

MEASURING THE PRECISION OF FRET ASSAYS: S/N AND Z'

S/N and Z' are useful indices of assay precision for FRET assays, and incorporate the same assay response parameters. The choice between them should be based on the needs of the investigator: Z' is particularly sensitive in discriminating between assays with poor precision; S/N provides clearer distinctions between higher precision assays.

Early in the development and promotion of FRET assays for the detection of molecular binding events, the robustness of assay results was often characterized by the signal-to-background (S/B) ratio of the resulting data. Frequently, the terms S/B and signal-to-noise (S/N) were used interchangeably to refer to the S/B result.

This blurring in the distinction between the weakly related S/B and S/N concepts was clearly a boon to the promotion of a specific FRET technology, time-resolved FRET (TR-FRET), which provides results characterized by extremely low background counts. While there are certain benefits to low background counts, the resulting high S/B was erroneously linked to or equated with a high S/N. (The S/B and S/N concepts and their relationships are discussed in greater detail in TechNote TNPJ100.02 *Precision of FRET Assays: S/N vs. S/B.*)

More recently, practitioners of HTS assays, whether employing FRET or other methodologies, have refocused assay evaluation on the S/N concept—that is, the relationship between net assay response to the event of interest and the statistical uncertainty of that result.

Many different ways exist to mathematically characterize the relationship of a signal to its variability; the most widely accepted is Z' (sometimes also called Z or the Z-factor), defined as:

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

where STD is the sample standard deviation of the indicated control.

Z' has become a popular measure of assay robustness since it provides a fixed upper value, approaching a value of 1 when there is no statistical uncertainty.

In TechNote TNPJ100.04 *FRET Calculations*, assay variability is assessed using the S/N ratio, defined as:

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

because it more directly reflects the relationship between the real assay response and its statistical variability.

A comparison of the equations for Z' and S/N reveals, most importantly, that they summarize the same underlying variables. The “signal” portion is identical in both equations: the difference between the mean positive control and the mean negative control (blank).

“Noise”, however, is characterized by the sum of the standard deviations in Z', but by the square root of the sum of their squares for S/N. The latter formulation is more statistically rigorous: since the “signal” is the

difference between the positive and negative

controls, “noise” is the variability of that *difference*, which is properly calculated using the square root of the sum of the squares.

In practice, however, the differences between these calculations of pooled deviations is not large and can effectively be ignored; while it would be possible to reformulate the calculation of Z' to incorporate the more rigorous noise calculation, minimal benefit would be derived.

Finally, in the equation for Z' , the variability measure is multiplied by 3 to approximate a confidence interval. Thus, Z' attempts to define the relative size of a “window” between the positive and negative controls in which statistically meaningful results can be found. However, since the multiplier 3 is applied independent of sample size, it does not in fact add meaningful information to the calculation.

Clearly, the intent of the two calculations differs somewhat, since Z' attempts to define the size of the reading window for the assay, while S/N is intended to be merely descriptive of the relationship between the assay response and the attendant statistical variability.

The mathematical relationship between Z' and S/N is illustrated in Figure 1. The curve shown is generated with the assumption that the standard deviations for the positive and negative controls are equal; however, sensitivity analysis shows that the shape of the curve changes only slightly when the relative magnitudes of the standard deviations were varied. Typical data lie very close to the calculated curve.

It is clear from the curve in Figure 1 that at low values of S/N , the Z' value is very sensitive to changes in S/N , while at higher values, Z' is quite insensitive. This is because as S/N becomes large, Z' approaches 1 asymptotically; increases in S/N only “split the difference” between Z' and 1.

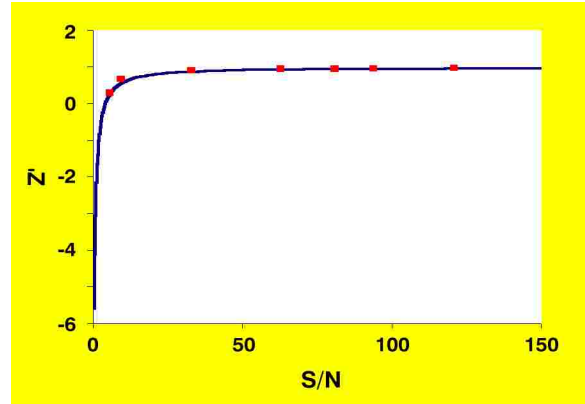


Figure 1 - Relationship between S/N and Z'
Solid line: calculated for the case where standard deviations of the positive and negative controls are equal. ■ - values calculated from actual data presented in TNPJ100.02 and TNPJ100.10.

A common rule of thumb in assay evaluation is that Z' should be greater than 0.5. This equates to S/N of about 8. Z' of 0.9 is achieved at S/N of a little over 40.

Both Z' and S/N are valid indicators of assay variability, and both are useful depending on the intent of the investigator. In general, Z' is a good indicator of performance in “problem” assays, where statistical variability is high. However, it tends to minimize differences between low-noise assays.

These characteristics of the Z' assay, however, can in many cases be consistent with reasonable criteria for assay acceptance, since beyond a certain level further improvements in S/N do not translate into meaningful improvements in assay performance.

In other cases however, such as for the data in Figure 1, Z' analysis can, in fact, obscure meaningful differences in performance. The example data in Figure 1 represent results for the detection portion of a tyrosine kinase assay—the portion of the assay that measures the concentration of phosphorylated peptide. Z' analysis suggest that the top four or five treatments represented are virtually

equivalent, while S/N analysis suggest that improvements of more than 2x can be attained with these same treatments. While these changes may not be of importance when the detection portion of the assay is viewed alone, a doubling of the S/N for detection may be extremely important when it is coupled with the potentially noisier phosphorylation portion of the assay.

In general, in ProZyme literature S/N is reported as the more sensitive measure of assay performance, and used in optimization studies. In comparative studies, both parameters are reported.

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