

## MICROPLATE COLOR COMPARISON IN A TRF ASSAY

Time-resolved FRET (TRF) assays were originally developed to overcome problems with sample background autofluorescence in proximity assays. Through the use of long lifetime fluorescence donors, detection is delayed until fluorescence from short-lived sources subsides, thereby eliminating most background. While the elimination of this source of interference is of value in some circumstances, techniques developed for the execution of these assays came to stress the reduction of background **at all costs**. As a result, recommended protocols with low background *as their sole objective* have actually lead to reductions in assay sensitivity.

*NOTE: It is vital to understand that Signal:Noise (S/N), not Signal:Background (S/B), must be optimized to obtain maximal assay sensitivity. These concepts are described in detail in TechNote TNPJ100.02 Precision of FRET Assays: S/N vs. S/B.*

TRF protocols sacrifice signal in their quest for reduced background in many ways (see TechNote TNPJ100 *Techniques for Optimizing the Sensitivity of Fret Assays*.) One of the clearest examples is the recommendation by manufacturers that TRF assays should be run in black-colored microplates.

Black plates create a one-pass illumination environment: the light beam passes through the sample once and is, for the most part, absorbed by the wall of the well. Only the portion of the well directly illuminated by the beam receives excitation light.

Alternatively, assays can be run in white plates, which provide a diffuse reflection of the incoming beam, followed by a complex pattern of escape through the surface, internal surface reflection, and additional reflection by the wall of the well.

The overall effect is for average well illumination to be highest in white plates. This should, in theory, lead to higher detector counts for both signal and background, which should be increased by roughly the same proportion. Since higher counts typically have lower relative standard deviations, higher S/N should result from the use of white plates.

To test whether this expected result could be achieved in practice, a tyrosine kinase assay was performed according to recommended procedures, with the single exception that assays were run in black and white plates.

### Experimental

A tyrosine kinase assay (see Table 1 for assay details) was run in both black and white plates on the Wallac Victor<sup>2</sup> 1420 Multilabel Counter (PerkinElmer). All necessary blanks and controls were included, as described in TechNotes TNPJ100.03 *Background Correction and Spectral Overlap Compensation in Fret Assays* and TNPJ100.04 *Fret Calculations*.

**Table 1 - Assay Conditions**

Donor	PY20-Eu, 6.0 nM
Acceptor	SA-APC. 2.3 nM
Incubation	2 hours
Excitation	340/50
Emission A	665/15
Emission B	615/8.5
Flash interval	1000 $\mu$ sec
Read duration	100 $\mu$ sec
Read delay	50 $\mu$ sec
Iterations	95 - 100x
Count time	100 msec

## Results & Discussion

Overall counting levels were higher in the white plates than in the black plates by a factor of 7 (see Table 2 for all results discussed here). These higher counting levels resulted in increased S/N for each of the calculated FRET response parameters (Net FRET, Quench, and A/B – see TechNote TNPJ100.04 *FRET Calculations* for definitions of these parameters and details of their calculation.) This improvement occurs because higher counts have lower relative standard deviations when all other factors are equal, as they were here.

Overall, S/B decreased slightly for white plates as compared to black, since background counts increased by a slightly higher proportion (about a factor of 8) than positive control counts (about factor of 7). While this result was unexpected, it demonstrates the absence of any correlation of S/N and S/B, since S/N increased despite the reduction in S/B.

This assay is a “good” assay for conventional TRF: the starting value of S/N for Net FRET of 33 is within an acceptable range. The

improvement due to white plates by a factor of not quite two, to 60, is a meaningful improvement, but not dramatic. If A:B is the only measure being used, the improvement in S/N shown here (63 to 79) is probably of little practical significance (although still enough to support the use of white plates).

The successful development of assays such as this one, in which recommended procedures give acceptable results, lent early support to the incorporation of protocols that reduced background at all costs. However, the real costs, in terms of sensitivity, of such poor choices—in this case, the use of black plates—become more apparent when the assay presents a greater challenge: the poorer the initial assay, the more the increased counts from using white plates will help. For instance, the calculated value for quench shown here, which is inherently more “noisy” and thus less sensitive than Net FRET, shows that white plates provide a significant increase in S/N from the black-plate value of about 6 to a white-plate value of 25.

Thus, white plates will help most for assays that are weakest to begin with. The kinase assays were all run at our “positive control” level for the assay (*i.e.* the high end of the range). Had a lower peptide concentration been chosen, or if for any other reason the assay had been weaker (*e.g.* lower affinity interactions), the improvements due to the selection of white plates would have been more dramatic.

More counts are almost always good. Various approaches to increase counts are summarized in TechNote TNPJ100 and discussed in more detail in the TechNotes referred to therein. Some of these techniques, such as the use of white plates, increase signal and background in direct proportion, and can be introduced with very little need for analysis.

Other techniques, such as changes in the detection window, may increase background by a larger factor than they increase signal, but are often still very beneficial in improving the signal:noise ratio of an assay.

**Table 2 - Measured and Calculated Values for White vs. Black Microplates**

Means of five replicate counts of twelve replicate wells for each value.

Plate Color	White			Black		
	Mean	STD	Rel STD	Mean	STD	Rel STD
<b>Measured Values</b>						
T <sub>a</sub> - Test, Channel A	52,975	1,756	3.3%	7,938	417	5.3%
C <sub>a</sub> - Assay Control, Channel A Both Donor and Acceptor present but no FRET <i>i.e.</i> no peptide or no kinase	765	26	3.4%	96	9	9.7%
A <sub>a</sub> - Acceptor Blank, Channel A All reagents except donor	35	6	17.0%	8	3	39.4%
D <sub>a</sub> - Donor Blank, Channel A All reagents except acceptor	701	27	3.8%	96	10	10.0%
I <sub>a</sub> - Instrument/Buffer Blank, Channel A	27	5	17.5%	7	3	48.9%
T <sub>b</sub> - Test, Channel B	162,573	1,552	1.0%	23,589	781	3.3%
C <sub>b</sub> - Assay Control, Channel B Both Donor and Acceptor present but no FRET <i>i.e.</i> no peptide or no kinase	198,943	1,662	0.8%	27,926	616	2.2%
A <sub>b</sub> - Acceptor Blank, Channel B All reagents except donor	32	7	20.0%	16	5	29.9%
D <sub>b</sub> - Donor Blank, Channel B All reagents except acceptor	201,049	1,667	0.8%	28,907	671	2.3%
I <sub>b</sub> - Instrument/Buffer Blank, Channel B	35	6	17.9%	14	4	28.1%
<b>Calculated Values</b> (see TechNote TNPJ100.04 <i>FRET Calculations</i> )						
Net FRET Counts (F)	52,332	1,698	3.2%	7,856	380	4.8%
Quench Counts (Q)	36,369	365	1.0%	4,336	200	4.6%
Quench %	18.3%			15.5%		
Raw Channel A/Channel B	0.322	0.008	2.5%	0.333	0.011	3.2%
Signal/Noise (S/N) of Net FRET	60	4	6.6	33	3	9.0%
Signal/Noise (S/N) of Quench	25	3	12.8%	5.8	1.1	18.7%
Signal/Noise (S/N) of A:B	78	5	5.9%	63	19	30%
Raw Signal/Background (S/B) of Net FRET	69	1.1	1.7%	83	3.7	4.5%
Raw Signal/Background (S/B) of A:B	85	1.4	1.7%	98	4.8	4.9%

## REFERENCES

TNPJ100 Techniques for Optimizing the  
Sensitivity of FRET Assays

<http://www.prozyme.com/pdf/tnpj100.pdf>

TNPJ100.02 Precision of FRET Assays: S/N vs.  
S/B

[http://www.prozyme.com/pdf/tnpj100\\_02.pdf](http://www.prozyme.com/pdf/tnpj100_02.pdf)

TNPJ100.03 Background Correction and  
Spectral Overlap Compensation in FRET  
Assays:

[http://www.prozyme.com/pdf/tnpj100\\_03.pdf](http://www.prozyme.com/pdf/tnpj100_03.pdf)

TNPJ100.04 FRET Calculations:

[http://www.prozyme.com/pdf/tnpj100\\_04.pdf](http://www.prozyme.com/pdf/tnpj100_04.pdf)

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