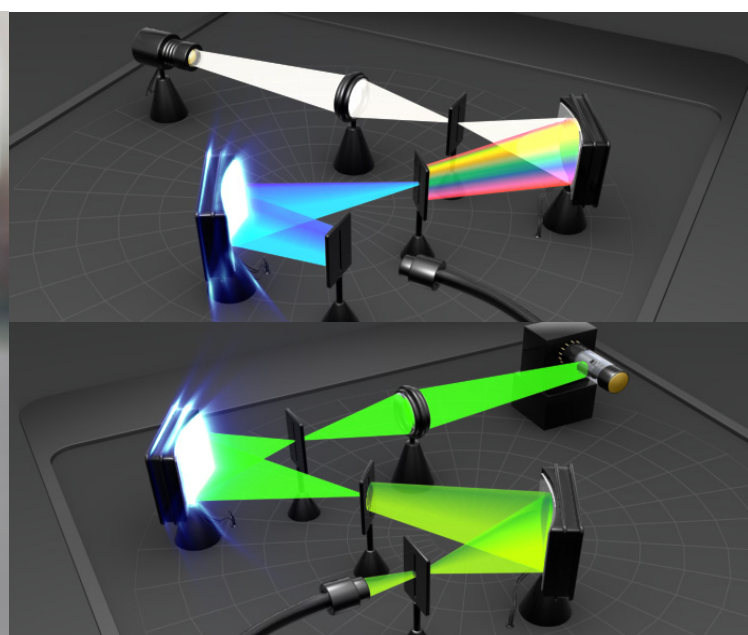
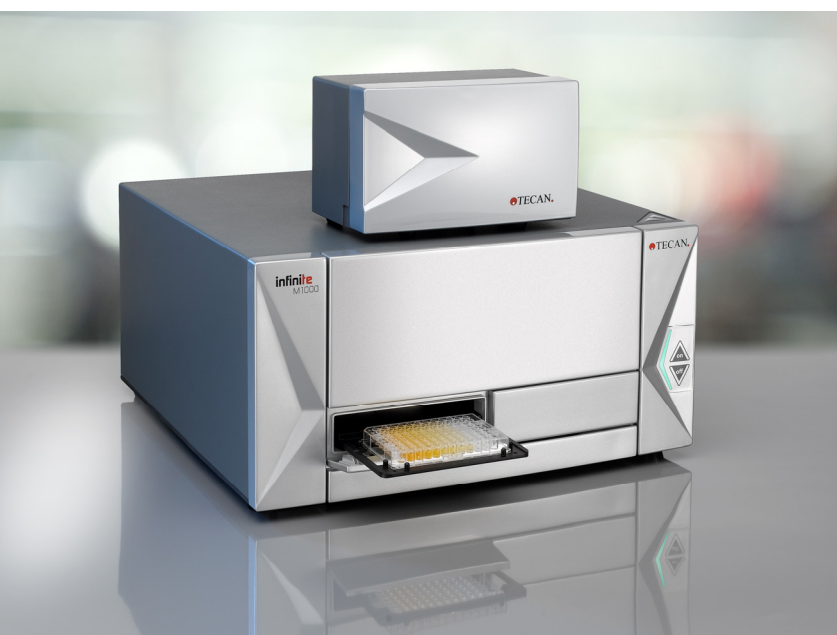


Impact of Extended Adjustable Monochromator Bandwidth in Fluorescence Based Application Technologies

Tecan Infinite[®] M1000



Introduction

This application note investigates how extended adjustable bandwidth settings maximize the sensitivity and reproducibility of the signal by fine-tuning the amount of excitation and emission light in a variety of fluorescence-based measurements.

With the Infinite M1000, Tecan launched the *premium* Quad4 Monochromators[™] technology, which provides more light to the sample and the detector and reduces stray light down to 2×10^{-7} .

The Infinite M1000 offers the possibility of continuous monochromator bandwidth adjustments for all fluorescence based measurements, i.e. fluorescence intensity (FI) including fluorescence scans (excitation, emission, 3D), fluorescence polarization emission (FP), fluorescence resonance energy transfer (FRET), time-resolved fluorescence (TRF) and time-resolved fluorescence resonance energy transfer (TR-FRET).

Tecan has a patent on the mechanism of stepless adjustable bandwidth setting (patent US 7,123,398).

The **excitation** slit width can be selected between 2.5 and 10 nm (in 0.5 nm steps) for wavelengths between 230 and 300 nm and between 5 and 20 nm (in 1 nm steps) for wavelengths between 301 and 850 nm (figure 1), resulting in 16 possible bandwidth selections for each excitation wavelength.

For fluorescence **emission** the monochromator slit width can be selected between 5 to 20 nm (in 1nm steps) for wavelengths between 280 and 850 nm and also features 16 possibilities for each emission wavelength selection.

Therefore, the user can choose between 256 bandwidth-possibilities for each fixed excitation/emission wavelength pair, offering the opportunity to optimize and run demanding assays in particular fluorophores with small stoke-shifts, advanced assays like FRET and TR-FRET assays or multiplexing FI-based assays without the need to use any fluorescence filters.

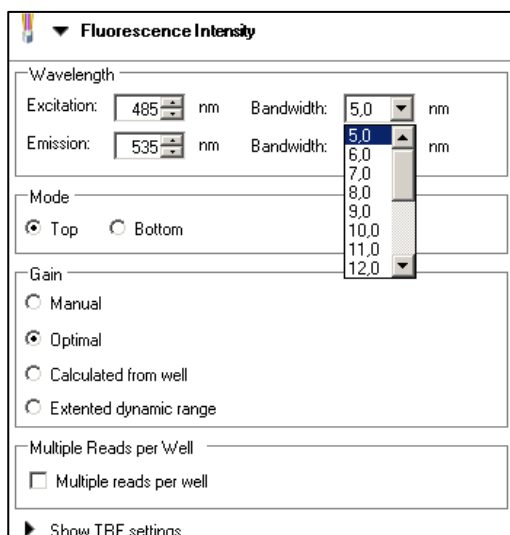


Figure 1: Section of Tecan's i-control™ software showing the selectable excitation monochromator slit widths from 5 to 20 nm in 1 nm steps.

In common cuvette-based spectrofluorometers, the monochromator slit widths are generally continuously selectable for fluorescence excitation and emission. The light intensity that passes through a monochromator is approximately proportional to the square of the slit width. Larger slit widths lead to increased signal intensities resulting in higher signal-to-noise ratios. On the other hand, smaller slit widths yield a higher resolution, but at the expense of light intensity. If the excitation slit is already wide enough to accept the focused image of the arc, then the intensity will not be increased significantly by further widening the slit width. If photobleaching of the sample is an issue, this might be minimized by decreasing the excitation intensity, i.e. by decreasing the excitation bandwidth. The lowering of the emission slit width enhances the resolution of the fluorescent signal [1].

Moreover, it is advantageous to have continuously adjustable excitation and emission slit widths for the optimization of FRET and TR-FRET based assays. In order to maximize the energy transfer and to avoid cross talk effects, an ultra fine-tuning of the excitation and emission wavelength and their respective bandwidths is of particular importance. Therefore the number of adjustable slit settings is a direct function of the flexibility of the instrument to fine tune the amount of excitation and emission light.

To investigate the impact of adjustable bandwidths the following experiments have been performed.

Fluorescence emission scans:

In the first set of measurements, a number of fluorescence emission scans with fixed excitation wavelength / bandwidth and varying emission bandwidths were performed in order to

show the relation between the monochromator slit width and the light arriving at the instruments detector.

DNA Quantitation via Quant-iT PicoGreen® assay:

In the second set of measurements, the influence of the monochromator bandwidth on the sensitivity of a common fluorescent stain (i.e. Invitrogen's PicoGreen DNA quantitation reagent) was demonstrated. Measurements were performed using fixed excitation and emission wavelength with varying excitation and emission bandwidths.

TR-FRET technology – HTRF® Reader Control Kit:

In the third set of measurements, a TR-FRET based assay, HTRF by Cisbio, was measured with fixed excitation and emission wavelengths and varying excitation and emission bandwidths in order to evaluate the impact of varying adjustable bandwidth settings on the TR-FRET performance of the Infinite M1000.

All data shown in this technical note are representative measurement data and therefore exemplify *typical performance values* and not instrument specifications.

Material and Methods

Instrument

- Infinite M1000 equipped with Tecan's premium Quad4 Monochromators (Tecan Austria)

Microplates

- Greiner® 384 well white with flat bottom; filling volume 100 µl (Greiner, Germany)
- Greiner 384 well black with flat bottom; filling volume 100 µl (Greiner, Germany)
- Greiner 96 well black with flat bottom; filling volume 200 µl (Greiner, Germany)
- Greiner 96 half area white; filling volume 100 µl (Greiner, Germany)

Reagents

Fluorescence emission scans:

- Fluorescein – 1 nM diluted in 10 mM NaOH (Sigma-Aldrich, Austria)
- 5-TAMRA – 5 nM diluted in 1 x PBS (Sigma-Aldrich, Austria)

- Europium – 1 nM diluted in Enhancement solution (PerkinElmer, USA)
- 1 x PBS
- Delfia® Europium Enhancement solution (PerkinElmer, USA)
- 20 x TE (Invitrogen, USA)
- Nuclease free water (Fluka BioChemika, Switzerland)

DNA Quantitation via Quant-iT PicoGreen assay:

- Quant-iT PicoGreen quantitation reagent (Invitrogen, USA)
- Nuclease free water (Fluka BioChemika, Switzerland)
- Herring Sperm DNA, 10 mg/ml stock solution (Promega, USA)
- 20 x TE (Invitrogen, USA)

TR-FRET technology – HTRF Reader Control Kit:

- HTRF Reader Control Kit (Cisbio bioassays, France)

20 x TE was diluted with nuclease free water to 1 x TE prior to use.

Reagents for the Quant-iT PicoGreen assay were prepared as described in the package insert [2].

Reagents for the HTRF Reader Control kit were prepared as described in the package insert [3].

Sample Preparation

Fluorescence emission scans:

Fluorescein was diluted to a final concentration of 1 nM in 10 mM NaOH. 100 µl of the fluorescein solution was pipetted into one well of a black 384 well microplate. For blank reduction 100 µl of the buffer (10 mM NaOH) were pipetted into another well. Both wells were measured as described below.

5-TAMRA was diluted to a final concentration of 5 nM in 1 x PBS. 100 µl of the 5-TAMRA solution was pipetted into one well of a black 384 well microplate. For blank reduction 100 µl of the buffer (1x PBS) were pipetted into another well. Both wells were measured as described below.

Europium was diluted to a final concentration of 1 nM in PerkinElmer's Enhancement solution. 100 µl of the Europium solution was pipetted into one well of a white 384 well microplate. For blank reduction 100 µl of the buffer (Enhancement solution) was pipetted into another well. Both wells were measured as described below.

DNA Quantitation via Quant-iT PicoGreen assay:

A 10-data-point DNA dilution series ranging from 50 ng/ml to 2.54 pg/ml was prepared in TE. 100 µl of each concentration was pipetted into a black 96 well plate (8-fold replicates). 100 µl of the dye solution was added to each sample. For blank samples, 100 µl TE + 100 µl PicoGreen dye were used. After 5 min incubation at room temperature the microplate was measured as described below.

TR-FRET technology – HTRF Reader Control Kit:

Samples and microplate were prepared according to the manufacturer's package insert (Document reference: 62RCLPEA). Measurements were performed as described below.

Measurement settings

Fluorescence emission scans:

Measurement parameter	Instrument settings
Plate	[GRE384fw.pdf]
Part of plate	A6 – A7
Mode	FI top emission scan
Excitation wavelength	345 nm
Excitation bandwidth	20 nm
Emission wavelength start	580 nm
Emission wavelength end	650 nm
Emission bandwidth	20 nm, 10 nm, 5 nm
Step width	1 nm
Number of flashes	20
Flash frequency	100 Hz
Integration time	400 µs
Lag time	100 µs
Gain	Manual: 200
Z-Position	Manual: 24493 µm
Settle time	0 ms

Table 1: Measurement settings for Europium emission scan. The plate was measured three times with varying emission bandwidths.

Measurement parameter	Instrument settings
Plate	[GRE384fb.pdf]
Part of plate	A14 – A15
Mode	FI top emission scan
Excitation wavelength	460 nm
Excitation bandwidth	20 nm
Emission wavelength start	495 nm
Emission wavelength end	570 nm
Emission bandwidth	20 nm, 10 nm, 5 nm
Step width	1 nm
Number of flashes	20
Flash frequency	100 Hz
Integration time	40 µs
Lag time	0 µs
Gain	Manual: 150
Z-Position	Manual: 24704 µm
Settle time	0 ms

Table 2: Measurement settings for Fluorescein emission scan.
The plate was measured three times with varying emission bandwidths.

Measurement parameter	Instrument settings
Plate	[GRE384fb.pdf]
Part of plate	A11 – A12
Mode	FI top emission scan
Excitation wavelength	520 nm
Excitation bandwidth	20 nm
Emission wavelength start	550 nm
Emission wavelength end	620 nm
Emission bandwidth	20 nm, 10 nm, 5 nm
Step width	1 nm
Number of flashes	20
Flash frequency	100 Hz
Integration time	40 µs
Lag time	0 µs
Gain	Manual: 140
Z-Position	Manual: 24704 µm
Settle time	0 ms

Table 3: Measurement settings for 5-TAMRA emission scan.
The plate was measured three times with varying emission bandwidths.

DNA Quantitation via Quant-iT PicoGreen assay:

Measurement parameter	Instrument settings
Plate	[GRE96fb_chimney.pdf]
Mode	FI top
Excitation wavelength	483 nm
Excitation bandwidth	20 nm, 10 nm, 5 nm
Emission wavelength	535 nm
Emission bandwidth	20 nm, 10 nm, 5 nm
Number of flashes	20
Flash frequency	100 Hz
Integration time	40 µs
Lag time	0 µs
Gain	Optimal
Z-Position	Calc. f. well (max signal): 21862 µm
Settle time	0 ms

Table 4: Measurement settings for Quant-iT PicoGreen assay.
The plate was measured three times with varying excitation and emission bandwidths.

TR-FRET technology – HTRF Reader Control Kit:

Measurement parameter	Donor settings	Acceptor settings
Plate	[GRE96fw.pdf]	[GRE96fw.pdf]
Mode	Fluorescence Top	Fluorescence Top
Excitation wavelength	317 nm	317 nm
Excitation bandwidth	20 nm, 9 nm	20nm, 9 nm
Emission wavelength	620 nm	665 nm
Emission bandwidth	9 nm, 10 nm, 13 nm, 15 nm	9 nm, 10 nm, 13 nm, 15 nm
Gain	Optimal	Optimal
Number of flashes	125	125
Flash frequency	100 Hz	100 Hz
Integration time	500 µs	500 µs
Lag time	60 µs	60 µs
Settle time	0 ms	0 ms
Z-Position	Calc. f. well (containing Eu)	Same as Label 1

Table 5: Measurement settings for HTRF Reader Control Kit.
The plate was measured several times with varying excitation and emission bandwidths for donor and acceptor fluorescence.

Data Evaluation

Fluorescence emission scans:

For each wavelength the RFU value of the blank was subtracted from the respective RFU value of the sample.

DNA Quantitation via Quant-iT PicoGreen assay:

For the blank and for each dilution the average and the standard deviation (SD) were calculated. The average blank was subtracted from the average sample signal. The SD for each dilution after blank reduction (SD_{corr}) was calculated using the following equation:

$$SD_{corr} = \sqrt{SD(blank)^2 + SD(sample)^2}$$

The sensitivity of Quanti-iT PicoGreen assay measured on Infinite M1000 was calculated using the following formula:

$$\text{Sensitivity (pg/ml)} = \frac{3 * SD(blank) * 50000}{(aver(sample) - aver(blank))}$$

TR-FRET technology - HTRF Reader Control Kit:

Data reduction and evaluation was performed according to the manufacturers package insert (Document reference: 62RCLPEA).

Results and Discussion

Fluorescence emission scans – the influence of the monochromator bandwidth on the light arriving at the detector:

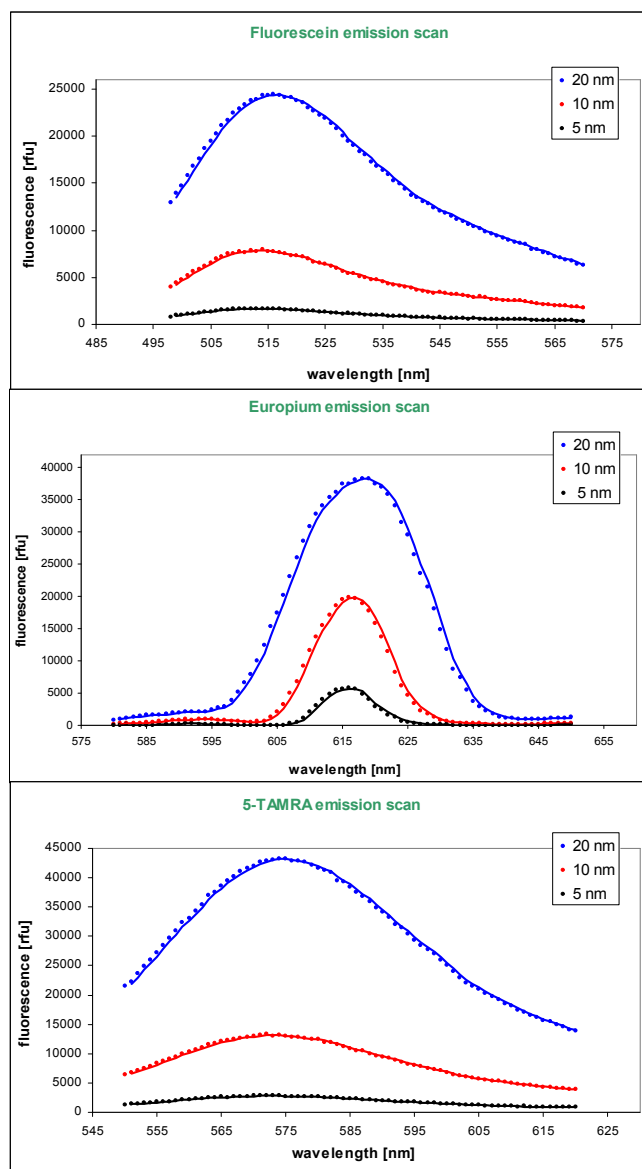


Figure 2: Emission Scans with three different fluorophores and variable emission bandwidths.

Three different fluorophores, Fluorescein, 5-TAMRA and Europium, were analyzed in order to show the influence of the bandwidth on the amount of light arriving at the photo multiplier tube (PMT) in the green and red wavelength range as well as with long-lifetime fluorophores.

Each fluorophore was scanned with three different emission bandwidths: 20 nm, 10 nm and 5 nm, respectively. The instrument gain value, which describes the amplification of the PMT, as well as the z-position of the optical head, which determines the focus of the light beam, was set to a constant value for all three measurements.

Figure 2 shows the measured emission spectra of Fluorescein, Europium and 5-TAMRA. An emission bandwidth of 20 nm results in a maximum of light and therefore an optimized signal. When measuring with a bandwidth of 10 nm, the signal decreases dramatically indicating the loss of light at the PMT. With a bandwidth of 5 nm the amount of light is minimized resulting in a defective emission spectrum with an inaccurate peak maximum.

It can be concluded that the monochromator slit widths have a direct impact on the amount of light arriving at the instruments detector, and maximizing the slit width is optimal for the performance of fluorescence scans.

DNA Quantitation via Quant-iT PicoGreen assay – the influence of monochromator bandwidth on the instruments gain value and sensitivity assessments:

When measuring fluorescence scans the impact of the bandwidth to the amount of light arriving at the detector could be compensated by the instruments PMT. An optimized amplification of the PMT (“optimal gain”) for each different emission bandwidth would have diminished the differences in emission spectra to certain extent resulting in comparable RFU values for each wavelength (data not shown). Higher gain values result in significantly higher background values, because of the increased electronic background level of the PMT. For fluorescence scans the background signal is not of crucial importance, but if determination of assay- and instrument sensitivity is the objective, the background noise level (which is reflected by the standard deviation (SD) of the assay-blank), is of crucial importance as it is directly proportional to the sensitivity:

$$\text{Sensitivity (pg/ml)} = \frac{3 * \text{SD}(\text{blank}) * 50000}{(\text{aver}(\text{sample}) - \text{aver}(\text{blank}))}$$

The equation clearly reflects that a high standard deviation of the blank value results in a low sensitivity.

The correlation between the instruments monochromator bandwidth, the instruments gain value and sensitivity determinations is demonstrated in figure 3 and table 6. A DNA dilution series was prepared and measured on the Infinite M1000 using Invitrogen’s PicoGreen assay. For this experiment the excitation and the emission slit width was set to either 20 nm, 10 nm or 5 nm and the gain value was set to “optimal”.

The absolute RFU value and the linearity do not differ significantly regardless of the respective excitation and emission slit width (figure 3 and table 6). The average of the blank (Aver(blank)) is the same for all three different bandwidth settings, but the standard deviation of the blank (SD(blank)) rises with increasing gain values, which leads to a dramatic loss in sensitivity.

The results clearly demonstrate that adjustable monochromator slit widths ensure maximized assay sensitivity and accuracy, e.g. in fluorescence based DNA quantitation.

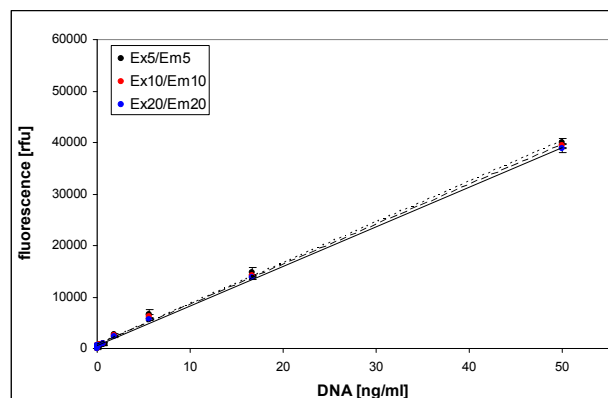


Figure 3: DNA dilution series quantified with Quant-iT PicoGreen assay.

Bandwidth (nm _{Exc.} /nm _{Emi.})	Instrument Gain value	Aver (blank)	Sensitivity (pg/ml) DNA	Linearity (R ² value)
		SD (blank)		
5 / 5	192	1259	504,67	0.997
		133.95		
10 / 10	128	1250	137,52	0.998
		36.01		
20 / 20	92	1298	34,93	0.999
		9.01		

Table 6: DNA dilution series quantified with Quant-iT PicoGreen assay - a comparison of the evaluation parameter.

TR-FRET technology – HTRF Reader Control Kit – the influence of monochromator bandwidth on advanced assay systems like TR-FRET:

For common fluorescent stains like the Quant-iT PicoGreen assay, maximized excitation and emission bandwidths in combination with an optimized instrument gain value lead to a minimized background noise and therefore to high sensitivity

levels. This equation becomes more complex when measuring more advanced detection technologies like TR-FRET based assays.

Table 7 displays the results of HTRF Reader Control Kit measured on Infinite M1000 with varying excitation- and emission slit widths.

In the first step, measurements were performed using the optimized bandwidth settings for the Infinite M1000, which is 20 nm for the excitation monochromator at 317 nm and 10 nm for both, the emission of the donor at 620 nm and the emission of the acceptor at 665 nm. These settings were defined during the instrument validation at Cisbio Bioassays and provide sufficient light as well as a high signal resolution. This maximizes the signal/blank ratio (S/B) and the dynamic range and minimizes the background noise, which is reflected by the low CV % of the standard 0 (negative) control (Std0 = 2.17) and the Z' value of 0.92.

In the second step, measurements were performed using bandwidth settings that are available on other monochromator based microplate readers (example 1-4).

In example 1, a 9 nm excitation bandwidth and a 15 nm emission bandwidth was used. Such bandwidth settings are suboptimal in the Infinite M1000, which is reflected by the performance values listed below (table 7). The S/B ratio, delta F high and delta F low are significantly lower compared to the measurements performed with the optimized bandwidth settings. Concurrently, the CV% of the standard 0 control, as well as of the low and the high control is much higher. This can be explained by the relatively small excitation bandwidth, which limits the energy for the excitation of the donor molecule and is reflected by the small S/B ratio combined with a relatively high gain value for donor emission (255 is the maximum gain available in Infinite M1000).

HTRF Validation Parameter		Infinite® M1000	Example 1	Example 2	Example 3	Example 4
		Excitation 317: 20 Emission 620: 10 Emission 665: 10	Excitation 317: 9 Emission 620: 15 Emission 665: 15	Excitation 317: 20 Emission 620: 9 Emission 665: 9	Excitation 317: 20 Emission 620: 13 Emission 665: 13	Excitation 317: 20 Emission 620: 13 Emission 665: 9
S/B	aver	345.60	194.52	261.11	349.23	363.71
	SD	8.14	22.00	24.79	23.43	9.98
CV (%) Std0	aver	2.17	5.11	5.54	2.82	4.30
	SD	0.57	0.79	0.17	0.23	0.68
CV (%) low control	aver	2.23	4.81	3.64	2.92	4.96
	SD	0.63	0.72	0.91	0.80	1.57
CV (%) high control	aver	1.97	2.37	5.46	1.76	2.46
	SD	0.29	1.02	1.17	0.19	0.39
Delta F low (%)	aver	21.00	18.80	23.71	19.63	26.28
	SD	0.71	2.02	1.34	1.99	0.99
Delta F high (%)	aver	969.66	682.57	923.79	769.56	965.16
	SD	6.32	25.36	14.05	22.70	21.41
Z'	aver	0.92	0.89	0.82	0.91	0.90
	SD	0.01	0.04	0.04	0.01	0.01
Gain Label 620		208	224	212	198	199
Gain Label 665		230	247	250	216	249

Table 7: HTRF Reader Control Kit measured with different combinations of excitation and emission bandwidths. Even very small modifications cause significant changes in performance values. Average and SD are calculated out of three independent measurements.

In example 2 the excitation bandwidth at 317 nm was set to 20 nm in order to achieve a maximized excitation of the donor molecule. The emission bandwidth for the donor emission at 620 nm as well as the acceptor emission at 665 nm was set to 9 nm. This is only 1 nm difference for both labels compared to the optimized bandwidths for the Infinite M1000, which are 10 nm, but still has a dramatic impact as outlined by the data presented in table 7. The S/B is lowered about 25%, the CV% of the negative control rises from 2.71% to 5.54%, which results in a significant increase in the background noise. This is reflected by the Z' value, which is decreased to 0.82 compared to 0.92. The dynamic range is still good, but the delta F high value is obviously lower compared to "optimized" bandwidth settings.

In example 3, both emission bandwidths were set to 13 nm. In this case the S/B is at the same level as with optimized bandwidth settings. Likewise the CV% of the standard 0 control is very low. This can be explained by the increase in the emission bandwidth of both labels, which generates enough light to guarantee an optimized excitation of the acceptor molecule and therefore a small background noise. But still the emission slit for the acceptor emission was too big, leading to a suboptimal resolution of the signal, resulting in a small dynamic range (delta F low and delta F high are significantly lower than with 10 nm bandwidth).

In example 4, a bandwidth of 13 nm was used for the 620 nm emission and a bandwidth of 9 nm for the 665 nm emission. This would theoretically generate enough light for the acceptor excitation and a good resolution for the acceptor signal. Indeed, example 4 worked well when considering the dynamic range and the S/B ratio, but when taking a closer look to the CV% of the standard 0 control, it becomes obvious that the Infinite M1000 performs best with 10 nm for the acceptor emission at 665 nm. This seems to be mandatory to optimize the ratio of background noise and signal resolution. With 13 nm at 620 nm and 9 nm at 665 nm sufficient light excites the donor. The signal resolution for the acceptor is good enough to provide a satisfying S/B and dynamic range, but with only 1 nm change in the bandwidth settings the SD of the standard 0 would be significantly lowered!

It can be concluded that the extended adjustable bandwidths have a crucial impact on TR-FRET based measurements. Whereas maximizing the slit was ideal on the excitation side, an exact setting of 10 nm on the emission side was mandatory to achieve the best precision, especially when working with small signal intensities (standard 0).

Conclusion

The Infinite M1000 premium Quad-4 monochromator-based multimodal microplate reader is equipped with an extended range of adjustable monochromator bandwidth selections for all fluorescence based readouts. This feature offers enormous flexibility to control the amount of light and to fine-tune the sensitivity for fluorescence based measurements. This

application note evaluates the importance of extended adjustable bandwidth settings on a number of different applications.

For basic applications like fluorescence scans the flexibility of the bandwidth selection enables superb data quality and high spectra-resolution.

When it comes to determinations of the detection limit in common fluorescence intensity-based applications, maximizing the adjustable bandwidth yields an optimal amplification of the PMT, which results in outstanding signal to blank values and detection limits in the example experiment.

For advanced assay technologies like TR-FRET based measurements, here exemplified by HTRF, the Infinite M1000 offers the possibility to *ultra fine-tune* the bandwidth for donor and acceptor emission, in order to optimize the ratio between the amount of light and the signal resolution.

Due to the ultra fine-tuned adjustable bandwidth setting possibility, combined with the premium Quad4 Monochromators of the Infinite M1000, the instrument provides outstanding flexibility to be assay-ready for a wide range of fluorescence-based assays and applications, without the need to use fluorescence filters. It is a great tool for assay development in academic research, life-science and the pharmaceutical industry.

List of Abbreviations

Aver	average / mean value
BSA	bovine serum albumin
CV	correlation value
DAS	diode array system
DNA	deoxyribonucleic acid
FI	fluorescence intensity
FP	fluorescence polarization
HTRF	homogenous time resolved fluorescence
PBS	phosphate buffered saline
PMT	photomultiplier tube
SD	standard deviation
TE	Tris-EDTA
TRF	time resolved fluorescence
TR-FRET	time resolved fluorescence energy transfer

Groedig, August, 2009

Literature

- [1] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, chapter 1, page 1-25, Springer Science & Business Media, 3rd edition, 2006
- [2] www.invitrogen.com
- [3] www.htrf.com

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