will be a real difference, even after normalization, in the signal resulting from those wells. Some level of such experimental error is to be expected. Using the pre-blanking data normalization routine, however, minimizes the contribution of measurement error.

Comments

Subtracting the baseline signal won't alter "rank order" trends in the data, but it will artificially exaggerate the relative magnitudes of differing signals, depending upon the gain setting. Consider, for instance, the two following scenarios, identical in every way except for the gain setting on the reader.

In Case #1, the baseline fluorescence is 500 units, and experimental wells 1 and 2 give signals of 600 and 1,200 units, respectively. In Case #2, this same experiment would give signals of 2,000, 2,400 and 4,800. By normalization, it can be seen that in either case, values of 1.2 and 2.4 NRFU will result for wells 1 and 2, respectively.

By contrast, subtraction would yield 100 and 700 for Case #1, but 400 and 2,800 for Case #2. Not only do the different cases yield different numbers, they also artificially exaggerate the difference between the two wells, relative to the baseline.

In addition to being consistent with theory, normalizing by division also puts all experimental data onto the same dimensionless scale, facilitating comparisons of data from different machines or different gain settings.

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