Tech Note

Fluorescence Determinations Using the Extended Dynamic Range Measurement Option of the Synergy™ Neo2

Results over 7 Orders of Magnitude in a Single Read Step

Research microplate readers used for low light measurements such as fluorescence or luminescence typically employ photomultiplier tubes (PMTs) to amplify and measure the emitted light source. Readers use physical parameters such as probe z-height and well centering, along with collection time to optimize the signal collection. In addition to the physical parameters these devices also allow the researcher to alter the gain setting on the PMT as a means to increase or decrease the signal amplification. One of the more difficult concepts to grasp when converting an absorbance based assay to one that uses fluorescence or luminescence based detection is the selection of the gain or sensitivity settings associated with a PMT.

Fluorescence and luminescence readers use PMTs to transduce photons into an electrical current that can be measured. PMTs are vacuum phototubes that are typically constructed with a glass housing containing a photocathode, several dynodes, and an anode (Figure 1). Incident photons strike the photocathode material, which is usually a conducting layer on the inside of the entry window of the device. Electrons are ejected from the surface as a consequence of the photoelectric effect. These electrons are directed by the focusing electrode toward the electron multiplier, where electrons are multiplied by the process of secondary emission.

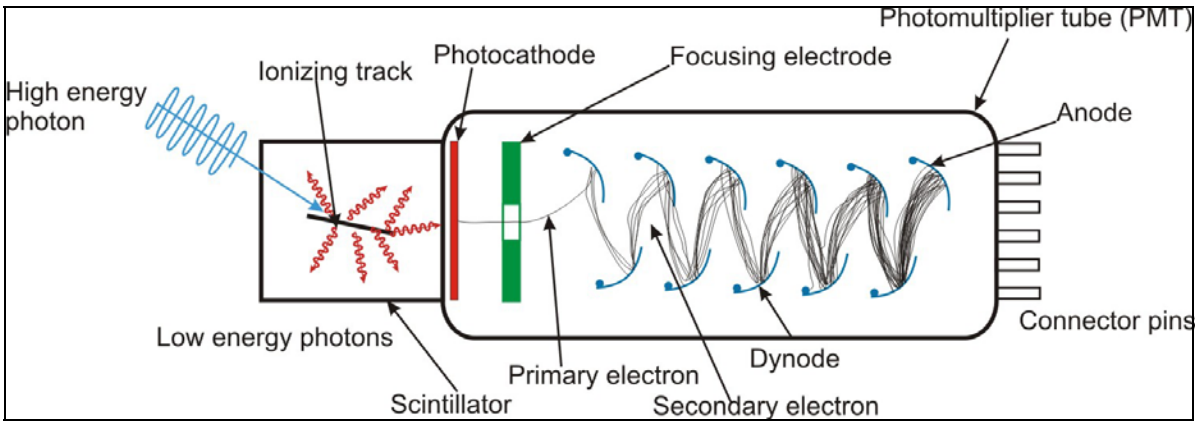

Figure 1. Schematic drawing of a photomultiplier tube transducing a photon to an electron followed by multiplication of the primary electon into a measurable current at the anode.

The electron multiplier consists of a number of electrodes called dynodes. Each dynode is held at a more positive potential than the preceding one. A small group of primary electrons is created by the arrival of a group of initial photons on the cathode. As the primary electrons move toward the first dynode they are accelerated by the electric field, with each arriving with kinetic energy imparted by the potential difference. Upon striking the first dynode, more low energy electrons are emitted, and these electrons are in turn accelerated toward the second dynode. The geometry of the dynode chain is such that a cascade occurs with an exponentially-increasing number of electrons being produced at each stage. This last stage is called the anode. This large number of electrons reaching the anode results in a sharp current pulse that is easily detectable.

The degree of signal amplification is proportional to the voltage potential applied to the PMT. For low signal detection a high voltage is required to amplify the signal sufficiently, while high light levels requires less voltage. As stated previously, with many PMT based devices the voltage is variable and can be set by the researcher. This feature is usually referred to as the gain or sensitivity setting. In addition to different gain settings resulting in different response values, PMTs have variable responses to different light wavelengths. For these reasons, light detection, as compared to absorbance determination, has no true standard and measurements are considered relative to one another.

With most PMTs a single gain setting can return signal values over 4-5 orders of magnitude. Determinations that have signals ranging greater than 5 decades typically utilize multiple determinations with different PMT gain settings to address the different signal outputs. Because interpolation of standard curves to determine unknown sample concentrations are only valid with readings made with the same PMT gain setting, multiple standard curves or sample dilutions may be necessary to coordinate unknown sample results with the appropriate standard curve. For a fixed incubation assay with a stable signal, reading the samples multiple times at different gain settings is a viable option. However, when one does not have sufficient information regarding the signal or the signal changes substantially over time with many kinetic assays, the possibility of signal over- or under-range is a definite possibility. In order to alleviate this problem BioTek has developed an extended dynamic range PMT gain feature for their newest readers. This feature seamlessly automates the use of different PMT gain settings within a single read step and selects the appropriate gain setting for the sample measurement. In addition the software automatically integrates the returned signal to seamlessly record measurements over 7 orders of magnitude.

Materials and Methods

The 96-well microplates, catalogue numbers 3915 and 3912, were purchased form Corning Life Sciences, (Cambridge, Massachusetts). Fluorescein Di-β-D-galactopyranoside (FDG), cat# F-1179 was purchased from Life Technologies. Sodium fluorescein, cat # 98155, was from BioTek Instruments. CellTiter-Glo reagent, cat. G7572, was purchased from Promega (Madison WI.), β-Galactosidase enzyme cat.# G-6008, 4-methyl umbelliferone (MUB), cat # M1381, CBB buffer capsules, cat # C3041, sodium phosphate, magnesium chloride and 2-mercaptoethanol were obtained from Sigma-Aldrich.

Sodium Fluorescein powder was dissolved in Milli-Q water at a concentration of 0.5 mg/mL. Subsequent dilutions were made in PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4, 10 mM, KH_2PO_4, 1.8 mM pH 7.4). After dilution, 100 µL aliquots were pipetted into a solid black microplate.

Fluorescence was determined using a Synergy™ Neo2 using either monochromator (Ex. 485, Em 528) or with filters (Ex 485/20, Em 528/20), configured with extended dynamic range measurement. 4-Methylumbelliferone powder was dissolved in methanol to a concentration of 1 mg/mL. Subsequent dilution were made using CBB buffer (50 mM carbonate-bicarbonate pH 9.5). After dilution, 100 µL aliquots were pipetted into a solid black microplate. Fluorescence was determined using a Synergy Neo2 using either monochromator (Ex. 360, Em 460) of with filters (Ex 360/40, Em 460/40), configured with Extended dynamic range measurement.

The β-galactosidase assay was performed according to Sanbrook et.al [1], 100 µl aliquots of samples or standards diluted in distilled water were placed in each well of a 96-well microplate. The assay was initiated by the addition of 100 µl of 2X assay buffer. Assay buffer (1X) consists of 100 mM sodium phosphate, pH 7.0; 1 mM $MgCl_2$; 50 mM β-mercaptoethanol; and 0.5 mg/ml FDG in distilled water. Assay buffer was prepared previously as a 2X stock solution and stored frozen at -20°C. Lyophilized β-galactosidase enzyme was reconstituted with distilled water to stock concentration of 1000 U/ml. Enzyme dilutions were made fresh daily and stored on ice until assayed. A series of enzyme dilutions ranging from 0 to 10 U/ml of β-galactosidase (β-gal) were then made using distilled water as the diluent.

Luminescence measurements were performed using dilutions of Adenosine triphosphate (ATP) with CellTiter-Glo reagent. ATP solutions from 0 to 100 µM were prepared with Milli Q water as the diluent. Aliquots (100 µL) were pipetted into a solid while 96-well microplate in replicates of 4 per concentration. To each, 100 µL of reconstituted CellTiter-Glo reagent was added and the plate incubated in the dark for 5 minutes. After incubation the luminescence was determined using a Synergy Neo2 Multi-Mode Microplate Reader (BioTek Instruments, Inc.).

Read Step Procedure:

The extended dynamic range setting is accessed from the Read Step by selecting the “Edit” moniker in the Read step parameters window (See arrow in Figure 2). Selection opens the Measurements Options window where a number of different read parameters can be optimized.

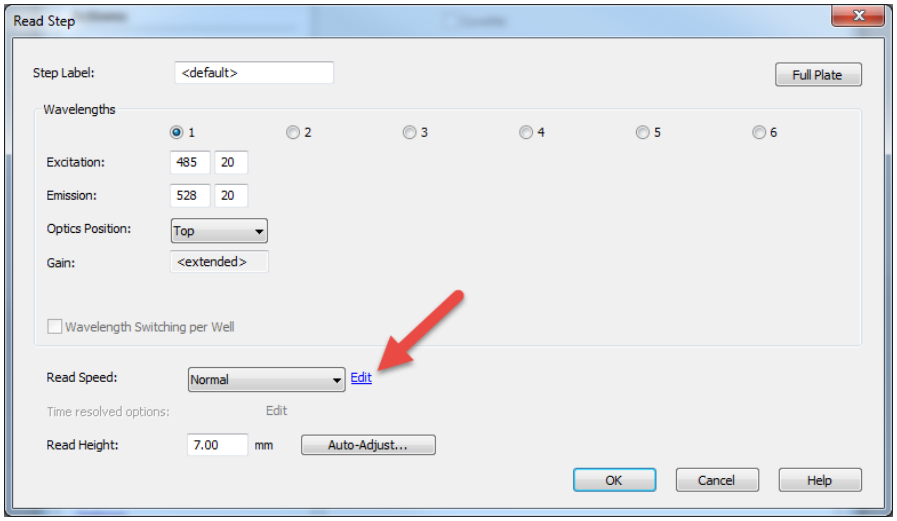

Figure 2. Selection of the Edit Moniker in the Read Step Parameters Window.

The selection of the “Extended” radio button results in a signal range from 0 to 9,999,999 (Figure 3). Once all of the measurement parameters have been selected, pressing the “OK” button saves the selections and closes the window.

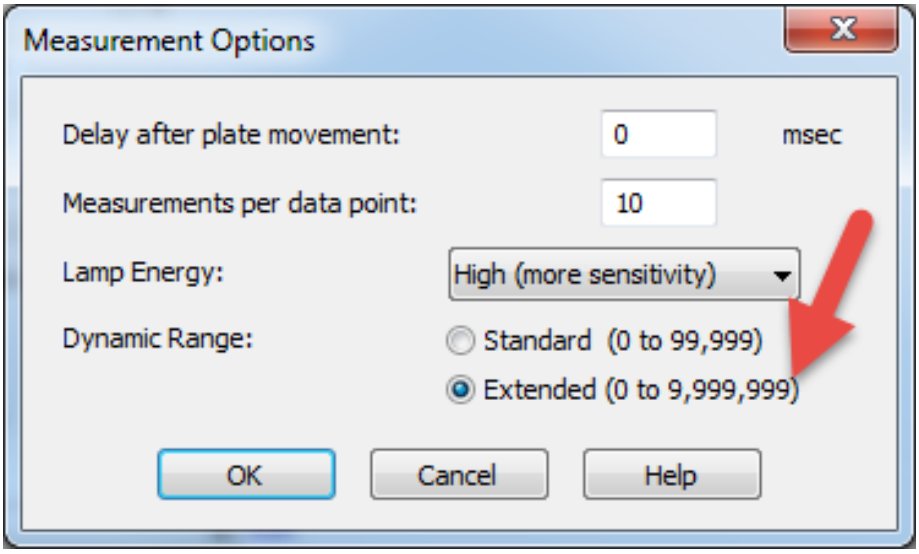

Figure 3. Selection of the extended dynamic range button in the Measurements Options Window. Among the number of read parameters can be altered is the Dynamic range, where Standard and Extended dynamic range are available.

Once the Measurements Options window is closed the user is returned to the Read step and the Gain setting should indicate that the extended dynamic range has been enabled (red arrow Figure 4).

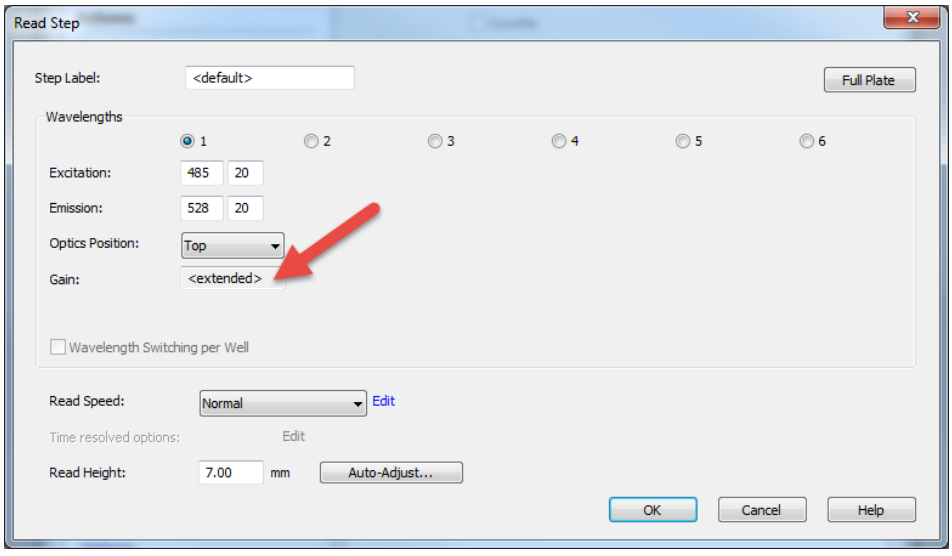

Figure 4. Extended Gain Dynamic Range Setting Selected in Read Step.

The Extended dynamic range is an option for fluorescence intensity, luminescence, and time-resolved fluorescence read methods.

Results

The fluorescence generated by a series of sodium fluorescein dilutions demonstrates the capability of the extended dynamic range feature. As demonstrated in Figure 5, fluorescence signal spanning 7 orders of magnitude can be obtained with a single determination. In this example, fluorescein concentrations ranging from 100 µM to 10 pM were determined and the relative fluorescence plotted without the need to alter PMT gain. These data had values from 1 to 10,000,000 (7 decades). During the read step the reader interpolates the signal and automatically adjusts PMT gain to accommodate for the amount of fluorescence being generated. The raw data is then seamlessly integrated such that broad concentration ranges with significant differences in detected signal can be measured on the same plate with a single plate read step. Using fixed PMT gain detection fluorescent samples require multiple determinations each at a different PMT gain setting to provide viable measurements across large concentration ranges (Figure 6). As shown in Figure 6 lower gain settings provide linear results for the higher fluorescein concentrations, while the lower dye concentrations require an increase in PMT gain to return measurable signal. From these data it is apparent that fixed PMT gain measurement requires at least two different PMT gain settings to provide measurements that span the concentration range of fluorescein.

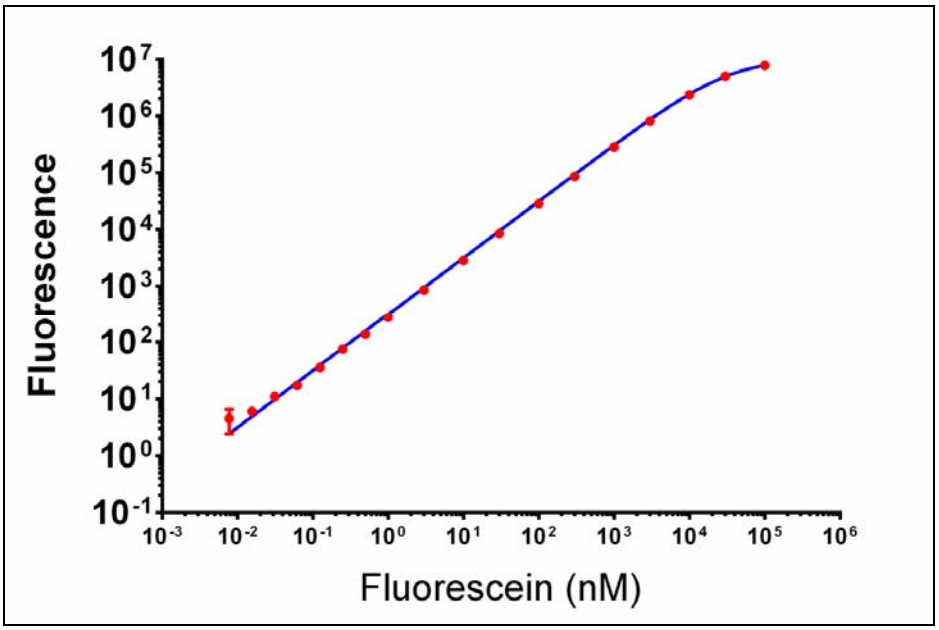

Figure 5. Fluorescein titration measured using extended dynamic range. Log-log plot of fluorescence from a sodium fluorescein titration. Data represents the mean and standard deviation of 4 determinations at each concentration.

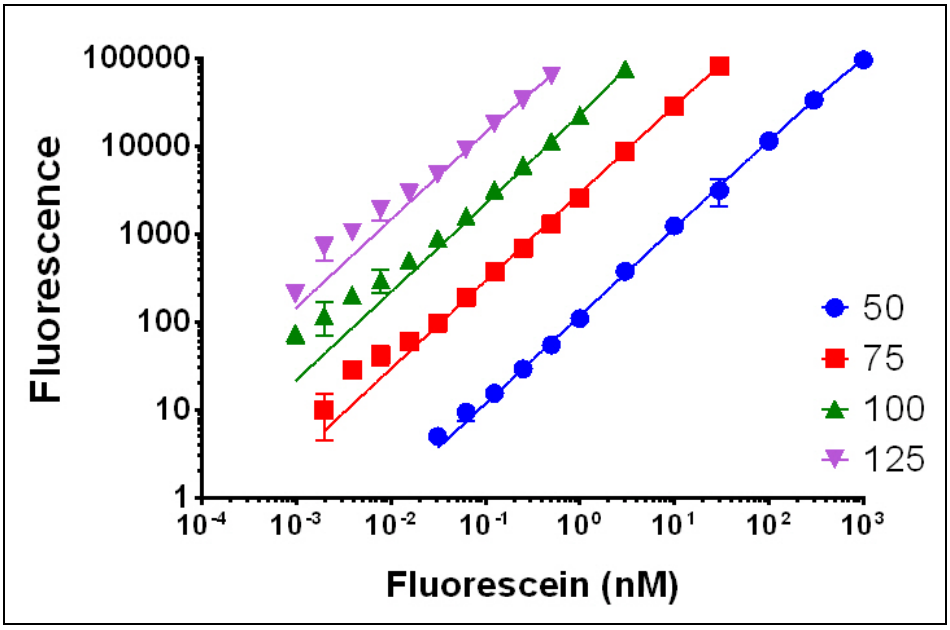

Figure 6. Fluorescein titration measured using fixed gain. The fluorescence signal at different PMT gain settings from a single sodium fluorescein titration was graphed using Log scale axis. Data represents the mean and standard deviation of 4 determinations at each concentration.

The extended range feature can be used for any wavelength that is valid for the installed PMT. As demonstrated in Figure 7, the fluorescence for titrations from 0 to 1 µM of 4-methyl-umbelliferone can be measured using a single read step. These data also demonstrate the linearity of signal from 0 to 10,000,000 RFU values using the Synergy Neo2 with the extended dynamic range enabled.

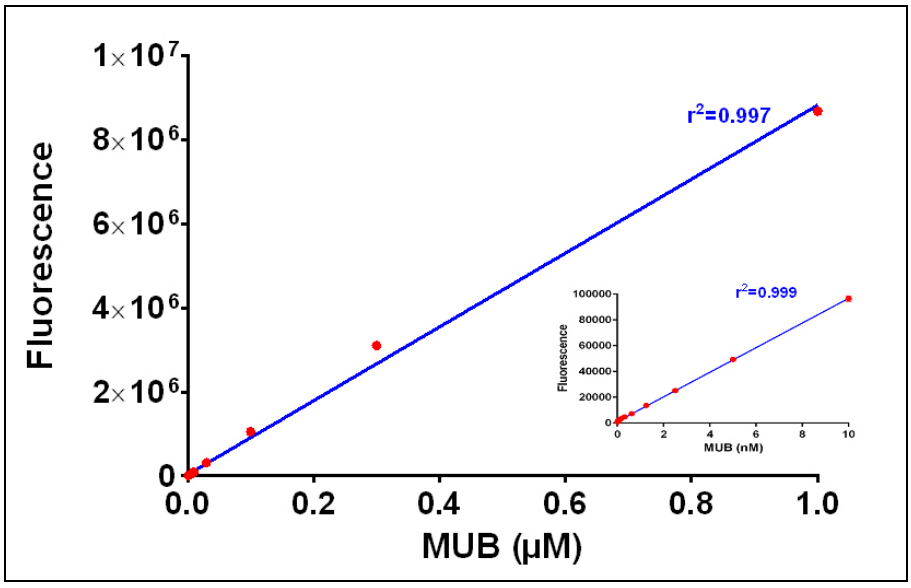

Figure 7. 4-Methylumbelliferone titration measured using extended dynamic range. The linear regression analysis of fluorescence from 0 to 1 µM concentrations of MUB was plotted on linear axis. Inset depicts results for low concentrations (0-10 nM) of MUB. Data represents the mean and standard deviation of 4 determinations at each concentration, with r^2 values of each linear regression shown.

One of the most important capabilities of the extended range gain setting is the ability to perform kinetic assays. Figure 8 demonstrates the ability of the extended dynamic range feature to accommodate the large fluorescent signal changes that can occur with kinetic fluorescent assays. β-Galactosidase enzyme catalyzes the hydrolysis of the non-fluorescent compound fluorescein Di-β-D-galactopyranoside substrate into galactose and fluorescein, which is fluorescent. The stoichiometry of this reaction is such that two molecules of fluorescein can be liberated from each molecule of FDG. With continual enzymatic activity large amounts of fluorescent product can be produced over time. Only enzyme protein inactivation or the exhaustion of the substrate will result in curtailing the increase in fluorescence. As shown in Figure 8, ß-galactosidase activity results in substantial amounts of fluorescence over time, with the increase in fluorescence being both enzyme concentration and time dependent.

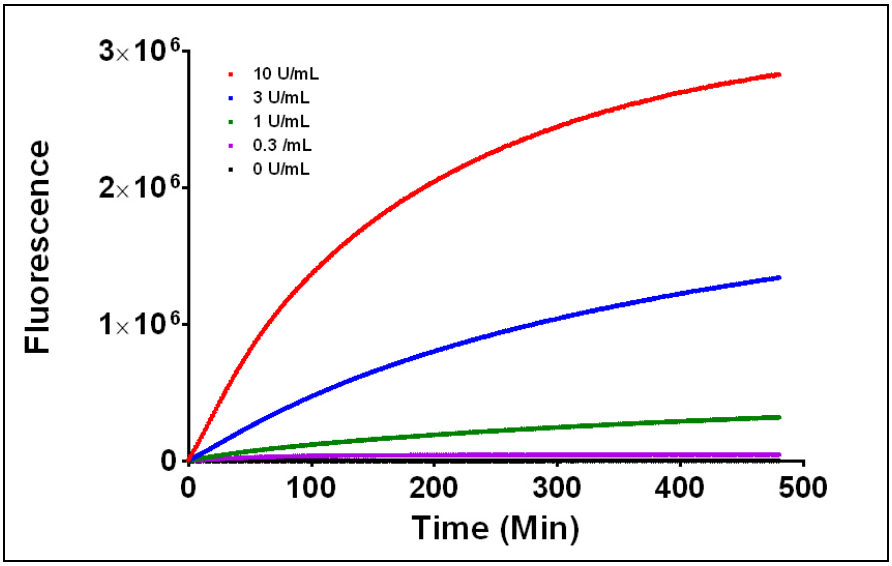

Figure 8. Kinetic measurement of fluorescence generated by β-galactosidase enzyme at various concentrations. Dilutions of β-galactosidase were incubated with FDG substrate and the fluorescence recorded every 5 minutes for a total of eight hours.

A similar expansion of the dynamic signal range is automatically performed with luminescence measurements. The data shown in Figure 9 shows the mean luminescence intensity over 10 minutes produced from incubating ATP with CellTiter-Glo reagent. The signal from these data varies over 6 orders of magnitude with ATP concentration.

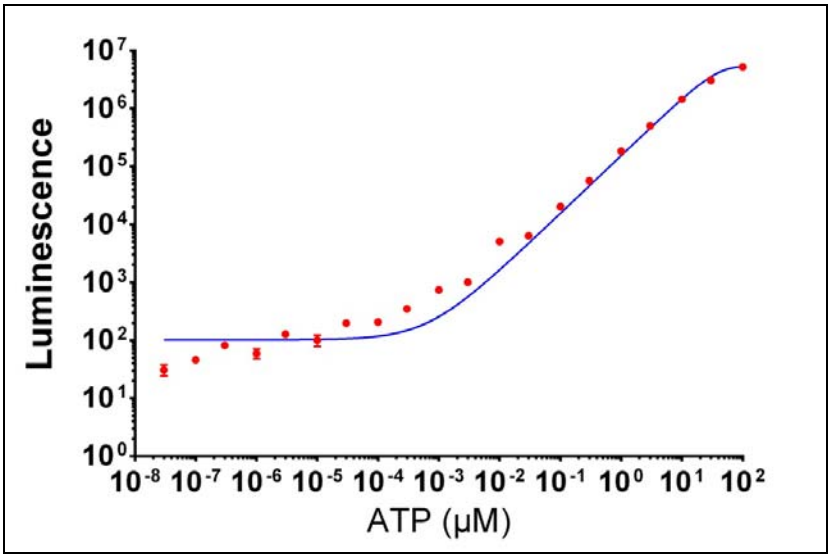

Figure 9. Luminescence of ATP titration produced with a Cell Titer-Glo assay. Various concentrations of ATP (100 µL) were incubated with Cell Titer Go reagent (100 µL) for 10 minutes and the luminescence was determined. 5-Parameter logistic fit was performed on the data. Data points represent the mean of 4 determinations.

Discussion

The electronics of photomultiplier tubes used for the majority of low level light detection such as fluorescence and luminescence requires that a fixed voltage differential is applied resulting in relative signal differences between samples. Because the assays used in many biochemical assays can result in very large signal variation it is typical for most instrumentation to allow the user to vary the voltage differential or gain of the PMT. Determining the most appropriate gain value is often made through a series of iterative empirical tests much like *Goldilocks and the Three Bears*, where first the value is too high and then the value is too low. Auto-scaling routines eliminate the need for manual intervention for PMT gain selection by scaling the gain setting to the highest well, which insures that all wells will not over or under range depending on a high well or low well scaling selection. While effective in their own right this type of scaling makes a single gain setting selection without eliminating the need to provide extended dynamic ranges to accommodate large signal ranges. These data demonstrate the utility of the extended dynamic range for measuring fluorescence over a broad range of signal.

Luminescence measurements in particular present a challenge with PMT based detection. Unlike fluorescence detection there is no excitatory light source that can be regulated to lower signal intensity with luminescence chemistry. With sufficient substrate reagents, light intensity is a function of the analyte in question. With large amounts of analyte, signal intensity can be very high, so the potential for over-ranging the dynamic range is a real possibility without an extended dynamic range feature. Many of the luminescence based chemistries involve a very rapid reaction, often occurring in a little as 10 seconds or less. For these chemistries built in reagent injectors are often used to add trigger reagent immediately before the luminescence determination. Because the chemistry consumes the analyte these reactions only allow a single measurement attempt. Failure to select the appropriate gain setting results in unusable data and the experiment needs to be repeated.

Kinetic assays often present problems when selecting a PMT gain due to the dynamic nature of the signal response. If the gain is set too high the investigator runs the risk of having the signal move outside the valid response range of the PMT for some wells. If the gain is set too low, there will be little to no response from many of the wells for the lag between the initiation of the assay and meaningful results is too long. In either scenario the assay has to be repeated with a different gain setting selected. This iterative process can be labor intensive, time consuming and frustrating. Using extended dynamic range for PMT selection eliminates the guesswork from PMT gain selection, thus decreasing not only the time and labor of developing a new assay, but also the frustration often associated with it as well.

The extended dynamic range feature of the Synergy Neo2 provides the researcher with an easier path during assay development. By expanding the valid dynamic measurement range the likelihood of a successful PMT based determination is greatly improved. This results in a savings of not only time, but also expense as reagents need not be used on repetitive experiments.

References

(1) Sanbrook J. E.F. Fritsch, and T. Maniatis (1989) Molecular Cloning: A Laboratory Manual 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

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July 1, 2021