

## DETECTING INTERFERENCE IN PB-FRET™

*PB-FRET results that may be compromised due to candidate absorbance or fluorescence are readily identified by Quench-FRET analysis. The cost of the additional controls is minimal compared to the improved discrimination capability.*

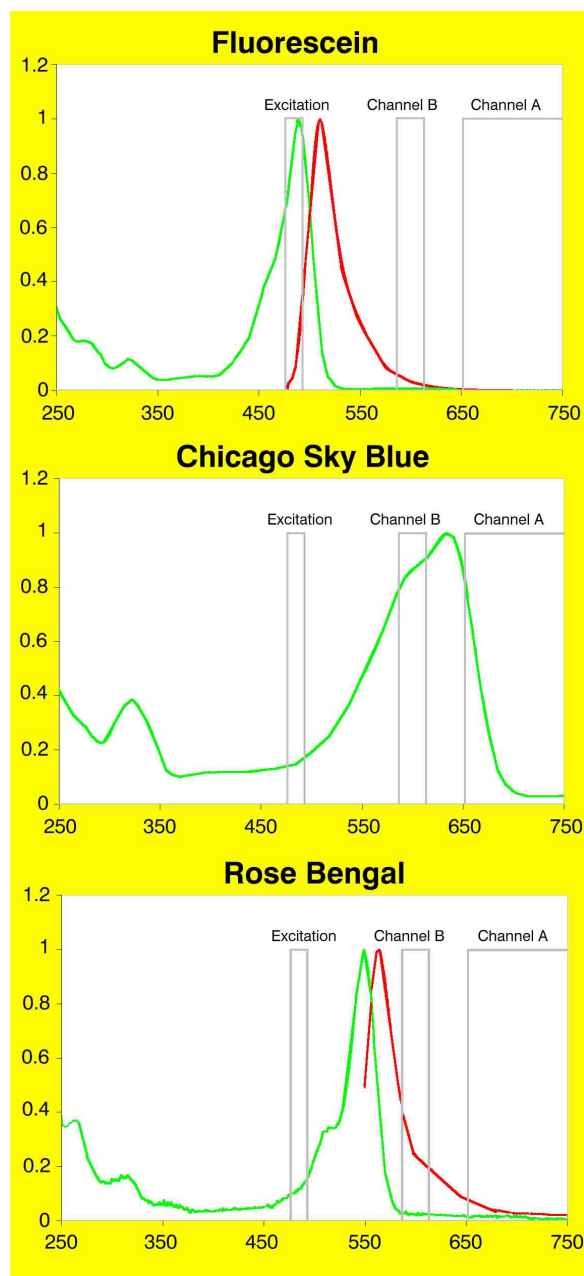
(Fundamentals of Quench-FRET analysis are discussed in TechNote TNPJ100.05 *Dissecting FRET Data: Quench-FRET Analysis*.)

FRET assays use fluorescence for detection so their ability to reliably measure molecular interactions may be impaired by candidates that fluoresce or absorb light. In screening assays, it is necessary to identify these results and flag them as potentially unreliable.

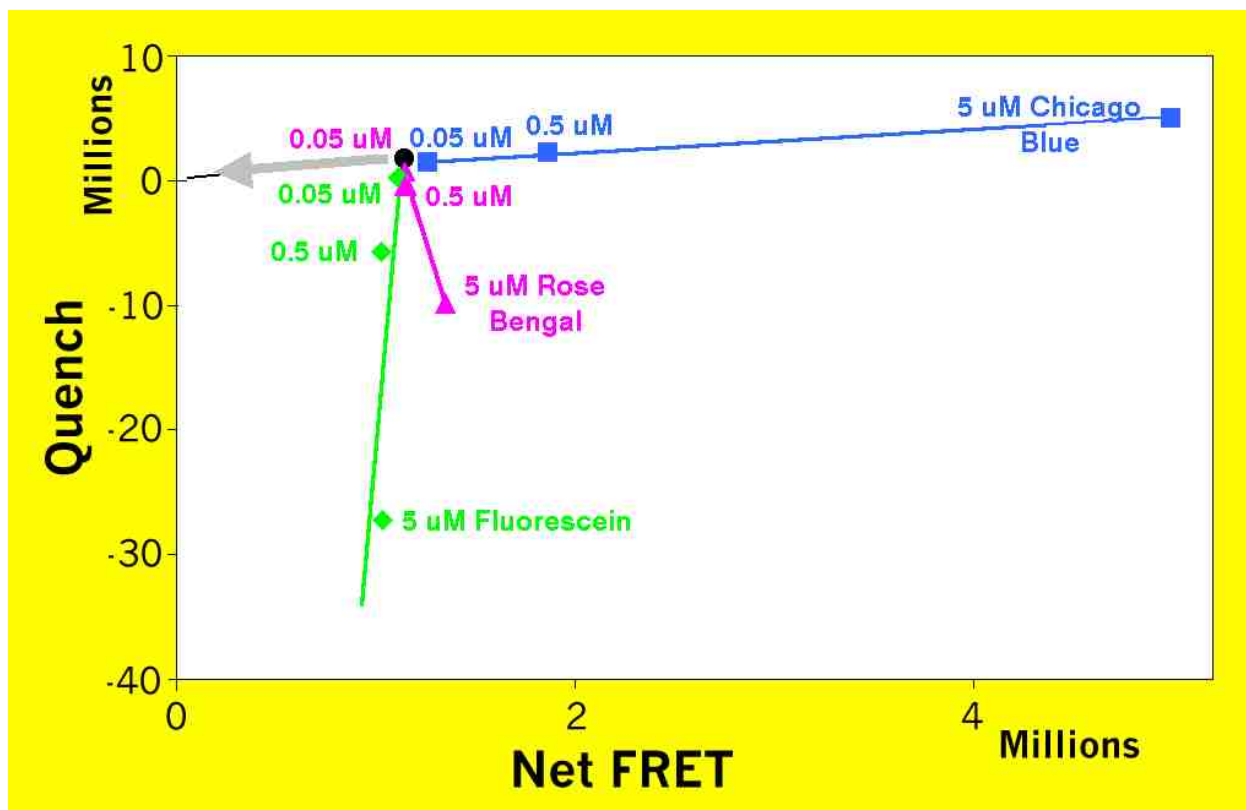
To test the effects of candidate absorbance and fluorescence, three compounds known to interfere with fluorescent assays (Fluorescein, Chicago Sky Blue and Rose Bengal) were tested in a model kinase assay and the results assessed using Quench-FRET analysis. These dyes make particularly good candidates for testing interference in PB-FRET assays because their absorbance/fluorescent spectra overlap the Excitation, Channel B or Channel A windows of the donor/acceptor pair (Figure 1).

In the assay, kinase activity is reflected in the level of phosphopeptide in the final assay mixture when the reaction is stopped; inhibition is reflected in a lower final concentration of phosphorylated peptide. Results from assay test wells are divided into three types:

- wells where no inhibition is demonstrated
- wells that demonstrate inhibition within the expected range of the assay
- indeterminate wells where results are identified as unreliable due to interference by assay components, *i.e.* candidate absorbance and/or fluorescence has masked the activity or inactivity of the candidate.



**Figure 1** - Absorbance (green) and fluorescence (red) spectra of selected dyes. (Fluorescence of Chicago Sky Blue was diffuse.)



**Figure 2 - PB-FRET™ Assay Results Plotted on the Quench-FRET Plane** (← indicates inhibition response, ● indicates the assay control values without added compounds.)

## METHODS

Assays were performed using the optimized protocol described in TechNote TNPJ100.15 *Improving S:N Ratios in a PB-FRET Assay*. The dyes were added at three levels (0.05  $\mu\text{M}$ , 0.5  $\mu\text{M}$  and 5  $\mu\text{M}$ ).

## RESULTS

Figure 2 shows the PB-FRET assay results plotted in the Quench-FRET plane, *i.e.* *apparent* Quench-FRET assay results are compared to the expected Quench-FRET correlation line obtained from control wells (gray arrow). The individual dyes effect assay results as follows:

**Fluorescein** absorbs excitation illumination, and fluoresces in Channel B. Absorbance of excitation light is equivalent to absorbance in both Channel A and Channel B. The effect of increasing fluorescein concentrations is

described by a line in the Quench-FRET plane (green line in Figure 2) that points downward and slightly to the left. The downward direction indicates that Channel B fluorescence significantly outweighs Channel B absorbance, while the leftward shift reflects reduced donor excitation due to absorbance at the donor excitation wavelengths.

**Rose Bengal** fluoresces in Channel B, and fluoresces less strongly in Channel A. The line representing increasing concentration of the dye slopes downward and to the right (rose line in Figure 2), the downward slope representing Channel B fluorescence and the rightward trend representing Channel A fluorescence.

**Chicago Sky Blue** absorbs in Channel B and less strongly in Channel A while fluorescing in Channel A. Deflection is to the upper right (blue line Figure 2), reflecting the dominant Channel A fluorescence over Channel A absorbance for the right-hand deflection and Channel B absorbance for the upward

deflection. Interference by Chicago Sky Blue is an example of the only type of interference that cannot be fully resolved by Quench-FRET analysis, since the deflection line due to its interference is nearly parallel to the original Quench-FRET correlation line, in the opposite direction from the vector due to inhibition of the phosphorylation reaction. In all cases where the deflection due to color is greater than that due to inhibition, a positive result for inhibition will be masked.

One additional case in which false positive results due to interference by sample color can be obtained is when there is Channel A absorbance and Channel B fluorescence in a sample. However, in FRET analyses, Channel A is normally at a higher wavelength than Channel B, and the physics of fluorescence requires that emission occurs at a higher wavelength than absorbance. Thus, such a combination is not expected in any single colored substance, and is only possible (albeit improbable) when multiple colored substances are present in the sample being screened.

## CONCLUSIONS

Quench-FRET analysis was able to readily identify data compromised by three dyes known to interfere with FRET assays.

Interfering substances that fluoresce in Channel A and quench in Channel B in the same proportion as the Quench-FRET ratio would not always be identified by this analysis. However, substances of this sort will be rare.

## TECHNICAL SERVICE

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

<http://www.prozyme.com/technical/index.html#technotes>

ProZyme customers are an important source of information regarding advanced or specialized uses of our products. We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.



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