Top tips and tricks: Performing assays in microplates

Choose the best microplate reader for your work, optimize current processes, and avoid setbacks with this expert guide

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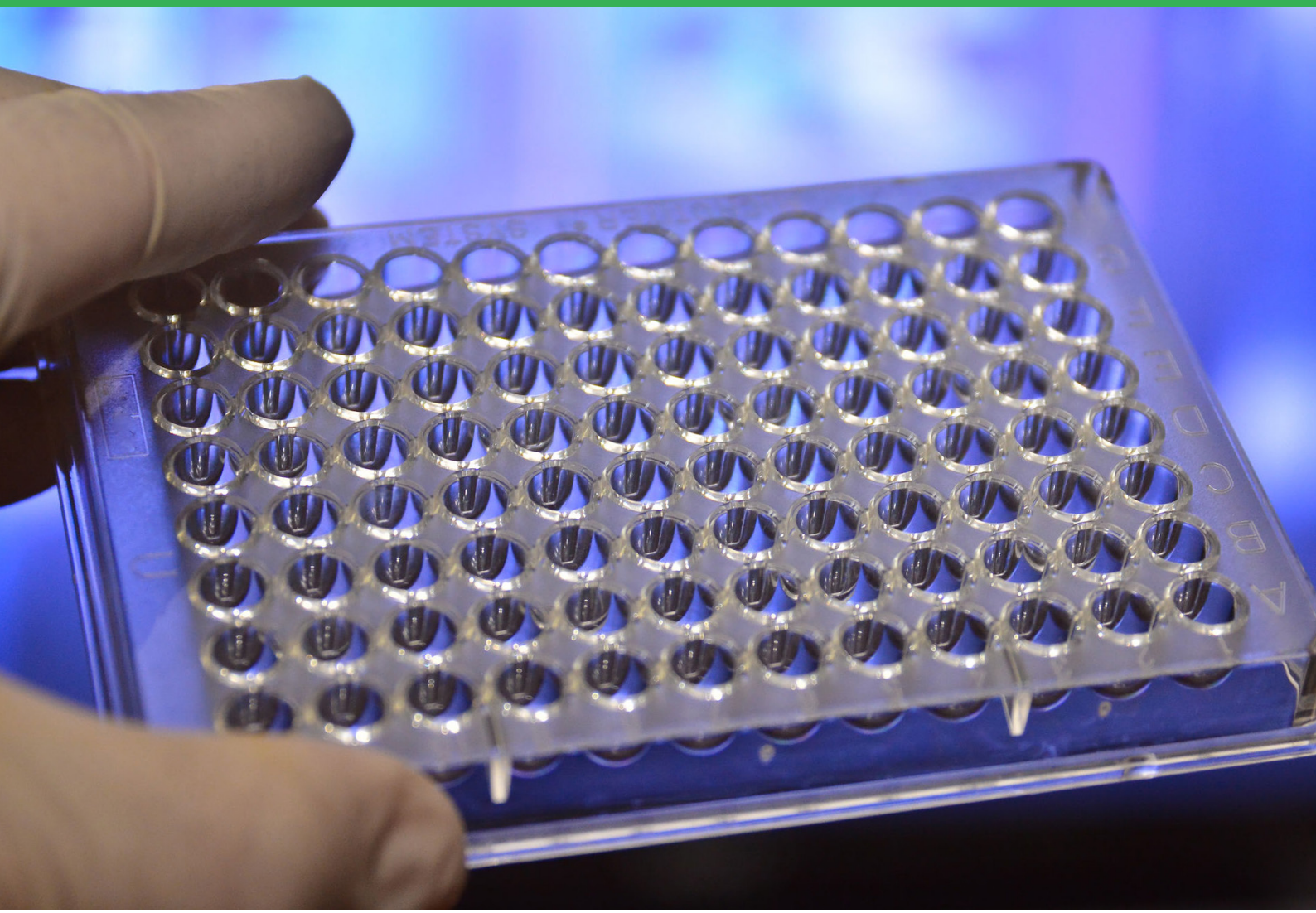

Introduction

Microplate assays are now a standard analytical tool in many life science experiments, matched by the corresponding growth of the types of microplate readers available to conduct critical measurements.

From absorbance and fluorescence to protein-protein interactions and the quantification of difficult-to-measure proteins, technology providers are developing microplate readers to overcome common problems and offer gains in measurement sensitivity, reliability, speed, and automation. Choosing the right assay is vital, but equally important is an understanding of how microplates themselves can affect measurement through phosphorescence, or how detection technologies which operate in the center of wells can be unreliable for any non-homogeneous sample, unless

Contents

corrective measures are taken. Mastering common setbacks with the right technology is the key to fast, reliable, and accurate microplate assays.

In this expert guide, we explore a selection of microplate readers from Berthold Technologies through different assay case studies, explain the basics of assay measurements, and outline common pitfalls. We will also reveal how choosing the right microplate reader for a given assay is vital to overall assay success, and share top tips from experts to help you make the right purchasing decision for you.

Critical considerations: Choosing the right microplate reader

The microplate reader has become an essential instrument used extensively within many laboratories across the globe. These small, yet powerful tools, are now used within a whole range of applications - from ELISA tests and gene expression to DNA quantification. Exploring the microplate readers on the market and deciding which reader to purchase can be a significant challenge for any lab worker, with so many options and features now available. Before users perform any assay within a microplate, they must choose a microplate reader that is not only suitable for their application, but also within budget, easy-to-use, and reliable.

Since every lab is unique, it is important that an appropriate microplate reader is chosen to meet a particular **application need**. There are many considerations one

TOP TIPS FROM THE EXPERTS AT BERTHOLD:

- **Pick a reader with the detection technologies you are going to use within the next few years.**
- **Choose a reader with filters if you need sensitivity, or a reader with a monochromator if you need flexibility. Alternatively, choose a reader with both if you have the option.**
- **Configure your reader with temperature control and bottom reading if you plan to perform cell-based assays.**
- **Add reagent injectors to measure flash luminescence or for a timely start of a kinetic measurement by adding a reagent.**

Microplate reader selection: Key questions to ask yourself:

- Do you need filters or monochromators? Monochromators are often used to select the desired wavelength and can have a direct impact on reader performance. Filters are for fixed wavelength detection and offer greater sensitivity.
- Is speed essential to the work? Consider the read speed offered by the microplate reader.
- Are you handling ultralow quantities? If so, sensitivity could be an important factor for your work, and therefore you need to select a reader designed for high sensitivity.
- What type of read mode do you need? Multi-mode readers provide greater flexibility, but it may be more cost-effective to purchase a reader dedicated to your main application. For those interested in one platform, the “single-mode” microplate reader works by either absorbance, fluorescence, or luminescence detection. However, for more diverse lab work, you may benefit from a “multi-mode” microplate reader, which can perform any of the three reading modes.
- Is the microplate reader user-friendly for all potential users and within budget?

must consider before selecting a microplate reader, such as throughput, reliability, and/or flexibility. For instance, for a lab where throughput is important, the user should buy a reader designed to screen many microplates within a short amount of time. Users must also consider the type of detector technology used, along with the read mode – such as absorbance, fluorescence, and luminescence. Fortunately, the experts at Berthold Technologies have developed a range of microplate readers designed to accommodate a whole range of research needs, from multimode and luminescence readers to enzyme-linked immunosorbent assay (ELISA) and fluorescence readers.

Application in mind: Select the right reader for your unique work

Microplate readers are used for many applications within a range of disciplines including life sciences, drug discovery, quality control, drug safety, and pharmaceutical manufacturing. As a result, there are now an expansive range of microplate readers available on the market, offering greater functionality, flexibility, speed, and throughput.

In addition to all the considerations above, it's vital that you consider the application and what you will be using the microplate reader for. Afterall, this tool will be your laboratory workhorse, and therefore you need to ensure it meets all your requirements, such as reliability, selectivity, and speed. For instance, will you be using your microplate reader for protein quantification or an ELISA? For protein quantification, this application note introduces the Apollo LB 917 absorbance reader from Berthold, a reader designed for the simple, convenient, and straightforward measurements of protein concentration, ELISA, and more.

Using the right microplate is as important as choosing the right reader: White microplates are used for luminescence and bioluminescence resonance energy transfer (BRET) applications, while black microplates are often used for fluorescence applications, as their material minimizes light scattering and reflection of excitation light.

TOP TIP: **Are you noticing unexpected results in your measurements? Make sure to check if you are using the right type of microplate. In case of doubt, compare the results of your readings performed within different types of microplates (for example, white vs black).**

Top tips: Measuring assays based on absorbance

Absorbance measurements in the UV-visible spectrum have become a standard technology for determining the concentrations of solutes. Formerly conducted in cuvettes, absorbance tests are mostly carried out in the microplate format, but there remain several challenges with generating reliable experimental data, in particular light scattering effects (and their causes) which prevent a signal reaching a detector. In this technical note, learn the importance of the spectral bandwidth of the incident light, and find out how to maximise reliability by using diluted solutions and creating calibration curves to avoid the effects of the different surface tension of solvents.

A popular application of absorbance measurements in microplates is the quantification of proteins. The accurate quantification of protein in samples is necessary for many applications, such as measuring enzyme activity and the development of immunoassays. Amongst the most popular protein concentration assays are three absorbance methods, which use the visible range and are performed in microplates: bicinchoninic acid (BCA), Coomassie Plus and Lowry. The Apollo LB917 Absorbance Reader from Berthold Technologies is ideally suited to these assays, yielding rapid measurement of 96-well plates in six seconds, and offering a wide dynamic range and intuitive touchscreen operation. In this resource, find out how the Apollo LB917 Absorbance Reader was used to reliably measure bovine serum albumin concentrations in BCA, Coomassie Plus and

Lowry assays, which produced ideal standard curves with high correlation coefficients.

Quantify nucleic acids using absorbance in microplate readers: Nucleic acid quantification in microplate readers is most often performed using fluorescent dyes, but nucleic acid quantification using absorbance has some of its own advantages, such as the estimation of purity of the sample. However, the minimum working volume of most microplates is many times larger than the sample volume required by microvolume spectrophotometers. A workaround for this is the use of microvolume microplates for DNA quantification. This type of microplate uses small sample volumes (usually 2 µL), is UV-transparent, and has a fixed pathlength that can be easily applied to the calculations without additional measurements. While they don't have as many sample positions as standard microplates (normally 16 instead of 96), they offer a good compromise between sample volume and throughput. For convenient quantification of DNA in microplate readers, Berthold Technologies offers the µDrop plate, including parameter files for easy measurement and calculation using the MikroWin software.

TOP TIP: **Check the microplate just before taking a measurement. Anything within the light path (such as bubbles, dust, fingerprints) would increase absorbance and could lead to inaccurate results.**

Top tips: Measuring assays based on luminescence & fluorescence

Is your assay type flash or glow? To optimize your luminescence measurement, it's important to know if it's a flash or a glow assay. Most luminescence-based assays can be classified as either flash or glow assays. Flash assays have fast kinetics (with reactions lasting a few seconds only) and this results in short, high-intensity signals, and highly sensitivity assays. However, they also require dispensing reagents using automatic injectors. Conversely, glow assays have slow kinetics (with reactions lasting minutes or even hours), which makes handling easier and allows reagents to be dispensed using a manual pipette. Nevertheless, this assay delivers lower sensitivity and is more prone to crosstalk problems. Some assays are available in both flash and glow versions. Choosing the right assay depends on your workflow and the specifications of the microplate reader you will use for the measurements.

TOP TIP: **Most luminescent assays are measured in endpoint mode. However, it is very useful to perform a kinetics measurement when starting work with a new assay, since this will help optimize injection and timing settings, if needed.**

Crosstalk:
Importantly, modern microplate readers can detect very low quantities of light, leading to interference (also referred to as crosstalk) in the measurement of signal from adjacent wells in a microplate. This magazine article explains how to best minimize crosstalk without wasting wells or compromising throughput, and helps to understand how different microplate plastics and colours can influence crosstalk. Plus, microplate readers from Berthold Technologies also incorporate optimized designs in their optical pathways that virtually eliminate sample-to-sample crosstalk.

Phosphorescence: Preventing setbacks
One frequent source of unwanted background noise is the phosphorescence of materials used to manufacture tubes and microplates. When exposed to bright light, they emit phosphorescence at a low intensity. This phosphorescence is not noticeable in plain sight but can be detected by sensitive luminescence readers. A simple way to check whether you are suffering from high background due to phosphorescence is to insert an empty plate in the instrument and

measure every 5-10 minutes. If the signal of the empty plate is higher than expected and clearly declines with time, the microplate is likely to be emitting phosphorescence. If this is the case and background is too high for your application, protect your plates from bright light. You can do so by working in an area without bright light sources, keep microplates in the dark, and expose them to ambient light only when necessary. Switching to microplates of a different model, manufacturer or material might also help, but many do exhibit some degree of phosphorescence, so avoiding exposure to bright light is the best way to solve the problem.

TOP TIP: **When performing luminescence or fluorescence measurements, avoid working under bright light conditions. This is helpful not only to reduce phosphorescence from tubes and plates, but also to protect light-sensitive reagents, which are often used in fluorescence and luminescence assays.**

Reporter gene assays:
Reporter gene assays are a very popular type of assays based on luminescence. Reporter genes are widely used in both academic and applied research to study the regulation of gene expression. The gene for firefly luciferase is a classic example, since the enzyme catalyses a reaction to produce light (luminescence), and the Berthold Centro LB963 Microplate Luminometer can be used to accurately measure the associated luminescence.

In this application note, find out how the Centro LB963 Microplate Luminometer was used to validate a popular commercial firefly luciferase assay, known as the Dual-Luciferase Reporter™ (DLR) Assay System from Promega – by meeting or exceeding all the test parameters required (tubing adsorption, enzyme quenching and consistency). Plus, learn how the Centro is an easy-to-use luminometer for both flash and glow assays, with superior sensitivity, negligible crosstalk, and automation compatibility.

TOP TIP: **There are two important sources of error when performing dual luciferase assays:**

1. Quenching of the Firefly signal requires perfect mixing when injecting the Stop & Glo solution. If you use reduced reagent volumes, check that the Firefly signal has been completely quenched by measuring a control without Renilla plasmid.

2. Check that Renilla signal is not affected by your experimental conditions. If it is, use a plasmid with a different promoter.

ATP & BRET assays:
Other highly popular luminescence assays are the adenosine triphosphate (ATP) assay, along with the BRET assay.

Adenosine triphosphate is present in all living cells and can be detected rapidly via light emission through the combined use of luciferase and a luminometer. This has been used to develop quick, simple, and highly sensitive assays, which can be used to assess cell/biomass quantity, cell proliferation and cell death, among others. ATP assays are available both in flash and glow versions.

TOP TIP: **Be careful to avoid contamination of samples and reagents, as ATP is present in most organic matter (for example, your fingerprints), and could lead to inaccurate results. It's useful to measure two blanks of each run, one at the beginning and one at the end of the sample series. If the signal of the last blank is clearly higher than that of the first one, this could mean some reagent or consumable has been contaminated while measuring the series.**

The BRET assay is a mechanism describing energy transfer between one light-emitting molecule (typically a luciferase) and a

light-sensitive molecule (typically a fluorescent protein). The BRET assay is often compared against fluorescence resonance energy transfer (FRET), one of the most widely used methods of fluorescence. In this method, the fluorescent donor protein is coupled to one of the proteins of interest and the acceptor to the other protein. If there is no interaction, only the fluorescence of the donor should be detected. However, if an interaction takes place, the fluorescent donor protein can excite the acceptor via resonance energy transfer, ideal for the visualization of molecular interactions of proteins and nucleic acids. On the other hand, BRET was developed to bypass some of FRET's background problems by using a luciferase as a donor, instead of a fluorescent protein. Since luciferases produce light through a chemical reaction, an external light source is not required for excitation. As a result, BRET has a small background and does not suffer from problems compared to FRET, such as autofluorescence, light scattering or photo bleaching. This handy resource explores the BRET assay in more detail, and introduces the NanoBRET™ assay - an assay designed to deliver increased signal and lower background. For the detection of the signal, a suitable plate reader called the Tristar Multimode Microplate Readers developed by Berthold Technologies was used.

DNA quantification using fluorescence

DNA quantification is an important pre-analytical method, typically based on UV-visible or fluorescence spectroscopy both of which have advantages and disadvantages. The range of high-performance microplate readers from Berthold Technologies can facilitate higher throughput, greater sensitivity, and improved specificity in DNA measurement. Learn from this application note how the Berthold Tristar Multimode Microplate Readers used in tandem with the Quant-iT™ PicoGreen® double-stranded DNA (dsDNA) reagent can permit the specific quantification of dsDNA in 96-well plates to a detection level below 0.1 pg/µL in under one minute.

TOP TIP: **When measuring fluorescence using the monochromator, you may want to optimize the bandwidth in the monochromator settings. A broad bandwidth gives more signal, while a narrower bandwidth generates higher specificity and lower background. Test several setting combinations and choose the combination giving the best signal/noise ratio.**

Top tips: measuring cell-based assays

When working with microplate readers, it is important to consider the benefits of top reading vs bottom reading, and which format you will choose to use. For instance, top reading delivers high sensitivity and a better signal-to-noise ratio, with reduced crosstalk between wells. Interestingly, reading fluorescence from the top is more sensitive because light being attenuated and scattered by the plastics of the well-bottom can increase the background and therefore decrease measurement efficiency. Nevertheless, bottom reading is also associated with its own benefits that you can take advantage of. For example, bottom reading is ideal when working with adherent cells growing at the bottom of the plate, which either express, bind, or secrete a fluorophore at or close to their bottom attachment side. Discover from this magazine article how top reading offers higher sensitivity and is particularly valuable for solution-based assays such as DNA and protein quantification. This article also explores bottom reading and explains why this approach is ideal for those working with adherent cells in clear bottom wells, while also highlighting the appropriate time a protocol may be needed to run both top and bottom reading.

Working with scattered samples:
It's important to consider whether you're measuring scattered or irregularly distributed samples. By default, in most detection technologies, measurements are performed

in the centre of the well. Performing measurements in the centre of a well is ideal for measuring homogeneous samples (for example, a cell lysate or fluorophore solution), however, if the signal is scattered or irregularly distributed through the well, signal in the centre will not be well representative of the rest of the well. This could be the case for larger wells, in particular, such as those of 24-well plates. This limitation can be solved by measuring in Scanning mode (often referred to as Area Scan or Well Scan). In Scanning mode, the well surface is divided into smaller areas (for example, in 3 x 3 or 10 x 10 pattern), with one measurement performed within each of them. The more measurements that are conducted means the more representative the result is, but also will cause the measurement to be slower. For example, measuring a 10 x 10 pattern means measuring 100 spots per well. This will increase the measurement time by 100 times. To save valuable time, it is recommended to test several different settings to find the pattern giving the most representative results in the shortest time.

Need to improve your results? It could be time to consider automation

The detection step is very important in any method involving a microplate reader measurement, but in some cases, performance of the assay has much to do with the previous steps. An example of such methods includes the ELISA, which requires several incubation, washing and dispensing steps before the absorbance measurement takes place. One way to increase the reliability and reproducibility of such methods, and reduce human error, is automation. For more information on the value of automation and ELISA, look at this valuable resource.

Automating ELISA and other assays involving washing, dispensing and incubation steps can improve the quality of results in several ways:

- Washing steps are critical for the performance of the assay, but the different wash technique of different technicians can lead to inconsistent results.
- The duration of incubations is important, but manual processing means the technician uses the incubation time to perform other tasks, which means abandoning the task and walking to the location of the plate when incubation time ends. This often means that the technician is not available immediately when the incubation time ends, and this results in different incubation times in different days. This way of working also increases the probability of mistakes either in the ELISA or the other tasks performed.
- If results are inaccurate and the assay was performed manually, it can be difficult to find out in which step something went wrong. Fortunately, the software of most automated solutions includes log files which document each step performed to make troubleshooting much easier.

For those working with ELISA, this resource outlines the automation of the APP ΔC31 ELISA kit, a complete, colorimetric, immunometric immunoassay kit designed for the quantitative determination of human APP ΔC31 (the stable amyloid precursor protein fragment) - which delivers results in just 2 hours.

TOP TIP: **Optimizing wash settings is often the most important factor when automating an assay in microplates. To reduce the time and cost of optimization, test wash settings with one single column of wash buffer only, and run an assay with standards and controls only before measuring real samples.**

Application Note

PROTEIN QUANTIFICATION USING THE APOLLO LB 917 ABSORBANCE READER

Abstract

Some of the most popular methods for protein quantification are BCA, Coomassie Plus (Bradford), and Lowry, and they are most often measured using a microplate absorbance reader. The suitability of the Apollo LB 917 to perform those assays was assessed and confirmed. Furthermore, the Apollo is easy to program and performing the assays is simple and straightforward.

Introduction

The quantification of the protein content of a sample is necessary for many applications, such as enzymatic activity measurement, western blotting, development of immunoassays, and more.

There are many protein quantification methods available, and the choice of the most suitable method depends on a range of factors: from the protein to be quantified (protein mixture or specific proteins), the presence of detergents in the buffer, the amount of sample available, and others. Some of the most popular methods are BCA, Coomassie Plus (Bradford), and Lowry [1]. All three are colorimetric methods which are quantified using absorbance in the visible range and are most often performed in microplates.

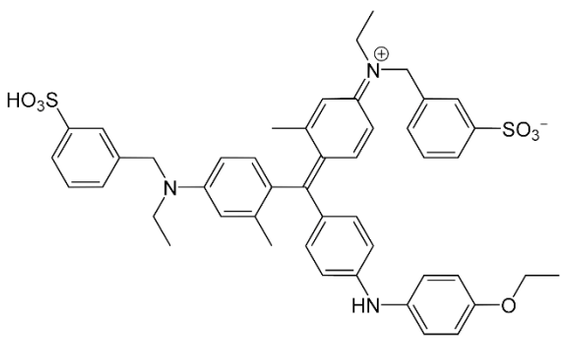

***Figure 1**: Coomassie brilliant blue G-250, the binding dye for the Bradford assay.*

The BCA assay combines the reduction of Cu^{+2} to Cu^{+1} by the peptide bonds in protein in an alkaline medium with the colorimetric detection of the cuprous cation (Cu^{+1}) using a reagent containing bicinchoninic acid (BCA) and is measured at 562 nm.

The Bradford/Coomassie Plus assay uses Coomassie dye. When it binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm, with a concomitant colour change from brown to blue.

Authors

Francesc Felipe – Berthold Technologies
http://www.berthold.com/bio

The Lowry assay is based on two chemical reactions: the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein, and reduction of the Folin & Ciocalteu's phenol reagent, which yields a purple colour. Absorbance of the coloured solution is read at a suitable wavelength between 500 nm and 800 nm.

This application note demonstrates the suitability of the Apollo absorbance reader for protein quantification.

Materials

- Apollo LB 917 absorbance reader from Berthold Technologies (73664-10) equipped with 560, 595 and 650 nm filters.
- Pierce™ BCA Protein Assay from ThermoFisher Scientific™ (23227).
- Total Protein Kit, Micro Lowry, Peterson's modification from Sigma-Aldrich® (TP0300).
- Pierce™ Coomassie Plus (Bradford) Assay Kit from ThermoFisher Scientific™ (23236).
- Pierce™ Bovine Serum Albumin Standard Ampules, 2 mg/mL (23209).
- 96-well clear microplates from ThermoFisher Scientific™ Nunc (269620).
- Deionized water (to prepare BSA standards).
- Pipettes and pipette tips (various models and volumes).

Methods

All reagents were prepared following the manufacturer's instructions. A standard curve of BSA was prepared with the following concentrations: 2000 (Standard 1), 1500 (Standard 2), 1000 (Standard 3), 750 (standard 4), 500 (Standard 5), 250 (Standard 6), 125 (Standard 7) and 25 (Standard 8) µg/mL, and deionized water was used as blank. In addition, 1 unknown sample was measured: it was prepared by mixing equal volumes of the 750 and 1000 µg/mL standards and had hence a theoretical concentration of 875 µg/mL. All measurements were performed in duplicate.

Each assay was performed following the manufacturer's instructions (see below). The BCA assay was measured at 560 nm, as a 562 nm filter was not available.

BCA protocol

1. Pipette 25 µL of each standard or sample to each well
2. Add 200 µL of working reagent to each well
3. Shake 30 seconds
4. Cover plate and incubate 30 minutes at 37° C
5. Measure absorbance at 560 nm

Micro Lowry protocol

1. Pipette 50 µL sample or standard to each well
2. Pipette 50 µL Lowry reagent to each well
3. Incubate 20 minutes at RT
4. Pipette 25 µL Folin & Ciocalteu's phenol reagent to each well and mix immediately
5. Incubate 30 minutes at RT
6. Measure absorbance at 650 nm

Coomassie Plus (Bradford) protocol

1. Pipette 10 µL of standard or sample to each well
2. Pipette 300 µL Coomassie Plus reagent to each well
3. Shake 30 seconds
4. Incubate 10 minutes at RT
5. Measure absorbance at 595 nm

The positions of standards, blank and samples were entered in the Apollo built-in software. The plate layout used is displayed in Figure 2:

***Figure 2**. Plate layout programmed in the Apollo software. Wells in green are the standards (Standard 1 to Standard 8), wells in white are the blanks, and the rest of the plate is left for samples. The unknown sample was pipetted in duplicate in wells C3 and D3.*

To perform the calculations, 4 Parameter Logistic was selected as curve fitting algorithm (labelled "Logistic" in the software). Calculated protein concentrations were directly obtained from the built-in software. Data were exported to CSV format using a USB drive for further processing (reporting and publication of results). Curve fitting graphics were exported to BMP format using a USB drive.

Instrument settings

Common settings:

- Mode: Normal
- Plate: 96 wells (see Figure 2)
- Shake: Off
- Preprocess: Off
- Kinetic: Off
- Curves: On
- Interpret: Off
- Quality: Off

BCA settings:

- Filter: 650/Off

Coomassie Plus settings:

- Filter: 595/Off

Micro Lowry settings:

- Filter: 560/Off

Results

As expected, the standards produced curves of slightly different shapes for each assay, with shapes very similar to the examples displayed in the user manual of each assay. In all cases, correlation was excellent (R^2 ≥0.997). All values were within the dynamic range of the Apollo reader (0.0-4.0 OD). Obtained standard curves are displayed in Figure 3.

The calculated concentrations of the unknown sample were quite close to the expected concentration: within a 5% difference for the Coomassie Plus and Lowry assays, and a 6.84% lower for the BCA assay. Numerical results are summarized in Table 1.

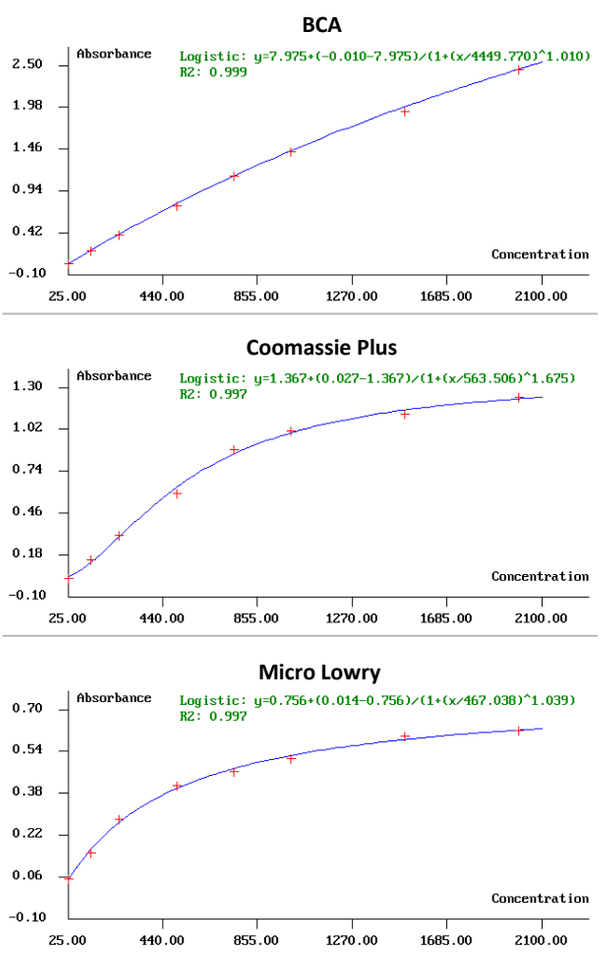

***Figure 3**. Standard curves generated by the Apollo built-in software using the Logistic function (4PL). Graphs were exported as BMP files.*

Standards			
µg/mL	OD		
BSA Standard	BCA	Coomassie Plus	Lowry
2000	2.447	1.232	0.620
1500	1.931	1.122	0.599
1000	1.421	1.012	0.513
750	1.124	0.888	0.463
500	0.751	0.590	0.409
250	0.392	0.307	0.281
125	0.197	0.146	0.151
25	0.031	0.022	0.052
0	0.000	0.000	0.000
Unknown sample (875 ug/mL)			
	BCA	Coomassie Plus	Lowry
OD	1.208	0.955	0.510
Calculated concentr. (µg/mL)	815.1	915.1	918.8
Difference from expected (%)	-6.84	4.58	5.00

***Table 1.** Numerical values obtained for the standards (top) and unknown sample (bottom). All values are the average of duplicate measurements.*

As the BCA assay should be measured at 562 nm but only a 560 nm filter was available, the impact of this difference in the measurement wavelength was assessed. To test this, the same plate was measured with a monochromator-based microplate reader (Tristar 5), both at 562 nm and at 560 nm. Measurements at 560 nm were a 0.86% lower as average than the measurements at 562 nm (data not shown).

Conclusions

Using the Apollo LB 917 to measure the BCA, Coomassie Plus/Bradford and Lowry assays was simple and straightforward thanks to the intuitive built-in software, which allowed to easily set measurement settings, standard positions and concentrations, and curve fitting functions. The standard curves produced were similar to the examples provided with the kit's instructions and had a high correlation coefficient. Although a 562 nm filter was not available to measure BCA, the impact of measuring it at 560 nm instead was negligible. The results obtained for the unknown sample were very close to the expected values, with differences which can be explained mostly by pipetting error.

Concerning the comparison of the different assays tested, the closest concentration to the expected one was obtained with Coomassie Plus (only 4.58% higher than expected) and the most different with BCA (6.84% lower than expected), but it's difficult to assess at what extent these differences are due to the performance of the assay or to pipetting error.

The obtained results confirm that the Apollo LB 917 is a suitable reader for convenient and reliable measurement of protein concentrations.

References

1. Goldring, L.P.D. Protein Quantification Methods to Determine Protein Concentration Prior to Electrophoresis. Methods Mol Biol (2012), 869: 29-35.

Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

BERTHOLD

Technical Note

QUANTITATIVE ABSORBANCE MEASUREMENTS IN MICROPLATES

Beating the most common challenges

UV-VIS absorbance measurements have emerged as a standard technology to determine concentrations of substances in solution (or even in gas phase). As this technology provides absolute values, it is generally independent from the instrument or individual experiment setups. After having been used macroscopically in cuvette-based studies for decades, absorbance tests in the microplate format have gained increasing importance. Although absorbance studies appear to be straight forward at first glance, there are several challenges one needs to master in order to generate reliable experimental data. A deep understanding of the physical background of absorbance measurements and awareness of the strict boundary conditions that have to be fulfilled for a valid experiment are key for planning your experiment to result in accurate and reproducible data.

The physics of absorbance measurement

The basic principle of absorbance measurements is described by Lambert-Beer's law. Absorbance measurements are typically based on a transmission configuration[i] to determine the magnitude of intensity attenuation of incident light by an analyte [2]. The absorption of light with an initial intensity I_0 penetrating a diluted solution

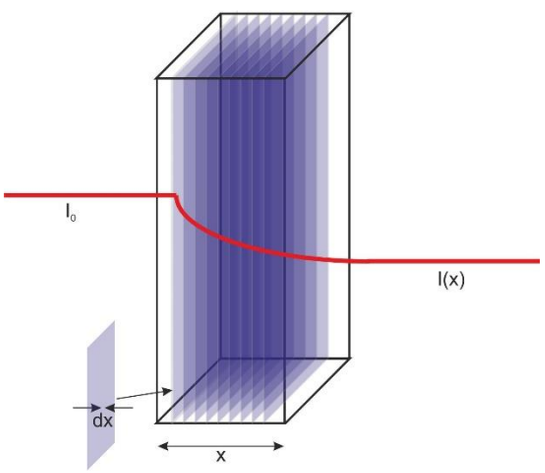

Figure 1: Light attenuation by passing through a solution of a given substance.

of a given substance over a length of x can be described by cutting the light path into infinitesimally small sheets of the width dx (Figure 1).

[i] Strictly speaking, the Lambert-Beer's law describes the wave propagation of radiation through a medium [1]. For convenience in this technical note we refer to a transmission configuration, which is commonly used in absorbance studies.

Dr. Frank Schleifenbaum &
Dr. Thomas A. Schild
Berthold Technologies GmbH, Bad Wildbad, Germany
http://www.berthold.com/bio

In each of these small sheets there may be molecules of the substance of interest present, which are equally distributed over the entire sample volume. To take into account that substances have different abilities to interact with irradiation of a certain wavelength, we may assign an area of a distinct diameter to each molecule and call this area the "absorbance cross-section" a (Figure 2). If light hits the cross-section area of a molecule, it will be completely absorbed[ii].

As a result of this process the light intensity is attenuated after it has passed through the sample, i.e. the number of photons that appear at the end of the sample volume is reduced compared to the number of photons entering the sample. To quantify this attenuation, we find that the light intensity depends on the number of molecules present in the light path n/A and on their absorbance cross-section. Hence, we can write for the local intensity in every of the small sheets

$$dI = -a \cdot n/A \cdot I \, dx$$

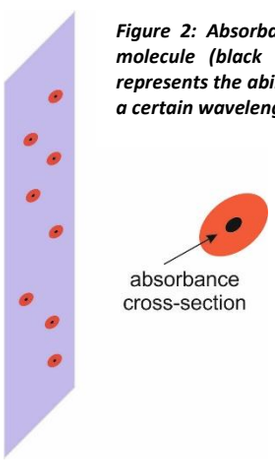

Figure 2: Absorbance cross-section (red area) of a molecule (black dot). The size of the red area represents the ability of a molecule to absorb light of a certain wavelength.

The density of the molecules in the complete sample, n/V, can be expressed as a molar concentration $c=n/N_A$ (N_A =Avogadro's number) and integration of this equation over x with the initial intensity I_0 yields:

$$lnI = -a \cdot c \cdot x + lnI_0$$

$$ln\left(\frac{I_0}{I}\right) = -a \cdot cN_A \cdot x$$

or $ln\left(\frac{I}{I_0}\right) = a \cdot cN_A \cdot x$, respectively.

Commonly, this expression is found in decade notation and the conversion factor 2,303 is expressed together with the absorbance cross-section as the molar extinction coefficient $\varepsilon(\lambda)$. With the path length x written as d[iii] we obtain the common expression of Lambert-Beer's law for the absorbance A:

$$\mathrm{A} = \log\left(\frac{I}{I_0}\right) = \varepsilon(\lambda) \cdot c \cdot d$$

Although appearing rather straight forward, Lambert-Beer's law is only valid under well-defined conditions and there are several challenges when applying the law in practice.

Wavelength dependency

Obviously, solutions of different molecules may appear in different colours. Accordingly, the selection of the measurement wavelength must be specified precisely [3]. Typically, one will choose a wavelength where the absorbance is maximal to obtain the highest sensitivity. Of similar importance is the spectral bandwidth $\Delta\lambda$ of the light. Let's consider a substance with a molar extinction coefficient $\varepsilon(\lambda)$ at a wavelength λ_1 which

[ii] The model of a complete absorption of light by a single molecule accounts for the quantum nature of light. Here, single traveling photons are considered which can either be absorbed or may travel unaffected.

[iii] Note that for historic reasons d is given in centimeters (10^{-2} m).

is irradiated with the intensity $I_{0,1}$ and a different molar extinction coefficient at a second wavelength λ_1 irradiated with the intensity $I_{0,2}$. For the two wavelengths Lambert-Beer's law yields:

$$I_1 = I_{0,1} \cdot 10^{-\varepsilon(\lambda_1)cd}$$

$$I_2 = I_{0,2} \cdot 10^{-\varepsilon(\lambda_2)cd}$$

The detected intensity represents the sum of the intensity of all wavelengths and one obtains:

$$A = log \frac{I_1 + I_2}{I_{0,1} + I_{0,2}}$$

$$= log \frac{I_1 + I_2}{I_{0,1} \cdot 10^{-\varepsilon(\lambda_1)cd} + I_{0,2} \cdot 10^{-\varepsilon(\lambda_2)cd}}$$

As a consequence, $A = \varepsilon(\lambda) \cdot c \cdot d$ is only true for $\varepsilon(\lambda 1) = \varepsilon(\lambda 2)$ and, strictly speaking, Lambert Beer's law is only valid for monochromatic light of one single wavelength as it is provided e.g. by laser sources. In practice, a spectral bandwidth of up to 10-15 nm nanometres is acceptable, especially if the absorbance spectrum is rather flat in the particular spectral window. For spectra exhibiting very sharp peaks it is recommended to choose a wavelength where the spectrum is smoother even if the absorbance is weaker at this wavelength (Figure 3).

As a rule of thumb, the FWHM[iiii] of the absorbanceband $\Delta\lambda_{H,\varepsilon}$ should be greater than the FWHM of the incident light $\Delta\lambda_{H,meas}$ by a factor of 5. However, if a monochromator is used for wavelength selection, the spectral bandwidth might be set to the smallest value. It is important to note, that the maximum accessible OD value is reduced for small bandwidths for the benefit of a dramatic increase in the precision of concentration determination.

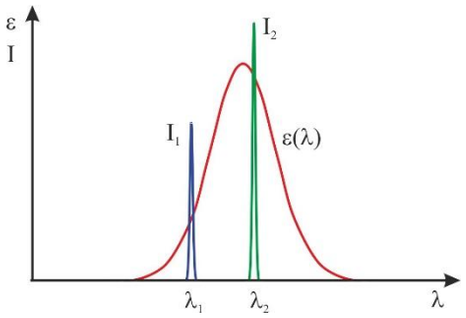

Figure 3: Influence of the spectral bandwidth of the irradiation light for different wavelengths.

Concentration dependency of ε

One highly important characteristic of the Lambert-Beer's law is that it is only valid for diluted solution with absorbance-values A $\lesssim$ 1.5 corresponding to concentrations of about c $\lesssim$ 0.01 mol/l for typical organic molecules. The reason for this limitation is given by the derivation Lambert-Beer's law as outlined above. Here, it was assumed that every molecule has the identical probability to absorb light. For higher concentrations, the mean free path (i.e. the average distance a molecule travels until it hits another molecule of its kind) between two analyte molecules is too small. This causes interactions between these molecules that may lead to e.g. aggregation, dissociation or the formation of complexes. Also, shadowing effects can have an impact on the measurement. Molecules can be shielded by neighbouring molecules so that they do not "see" the impinging light. Hence, reliable absorbance measurements should only be performed using diluted solutions and great care should be taken that the absorbance is not exceeding values of 1.5. Else, only qualitative conclusions may be given and exact information on concentration is no longer revealed.

[iiii] Full width at half maximum

Scattering and reflection

Another challenge in quantitative absorbance studies is a result of Lambert-Beer's law idealising the processes that occurs during a transmission measurement by considering exclusively light attenuation by molecular absorption. Especially losses due to scattering are not included in the theoretical description [6]. In general, scatter deflects part of the light beam so that it is not reaching the detector at all or at least not in a straight way. This scattering may occur at any objects in the beam path, e.g. at optical components such as lenses or shutters but also particulate contaminations on surfaces or in the sample solution itself can be the origin of optical scattering. Moreover, any dielectric surface partly reflects impinging light, causing an attenuation of e.g. up to 10% for glass surfaces. Therefore, in absorbance studies the unattenuated intensity I_0 is determined typically in a way that the beam path contains all elements of the actual measurement setup, including solvent and sample container, except for the actual analyte substance. This procedure guarantees that the measurement parameters are exactly the same as in the actual measurement. For classical photospectrometers using a cuvette as a sample container, this is accomplished either by providing a separate reference channel or by two successive measurements.

In instruments using microtiter plates as sample containers the situation is more complex. To facilitate an absorbance measurement in any of the plate wells the I_0 measurement is performed "through the air" by default. This means, the complete sample is bypassed and the unattenuated irradiation is measured. Although widely accepted, this procedure is not suited to determine absolute concentrations with highest accuracy. Thus, it is recommended to use a well in the microtiter plate that contains the solvent only, e.g. buffer, to measure I_0. In that case, the instrument takes the absorbance of that particular well as the I_0 value to calculate the concentration. This approach guarantees much more reliable data. However, the accuracy of a cuvette-based measurement still cannot be obtained. The reason for this restriction is due to the surface tension of the different solvents.

In Figure 4 different surface tensions are indicated as they may occur for different solvents. Due to the differences in curvature the effective light pathway varies for the 4 examples shown in Figure 4.

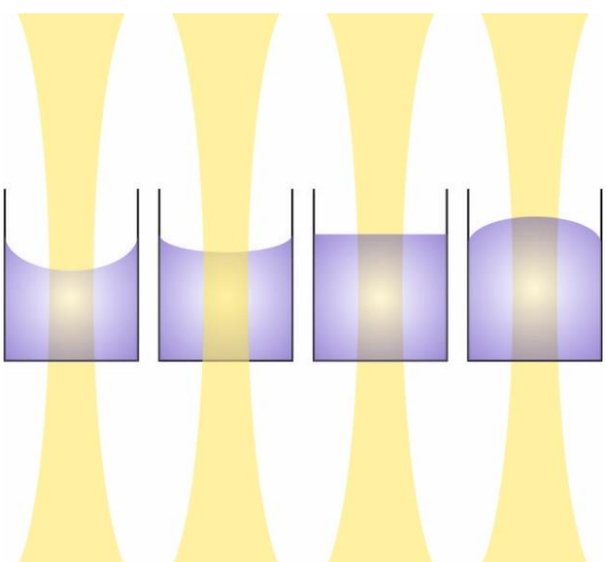

Figure 4:
Effect of surface tension on the effective light pathway.

Using the absorbance values to determine the concentration of the analytes in the sample wells would lead to inaccurate results, because the value for the path length d in Lambert-Beer's law is not constant. Whereas the mean filling height can be determined by electronic means, curvatures in the liquid column cannot be corrected for. These curvatures, however, act like an optical lens that collimates or defocuses the light, respectively.

These effects can hardly be foreseen and corrected for. Thus, we recommend for concentrations measurements with highest accuracy to record a calibration curve prior to the actual measurement, using a dilution series of the analyte. Alternatively, Berthold Technologies microplate readers also offer the possibility to use conventional cuvettes for absorbance measurements. Due to precisely parallel aligned glass surfaces the geometric limitations as discussed above are no longer valid and highly accurate absorbance measurements can be performed.

Important considerations summary

- Choose a measurement wavelength where the absorbance is maximal to obtain the highest sensitivity
- Use monochromatic light or light with a spectral bandwidth of <10-15 nm only
- The solution to be analysed should be diluted, resulting in absorbance values $A \lesssim 1.5$ or concentrations of about $c \lesssim 0.01$ mol/l
- Avoid scattering of light, e.g. due to pollutions in the sample
- When using microtiter plates run a buffer control
- For highest accuracy record a calibration curve prior to the actual measurement

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Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

Application Note

PROTEIN QUANTIFICATION USING THE APOLLO LB 917 ABSORBANCE READER

Abstract

Some of the most popular methods for protein quantification are BCA, Coomassie Plus (Bradford), and Lowry, and they are most often measured using a microplate absorbance reader. The suitability of the Apollo LB 917 to perform those assays was assessed and confirmed. Furthermore, the Apollo is easy to program and performing the assays is simple and straightforward.

Introduction

The quantification of the protein content of a sample is necessary for many applications, such as enzymatic activity measurement, western blotting, development of immunoassays, and more.

There are many protein quantification methods available, and the choice of the most suitable method depends on a range of factors: from the protein to be quantified (protein mixture or specific proteins), the presence of detergents in the buffer, the amount of sample available, and others. Some of the most popular methods are BCA, Coomassie Plus (Bradford), and Lowry [1]. All three are colorimetric methods which are quantified using absorbance in the visible range and are most often performed in microplates.

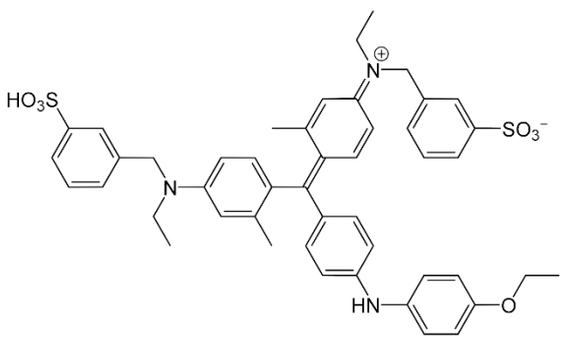

***Figure 1**: Coomassie brilliant blue G-250, the binding dye for the Bradford assay.*

The BCA assay combines the reduction of Cu^{+2} to Cu^{+1} by the peptide bonds in protein in an alkaline medium with the colorimetric detection of the cuprous cation (Cu^{+1}) using a reagent containing bicinchoninic acid (BCA) and is measured at 562 nm.

The Bradford/Coomassie Plus assay uses Coomassie dye. When it binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm, with a concomitant colour change from brown to blue.

Authors

Francesc Felipe – Berthold Technologies
http://www.berthold.com/bio

The Lowry assay is based on two chemical reactions: the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein, and reduction of the Folin & Ciocalteu's phenol reagent, which yields a purple colour. Absorbance of the coloured solution is read at a suitable wavelength between 500 nm and 800 nm.

This application note demonstrates the suitability of the Apollo absorbance reader for protein quantification.

Apollo Absorbance Reader

Reliable ELISA & absorbance analysis in just 6 seconds

The Apollo Absorbance Reader is an intuitive and reliable filter-based microplate reader that can be used for a wide variety of research and routine applications. The system has been designed to help you accelerate your research combining fast measurement of 96-well plates in just 6 seconds with intuitive 7-inch colour touchscreen stand-alone operation.

Apollo benefits at a glance:

- Wide variety of applications: With its wavelength range from 340 to 750 nm it is ideal for ELISA, cytotoxicity assays, protein colorimetric assays, endotoxin assays and more.
- Fast measurement: Read your plate in just 6 seconds (fast mode).
- Trust your data: Accuracy of ±0.005 OD or ±1% (whichever is greater) at 0-3 OD.
- Intuitive operation: 7'' colour touchscreen and preprogrammed protocols simplify operation of the system.
- Large dynamic range: The system´s dynamic range of 4.0 OD is large enough to cover any assay´s requirements.

Materials

- Apollo LB 917 absorbance reader from Berthold Technologies (73664-10) equipped with 560, 595 and 650 nm filters.
- Pierce™ BCA Protein Assay from ThermoFisher Scientific™ (23227).
- Total Protein Kit, Micro Lowry, Peterson's modification from Sigma-Aldrich® (TP0300).
- Pierce™ Coomassie Plus (Bradford) Assay Kit from ThermoFisher Scientific™ (23236).
- Pierce™ Bovine Serum Albumin Standard Ampules, 2 mg/mL (23209).
- 96-well clear microplates from ThermoFisher Scientific™ Nunc (269620).
- Deionized water (to prepare BSA standards).
- Pipettes and pipette tips (various models and volumes).

Methods

All reagents were prepared following the manufacturer's instructions. A standard curve of BSA was prepared with the following concentrations: 2000 (Standard 1), 1500 (Standard 2), 1000 (Standard 3), 750 (standard 4), 500 (Standard 5), 250 (Standard 6), 125 (Standard 7) and 25 (Standard 8) µg/mL, and deionized water was used as blank. In addition, 1 unknown sample was measured: it was prepared by mixing equal volumes of the 750 and 1000 µg/mL standards and had hence a theoretical concentration of 875 µg/mL. All measurements were performed in duplicate.

Each assay was performed following the manufacturer's instructions (see below). The BCA assay was measured at 560 nm, as a 562 nm filter was not available.

BCA protocol

1. Pipette 25 µL of each standard or sample to each well
2. Add 200 µL of working reagent to each well
3. Shake 30 seconds
4. Cover plate and incubate 30 minutes at 37° C
5. Measure absorbance at 560 nm

Micro Lowry protocol

1. Pipette 50 µL sample or standard to each well
2. Pipette 50 µL Lowry reagent to each well
3. Incubate 20 minutes at RT
4. Pipette 25 µL Folin & Ciocalteu's phenol reagent to each well and mix immediately
5. Incubate 30 minutes at RT
6. Measure absorbance at 650 nm

Coomassie Plus (Bradford) protocol

1. Pipette 10 µL of standard or sample to each well
2. Pipette 300 µL Coomassie Plus reagent to each well
3. Shake 30 seconds
4. Incubate 10 minutes at RT
5. Measure absorbance at 595 nm

The positions of standards, blank and samples were entered in the Apollo built-in software. The plate layout used is displayed in Figure 2:

***Figure 2**. Plate layout programmed in the Apollo software. Wells in green are the standards (Standard 1 to Standard 8), wells in white are the blanks, and the rest of the plate is left for samples. The unknown sample was pipetted in duplicate in wells C3 and D3.*

To perform the calculations, 4 Parameter Logistic was selected as curve fitting algorithm (labelled "Logistic" in the software). Calculated protein concentrations were directly obtained from the built-in software. Data were exported to CSV format using a USB drive for further processing (reporting and publication of results). Curve fitting graphics were exported to BMP format using a USB drive.

Instrument settings

Common settings:

- Mode: Normal
- Plate: 96 wells (see Figure 2)
- Shake: Off
- Preprocess: Off
- Kinetic: Off
- Curves: On
- Interpret: Off
- Quality: Off

BCA settings:

- Filter: 650/Off

Coomassie Plus settings:

- Filter: 595/Off

Micro Lowry settings:

- Filter: 560/Off

Results

As expected, the standards produced curves of slightly different shapes for each assay, with shapes very similar to the examples displayed in the user manual of each assay. In all cases, correlation was excellent (R^2 ≥0.997). All values were within the dynamic range of the Apollo reader (0.0-4.0 OD). Obtained standard curves are displayed in Figure 3.

The calculated concentrations of the unknown sample were quite close to the expected concentration: within a 5% difference for the Coomassie Plus and Lowry assays, and a 6.84% lower for the BCA assay. Numerical results are summarized in Table 1.

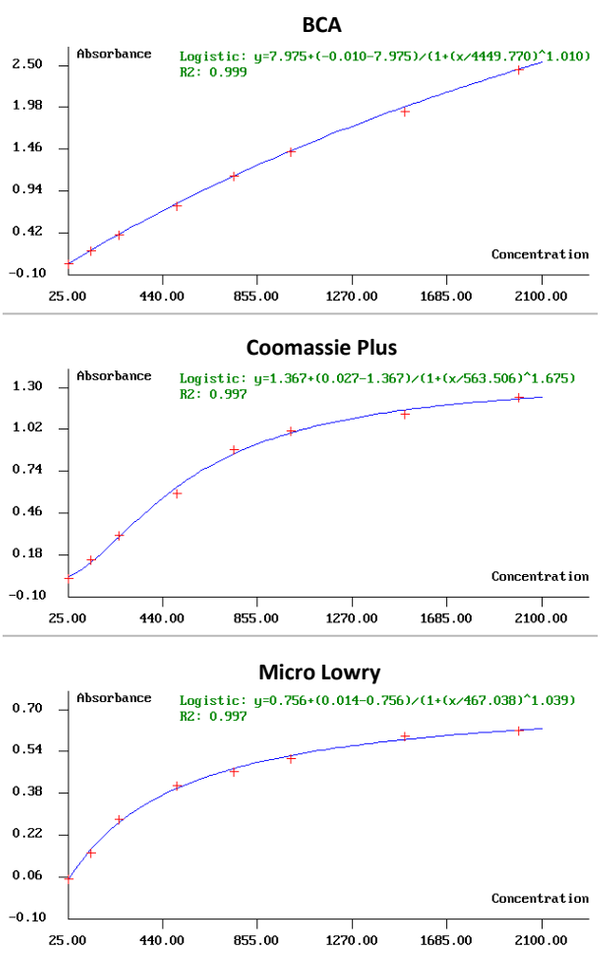

***Figure 3**. Standard curves generated by the Apollo built-in software using the Logistic function (4PL). Graphs were exported as BMP files.*

Standards			
µg/mL	OD		
BSA Standard	BCA	Coomassie Plus	Lowry
2000	2.447	1.232	0.620
1500	1.931	1.122	0.599
1000	1.421	1.012	0.513
750	1.124	0.888	0.463
500	0.751	0.590	0.409
250	0.392	0.307	0.281
125	0.197	0.146	0.151
25	0.031	0.022	0.052
0	0.000	0.000	0.000
Unknown sample (875 ug/mL)			
	BCA	Coomassie Plus	Lowry
OD	1.208	0.955	0.510
Calculated concentr. (µg/mL)	815.1	915.1	918.8
Difference from expected (%)	-6.84	4.58	5.00

***Table 1.** Numerical values obtained for the standards (top) and unknown sample (bottom). All values are the average of duplicate measurements.*

As the BCA assay should be measured at 562 nm but only a 560 nm filter was available, the impact of this difference in the measurement wavelength was assessed. To test this, the same plate was measured with a monochromator-based microplate reader (Tristar 5), both at 562 nm and at 560 nm. Measurements at 560 nm were a 0.86% lower as average than the measurements at 562 nm (data not shown).

Conclusions

Using the Apollo LB 917 to measure the BCA, Coomassie Plus/Bradford and Lowry assays was simple and straightforward thanks to the intuitive built-in software, which allowed to easily set measurement settings, standard positions and concentrations, and curve fitting functions. The standard curves produced were similar to the examples provided with the kit's instructions and had a high correlation coefficient. Although a 562 nm filter was not available to measure BCA, the impact of measuring it at 560 nm instead was negligible. The results obtained for the unknown sample were very close to the expected values, with differences which can be explained mostly by pipetting error.

Concerning the comparison of the different assays tested, the closest concentration to the expected one was obtained with Coomassie Plus (only 4.58% higher than expected) and the most different with BCA (6.84% lower than expected), but it's difficult to assess at what extent these differences are due to the performance of the assay or to pipetting error.

The obtained results confirm that the Apollo LB 917 is a suitable reader for convenient and reliable measurement of protein concentrations.

References

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Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

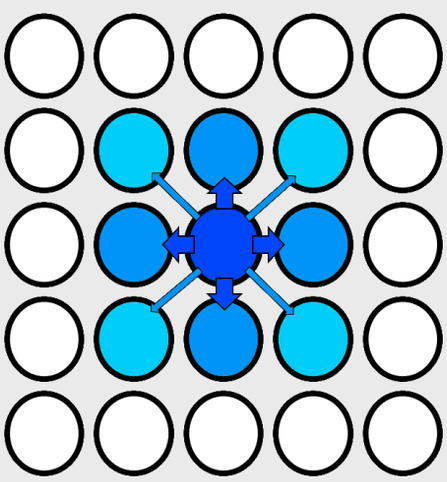
Magazine

MINIMISING CROSSTALK

Today, luminescence as well as fluorescence assays and reagents are getting widely used in drug discovery, cosmetics, food and environmental testing, as well as many other areas of research. Modern microplate readers can detect very low quantities of light. This has led to an increasing focus on optical crosstalk.

What is crosstalk?

In the field of microplate measurements, crosstalk is whether or not a signal produced in a well of a microplate stays in that particular well without interfering with the signals of the adjacent wells. This can result in artificially elevated signals and presents a major issue when analysing low signal samples adjacent to high-signal samples. The typical workaround is trying to separate high-signal samples from the other ones to avoid crosstalk as much as possible, but this is wasting precious wells and is not an option when your throughput is high. So, what should you consider to minimize the crosstalk in your assay?



Assay influence on crosstalk

In general, the signal strength of your assay is an important consideration. Assays like chemiluminescent assays can generate relatively high signals, leading to significant crosstalk. Also, the wavelength of the light emitted is another

Authors

Thomas Schild – Berthold Technologies GmbH
http://www.berthold.com/bio

consideration to make. The shorter the wavelength of the emission, the higher is its energy level, which will make your assay more prone to crosstalk.

On the other hand, fluorescence assays are less prone to crosstalk, as normally only the measured well is illuminated by excitation light and fluorescence lifetime is very short (in the order of nanoseconds for prompt fluorescence assays).

There are two other important factors that affect crosstalk: on the one hand, the microplate used can have a significant impact on the level of crosstalk you will observe; on the other hand, there are several instrument design considerations that will affect the level of crosstalk.

Influence of microplates on crosstalk

Microplates for use in assay development and High Throughput Screening are usually manufactured from a polystyrene polymer. For white plates an optical brightener is added, titanium dioxide. This is to increase the reflectivity with a very smooth bright surface inside the wells. The following summarizes general plate design considerations that will impact the level of crosstalk you will observe:

Microplate plastic and colour

- Black microplates exhibit the lowest amount of crosstalk, followed by light-grey microplates
- White microplates crosstalk potential varies with the level of titanium dioxide used, but is typically medium
- Clear microplates give the highest crosstalk, as light can freely cross the walls, and should never be used for any luminescence assay

Microplate design

The well-geometry, the distance from well to well as well as the thickness of the wall of adjacent wells and the bottom of the well are having an influence on the level of crosstalk one will observe.

Berthold Technologies provides microplates best suited for luminescence, fluorescence, and absorbance measurements.

Instrument influence on crosstalk

High-performance microplate readers typically have technologies that reduce crosstalk to negligible levels. In general, the alignment of the various components of the optical pathway has been optimised to avoid that signal from adjacent wells is getting measured in the well that is analysed. In addition, physical masking devices have been applied to isolate the well getting measured from adjacent wells.

At Berthold Technologies we have patented designs to eliminate signals from not-measured wells that are positioned automatically according to the plate

format selected. This feature is in all our microplate readers. The crosstalk specification for most of our plate readers is 10^{-6}. This equates to 1,000,000 counts in a particular well and only 1 count in all adjacent wells. In plate readers from other manufacturers, we have seen counts vary from 100 to over 5000 counts in the adjacent wells. As you can see, smart instrument design can effectively minimize the physical crosstalk between adjacent wells to virtually no sample-to-sample crosstalk.

Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

Application Note

DUAL-LUCIFERASE® REPORTER (DLR™) VALIDATION OF THE CENTRO LB 963

Abstract

The Dual-Luciferase Reporter® (DLR™) Assay System from Promega is a popular commercial assay using firefly luciferase as reporter for a promoter of interest and renilla luciferase as internal control reporter. Promega's DLR assay had been validated on the Centro LB 963 microplate luminometer.

Introduction

Reporter genes have become an invaluable tool in studies of gene expression. They are widely used in biomedical and pharmaceutical research and also in molecular biology and biochemistry.

The main purpose of the reporter gene assay is to investigate the promoter of a gene of interest, i.e., the regulation of its expression. This can be done by linking the promoter of interest to an easily detectable gene, such as the gene for firefly luciferase, which catalyses a reaction that produces light.

Reporter gene assays based on luminescence are very popular for several reasons:

Authors

Francesc Felipe, Timo Staab
Berthold Technologies GmbH
www.berthold.com/bio

- They have a high sensitivity (typically 10-10,000 times higher than methods absorbance or fluorescence).
- Most cell types do not have endogenous luciferase activity.
- Luminescence assays have a large dynamic range.
- They are quick to perform.
- Their costs are relatively low.

In order to minimize experimental variability caused by random factors (such as differences in cell number, cell viability or transfection efficiency), dual reporter systems can be used. In such systems, two different luciferase reporter enzymes are expressed simultaneously in each cell: one is controlled by the promoter of interest and the other one is controlled by a promoter that gives a stable expression and does not change with the experimental conditions, which is used as internal control for normalization [1].

The Dual-Luciferase Reporter® (DLR™) Assay System from Promega is a popular commercial assay using firefly luciferase as reporter for the promoter of interest and renilla luciferase as internal control reporter. In a first step, a reagent containing the substrate of firefly luciferase (LAR II reagent) is dispensed, and the firefly luminescence is measured; in a second step a reagent is dispensed, which quenches the firefly luminescence and starts the renilla luminescence (Stop & Glo® reagent), and the renilla luminescence is measured [1].

Centro LB 963 Microplate Luminometer

The Centro is a high-performance, easy to use microplate luminometer for both, flash, and glow luminescence applications.

The optimized design provides excellent performance and flexibility:

- Superior sensitivity (<1.8 zmol firefly luciferase)
- Negligible crosstalk (10^{-6})
- Built-in shaker
- JET-injectors
- Temperature control (model-dependent)
- Ergonomic design
- Automation compatibility

The Centro is ideally suited for all luminescent reporter gene assays, immunoassays (LIA, ILMA), cell-based, and biochemical assays.

To meet your compliance requirements, a set of validation tools and optional software providing 21 CFR part 11 compliance are available.

Materials

- Centro LB 963 Microplate Luminometer from Berthold Technologies (Id. Nr. 70325-10).
- Renilla luciferase, 0.78 mg/mL, from Promega (Part # E359).
- Firefly luciferase - QuantiLum® Recombinant Luciferase, 12.4 mg/mL, from Promega (Cat# E1701).
- Bovine Serum Albumin, Acetylated (BSA), 10 mg/mL, from Promega (Cat# R3961)
- Dual-Luciferase® Reporter Assay System from Promega (Cat# E1910)
- 96 well white plates from Costar (Cat # 3912).
- Nuclease-Free Water from Promega (Cat# P1193)
- Pipettes and pipette tips (various volumes).

Instrument settings

The following settings were programmed in the ICE software and used in all tests:

1. Dispense 100 µL LAR II, injector 1, speed: middle, by well.
2. Delay 2 s, by well.
3. Endpoint luminescence, counting time 10 s, by well.
4. Dispense 100 µL Stop & Glo®, injector 2, speed: middle, by well.
5. Delay 2 s, by well.
6. Endpoint luminescence, counting time 10 s, by well.

BERTHOLD

Methods

Reagents and luciferase dilutions were prepared according to the manufacturer's instructions.

In order for a microplate reader to be validated for the DLR™ Assay System, the instrument has to pass 3 different tests:

1. Tubing adsorption: this test shows whether the tubing used in the instrument injectors has an effect on the DLR assay over time. The test passes if signal after 10 minutes with reagents standing in the tubing is ≥95% of the signal before incubation.
2. Firefly luciferase quenching: this test shows if the injection system provides enough mixing for signal of firefly luciferase to be quenched. The test passes if firefly signal after dispensing the Stop & Glo® reagent is quenched at least 10,000 times.
3. Consistency: this test shows if results are consistent in 24 replicates with 2 different firefly:renilla ratios (50:1 firefly:renilla and 50:1 renilla:firefly). The test passes if CV of the measurement is ≤5% both for firefly and renilla luciferases.

Results and conclusion

Results of all 3 tests are summarized in **Table 1**.

Test 1 passes if signal after 10 minutes with reagents standing in the tubing is ≥95% of the signal at time = 0. Results obtained with the Centro were 98.5% for the firefly measurements and 102.7% for the renilla measurements, indicating virtually no tubing adsorption.

Test 2 passes if quenching is at least 10,000. Quenching obtained with the Centro was above 100,000 (table 1), exceeding the required quenching by more than 10 times. Looking at the values of individual wells, the lowest quenching obtained was 23,435 (data not shown), also exceeding the required value of 10,000.

Test 3 passes if CV of the measurement is ≤5%. CV of the measurements performed with the Centro was <3% in all cases.

Taking all results into account, the Centro meets or exceeds all parameters required for the validation of the Dual-Luciferase Reporter® (DLR™) Assay System and is thus an excellent instrument to perform reporter gene assays using this system.

Test 1		
	Firefly	Renilla
Average t=0	123.695.545	3.536.743
Average t=10 min	121.830.671	3.631.599
Activity (%)	**98,5**	**102,7**
Test 2		
	Firefly	Renilla
Average	132.401.691	1.679
Quenching		**109.811**
Test 3		
50:1 Firefly:Renilla	Firefly	Renilla
Average	131.495.776	3.526.940
Std. Dev	3.118.096	101.652
CV (%)	**2,37**	**2,88**
50:1 Renilla:Firefly	Firefly	Renilla
Average	2.591.584	64.433.715
Std. Dev	56.652	3.211.533
CV (%)	**2,19**	**1,95**

Table 1: Results of tests 1, 2 and 3. Number of replicates: 12 in test 1, 24 in tests 2 and 3. Results in RLU unless otherwise indicated

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Not for use in diagnostic procedures.

Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

Application Note

MONITORING MOLECULAR INTERACTIONS USING THE PROMEGA NANOBRET™ PROTEIN:PROTEIN INTERACTION SYSTEM AND THE TRISTAR MULTIMODE MICROPLATE READERS

Abstract

Proteins perform a variety of functions in living cells and organisms and work together in a complex and coordinated way. The understanding of protein function requires analysis of protein interactions within the cellular context. The proximity-based BRET (Bioluminescence Resonance Energy Transfer) assay is an established technique to study protein:protein interactions, signal transduction pathways and receptors. The NanoBRET™ assay is a progression of this technology resulting in increased signal and lower background. For the detection of the signal, a suitable plate reader is required, such as the Tristar Multimode Microplate Readers developed by Berthold Technologies. In order to confirm the compatibility of the Promega NanoBRET™ System with the Tristar microplate readers, HEK293 cells were transiently transfected with the NanoBRET™ Positive Control Vector. It encodes a NanoLuc® and HaloTag® fusion protein that ensures energy transfer, which was detected using the NanoBRET™ Nano-Glo® Detection System. The results confirm that Tristar Multimode Readers are suitable devices for use with the Promega NanoBRET™ Protein:Protein Interaction System.

Muhammad Rafehi

Pharmaceutical Institute, University of Bonn, Germany

Introduction

The NanoBRET™ System is an experimental set-up that enables the monitoring of protein interactions in live cells. The underlying principle is that of bioluminescence resonance energy transfer (BRET): The two proteins that are investigated are tethered to a NanoLuc® fusion protein as the energy donor and a fluorescently labelled HaloTag® fusion protein as the energy acceptor. In the presence of appropriate substrate, the NanoLuc® luciferase forms a luminescent product, which in turn excites the fluorescent protein acceptor if the two proteins are in close proximity. The optimized blue-shifted NanoLuc® donor paired with the red-shifted HaloTag® acceptor minimizes spectral overlap within the assay, resulting in an improved signal-to-background ratio when calculating the NanoBRET™ ratio.

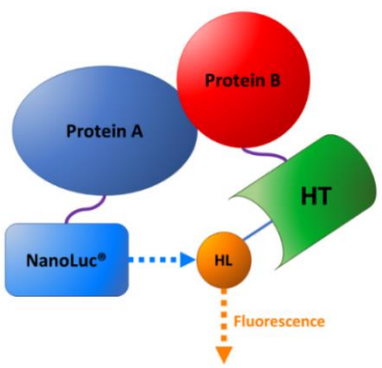

Figure 1: Basic principle underlying the NanoBRET™ protein:protein interaction assay. Protein A is tethered to a bioluminescent protein donor that excites the fluorescent acceptor fused to protein B if both proteins are in close proximity. HL: HaloTag® NanoBRET™ 618 ligand; HT: HaloTag® protein.

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- Time-Resolved FRET (TR-FRET / HTRF®)
- Fluorescence Polarization
- AlphaScreen®
- Top and Bottom Reading
- Incubation

Materials

- Berthold Technologies TriStar² S LB 942 Multimode Microplate Reader with extended spectral range luminescence module
- Promega NanoBRET™ positive control vector (catalogue no. N1581)
- Promega NanoBRET™ Nano-Glo® Detection System (catalogue no. N1661)
- FuGENE® HD Transfection Reagent (catalogue no. E2311)
- Sterile six-well plate with lid (Greiner 657160)
- White, opaque, sterile 96-well microplate (Berthold 51838)
- Human embryonic kidney (HEK) 293 cells and cell culture equipment and reagents
- Dulbecco's Modified Eagle's Medium (DMEM; Gibco catalogue no. 11995)
- Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies catalogue no. 11058021)
- Fetal bovine serum (Sigma catalogue no. F0804)
- Penicillin/streptomycin solution (Sigma catalogue no. P4333)
- 0.05 % Trypsin/EDTA (Invitrogen catalogue no. 25300)
- DPBS (Invitrogen catalogue no. 14190)
- Dimethylsulfoxide (DMSO; Sigma catalogue no. 2650)

Instrument settings

- Excitation filter: none
- Emission filter donor: 460-70*
- Emission filter acceptor: 600-LP*
- Reading mode: luminescence
- Counting time: 1 s

* Included in NanoBRET™ filter package, ID-Number 63140.

Methods

Two days prior to the assay, cultured HEK 293 cells were trypsinized and diluted in cell culture medium to a final density of 4×10^5 cells/ml. Subsequently, 2 ml of cell suspension (800,000 cells) was plated into a well of a sterile six-well plate and incubated for 5 h at 37 °C and 5 % CO_2. 2 µg of Transfection Carrier DNA was mixed with 0.002 µg of the NanoBRET™ Positive Control Vector diluted in water and 100 µl of Opti-MEM® I Reduced Serum Medium was added to the transfection mixture. Next, 8 µl of FuGENE® HD Transfection Reagent was added and the mixture incubated at room temperature for 10 min. The transfection mixture was given to the cells, and these were incubated for 20 h at 37 °C and 5 % CO_2. The transfection mixture was subsequently removed and the cells rinsed with 1 ml of phosphate-buffered saline. The cells were trypsinized and resuspended in 2 ml of cell culture medium. Following centrifugation at 125 × g for 5 minutes, the supernatant was discarded and the cells resuspended in Opti-MEM® I Reduced Serum Medium with 4 % fetal bovine serum at a final density of 2×10^5 cells/ml. To half of the cells, 1 µl of 0.1 mM HaloTag® NanoBRET™ 618 Ligand per milliliter of cells (100 nM final concentration) was given, while the other half of cells were treated with 1 µl of DMSO per millilitre of cells (0.1 % DMSO final concentration) as no-ligand control. 100 µl of both cell suspensions were dispensed into separate wells of a sterile white 96-well microplate and the plate was incubated for 20 hours at 37 °C and 5 % CO_2. Subsequently, a 5x solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM® I Reduced Serum Medium was prepared and 25 µl given to each well. The plate was shaken for 30 sec and luminescence measured using the Tristar² S LB 942 Multimode Microplate Reader.

Results

To determine the corrected NanoBRET™ ratio, the luminescence signal for 3-4 wells each of HaloTag® NanoBRET™ 618 Ligand and DMSO as no ligand control was measured. The results are shown in Table 1.

Note: absolute values can be different depending on the specific Tristar reader used (for example, they will be lower using a Tristar 3 or 5 compared to the TriStar^2 S), but ratios obtained with the same filters and settings should be very similar.

	Donor Emission (mean RLU)	Acceptor Emission (mean RLU)	NanoBRET™ ratio (mBU)
Ligand	9,696,667	2,133,333	219.6
No ligand	15,600,000	94,980	6.1
Corrected NanoBRET™ ratio			**213.5**

Table 1: Luminescence values determined for the HaloTag® NanoBRET™ 618 Ligand and DMSO (no ligand). Data are mean values of 3-4 wells.

To account for donor-contributed background or bleedthrough, the NanoBRET™ ratio for the no-acceptor (DMSO) control is subtracted from the NanoBRET™ ratio calculated for the HaloTag® 618 Ligand. The resulting corrected NanoBRET™ ratio is 213.5 mBU. The Z' factor calculated from these results is 0.97, indicating a highly robust assay.

The assay was also tested using the standard luminescence module instead of the extended spectral range one; in this case the emission filter donor used was ID-Number 40272 (460-25), as it provided the best results (data not shown). Even though the NanoBRET™ ratio was lower than with the extended spectral range module (98.8 mBU instead of 213.5), the Z' factor was also excellent at 0.96.

These results demonstrate the high performance of the Tristar² S LB 942 Multimode Microplate Reader for the Promega NanoBRET™ Protein:Protein Interaction System.

Conclusions

A corrected NanoBRET™ ratio of 213.5 mBU and a Z' factor of 0.97 were obtained, confirming that the Berthold Technologies Tristar² S LB 942 Multidetection Microplate Reader is ideal for detection of the Promega NanoBRET™ Protein:Protein Interaction System. Both the standard and the extended spectral range luminescence modules are suitable for this application, but the extended spectral range one provides the best results.

As obtained ratios must be very similar using other microplate readers of the Tristar series, it can be concluded that all readers in the Tristar series are suitable for the measurement of the Promega NanoBRET™ Protein:Protein Interaction System.

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Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

Application Note

QUANTIFYING DNA WITH THE QUANT-IT™ PICOGREEN® dsDNA KIT AND BERTHOLD TRISTAR MULTIMODE READERS

FAST, SIMPLE, AND ACCURATE NUCLEIC ACID QUANTIFICATION

Abstract

DNA quantification is mostly done by absorbance at 260 nm, but suffers from low throughput, sensitivity, and specificity. The Quant-iT™ PicoGreen® dsDNA reagent is specific for dsDNA and is suitable for microplate readers. In combination with the Tristar 3 and Tristar 5 Multimode Microplate Readers, it allows the specific quantification of dsDNA in 96-well plates achieving a limit of detection below 0.1 pg/µL.

Introduction

DNA quantification is an important pre-analytical method, which is of great importance for many molecular biological analysis methods and can even determine their success. It is also a routine technique in procedures for translational research such as Next-Generation Sequencing (NGS), Polymerase Chain Reaction (PCR) or Real-Time PCR (quantitative PCR; qPCR), cloning or transfection, which initiates the subsequent workflow.

Francesc Felipe
Berthold Technologies GmbH
www.berthold.com/bio

The most popular DNA quantification methods are based on UV-Vis- or fluorescence spectroscopy. Both methods have advantages and disadvantages.

Quantification using absorbance

Absorbance at 260 nm has been the method of choice for routine quantification of DNA and RNA since decades. It is simple and convenient to use as no further sample treatment (other than DNA extraction) is required. However, it is not very specific (it measures all nucleic acids as a whole) and it is sensitive to contaminants, so it demands very pure DNA to be accurate. Many of those contaminants can be estimated by measuring the absorbance of the sample at wavelengths other than 260 nm (usually at 230, 280 and 340 nm).

Absorbance of DNA samples at 260 nm is currently most often measured using a microvolume spectrophotometer, but it is also possible to use a microplate reader. Microplate readers can measure many samples in a short time (typical plate formats are 96- and 384-well), but they require larger sample volumes for the measurement than a microvolume spectrophotometer (up to 50 µL in standard 96-well plates, less in other microplate formats). However, microvolume microplates are available which use small sample volumes (usually 2 µL). While they don't

have as many sample positions as standard microplates (normally 16 instead of 96), they offer a good compromise between sample volume and throughput.

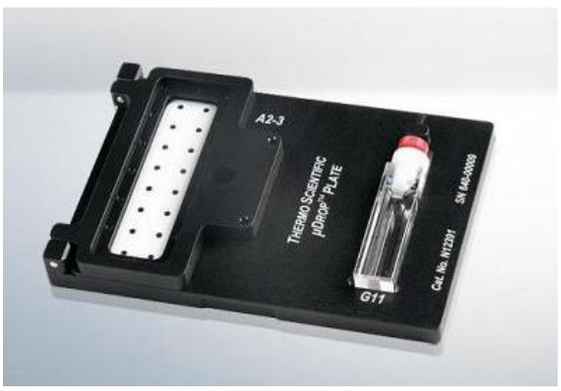

Figure 1: The µDrop plate can be used for DNA quantification using absorbance in a microplate reader using only 2 µL of sample and features also a cuvette port for increased flexibility.

Quantification using fluorescence

The use of fluorescent dyes permits the quantification of DNA with much higher sensitivity than measuring absorbance of DNA itself (typically 10-1000 times higher, depending on the specific methods compared). In addition, specific dyes can be used to stain only specific types of nucleic acid, such as dsDNA or RNA, thereby increasing the specificity of the quantification and reducing the effect of contaminants. However, fluorescence-based methods are more expensive than measuring absorbance at 260 nm, often require a standard curve to be prepared, and do not provide a direct estimate of the presence of contaminants (which may be important, for example, to evaluate the possible effects on downstream methods). Fluorescence measurement is performed using a microplate reader or a single-tube fluorometer.

Quant-iT™ PicoGreen® dsDNA reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution. Detecting and quantitating small amounts of DNA is extremely important in a wide variety of biological applications. The PicoGreen dsDNA quantitation reagent and kits are ideal for PCR-based assays, microarray samples, DNA damage assays, enzyme activity assays, genomic DNA quantitation, measuring dsDNA in complex mixtures, and viral DNA quantitation.

In this Application Note we report the suitability of the Tristar Multimode Microplate Readers to quantify dsDNA using the Quant-iT™ PicoGreen® dsDNA reagent and the recommended settings for this method.

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- Top and Bottom Reading
- Incubation

Materials

- Tristar 3 Multimode Microplate Reader from Berthold Technologies (Id. Nr. 69173-30).
- Tristar 5 Multimode Microplate Reader from Berthold Technologies (Id. Nr. 69185-15).
- Quant-iT™ PicoGreen® dsDNA Assay Kit from Invitrogen™ (Cat. # P7589).
- Black 96-well microplates from Berthold Technologies (Id. Nr. 23302).
- Tubes of various volumes.
- Pipettes and pipette tips (various volumes).

Instrument settings

- Reading mode: Fluorescence Endpoint
- Excitation filter: 485/14
- Emission filter: 535/25
- Counting time: 0.1 s
- All other settings with default values

Methods

Reagents were prepared following the manufacturer's instructions. Using the DNA standard included with the kit, two different standard curves were prepared: a high range standard curve, which could be used to quantify high concentration samples, and a low range standard curve, which could be used to quantify low concentration samples.

Equal volumes of standard and Quant-iT™ PicoGreen® reagent were mixed for each standard point. The final concentrations of each curve were the following ones:

- High range: 1, 10, 100, 250, 500, 750 and 1000 pg/µL dsDNA.
- Low range: 0.025, 0.25, 2.5, 5, 10, 20 and 25 pg/µL dsDNA

The mix was incubated for 5 minutes at room temperature in the dark. 200 µL of each mix were pipetted in triplicate in the wells of a black 96-well TE buffer was used as blank.

The plate was inserted in the multimode reader and measured with the settings detailed above. Blank values were subtracted from the values of the standard. Data were exported from the ICE software to xls format, and standard curves were drawn in Excel.

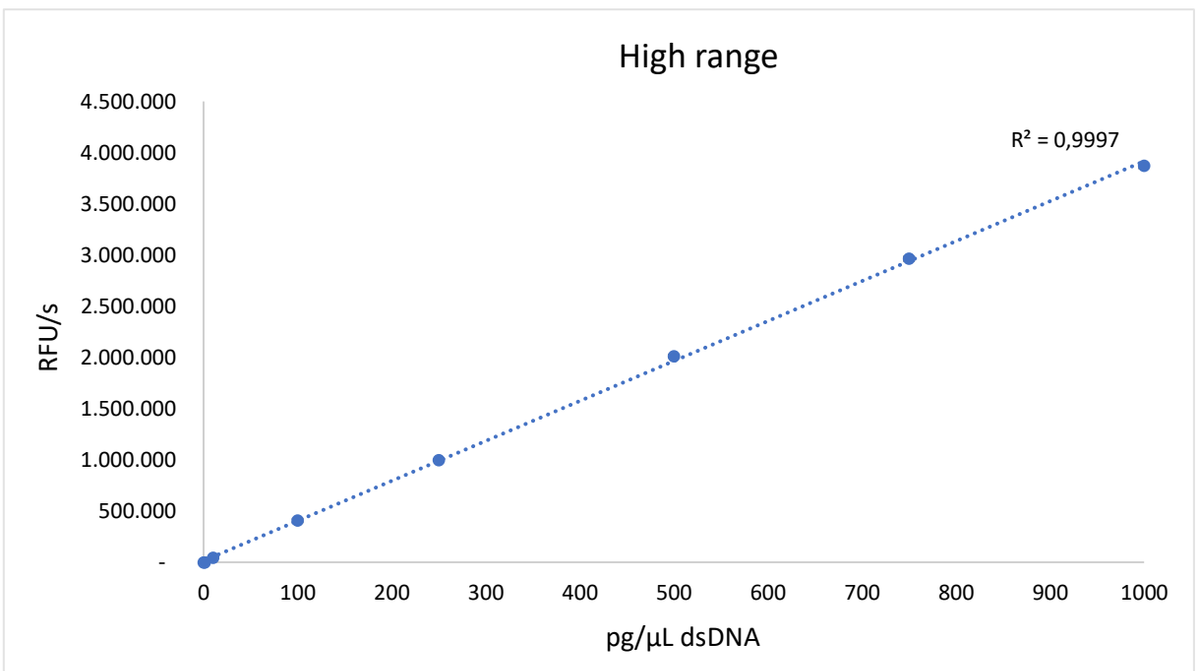
Results

The High Range and Low range standard curves of the Tristar 3 are displayed in Figure 1. Both curves exhibit excellent linearity. Standard curves looked very similar in the Tristar 5 (data not shown).

The calculated Limit of Detection was 0.069 pg/µL dsDNA (13.8 pg/well) for the Tristar 3 and 0.094 pg/µL dsDNA (18.8 pg/well) for the Tristar 5.

When the measurement was performed in 384-well plates, the calculated Limit of Detection was 0.272 pg/µL dsDNA (19.0 pg/well) for the Tristar 3 and 0.029 pg/µL dsDNA (2.0 pg/well) for the Tristar 5.

With the settings used, a full 96-well plate can be measured in 33 seconds.



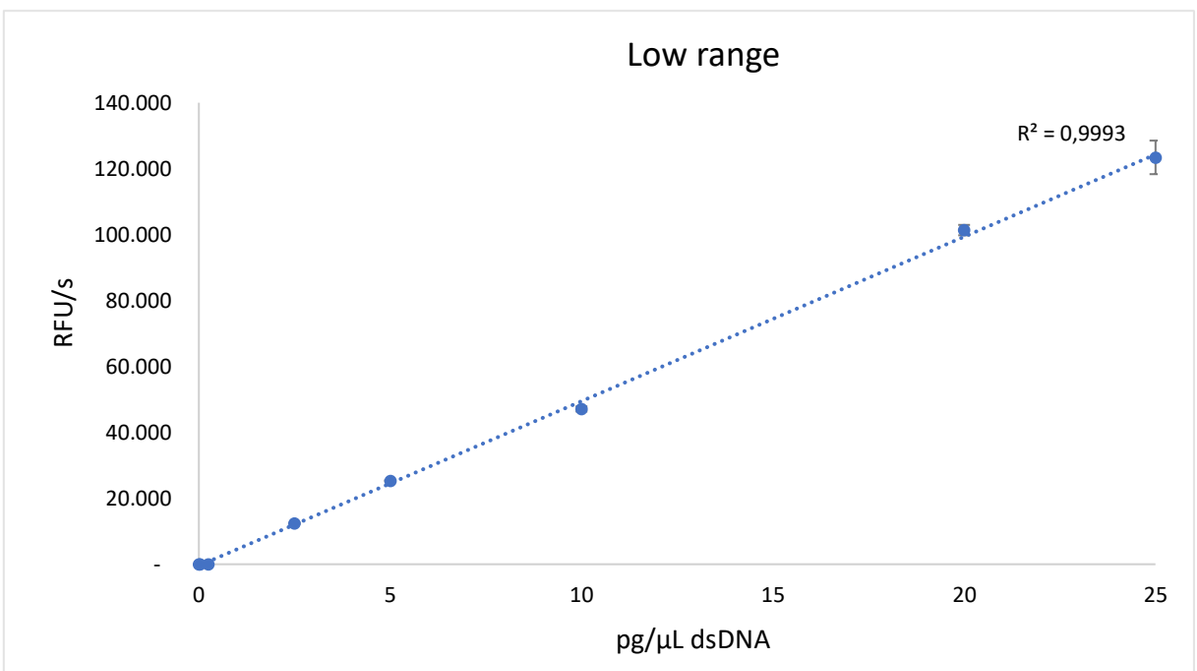


Figure 1: High (top) and Low (bottom) range dsDNA standard curves measured with a Tristar 3 Multimode Reader with standard fluorescein filters. All measurements in triplicate.

Discussion and conclusions

Quantification of dsDNA is very often performed using a microvolume spectrophotometer. However, this method suffers from low throughput, as samples have to be measured one by one, and of low sensitivity, as the limit of detection is typically 2 ng/µL dsDNA (2000 pg/µL). While using the Quant-iT™ PicoGreen® dsDNA reagent involves some increase in costs and preparation time (as a standard curve has to be prepared and measured), when used to measure dsDNA concentrations in the Tristar Multimode Microplate Readers, it allows to measure 96 wells under a minute while achieving a limit of detection below 0.10 pg/µL. This represents an improvement of sensitivity over the detection limit of a microvolume spectrophotometer of 20,000 fold. The limit of detection obtained with Tristar instruments is clearly better than the typical result for microplate readers which, according to the kit insert, is of 0.25 pg/µL.

The results demonstrate the high performance of the Tristar Multimode Microplate Readers for the quantification of dsDNA using the Quant-iT™ PicoGreen® dsDNA reagent.

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75323 Bad Wildbad
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Phone: +49 7081 177 0
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Magazine

WHAT ARE THE BENEFITS OF TOP READING VS BOTTOM READING?

Modern fluorescence microplate readers offer typically the option to read the fluorescence signal both, from the top and from the bottom. Top reading instruments rely on measuring reflected light above the wells. Bottom reading platforms illuminate and detect the sample from below to measure the absorption or fluorescence/ luminescence emission. When should you use each mode?

Top reading is usually more sensitive

Top reading usually provides better signal-to-noise ratios for solution-based assays such as DNA quantification or protein quantification. In general, reading fluorescence from the top is more sensitive than reading from the bottom. This is a result of the light being attenuated and scattered by the plastics of the well-bottom which can increase the background and decrease the efficiency of the measurement. Thus, the quality and thickness of the bottom clear plastic and the type of fluorophore are important considerations when designing your assay.

Authors

Thomas Schild – Berthold Technologies GmbH
http://www.berthold.com/bio

Microplate type is critical for bottom reading

Another factor to consider is of course the clear-bottomed plate type you are using – are you using an opaque-walled tissue-culture plate? Is the plate white or black? and, is the clear bottom made of either polystyrene or glass?

Answering the questions above is closely linked to the light wavelength of interest. If you are trying to detect visible light (350-900 nm) typically a plastic bottom will do. However, detecting below 350 nm requires a bottom made of UV-transparent material, for example quartz or modern COP/COC polymers. As a last resort, the more expensive optical glass bottom plates can be used allowing for detection from both, the top and bottom, as well as microscopic viewing of the cells with low background.

The key difference between white and black plates is their reflective properties. White plates reflect light and will maximize the light output signal. On

the other hand, black plates absorb light and reduce background and crosstalk. Thus, white plates are commonly used for luminescent assays while black plates are used for fluorescent assays.

When does bottom reading provide better results?

- When working with adherent cells that grow at the bottom of the plate and either express, bind, or secrete a fluorophore at or close to their bottom attachment side only.
- Chemotaxis assays monitoring the migration of cells through a membrane. This is analysed typically by a combination of both, top and bottom reading.
- Certain cell-based assays (e.g. GeneBLAzer® or QBT™ Fatty Acid Uptake) cannot be read from the top as the optical properties of the buffers used in the assay interfere with the excitation or emission of the fluorophore.

If still in doubt which reading technology to use, you can program your microplate reader to run a protocol including both, top and bottom reading and compare the results to make your final choice.

Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

www.berthold.com

APPLICATION NOTE

AUTOMATION OF THE ENZO APP ΔC31 ELISA KIT

Introduction

Unlike the β-amyloid and tau fibril formation pathways leading to amyloid plaques and tangles, APP ΔC31 reveals an alternative, unique pro-apoptotic mechanism leading to Alzheimer's disease. APP ΔC31 is the stable amyloid precursor protein fragment created from a caspase cleavage event of the APP695 molecule at Asp664 leaving a smaller 31-residue intracellular fragment. Both the APP ΔC31 and 31-residue fragments are pro-apoptotic and are present in 4-fold greater levels in Alzheimer's disease patients. Given that the smaller 31-residue fragment has a short half-life and is difficult to measure, the use of this APP ΔC31 ELISA provides for the first time a sensitive research tool to measure the levels of the APP caspase cleavage from tissue, biological fluids, and cells.

The APP ΔC31 ELISA kit is a complete, colorimetric, immunometric immunoassay kit for the quantitative determination of human APP ΔC31 in cell lysate, serum, plasma, and cerebral spinal fluid samples with results in just 2 hours.

Materials

- Crocodile miniWorkstation (Berthold Technologies)
- MikroWin module, quantitative and qualitative data reduction package (Titertek-Berthold)
- APP ΔC31 ELISA kit (#ADI-900-227, Enzo)
- ddH_20, pipette and tips, sample tubs, Vortex mixer

Methods

All reagents were brought up to room temperature for 30 minutes prior to use. According to manufacturer instructions wash buffer and 8 standards were prepared.

We changed the first steps in the assay procedure to have it more convenient for automation purposes: At the beginning 50µl of samples and standards in duplicate were transferred to the assay plate. For standard 0 (S0) 50µl of assay buffer was pipetted.

Automation with the Crocodile miniWorkstation was performed as shown on table 1 starting in dispensing antibody.

Adjust aspiration depth in your assay setup as to avoid cross-contamination through direct contact between aspiration needles and well surfaces.

The plate was read at 450 nm absorbance. After blanking the reader against the average blank OD, a standard curve was calculated by using MikroWin and fitted with four parameter algorithms.

Results

Standard	Concentration (pM)	OD average (Minus Blank OD)
Blank	-	0
S0	0	-0.002
S1	1500	2.135
S2	750	1.242
S3	375	0.602
S4	187.5	0.293
S5	93.75	0.139
S6	46.88	0.091
S7	23.44	0.036
S8	11.72	0.020

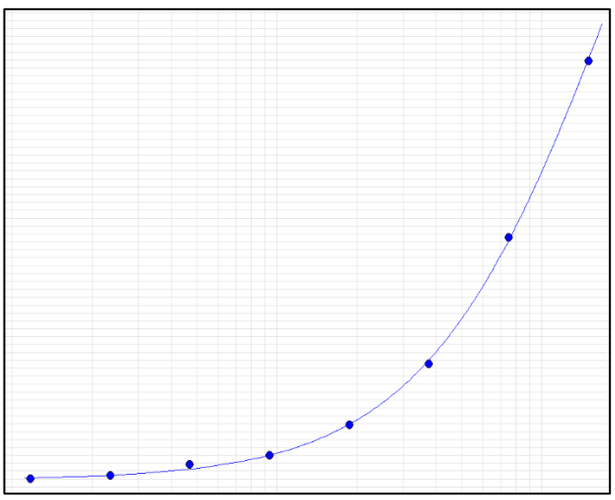

Figure 1. Standard curve fitted with four parameter algorithm. Y-axis linear, X-axis logarithmic.

Conclusion

The standard curve showed excellent fitting, so Crocodile provides a convenient and easy-to-use method for the automation of the Enzo APP ΔC31 ELISA kit. The assay procedure is extremely simple and involves only the addition of standards and controls while the instrument is processing all necessary dispense, wash, incubation and reading steps.

Changing the first steps in the assay procedure (pipetting antibody and standards/samples) doesn't affect the assay results at all.

Acknowledgement

Special thanks to Miriam Cortes-Caminero and Erica Brooks from Enzo for their support.

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Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
Phone: +49 7081 177 0
Email: bio@berthold.com

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Table 1: Assay program with Crocodile Control Software

#	Step Name	Description
1	**Prime Antibody**	**Dispensing** Volume 1000 µl Inlet 1 Label “Antibody” Method: Priming Well Count: 1
2	**Dispense Antibody**	**Dispensing** Volume 50ul Inlet 1 Label “Antibody” Method: Standard Well Count: 20
3	**Incubate 1**	**Shaking** for 01:00:00 at Incubator with 2mm Amplitude at 10 Hz
4	**Wash 1**	**Washing** Method: Standard Wash Solution Inlet: 1 Wash buffer Cycles: 3 Volume: 300 µl Delay: 2s Wait: 500ms Dispenser Depth: 1300 (Plate Offset 50) Aspiration Depth 2725 (Plate Offset 20) Sweep 5mm @1mm/s Well Count 96
5	**Prime Conjugate**	**Dispensing** Volume 1000 µl Inlet 2 Label “Conjugate” Method: Priming Well Count: 1
6	**Dispense Conjugate**	**Dispensing** Volume 100ul Inlet 2 Label “Conjugate” Method: Standard Well Count: 20
7	**Incubate 2**	**Shaking** for 00:30:00 at Incubator with 2mm Amplitude at 10 Hz
8	**Wash 2**	**Washing** Method: Standard Wash Solution Inlet: 1 Wash buffer Cycles: 3 Volume: 300 µl Delay: 2s Wait: 500ms Dispenser Depth: 1300 (Plate Offset 50) Aspiration Depth 2725 (Plate Offset 20) Sweep 5mm @1mm/s Well Count 96
9	**Prime Substrate**	**Dispensing** Volume 1000 µl Inlet 3 Label “Substrate” Method: Priming Well Count: 1
10	**Dispense Substrate**	**Dispensing** Volume 100ul Inlet 3 Label “Substrate” Method: Standard Well Count: 20
11	**Incubate 3**	**Shaking** for 00:30:00 at Incubator with 2mm Amplitude at 10 Hz
12	**Prime Stop**	**Dispensing** Volume 1000 µl Inlet 4 Label “Stop” Method: Priming Well Count: 1
13	**Dispense Stop**	**Dispensing** Volume 100ul Inlet 4 Label “Stop” Method: Standard Well Count: 20
14	**Measure**	**Reading** Single Wavelength Filter 1: 450nm (Pos:2) Well Count: 96

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Tristar Multimode Readers

Ease of use: ★★★★★ **After-sales service:** ★★★★★ **Value for money:** ★★★★★

Rating: ★★★★★

"We are very happy with the Tristar! Very efficient and high technical skills services."

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Centro LB 963 Microplate Luminometer

Ease of use: ★★★★★
After-sales service: ★★★★★
Value for money: ★★★★★

Rating: ★★★★★

"Easy to use luminometer with a very long lifetime. Great support from field staff."

Achmet Imam Chasan, University Clinics of Muenster

The Apollo Microplate Absorbance Reader

Ease of use: ★★★★★
After-sales service: ★★★★★
Value for money: ★★★★★

Rating: ★★★★★

"Great results, must buy product."

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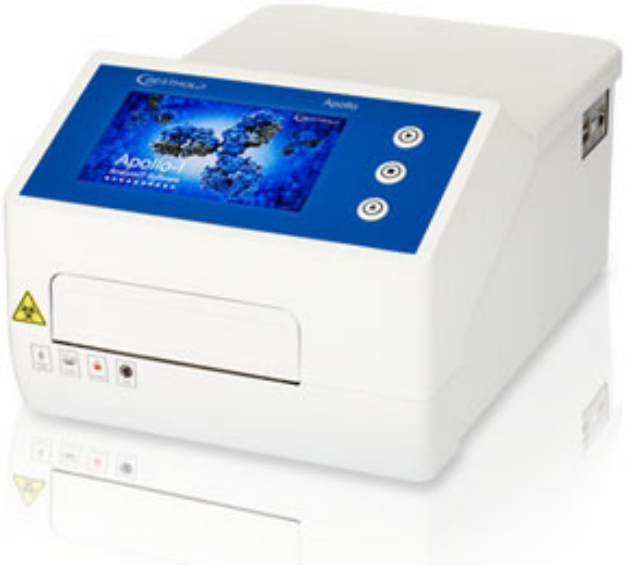

Crocodile 5-in-one LB 925 ELISA miniWorkstation