Redder Is Better: Far-Red PolarScreen™ FP Kinase Assay

Kevin L. Vedvik, Hildegard C. Eliason, Jennifer A. Fronczak, Melissa E. Krueger and Kurt W. Vogel
Invitrogen Corporation • 501 Charmany Drive • Madison, WI 53719 • USA

Why Is Redder Better?

Compound interference is the bane of HTS. Two forms of interference that affect fluorescence polarization assays are compoundfluorescence and light scatter. Compound fluorescence arises from the intrinsic fluorescence of some library compounds. Because compound libraries are typically screened at concentrations of 10 µM, and tracer concentrations are typically 1 nM, a library compound that has 1/10,000th (or less) of the fluorescent "brightness" of the tracer has the potential to interfere with the assay signal. Because most small molecules that fluoresce will emit a depolarized (low polarization) signal, this can lead to false positive or false negative results, depending on the assay configuration.

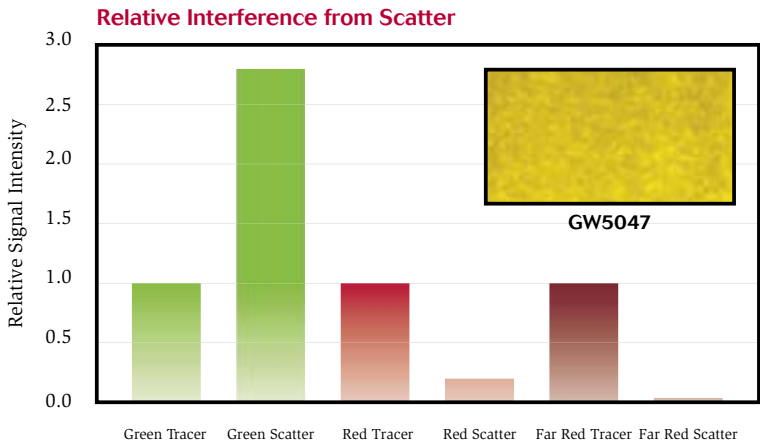
The ability of a molecule to absorb light in the "red" (longer wavelength) region of the spectrum depends largely on the degree of conjugation (adjacent double bonds and aromatic rings) within the molecule. In general, the more conjugation the molecule has, the better able it will be to absorb light in the red region of the spectrum. Because the ability to absorb light is a prerequisite to fluorescence, it follows that in order to fluoresce at "redder" regions of the spectrum a molecule needs more conjugated double bonds. The high degree of conjugation necessary for "red" fluorescence is incompatible with "druglike" properties, and therefore there are, in general, fewer "red" fluorescent compounds in most libraries.

The second interfering factor in FP assays arises from light scatter. Light scatter arises from precipitated compounds or other particulate matter (dust) in an assay well. Because light scatter is more efficient at lower (greener) wavelengths, and drops off in intensity relative to the 4th power of the wavelength, scatter is less of an interference at longer wavelengths. Scattered light is often polarized (this is the reason sunglasses are polarized—to block scattered light, or glare, from the horizon) and therefore interferes with an assay by giving an abnormally high polarization value.

Figure 1—Assay Interference Decreases at Longer Wavelengths

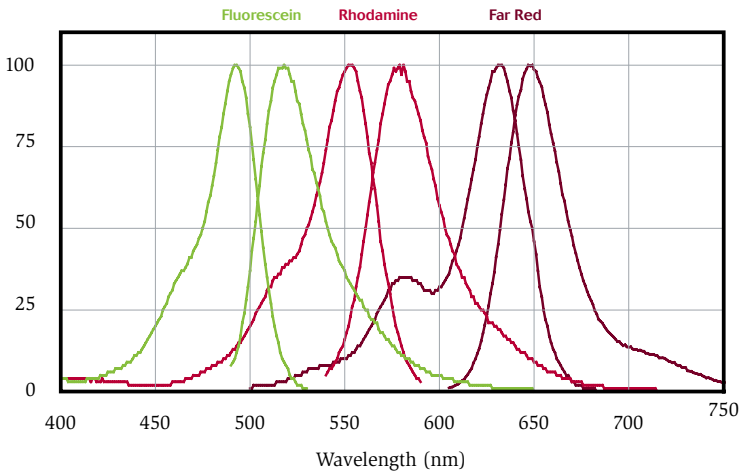
Compounds in LOPAC[1280] with greater than 50% signal intensity of 1 nM corresponding tracer		
Tracer	**λ (Ex / Em)**	**# of Compounds**
Green	485 / 535	19
Red	535 / 590	9
Far-Red	590 / 650	3

To determine an approximation of the amount of fluorescent interference that can be expected in a typical compound library at green, red, and far-red wavelengths, the LOPAC[1280] library was prepared at 10 µM in buffer and read in fluorescence intensity mode using filters typical to a green, red, or far-red tracer. Wells containing 1 nM of each tracer were placed on the assay plate so that signal intensity could be referenced to a typical tracer concentration.



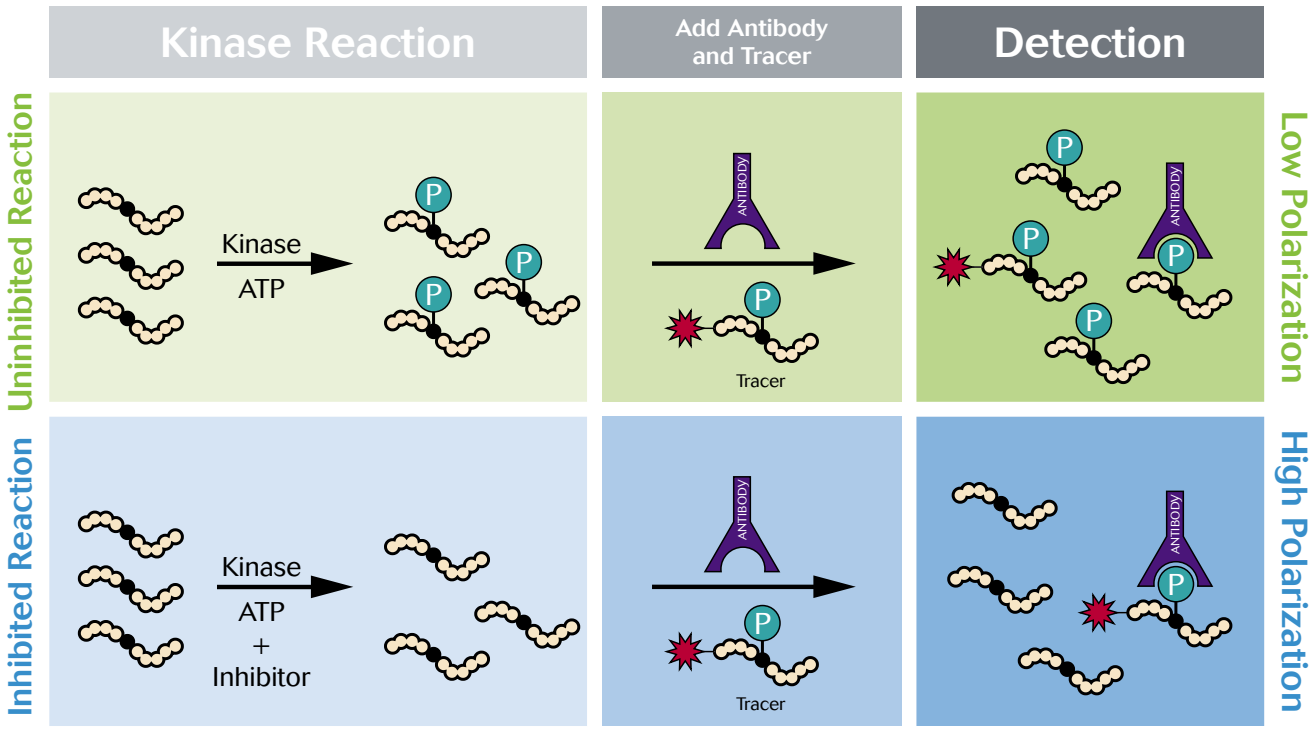
To demonstrate the effect of light scatter on assay interference, GW5047, a compound from the LOPAC Library prone to precipitation, was compared in signal intensity to 1 nM of green, red, and far-red tracers. The graph above illustrates negligible interference (< 0.4%) seen from GW5047 when using a far-red tracer. The inset shows a close-up of a vial containing the precipitated compound.

Figure 2—Excitation / Emission Spectra of Far-Red Tracers



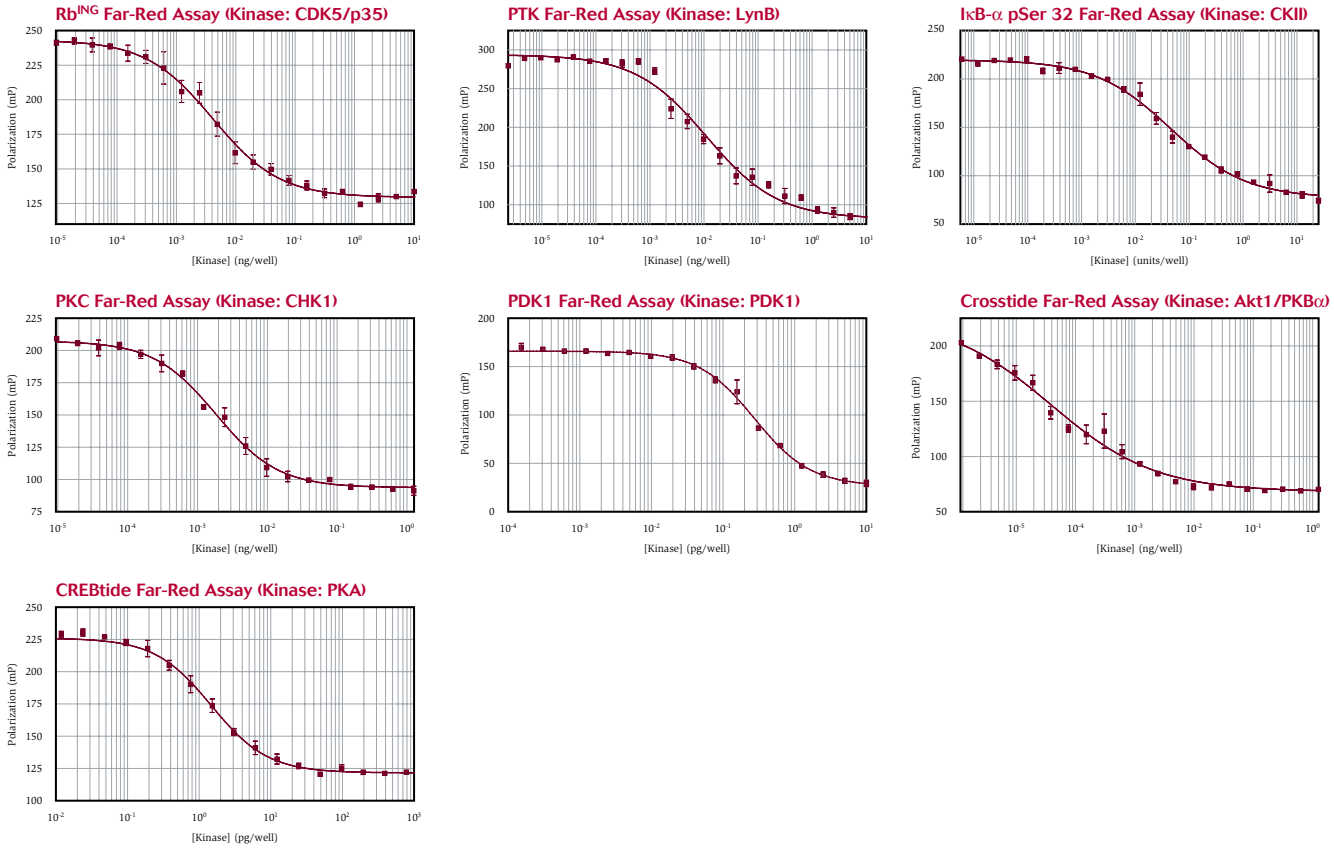
Excitation/Emission spectra of commonly-used fluorescein and rhodamine-based tracers compared to the spectra of Invitrogen's new far-red tracers. The excitation and emission maxima for the far-red tracer are red-shifted beyond 600 nm, allowing fluorescence polarization assays to be performed using a 610 nm excitation filter and 670 nm emission filters.

Figure 3—FP Kinase Assay Schematic



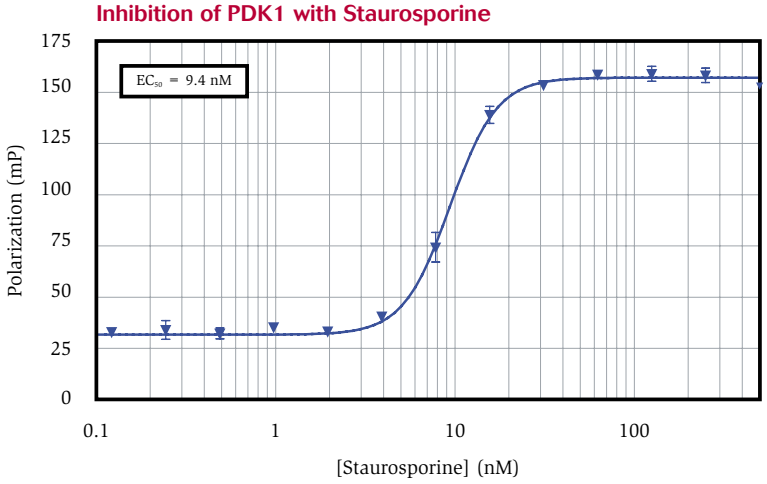
In a far-red FP kinase assay, kinase, substrate, and ATP are allowed to react in the presence of library compounds. After the reaction, antibody and far-red-labeled tracer are added. The amount of antibody that binds to the tracer is inversely related to the amount of phosphorylated product present. Thus library compounds that inhibit the reaction are identified as wells that have a high polarization value.

Figure 4—Far-Red FP Kinase Kits Address a Range of Kinase Targets



Seven far-red kinase assays are available to address a wide variety of kinase targets. The plots in Figure 3 show the results from kinase titrations in 10 µl, 90 minute kinase reactions under conditions of non-limiting substrate and ATP. In general, picogram to nanogram quantities of kinase will phosphorylate sufficient peptide substrate to effect an assay window of between 125 and 250 mP.

Figure 5—Inhibition of PDK1 by Staurosporine



Far-Red kinase assays can be used for both high-throughput screening as well as follow-up of hits to determine accurate EC_{50} values.

Conclusions

As the size of compound libraries increases, so does the cost of follow-up screening of false positive and false negative “hits” that are due to compound interference. The shift from “green” to “red” has been recognized as a valid strategy to overcome interference from autofluorescence or light scatter due to precipitated compounds. Invitrogen’s new Far-Red PolarScreen™ Assays employ a proprietary far-red fluorophore that gives excellent performance in fluorescence polarization assays. The fluorophore is highly water soluble, and unlike cyanine-based fluorophores, has a fluorescence lifetime that allows for large polarization shifts between free and bound tracer.