

PRECISION OF FRET ASSAYS: S/N vs. S/B

(Please see TechNotes TNPJ100 *Considerations for Development of FRET Assays* and TNPJ100.01 *Getting Started: PB-FRET™ for HTS* for background on the basic concepts of FRET, PB-FRET, and TR-FRET.)

Time-resolved fluorescence resonance energy transfer (TR-FRET) assays are distinguished from other FRET assays by their capacity to be operated with extremely low background counts; that is, in the absence of molecular proximity leading to FRET, very low fluorescence readings are obtained. These assays have frequently been evaluated, both one against another, and against other assay formats, on the basis of their raw signal-to-background ratio, *i.e.*, the factor by which the mean value of a positive assay control exceeds the value of the assay blank. Often, the term signal-to-noise has been used interchangeably with the term signal-to-background in this context.

Since the terms “signal”, “background”, and “noise” have been used in various ways in various fields over the years, it would not be correct to say that equating “background” and “noise” is categorically inappropriate; however, a vital element of FRET data interpretation—statistical variability—has gotten lost in the resulting confusion.

We have incorporated in this discussion (and a number of other TechNotes) an explicit set of definitions of these three terms, in which “background” and “noise” are clearly distinguished from one another, the former referring to assay blanks and the latter to statistical variability:

- Background - Raw signal when assay activity is zero.

- Noise - Statistical variability in the assay response from all sources, including: variation in actual quantities of reagents added or their concentration, instrument output variability, geometry of plates, uncertainty or variability in background, *etc.*
- Signal - The “real” response of an assay to activity in the sample, corrected for any background. “Raw signal” refers to the uncorrected counts in the positive control or any sample being analyzed.

When terms are defined as above, background and noise are not equivalent. Despite this, it could be argued that even if they are not equivalent, they are closely related to one another, and that assays with low background counts will also be likely to have low overall assay noise. The examples we present here show that this is not the case, and that in assay development and evaluation, noise should be examined directly rather than inferred from assay background.

CALCULATIONS

Figure 1 diagrams the various components involved in the calculation of signal, background, and noise. The simplest calculation is for background:

$$\text{Background} = \text{Mean Negative Control}$$

where a negative control is a well containing all assay reagents except the analyte. Signal is calculated as:

$$\text{Signal} = \text{Mean Positive Control} - \text{Background}$$

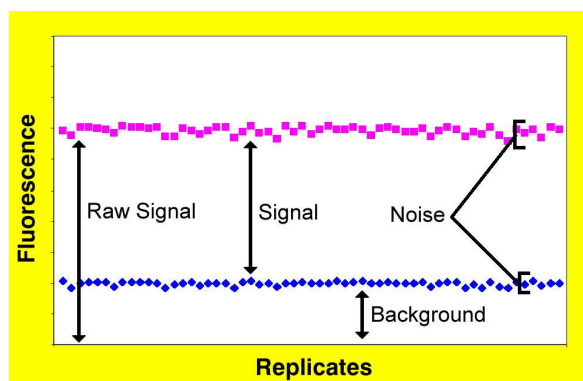


Figure 1 - Graphical Representations of Raw Signal, Signal, Background and Noise

where a positive control is a well containing a standardizing concentration of analyte that defines the upper end of the desired analytical range.

Since noise is defined as the variability in signal, the calculation of noise must incorporate the variability of both the positive and negative controls. Using standard deviation as an indicator of variability, the standard deviation (STD) of the difference between the positive and the negative control is calculated as:

$$STD = \sqrt{(Pos\ Control\ STD)^2 + (Neg\ Control\ STD)^2}$$

We then report the signal-to-noise ratio (S/N) as:

$$S/N = \frac{(Mean\ Pos\ Control - Mean\ Neg\ Control)}{\sqrt{(Pos\ Control\ STD)^2 + (Neg\ Control\ STD)^2}}$$

To be consistent with previous work on TR-FRET, we will report the raw signal-to-background ratio (S/B) as it has been calculated in the past, as:

$$S/B = \frac{Mean\ Pos\ Control}{Mean\ Neg\ Control}$$

An additional indicator of assay variability, Z', can be calculated as:

$$Z' = 1 - \frac{3(Pos\ Control\ STD + Neg\ Control\ STD)}{Mean\ Pos\ Control - Mean\ Neg\ Control}$$

This value provides for a quick assessment of assay performance, since it approaches a value of 1 for a “perfect” assay. It should be noted that the same four factors appear in the calculations of both S/N and Z'; that is, they are simply alternate ways of presenting the same data.

Each of the above calculations can be applied to several different values calculated for each FRET assay: Net FRET, Quench, FRET Ratio and A:B Ratio (see TechNote TNPJ100.11 for definitions of these quantities and for more information on Channels A and B). Here, we report values as they apply to the A:B ratio, the ratio of mean counts in Channel A (the acceptor emission channel), divided by mean counts in Channel B (the donor emission channel). Again, we chose the A:B ratio because it has commonly been employed in analyses of TR-FRET data. In many cases, however, it may be preferable to examine Channel A and Channel B data independently (TechNotes TNPJ100.19 *Evaluation of Sample Interference in FRET Results* and TNPJ.24 *Data Collection and Interpretation in FRET Assays*).

SAMPLE DATA

Several data sets, taken from work with a model assay (TechNote TNPJ100.11), are useful to illustrate the differences between S/B and S/N and to emphasize the main point of this discussion, *i.e.* that S/N (as well as the closely-related Z') and S/B are separate and largely independent characteristics of FRET assays.

The three panels in Figure 2 illustrate some of the ways in which S/B and S/N can vary. In Panel A, we show a TR-FRET assay in which a defective lot of reagent is responsible for very noisy results. In this case, the value of S/B at greater than 50 would seem to indicate that this is a reasonably “robust” assay but, obviously, this is in fact a very noisy assay, as is reflected in the poor S/N value of less than 10 and a Z' of 0.677. Even though the excessive noise in this assay is obvious to the eye when the data are plotted, an assessment of this assay in which only the S/B was

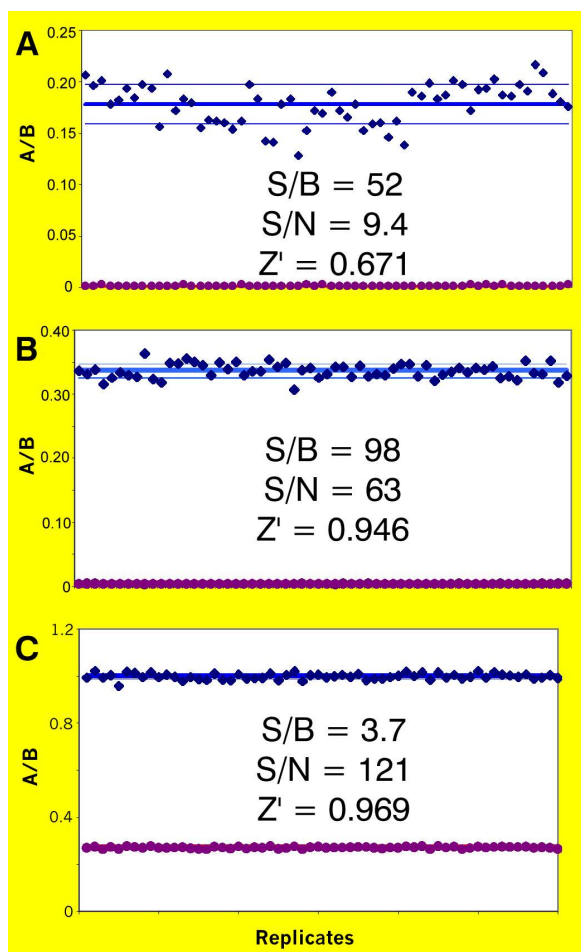


Figure 2 - Variability in Positive (◆) and Negative (●) Controls in FRET Assays
 Points represent individual replicates, heavy lines means, and lighter lines indicate \pm one standard deviation. A - TR-FRET Tyrosine Kinase assay with defective reagent. B - Same assay with new reagent. C - PB-FRET assay Tyrosine Kinase assay.

examined would have found it acceptable. The defective reagent would not have been detected if S/N were not examined¹.

In Panel B, we show the effect of replacing the defective reagent. A less than two-fold increase in S/B was accompanied by a more than sixfold improvement in S/N, and Z' improved to a much more satisfactory 0.946. To the eye, it is apparent that the reduction in noise with the new batch of reagent was substantial.

But while Panels A and B show that S/N and S/B are only poorly correlated, and that S/B cannot be relied on as an indicator of assay “performance”, Panel C demonstrates a case in which S/B is even less meaningful. This shows the results of a PB-FRET assay: the S/B ratio of 3.7 would at first seem to indicate that this is a poor assay, but in fact, both visual inspection of the results, the S/N of 121, and the excellent Z' value of 0.969 all confirm that this is a highly precise and low-noise assay. (For further comparisons of PB-FRET and TR-FRET, see TechNote TNPJ100.10 *PB-FRET vs. TR-FRET*)

DISCUSSION

It is clear from the above that S/N and S/B are poorly correlated, and that it is erroneous to take S/B by itself as an index of assay performance. To understand why this is so, it is necessary to examine individual parameters calculated from the summarized data presented above. Table 1 shows calculated values the data sets illustrated in Figure 2.

First, we contrast the data from Panels A and B, working backward to understand the origin of the result, a distressingly low S/N ratio (ID “I”). Since this is the ratio of the signal to its own standard deviation, we note that the relative standard deviation of signal (“II”) in Panel A is much higher than that for Panel B. Next, since the signal is the difference between the positive and negative controls, we look at these values and note that it is the standard deviation of the positive control A:B (“III”) that seems particularly high. Following the path backward to the counts that make up the ratio, we see that while the relative standard deviation of the counts in Channel B (“IV”) is very high, the relative standard deviation for the counts in Channel A is not. This result was highly reproducible, and suggests that the defect in the reagent lot affects only the counts in Channel B. A possible explanation is that the reagent contains significant amounts of highly aggregated donor (europium chelate) that

¹In fact, we used this reagent for some time while we were developing our Tyrosine Kinase assay. It was only when we developed our own data handling software, which calculates S/N, that this problem became apparent. Subsequently, we were able to explain results that previously had been puzzling.

does not participate in FRET, presumably because it is not active in binding substrate.

While the above example shows how an understanding of S/N can help in diagnosing faulty assays, comparison of Panels B and C contrasts fundamental characteristics of two different assays. The assay in Panel B is a TR-FRET assay that has been designed to exhibit very low background counts—achieved at the expense of low counting levels due to weak donor light absorption and restrictive (in time and wavelength) counting conditions. The PB-FRET assay in Panel C, on the other hand, is designed to maximize assay fluorescence at the expense of a high (but reproducible) background. Thus, the dramatically higher counts seen in Panel C data are the product of a fundamentally different approach to assay design.

The comparison between B and C is characterized by lower relative standard deviations for C in all counts and ratios, resulting in a S/N almost double that for Panel B. The lower standard deviations are in turn a product of the higher counting levels attained in the PB-FRET assay, since the relative variability of photomultiplier counts declines steadily with increasing counting level. In terms of assay precision then, the sacrifice of counting level for low background embodied in the TR-FRET approach has not paid off in terms of S/N. The opposite and incorrect conclusion would be (and has been) drawn if the S/B ratios of the two assays were compared. (The issue as to whether there is an inherent advantage to a “black” background in TR-FRET for purposes of avoiding interference due to sample autofluorescence is addressed elsewhere: TechNotes TNPJ00.10 and TNPJ100.19).

The advantage of assay C over assay B is less dramatic when Z' values, rather than S/N, are considered, since the Z' values for both assays come reasonably close to 1. The near-doubling of S/N in assay C only roughly “splits the difference” between the Z' of assay B and 1. This is not entirely misleading, however, since it is true that there is clearly some level of S/N beyond which further improvements have no significance to the usefulness of an assay.

CONCLUSIONS

When “noise” in FRET assays is defined explicitly as statistical variability in signal, signal-to-noise takes on meaning that is distinct from, largely uncorrelated with, and generally more useful than signal-to-background in assessing assay precision. S/N is useful in assessing performance differences both within and between assays.

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Table 1 - Values Calculated From Data in Figure 2

	ID	Panel A	Panel B	Panel C
Ratio Data				
Mean A:B, positive control		0.178	0.336	1.002
Standard deviation		0.0187	0.0057	0.0061
Relative standard deviation	III	10.5%	1.7%	0.6%
Mean A:B, negative control		0.0034	0.0034	0.272
Standard deviation		0.0005	0.0003	0.0012
Relative standard deviation		14.2%	9.5%	0.4%
Difference		0.175	0.333	0.730
Standard deviation		0.019	0.0057	0.0063
Relative standard deviation	II	10.8%	1.7%	0.8%
S/N	I	9.4	63	121
S/B		53	98	3.7
Z'		0.671	0.946	0.969
Counts				
Mean Channel A, positive control		6223	7938	181494
Standard deviation		122	245	1239
Relative standard deviation		2.0%	3.1%	0.7%
Mean Channel B, positive control		35307	23589	181710
Standard deviation		4323	697	969
Relative standard deviation	IV	12.2%	3.0%	0.5%
Mean Channel A, negative control		138	96	82560
Standard deviation		20	9	491
Relative standard deviation		14.5%	9.3%	0.6%
Mean Channel B, negative control		41095	27926	304658
Standard deviation		5306	329	1192
Relative standard deviation		12.9%	1.2%	0.4%



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