

FRET CALCULATIONS

The calculation of FRET results requires both correction for blanks and compensation for spectral overlap between channels (see TechNote TNPJ100.03 Background Correction and Spectral Overlap Compensation in FRET Assays). Moreover, the final results of FRET assays may be expressed in several different ways, either in terms of FRET counts or as ratios. Equations are provided for these various output parameters.

The precision of FRET assays, whether in the PB-FRET or TR-FRET format, can be improved by making the fullest possible use of the fluorescence data collected. To do this, both background and spectral overlap corrections (TNPJ100.03) are applied to the raw data, regardless of whether FRET counts or ratiometric data are the final objective.

DEFINITION OF TERMS

Instrument outputs (raw counts):

- T_a = test well, Channel A
- T_b = test well, Channel B
- I_a = instrument/buffer blank, Channel A (all reagents except donor and acceptor conjugates)
- I_b = instrument/buffer blank, Channel B (all reagents except donor and acceptor conjugates)
- A_a = acceptor blank in Channel A (all reagents except donor conjugate)
- D_a = donor blank in Channel A (all reagents except acceptor conjugate)
- D_b = donor blank in Channel B (all reagents except acceptor conjugate)
- C_a = negative assay control, Channel A (all reagents except FRET-mediating agent)
- C_b = negative assay control, Channel B (all reagents except FRET-mediating agent)

The equations presented here assume that the detection window has been chosen such that acceptor counts in Channel B are negligible. If this is not the case, many of the parameters described here will not be properly determinable (e.g. Net FRET, FRET Ratio, Donor Quench and A/B).

Calculated factors and assay response measures:

- F = Net FRET counts
- f = Uncompensated net FRET counts
- P = Proportionality factor for donor compensation
- Q = Donor quench due to FRET counts
- A/B = Ratio of acceptor channel counts to donor channel counts
- R = FRET ratio
- S/N = Assay signal-to-noise ratio
- S/B = Assay signal-to-background ratio
- Z' = Performance factor

CALCULATIONS

Net FRET (F) is the number of Channel A counts that are due specifically to FRET. It is determined by subtracting the acceptor background and the adjusted donor background from the total counts. Net FRET for each well is calculated as:

$$F = T_a - \overline{A_a} - P(T_b - \overline{I_b}) \quad [\text{Eq. 1}]$$

Note: Bars above symbols indicate average values for all determinations of that parameter. Average values of this sort are used when readings are unpaired. For example, in the above equation T_a and T_b were determined in the same test well, and thus are considered paired, while A_a and I_b were determined in separate wells, no one of which bears any special relationship to the test well, and thus are unpaired with the T values.

In Equation 1, the Proportionality Factor (P) between donor counts in Channel A and donor counts in Channel B is used to perform spectral overlap compensation of FRET results, correcting Channel A counts for the off-peak emission of the donor (see TechNote TNPJ100.03). Since there can be no significant acceptor counts in Channel B and the shape of the fluorescence emission spectrum for the donor will be conserved regardless of its magnitude, P is a constant that can be calculated as:

$$P = \frac{(\overline{D}_a - \overline{I}_a)}{(\overline{D}_b - \overline{I}_b)} \quad [\text{Eq. 2}]$$

For some applications, a value for FRET counts that is not spectrally compensated (f) may also be required:

$$f = T_a - \overline{A}_a \quad [\text{Eq. 3}]$$

Donor quench (Q) in FRET assays is the amount by which donor fluorescence is reduced when FRET takes place. (Since energy is transferred to the acceptor, less is available for donor fluorescence.)

$$Q = (C_b - \overline{I}_b) - (T_b - \overline{I}_b)$$

or

$$Q = C_b - T_b \quad [\text{Eq. 4}]$$

Frequently, FRET and Donor Quench values are combined into a single parameter by calculating a ratio involving the counts in both Channel A and Channel B. This enhances the dynamic range and hence the sensitivity of the output parameter, although it does so at the expense of certain information that can be obtained by examining quench and FRET independently (TechNote TNPJ100.24 *Data Collection and Interpretation in FRET Assays*).

On the other hand, A/B can be useful in partially eliminating the effects of well-to-well variability in reagent concentrations—for example, due to pipetting error—from calculated results:

$$A/B = \frac{T_a}{T_b} \quad [\text{Eq. 5}]$$

An alternative ratio, more fully corrected for background and other effects is the FRET ratio (R, also dimensionless)

$$R = \frac{F}{(T_b - \overline{I}_b)} \quad [\text{Eq. 6}]$$

For any assay design, the signal-to-noise ratio (S/N, dimensionless) is an important indicator of potential sensitivity. This ratio defines the factor by which values obtained exceed the statistical variability of such values, and is discussed in more detail in TechNote TNPJ100.02 *Precision of FRET Assays: Signal-to-Noise vs. Signal-to-Background*. For each of the response variables F, Q, A/B and R, signal-to-noise can be calculated as:

$$S/N = \frac{(\text{Mean Pos Control} - \text{Mean Neg Control})}{\sqrt{(\text{Pos Control STD})^2 + (\text{Neg Control STD})^2}} \quad [\text{Eq. 7}]$$

where STD is the sample standard deviation of the response variable. An additional assay characteristic of interest, the signal-to-background ratio (S/B), has been widely discussed in the TR-FRET literature. While it is generally less useful than the signal-to-noise ratio, it is reported here for comparative purposes:

$$S/B = \frac{\text{Mean Pos Control}}{\text{Mean Neg Control}} \quad [\text{Eq. 8}]$$

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

$$Z' = 1 - \frac{3(\text{Pos Control STD} + \text{Neg Control STD})}{\text{Mean Pos Control} - \text{Mean Neg Control}} \quad [\text{Eq. 9}]$$

PLEASE NOTE: The above equations form the theoretical basis for FRET calculations. The specific measurements taken and the details of recommended calculations vary with the type of assay employed.

A spreadsheet calculating these equations is available to customers. Please send an e-mail requesting a copy to:

info@prozyme.com

TECHNICAL SERVICE

This and other TechNotes are available on PROZYME's webpage located at:

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