

DISSECTING FRET DATA: QUENCH-FRET ANALYSIS

Quench-FRET analysis goes beyond standard FRET parameters (such as A/B ratio and Net FRET) by examining donor Quench, FRET and their ratio (Q/F). It is useful for detecting false positives and other artifacts produced by interference from absorbent/fluorescent sample compounds. Appropriate for both TR-FRET and PB-FRET™ assays, it is particularly suited to the latter because of the strong donor Quench and low noise in PB-FRET assays.

INTRODUCTION

Typically, when TR-FRET data analysis is performed according to the manufacturers' instructions, the output variable is the so-called A to B ratio (A/B), the ratio of:

- A - fluorescence counts in a detection window chosen to select fluorescence of the FRET acceptor ("Channel A") to
- B - fluorescence counts in a detection window chosen to select fluorescence of the FRET donor ("Channel B").

When FRET occurs, A increases while B decreases. A more dynamic response is obtained by using the ratio of the two. However, significant information conveyed by the absolute magnitude of the A and B values is lost in the calculation.

Sometimes the removal of signal magnitude may be beneficial; use of the A/B ratio can eliminate artifacts due to variation in reagent additions, non-specific sample absorbance (e.g. turbidity), and well-to-well variation in instrument counting sensitivity. On the other hand, failure to examine the individual components of the fluorescence signal can cause the investigator to overlook valuable information concerning the presence of interfering substances, such as absorbant or fluorescent compounds, in the assay mixture.

FUNDAMENTALS OF QUENCH/FRET ANALYSIS

We begin with the results of an assay calibration for FRET in standard A/B form, then break out this data into its individual components, showing how the effect of interfering substances can become progressively more apparent. The model system used for demonstration purposes was a TR-FRET assay for tyrosine kinase phosphorylated peptide; details of the assay are presented elsewhere (TechNote TNPJ100.10a *PB-FRET vs. TR-FRET: Sensitivity/Calibration*).

Figure 1 shows a conventional calibration curve for this assay, plotting A/B versus % phosphorylation level. This assay shows a very strong correlation between A/B and % phosphorylation, indicating that in the absence of interfering factors, good sensitivity can be obtained from this assay.

Figure 2 shows the values of the ratio components, counts in Channel A and counts in Channel B *vs.* % phosphorylation. Clearly, most of the variation in A/B is contributed by changes in Channel A; the relative variation in Channel B counts over the range is small, and its correlation to % phosphorylation is relatively weak. This means that the primary role of Channel B counts in the A:B ratio is normalization which, as mentioned above, may compensate for such factors as variable reagent additions or sample turbidity.

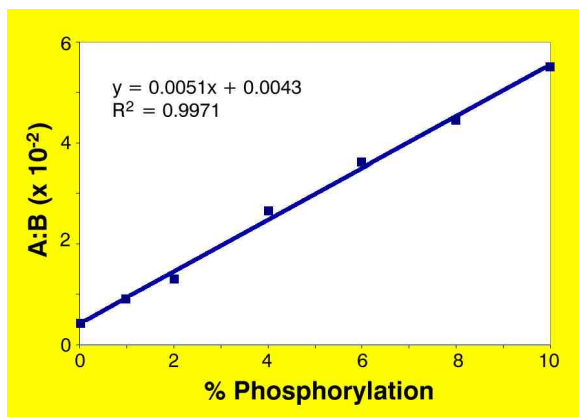


Figure 1 - Conventional (A/B) Calibration Curve

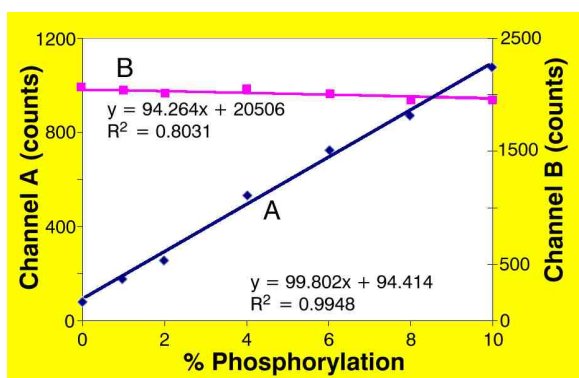


Figure 2 - A and B Counts Plotted Separately

Channel A counts, when corrected for various blanks and compensation factors, resolve to a value we refer to as Net FRET (F, TechNote TNPJ100.04 *FRET Calculations*). This value removes counts from all extraneous sources and leaves only counts due specifically to FRET fluorescence from the acceptor. Figure 3 shows that this value is still strongly correlated to peptide phosphorylation, as expected.

Similarly, Channel B counts resolve to a value referred to as Quench (Q), the reduction in fluorescence emission of the donor that is due to energy transfer to the acceptor. Thus, Quench is calculated as Channel B counts at 0 substrate concentration minus Channel B counts at the appropriate level of substrate (here, % phosphorylation), each corrected for all appropriate blanks and compensation. Figure 4 shows the correlation between Q and % phosphorylation. Since these values represent small differences between large

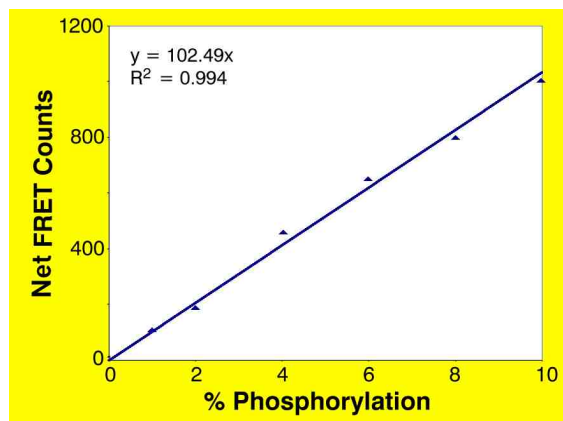


Figure 3 - Net FRET Calibration Curve

numbers (refer back to Figure 2), the results are significantly noisier than those for Net FRET, but there is still a significant positive correlation between the two variables.

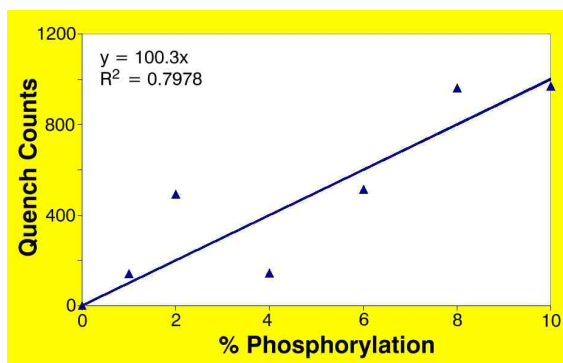


Figure 4 - Quench Calibration Curve

Since the donor is the sole source of excitation energy that results in acceptor FRET fluorescence, there should be a direct relationship between how much the donor is quenched and how much the acceptor fluoresces. This correlation is shown in Figure 5, although there is significant noise in the relationship. This noise is a consequence of the high noise in Q, seen in Figure 4.

While noisy quench data is a common characteristic of TR-FRET, there are other FRET approaches that are not encumbered by this shortcoming; Figure 6 shows the Quench-FRET relationship for PB-FRET data similar to the TR-FRET data set discussed here. The much closer correlation achieved

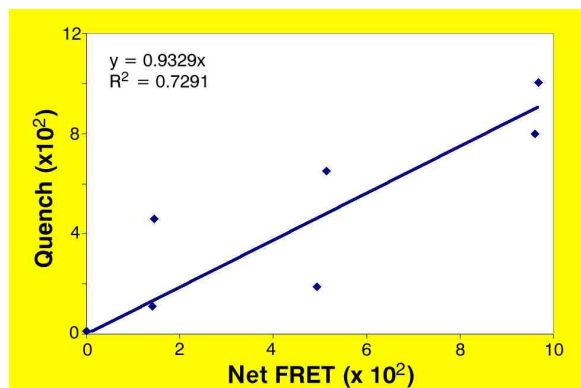


Figure 5 - Quench-FRET Correlation for TR-FRET

with PB-FRET is the direct result of the dramatically higher counts (see X- and Y-axes, Figure 6), which result in substantially less noise.

Even the very noisy Q-F relationship in TR-FRET can be adequate for the identification of many samples that are compromised due to sample absorbance or fluorescence. However, approaches such as PB-FRET can provide substantially improved sensitivity when using these sorts of approaches.

IDENTIFYING COMPROMISED RESULTS WITH QUENCH/FRET ANALYSIS

In the course of screening, it is not uncommon to encounter candidate compounds which possess color (for the purposes of this discussion, this means absorbance and/or fluorescence). Such samples alter the results of FRET assays in a manner that can either increase or decrease the *apparent* FRET signal (*i.e.* the calculated value for Net FRET in the presence of the compound, which is not equal to the actual level of FRET from the assay when colored/fluorescent compounds are present).

The technique presented here, Quench-FRET analysis, permits the identification of the vast majority of results that are compromised by sample color. The purpose of this process is to generate a *warning* when results are compromised in this fashion.

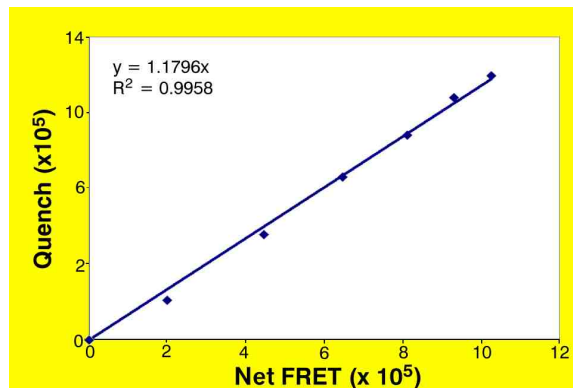


Figure 6 - Quench-FRET Correlation for PB-FRET

When Quench-FRET analysis generates a warning that data may be compromised, it is important to realize that, *a priori*, samples flagged in this manner may or may not possess the activity desired. The warning simply informs the investigator that the apparent result of the assay may not be relied upon to make this determination.

The investigator's response to such warnings depends on screening philosophy. Some investigators may choose to simply discard such flagged compounds as unsuitable for further investigation. Others may choose to continue the investigation of the compounds; where this is the case, rerunning the assay with the inclusion of additional controls can in many cases resolve the activity levels of the flagged compounds.

Four types of interference by colored compounds are possible:

- absorbance in Channel A
- absorbance in Channel B
- fluorescence in Channel A
- fluorescence in Channel B

Here, we discuss these effects individually, while recognizing that combined effects are also possible. When results are impacted by absorbance of excitation illumination, the effect is equivalent to absorbance in both Channel A and Channel B. We discuss the results for a model system in which a kinase reaction is allowed to proceed for a fixed period of time yielding a predictable level of phosphorylation (the Positive Control). In

such a system, an inhibitor would reduce the final phosphorylation level: we refer to cases in which the presence of a colored compound reduces the *apparent* result for phosphorylation as a *false positive* result.

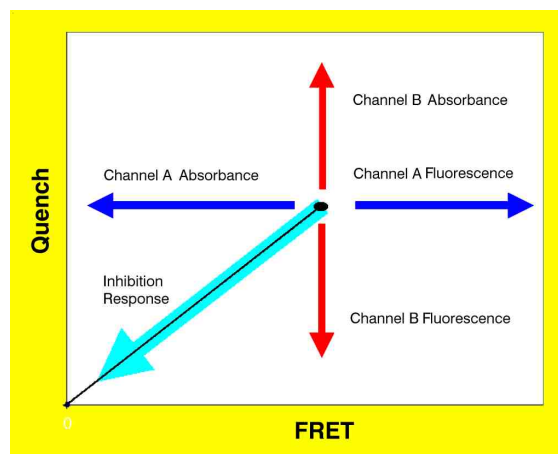


Figure 7 - Effect of Interfering Substances on Quench/FRET. (● indicates the assay control values without added compounds.)

Figure 7 shows the effects of each of these factors graphically. In each case, the interfering substance displaces the locus of the experimental result in the Quench-FRET plane (horizontal and vertical arrows) in a manner that is not consistent with the results that can be expected when no interference is acting (arrow superimposed on the Quench-FRET correlation line).¹ Thus, when screening results lie outside of the Quench-FRET correlation line (where the breadth of the arrow is determined by the level of noise in the Quench-FRET correlation), a warning is in effect, indicating the likelihood that interference is acting on the result.

Often, it is easiest to perform Quench-FRET analysis simply by examining results graphically, as in Figure 7. To identify the same results non-graphically, a test result to

¹The effect of interfering substances when there is no sample activity is shown in Figure 7. In the event that a sample had activity as well as interference, results would be displaced in the same directions from a point somewhere on the Quench-FRET correlation line.

which a warning must be attached is one that possesses *any one* of the following characteristics:

- Q lies outside the assay range
- F lies outside the assay range
- the test result lies significantly off of the Quench-FRET correlation line.

This last characteristic can be expressed as a single parameter, Q/F, which is Quench/Net FRET. A positive result, free of interference, will have an Q/F that is unchanged from the controls.

Of all the interfering processes, Channel A absorbance is the most problematic. It generates a false positive in A/B and FRET, and can only be identified through its effect on the Q/F ratio. That is, both A/B and FRET are reduced in a manner that would be consistent with the presence of a phosphorylation inhibitor, and the only indicator of a problem is that the Quench value remains unchanged, and thus the Q/F ratio increases.

For TR-FRET however, both Quench values and the Quench-FRET relationship are quite noisy. As a result, large changes in Quench or Q/F must occur before they become statistically significant; it is difficult to discriminate between false positives due to Channel A absorbance and true inhibition. This problem can best be resolved by utilizing the more sensitive PB-FRET assay in place of TR-FRET, but the ability of TR-FRET to resolve interference can also be improved by optimizing TR-FRET sensitivity (TNPJ100.16 *Optimizing S:N Ratios in TR-FRET*).

Table 1 summarizes the effects of these four types of interference on FRET results. The first highlighted row, A/B, shows that two of the four types of interference can be identified from those results alone, while the other two generate false positives. The second highlighted row, Q/F, generates warnings for all four types of interference.

There are highly improbable cases in which offsetting vectors for sample activity, absorbance, and fluorescence can generate

	Nature of Interference			
Response	Channel A Absorbance	Channel B Absorbance	Channel A Fluorescence	Channel B Fluorescence
Measured Channel A Fluorescence	↘	■	↗	■
Measured Channel B Fluorescence	■	↘	■	↗
A/B	↘ ✖	↗ ✖	↗ ✖	↘ ✖
Apparent Net FRET	↘ ✖	■	↗	■
Apparent Quench	■	↗ ✖	■	↘ ✖ or ✖
Q/F	↗ ✖	↗ ✖	↘ ✖	↘ ✖

↘ - Decreased counts, ↗ - Increased counts, ■ - Unaffected, ✖ - False Positive, * - Interference warning (further analysis required)

an apparent negative result when sample activity is present (TechNote TNPJ100.19 *Detecting Interference in PB-FRET*). However, Quench-FRET analysis will identify the vast majority of results that reflect sample interference.

TECHNICAL SERVICE

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