Method Sheet 16

Using a microplate reader to measure absorbance

Overview

All microplate readers are different in terms of the capabilities of the instrument and their software interface.

This method sheet gives broad advice on how to get the most from your own microplate reader when performing drug discovery screens with our natural product libraries.

Background to absorbance measurements

A microplate reader is a laboratory instrument designed to detect and quantify the products of biological or chemical reactions in samples contained within plastic microplates, typically in 96-well format. Microplate readers most often quantify biological activities by measuring the **fluorescence**, **luminescence** or **absorbance** values of the samples. Fluorescence refers to the emission of light of a particular wavelength from a sample after it has been illuminated with light of a different wavelength. Luminescence is the emission of light from a sample in the absence of any light source. Absorbance is the diminishment of light intensity as it passes through a sample. This guide will focus on practical advice for the measurement of absorbance values using a microplate reader.

The abundance of a specific molecule of interest in a sample can often be measured by quantifying the amount of light of a specific wavelength that is absorbed as it passes through the sample. In this mode, a beam of light of a defined wavelength is directed through each sample in turn, while a detector records how much light reaches the other side. The amount of absorbed light correlates with the concentration of certain molecules in the solution, according to the Beer-Lambert law.

Using a microplate reader to measure absorbance has several advantages over the use of single cuvette spectrophotometry. First, it enables high-throughput measurements of many samples simultaneously. Second, it reduces the sample volume requirements. Third, it is compatible with the format of most high throughput screening protocols, which makes it particularly valuable in drug discovery programmes.

Assays commonly based on absorbance measurements

Measuring light absorbance in this way is commonly used to investigate biological processes such as enzyme kinetics (e.g. cleavage of ONPG by β-galactosidase) and cellular metabolism (e.g. the MTT assay).

Another commonly used application of the absorbance assay is the quantification of stain remaining in a well after dyeing a cell culture with a coloured compound. This approach can be used to quantify the relative number of mammalian cells that remain adherent to the plate after washing (e.g. the crystal violet assay for cytotoxicity), or the proportion of bacteria that remain plate-bound (e.g. a biofilm formation assay).

Microplate readers can also be used to quantify the growth of microbial cultures by measuring the light absorbed at 600 nm - also commonly referred to as the ‘optical density’ at 600 nm, or OD600. In this application, the reduction in light reaching the detector is caused primarily by the scattering of light by bacterial cells in suspension, rather than by absorption by specific molecules.

Types of microplate

Not all formats of 96-well microplate are suitable for use in absorbance assays. Some microplates are formed entirely of non-transparent (often black or white) plastic. This type of plate is not suitable for absorbance assays as the light must be able to transmit from below the well through the sample to a detector above the plate. Instead, they are used primarily for luminescence and fluorescence assays where the non-transparent walls are necessary to prevent light spillover from neighbouring wells. Another common type of plate has a clear glass or plastic bottom, and non-transparent side walls. These allow for imaging of cells grown on the bottom of the plate by microscopy (e.g. in high content screening) and are also compatible with fluorescence and luminescence assays. They could technically also be used for absorbance assays, but as they are much more expensive than standard plates, they would not be economical to use.

Instead, the type of microplate most commonly used in absorbance assays is the polystyrene 96-well plate format in which all of the plastic is transparent (clear). These can be purchased in sterile and non-sterile formats. For assays in which bacterial or cell cultures are grown in each well before assay, the sterile format should be used. For assays such as enzyme activity assays and ELISA, there is no requirement for sterility and non-sterile plates can be used. Microplate wells can also be shaped in a V-bottom, U-bottom, or flat-bottom well format. The latter type is necessary for absorbance assays.

Use of a secondary wavelength for background correction

Some protocols for microplate absorbance measurement recommend measurement of absorbance at both the primary wavelength, (i.e. the wavelength at which the molecule of interest maximally absorbs light), and a secondary wavelength (also called a reference or correction wavelength) to improve accuracy and reliability of results. Some microplate readers record both wavelengths during a single scan of the plate, while others require two separate scans to be peformed.

Secondary wavelength measurements are sometimes used in this way to take account of various factors that can contribute to variability in measurements between wells, such as light scattering from cell debris, scratches on the plate, or background absorbance from coloured compounds in the medium. Absorbance from any of these could contribute to the signal measured at the primary wavelength and therefore confound accurate quantitation of the molecule of interest.

The secondary wavelength is selected on the basis of being where the analyte of interest does not absorb light, but where background interference (such as from turbidity or optical imperfections) is still detected. Subtracting the background contribution measured at the secondary wavelength from the signal measured at the primary wavelength helps to limit the variability caused by these imperfections.

Use of a secondary wavelength is particularly appropriate when there is no coloured compound or pigment present in the samples other than that of the primary molecule of interest, or alternatively, if such a confounding compound is present, it is there at equal concentrations in all wells of the plate.

Notably, this condition is often not met when screening a natural compound or natural extract library, since plant pigments or other coloured compounds are often present in such preparations. We therefore advise that you do not utilise correction against a secondary wavelength when screening the *Phytotitre* or *Puretitre* libraries.

Instead, the most appropriate method to account for variations in pigment between wells is to measure absorbance at two different timepoints - one just before beginning the reaction (or microbial culture period), and the other at one or more later timepoints. By measuring the signal at the primary wavelength before and after treatment or growth, it is possible to perform a background correction during the data analysis phase which accounts more correctly for the potential confounding properties of any coloured plant compounds or pigments, without further interference from absorbance at a secondary wavelength.

Technical tips

1) Remove the plate lid before inserting into the plate reader, as the lid may foul on the mechanism in some instruments.
2) Droplets of liquid caused by condensation are a common cause of high variability and poor reproducibility in microplate reading.
3) If any misting or spilled liquid is present on the bottom of the plate, use a piece of tissue to gently wipe it dry before placing in the instrument.
4) Condensation in the lid is also a common cause of poor reproducibility, so this is another reason why it is helpful to remove it before reading the plate.
5) It is easy to accidentally place the plate in the wrong orientation - ensure that well A1 is furthest from you and on your left as you place it in the instrument drawer.
6) The products of some stains or cellular reactions are sometimes quite patchy due to clumps of product forming, which if left in place gives rise to poor reproducibility.
7) To avoid such patchiness, inspect the plate visually and ensure the colour in each well is homogeneous (i.e. without clumps) - if it is not, tap the side of the plate gently to mix the contents of the wells until patchiness of colour is absent from the wells (do not do this too vigorously or cross-contamination of wells due to splashing may occur).
8) Before reading the plate, ensure that the instrument is set up for the correct wavelength that you want to use (e.g. 600 nm for bacterial growth assays, 420 nm for ONPG cleavage assays, 570 nm for crystal violet assays).
9) If your instrument is not capable of measuring absorbance at the ideal wavelength for that molecule, it is sometimes possible to achieve satisfactory results using a filter that is close to that wavelength (for example, crystal violet assays can be read anywhere between 540 nm and 600 nm).
10) Copy the data from the plate read onto a cloud or USB drive soon after reading for data analysis and to create a backup copy.

Notes

- The concentration of many coloured compounds can be quantified using the Beer-Lambert equation, which is given as: **$A = \varepsilon \cdot c \cdot l$**
- Where:

  A = absorbance (no units, since it's a logarithmic ratio)

  ε = molar absorptivity (units are $L.mol^{-1}cm^{-1}$)

  c = concentration of the absorbing species (units are $mol.L^{-1}$)

  l = path length of the light through the sample (units are cm)

- The value of ε is different for each different type of compound, and is also specific for a particular wavelength.

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