Technical Bulletin

GENios Multilabel Reader-- Frequently Asked Questions

What Plate should I use?

· **Absorbance Measurements-** Of course one has to use clear bottom plates, and most plates on the market work fine. If reading in the deep UV (230-300 nm) you need to use a plate which will not absorb in the UV (these plates are often called "UV Transparent"). These plates are available from Costar, Nunc and Greiner. It is always best to use a flat bottom plate. You can use the round bottom plates, but then you have to make sure your plate definition file is very accurate, since if you are not measuring in the middle of each well, the path length will be different, and the accuracy of your reads will be poor. If you use a round bottom plate on a Sunrise, you need to make sure the reader is in Center mode. It is advised to NOT use the flexible plates that Falcon makes, because of the plate-to-plate variability. They also get distorted by the clipping mechanism, which can result in sample being popped out of the plate.

· **Luminescence Measurements-** In most luminescence reads you will want to use a white plate to maximize the signal. The white surface reflects the signal up to the optics. Be sure to use the black cover mask which comes with the instrument, to prevent well-to-well cross talk.

· **Standard Fluorescence Measurements-** The basic rule of thumb is to use a solid black plate. There are of course exceptions to this rule, and the best thing for the user to do is to try several different types of plate to see which works best for them. In some cases a white plate may work best. It depends upon the assay. Generally the polystyrene plates autofluorescence more at shorter excitation wavelengths. Thus, when working with short wavelength excitation probes, black plates will help minimize the background. Of course, the worst plate to use is a clear plate. These tend to produce an edge-well effect (wells around the edge of the plate will have different readings from those in the middle of the plate). This results from the reflectance of the emission off the neighboring well walls. Since the edge wells on a clear plate do not have a full set of neighbors, they reflect the light differently than the wells in the middle of the plate.

General Comments- Be aware that the PDF files provided with XFluor or Magellan software are generated for polystyrene plates. If you need to use a polypropylene plate, you should generate a new PDF. Most polypropylene plates shrink some when they are removed from the molds, and so the PDF for the polystyrene plates will not be accurate. The clipping

mechanism of the fluorescence readers can also cause some warping of polypropylene plates, resulting in poor reads. If possible use a rigid frame for holding these in the plate carrier.

What Filters should I use?

Choosing the Right one

It is important to choose the correct filter for your application; do not base your selection solely on EX and EM maxima. This is fine if your EX and EM are well separated (>100 nm apart). But this is rarely the case. More frequently the EX and EM are only about 30-50 nm apart. This short Stokes shift (distance from EX max to EM max) can lead to an overlap of the two peaks and a high background if filters centered on the peak max are used. In other words, say you want to measure Fluorescein which has a EX max at 494 nm and EX max at 518 nm. If you use a 494 and 518 filter you will get a poor reading because the EX and EM peaks overlap, and so would your filters. Thus, the optimal filters for Fluorescein are actually 485+/-10 (475-495), and 535+/-12.5 (522.5-547.5). The best filters are "upstream" and "downstream" from the peaks, and have a separation of about 27nm.

So when you choose your filters, you need to consider how close the peaks are, and it might be best to go off peak with a wider bandwidth, than to choose the peak wavelengths with very narrow bandwidths. If you can look at scans of the Ex and Em peaks, then you can judge where they can get clean signal. If the probe you are using is from Molecular Probes in Oregon, their web site (www.probes.com) has many of the spectra posted there.

Absorbance vs. Fluorescence

There is a difference between the filters used for absorbance measurements and those used for fluorescence measurements. You can not use a filter designed for absorbance for fluorescence, or visa-versa. Doing so often results in a Bright Value Error, as the wrong amount of light is passing through the filter.

EX vs. EM

In most cases there is NOT a difference between the EX and EM filters and if you want to move a filter from one slide to the other you can. However, when you place an order you should order what you need. This minimizes the movement of filters. There is always a risk of damaging a filter when it is moved from slide to slide often, so it really is better to have enough slides and filters so that you do not have to move them around frequently.

Luminescence

No filters are used in luminescence measurements. You want to collect all the light possible, and do not care about wavelength.

Gain - What is it and how do I know what setting to use?

Gain is technically referring to the amount of charge applied to the Photon Multiplier Tube (PMT). The higher the gain, the higher the voltage, and the more amplification of the signal there is. This relationship is exponential. Thus a small increase in gain will result in a large increase in the signal (all signal, including background).

Manual gain allows you to set the gain at which you want to run. This is good if you want to be able to directly compare raw data from one run to another. This is also the option you will need if you are doing a kinetic assay where the signal is increasing. If you use optimal gain on a kinetic run where the signal increases, it would reset the gain as fluorescence increased resulting in a flat curve

Optimal gain refers to the gain setting at which the sample with the highest signal on your plate will be at the maximal range of the instrument. To determine the optimal gain the instrument starts with a low gain and reads the whole plate as fast as it can (1 flash). It then takes the highest signal and adjusts the gain based on that signal, so that it is at the maximal range of the unit. It then reads it again at the new gain, and checks to see how closely it extrapolated things. If it was close enough the next read cycle with be the measurement cycle. If the extrapolation was not very close, it resets the gain based on the second reading and reads again. These cycles are repeated until the optimal gain is set. Once the optimal gain is found, it reads the high well again with the settings you chose (flashes, delays), and confirms things, and then reads the whole plate with the settings you chose.

Calculated from well will have the instrument determine the optimal gain based upon a particular well. It goes to that one well, and does a series of measurements to determine what the gain should be. If you know where your highest signal should be this is the fastest way to have the instrument determine the optimal gain setting. If you do not know where the highest signal is, then you should use the full plate optimal gain determination.

Extended Dynamic Range will first find the optimal gain for the plate, and perform a read at that gain, it then increases the gain so that the signal increases about 100 fold, and reads again. The highest concentrations will often go INVALID, or OVER. It then looks for a linear relationship between the two sets of reads. If it sees a linear relationship, then it will extrapolate between the two sets of reads, and the maximal read becomes 6.5 million instead of 65,000. This is useful when working with samples with a wide range of signal. It will not work with all assay systems. Frequently, fluorophores will self-quench at higher concentrations, and thus there will not be a linear relationship between the two sets of reads. When no linear relationship exists, the second set of reads are tossed out, and just the first set are used.

Kinetic Assays must use Manual gain. If it is an assay where the signal is decreasing over the course of the reaction you can set the gain based on the starting fluorescence. Do a

quick pre-read of the wells with the highest signal, using optimal gain so the instrument will determine what an appropriate gain setting is. Then perform your kinetic reads manually setting the gain to the value you determined in the pre-read.

If the reaction results in an increase in signal, then you need to run a control before you run the assay. This control should yield the maximal signal you expect to see, and should be allowed to run to completion so that you have as much fluorescent signal as you expect from your most active sample. Do a read on this sample using Optimal gain to determine what gain setting to use for your actual assay.

How many Flashes should I use?

The number of flashes refers to how many reads it will take of each well during 1 read cycle. The higher the number the more accurate the reading will be, but it will also be slower, and could result in photobleaching of fluorescent samples. If you choose 1-3 flashes the plate is moving while the reads are taken. If you 4+ flashes, the reader stops at each well to take the reads. If speed is important use an on-the-fly measurement with 1-3 flashes. If sensitivity and accuracy are more important, use 10 or more flashes.

How come my 260 nm DNA reads are so low?

The Pathlength in a standard cuvette is 1 cm, the pathlength in a microplate is volume and plate dependant.

- **Absorbance 260 nm:** In a cuvette a 50 ug/ml solution has an OD of 1.Thus if the path length is 0.5 cm, the OD would be 0.5 for the 50 ug/ml solution. So a 5 ug/ml solution would have an OD of 0.05 in a 0.5 cm pathlength…
- **In a microplate** (Costar standard UV) a 200 ul sample has about a 0.5 cm pathlength. The average background on these plates is 0.04-0.06, so your detection limit would be above 5 ug/ml. Of course if you decrease the volume, the detection limit goes up. So a 100 ul sample can probably only get down to 10 ug/ml or 1 ug/well.

What is the minimum volume I can use?

In general the larger the volume the better the result, but if you are limited in the volume you can use, be aware that the quality of your results may decrease.

- **Fluorescence-** The liquid needs to at least cover the bottom of the well.
- **Absorbance-** The lower the volume, the more of an effect the meniscus will have on the results. If there is a deep meniscus in some wells and not others, it can cause large variation in the reads, especially at the lower volumes.

rev 12.03

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