The Next Step in Near Infrared Fluorescence:

IRDye® QC-1 Dark Quencher

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Non-fluorescent quenching dyes are an important component of probes for protease activity assays (1-6), nucleic acid hybridization (7, 8), and real-time PCR (9-11). Until recently, quenched probes have been mainly limited to visible fluorophores at wavelengths <700 nm. However, higher wavelength near-infrared dyes, such as IRDye 800CW, offer advantages in many biological applications.

Cells, tissues, plastics, blotting membranes, and chemical compound libraries all exhibit autofluorescence that can interfere with detection. However, in the near-infrared (NIR) spectral region (650 - 900 nm), autofluorescent background is dramatically reduced (12, 13). For this reason, NIR fluorophores such as IRDye infrared dyes are able to enhance detection sensitivity and signal-to-noise ratios in applications where autofluorescence had been limiting. This improvement has extended the benefits of fluorescent detection to new applications such as Western blotting and *in vivo* imaging, and can provide improved performance for cell-based assays, protein microarrays, microtiter plate assays, microscopy, and screening of small molecule libraries(14, 15).

LI-COR® Biosciences has developed a new non-fluorescent (dark), water-soluble quencher dye called IRDye QC-1. This novel quencher has the widest available quenching range, extending into the NIR, and has been shown to quench a range of commonly used fluorophores with a quenching efficiency >97% (16). IRDye QC-1 is also water-soluble, which simplifies the labeling and purification process, eliminates undesired non-specific binding with target biomolecules, and helps maintain the solubility of conjugated biomolecules in aqueous assay media. IRDye QC-1 can bring the advantages of the near-infrared region to quenched probe applications.

A review of the following studies:

X. Peng, H. Chen, D. R. Draney, A. Schutz-Geschwender, D. M. Olive, A nonfluorescent, broad range quencher dye for Förster resonance energy transfer assays. Analytical Biochemistry 388 (2009) 220-28.

X. Peng, D. R. Draney and W. M. Volcheck, Quenched near-infrared fluorescent peptide substrate for HIV-1 protease assay. Proc SPIE 6097 (2006) 60970F.

FRET Assays

One advantage of using quenched fluorescent substrates in real-time quantitative PCR, protease digestion assays or *in vivo* optical imaging is the ability to achieve a good signal/background ratio in the presence of excess unreacted or unbound fluorescent probe. In a simple fluorescence intensity assay, the unbound or unreacted fluorescent probe must be removed from the sample to measure the signal from the desired target, because every dye molecule can potentially contribute signal. Therefore, fluorescence intensity assays may require washing steps, column purification, or clearance of the unbound probe from the animal's system in optical imaging before a specific signal can be identified and quantified. An additional factor contributing to assay background is nonspecific binding of the probe. Depending on the assay, the chemical nature of a fluorescent dye can result in nonspecific binding interactions which will contribute background and negatively impact the limit of detection (LOD) for an application. Fluorescence polarization, Förster (or fluorescence) resonance energy transfer (FRET) and time-resolved methods were also developed to address these issues.

FRET is a particularly useful method that can be employed with commonly available fluorescent detection systems, whereas fluorescence polarization and time-resolved studies require instrumentation with special capabilities. Basic fluorescence occurs when a dye absorbs light energy at a short wavelength and then emits longer wavelength light as fluorescence (Figure 1). The difference between maximum absorption wavelength and maximum emission wavelength is known as the Stokes shift.

An excited fluorophore normally emits absorbed energy in the form of light. But when two compatible fluorophores are in close proximity, the energy can be transferred in a non-radiative process from a donor fluorophore to an acceptor fluorophore, which becomes excited. As a result of this energy transfer, donor fluorescence is quenched and acceptor fluorescence is generated. The acceptor fluorophore then emits light at its characteristic emission wavelength, which is easily distinguished from donor fluorescence. This process is called FRET. Not all fluorophore combinations emit light. Some acceptor fluorophores, known as quenchers, can dissipate the absorbed energy in molecular vibrations or heat (non-radiative processes) and the probe remains relatively dark. Both types of FRET probes are very useful for reducing background and simplifying assays.

In addition, quenching of a donor-acceptor pair can be enhanced through a non-FRET mechanism called static quenching or contact quenching. This type of quenching can occur even in supposedly "linear" oligonucleotide probes that have no defined secondary structure to bring the reporter and quencher pair together. When static quenching accompanies FRET quenching, the quenching efficiency of the system is enhanced.

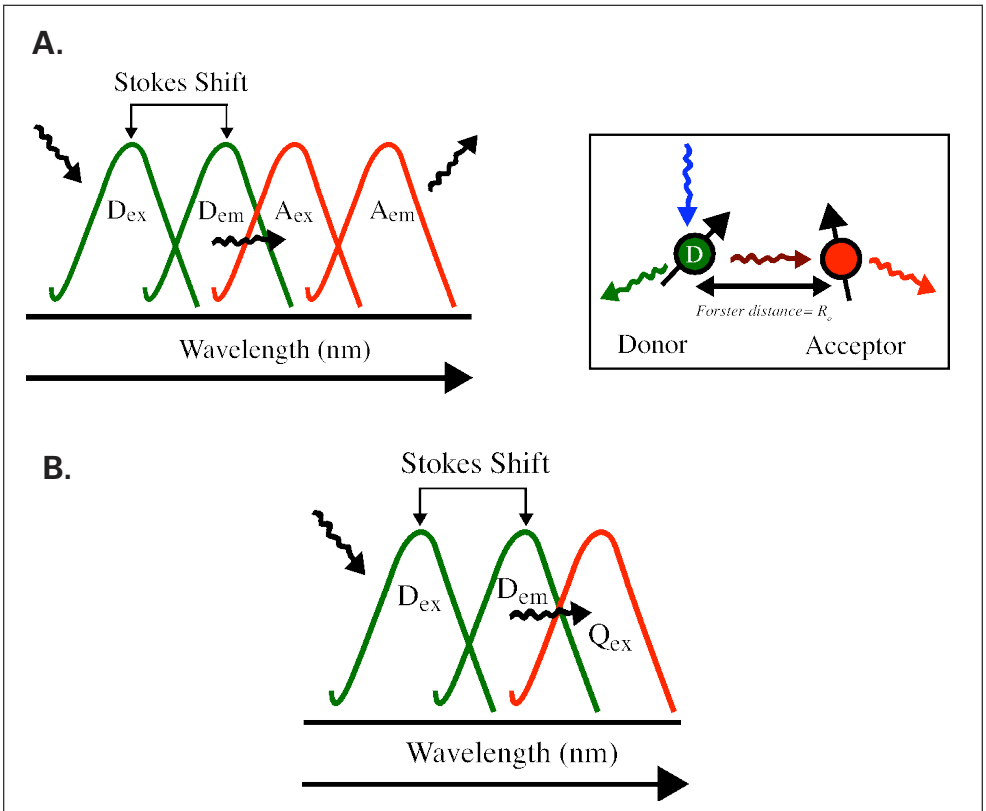

Figure 1. In FRET, the energy is transferred from the donor (D_{em})and emitted at the acceptor's wavelength (A_{em}; 1A). In the case of a quencher acceptor (Q), the light energy is not emitted but is dissipated as heat (1B).

For FRET to occur, two conditions must be satisfied simultaneously: the fluorophores must be spectrally compatible and within close proximity. Fluorophores are spectrally compatible when the absorbance spectrum of the acceptor overlaps the emission spectrum of the donor. Without spectral overlap, the energy from the donor will not excite the acceptor. If the absorbance spectrum of the acceptor does overlap the emission spectrum of the donor, energy from the donor will excite the acceptor molecule if the distance criterion is met. The distance separating the donor and acceptor molecules strongly affects the efficiency of the energy transfer process (by the inverse of the sixth power). Each fluorophore pair can be

characterized by its Förster radius (R_0), the calculated distance at which resonance energy transfer between a compatible donor and acceptor will drop to 50%. In practical terms, there is a maximum length for an oligonucleotide or peptide with a fluorophore at each end, beyond which FRET will not be sufficient for a reliable assay. The Förster distance is a useful guide for designing the spacing of the fluorophore pair in a quenched probe to obtain efficient quenching. As a guideline, an R_0 value of 60 to 80 angstroms is very good, while a value less than 40 angstroms indicates the pair will be less useful.

IRDye QC-1

The choice of the best quencher (acceptor) for a particular fluorophore will depend on several factors: 1) the spectral overlap with the acceptor; 2) the quenching efficiency of the donor/quencher pair; 3) the native fluorescence of the quencher; and 4) the chemical nature of the dye (including hydrophobicity and steric size). IRDye QC-1 has been assessed and shown to be an excellent quencher by these standards (16).

IRDye QC-1 was tested in protease assays for caspase-3, HIV and beta secretase. The quenched peptide substrates for these assays were designed with a donor and quencher placed on opposite sides of the protease cleavage site. Upon cleavage by the protease, the donor and quencher are separated beyond the required interaction distance and fluorescence emission is detected from the donor dye. This process is depicted schematically in Figure 2 for the HIV-1 protease. When the enzyme cleaves the IRDye 800CW-VSQNYPIVQNK-(IRDye QC-1)-COOH quenched peptide substrate, IRDye 800CW will fluoresce and signal will be detected around 780-820 nm.

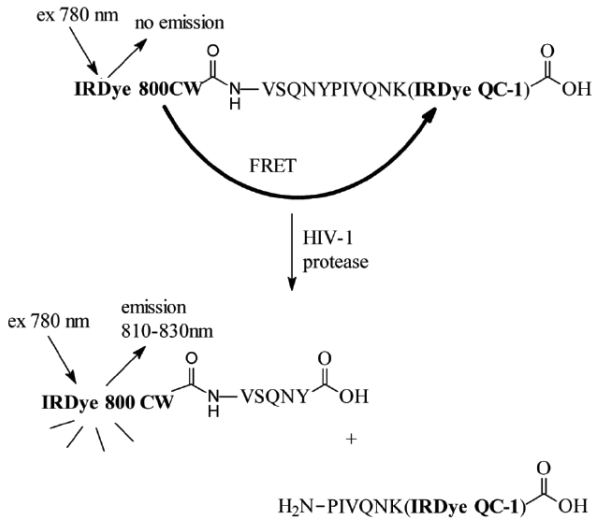

Figure 2. The principle of the IRDye HIV-1 Protease Assay. Upon cleavage, IRDye 800CW becomes fluorescent.

The reaction rate and amount of signal are directly proportional to enzyme activity.

The Odyssey® Infrared Imaging System or Aerius® Automated Infrared Imaging System are the best platforms for detection of IRDye infrared dyes in microplate assays. IRDye 800CW is detected in the 800 nm channel of these instruments, and the 700 nm channel can detect IRDye 700DX or IRDye 680. An example is shown in Figure 3, where the IC_{50} of Pepstatin A inhibition of HIV-1 protease was characterized. The IC_{50} of 62 nM for this inhibitor agreed with previously reported values (17).

A caspase-3 activity assay has also been evaluated with the octapeptide substrate IRDye QC-1-GDEVDGAK-(IRDye 800CW)-COOH (16). The sensitivity of this assay was evaluated with dilutions of human recombinant caspase-3 in a 96-well microplate format. The assay detected caspase-3 enzyme activity at levels as low as $1x10^{-5}$ units/ml, while maintaining a signal-to-noise ratio above 3. This limit of detection is approximately 7 times

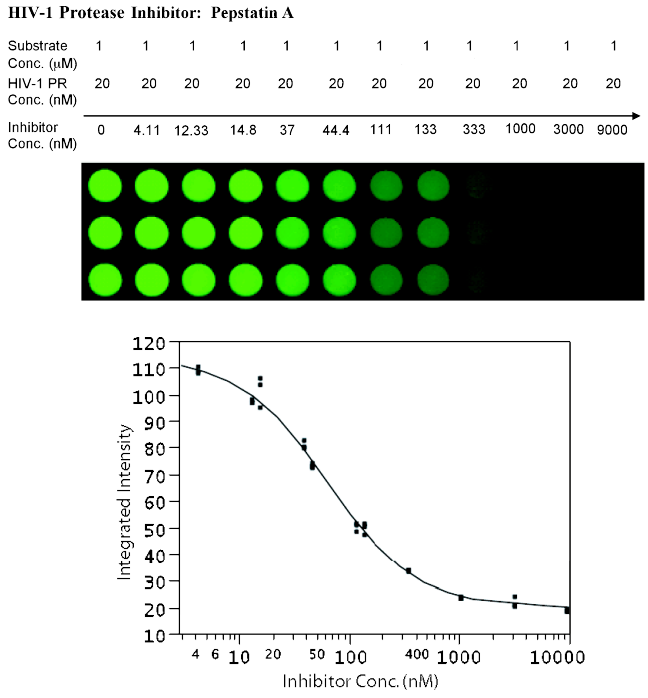

Figure 3. Inhibition of HIV-1 protease by a characterized inhibitor, Pepstatin A, in a microplate assay. Proteolysis of the quenched substrate (final concentration 1 μM) by HIV-1 protease (20 nM) was inhibited by the addition of increasing amounts of Pepstatin A (at final concentrations ranging from 4.11 nM to 9 μM, with no-inhibitor wells included as controls). After incubation at 37°C for 1 hr, the reactions were stopped with protease stop solution, and fluorescence intensity of IRDye 800CW was measured with an Aerius Automated Infrared Imaging System (17). The resulting data were fitted with a Rodbard model.

more sensitive than a similar bioluminescent assay using Z-DEVD-aminoluciferin and ~7000 times more sensitive than a fluorescent assay with Z-DEVD-AMC (8). The additional sensitivity demonstrates a clear advantage of IRDye infrared dyes.

Quenching range

As we have discussed, the quencher absorption spectrum should overlap significantly with the emission spectrum of the fluorophore for efficient quenching to occur. The first FRET assays used a portfolio of different quenchers with visible donor dyes (450 – 600 nm). Later quencher families, such as the BHQ, CyQ, QSY and QXL families, were developed where the members had similar structures and properties. For example, QSY 35, 7 and 9 can quench the fluorescence from blue and green, green and orange, green and orange fluorophores, respectively. BHQ-1, -2 and -3 can be used to quench from ~480 to 580 nm, ~559 to 670 nm and ~620 to 730 nm, respectively. Cy5Q is suitable for Cy3 fluorescence quenching and Cy7Q is suitable for Cy5 fluorescence quenching. A quencher with a broader quenching range like IRDye QC-1 allows more consistency in preparation and use of probes in multiplexed assays. An extensive list of quenchers and quenching ranges is found in Table 1.

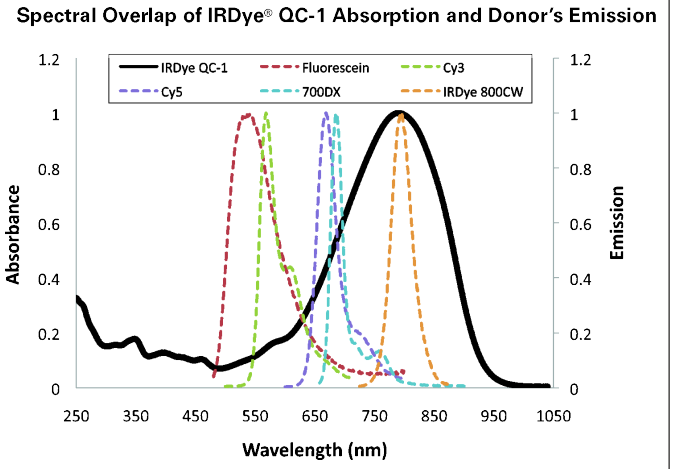

Figure 4. The normalized emission spectra for several commonly used fluorophores and the absorption spectrum of IRDye QC-1.

The broad absorption peak of IRDye QC-1 spans the entire visible region and into the near infrared region of the spectrum, with a maximum at 737 nm and a shoulder at 815 nm (Figure 4). The quenching range

Table 1. A list of fluorophores and spectrally compatible quenchers (shaded box = compatible) (18-22)

Fluorophore	E_{max}	Dabcyl	BHQ-1	BHQ-2	BHQ-3	Cy5Q	Cy7Q	Iowa Black FQ	Iowa Black RQ	IRDye QC-1	QSY35	QSY7	QSY21	QXL520	QXL570	QXL610	QXL680
Coumarin	472	■									■						
Alexa Fluor® 488-X	517	■	■					■			■			■			
Oregon Green 448-X	518	■	■					■			■			■			
Fluorescein	520	■	■					■		■	■	■		■			
Rhodamine Green-X	531		■					■		■		■					
Alexa Fluor 532	553		■			■		■	■	■		■					
Cy3	564		■	■		■		■	■	■		■			■		
BODIPY® 558/568	568		■	■		■		■	■	■		■	■		■		
Alexa Fluor 546	571		■	■		■		■	■	■		■	■		■		
TAMRA	583		■	■				■	■	■		■	■		■		
ROX	608			■				■	■	■		■	■		■	■	
Alexa Fluor 594	616			■				■	■	■			■			■	
Texas Red®-X	617			■	■			■	■	■			■			■	
BODIPY 630/650-X	653			■	■		■		■	■			■				
Cy5	668			■	■		■		■	■			■				■
Alexa Fluor 647	670			■	■		■		■	■			■				■
IRDye 680	702				■					■							■
IRDye 700DX	700				■					■							
Cy5.5	706				■					■							
Alexa Fluor 750	775									■							
IRDye 800CW	789									■							
IRDye 800	809									■							

was validated by measuring the quenching efficiency for the same caspase-3 octapeptide using six different visible and near infrared dyes in combination with the IRDye QC-1 quencher. Efficiency of fluorescence quenching (measured as the difference in fluorescence between the quenched and completely cleaved substrates) was expressed as a percentage. The data in Table 2 show IRDye QC-1 has excellent quenching efficiency over the entire range from fluorescein to IRDye 800CW in this system.

Native fluorescence

Ideally, a quencher should be "dark" or non-fluorescent so that the quenched, unreacted molecule does not contribute any fluorescence to the assay. Early FRET assays used a second fluorescent dye as an acceptor, such as the combination of fluorescein (donor) and rhodamine (acceptor). Since both the donor and the acceptor fluoresced, the acceptor also contributed signal and complicated the analysis of the assay. Dark quenchers, such as the BHQ and QXL families, were developed to eliminate this problem and these dye families have become widely adopted for major applications like quantitative PCR.

IRDye QC-1 is also a dark quencher. To demonstrate this property, the intrinsic fluorescence spectrum of solution IRDye 800CW was compared with that of IRDye QC-1. The results for dye solutions with the same absorbance at 700 nM are shown in Figure 5. IRDye QC-1 showed essentially no fluorescence, with an absorbance spectrum nearly identical to the methanol blank.

Steric issues

Because IRDye 800CW and IRDye QC-1 are larger molecules than visible fluorophores, it was important to determine whether or not they would sterically block interactions with proteins or alter kinetics. The kinetics (k_{cat}/K_M) of the cleavage reaction were compared for a quenched caspase-3 substrate peptide (GDEVDGAK) with a range of acceptor dyes on one end and IRDye QC-1 quencher on the other. The results are listed in Table 3, with values reported for some commonly used fluorescent caspase 3 substrates. IRDye conjugation had no

Table 2. Quenching efficiency of IRDye QC-1 paired with several common fluorophores on a caspase-3 octapeptide substrate (16).

Fluorogenic Caspase-3 Peptide (GDEVDGAK)	% Quenching Efficiency
IRDye QC-1 - Fluorescein	97.5
IRDye QC-1 - Cy3	98.1
IRDye QC-1 - Cy5	97.9
IRDye QC-1 - IRDye 680	98.8
IRDye QC-1 - IRDye 700DX	98.8
IRDye 800CW - IRDye QC-1	98.7

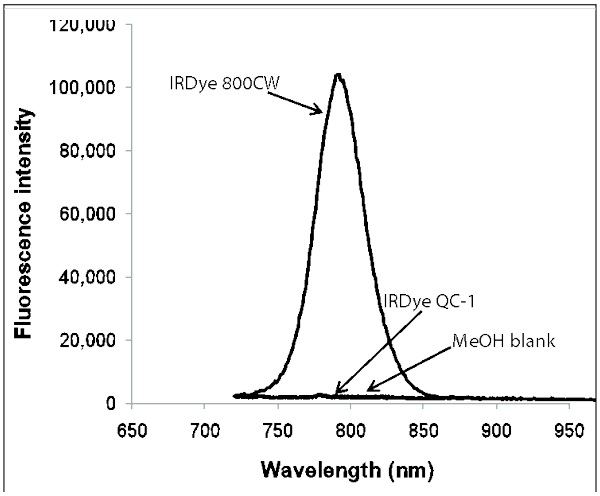

Figure 5. Fluorescence spectra of IRDye 800CW and IRDye QC-1 in methanol (each at 0.01 OD absorbance at 700 nm) following excitation by 700 nm light.

Table 3. Kinetic data for caspase-3 cleavage of several of IRDye QC-1 quenched substrates.

Fluorophore	Quencher	Substrate	K_{cat}/K_M ($\times 10^7$ $M^{-1}\cdot S^{-1}$)	Ref
Fluorescein	IRDye QC-1	GDEVDGAK	3.86	16
Cy3	IRDye QC-1	GDEVDGAK	4.65	16
Cy5	IRDye QC-1	GDEVDGAK	5.14	16
IRDye 680	IRDye QC-1	GDEVDGAK	2.71	16
IRDye 700DX	IRDye QC-1	GDEVDGAK	7.99	16
IRDye 800CW	IRDye QC-1	GDEVDGAK	3.84	16
AMC	None	DEVD	0.14 - 0.18	23
pNA	None	DEVD	0.02	23

significant effect on the cleavage kinetics when compared with visible dyes, and the kinetics were even better than some commonly used substrates.

Cell-based apoptosis assay

Many commercially available quencher dyes are hydrophobic molecules with poor water solubility, and are better suited for DNA probes than protein-based biological assays. Bioconjugates prepared with hydrophobic dyes are more difficult to prepare and purify; are often not sufficiently soluble in the aqueous assay medium; and may exhibit undesired non-specific binding with other biomolecules in the assay. To demonstrate the suitability of IRDye QC-1 quencher for biological assays, a caspase activity assay was developed for use in apoptotic cells (16).

Jurkat cells were grown in a 96-well microplate, treated with anisomycin or camptothecin to induce apoptosis, and then permeablized with Triton® X-100. IRDye 800CW or IRDye 680 quenched caspase-3 substrates were incubated with the cells, and the microplate was then imaged directly with an Aerius® Infrared Imaging System (16). Figure 6 shows the relative fluorescence signal obtained by incubating the substrates with serially diluted apoptotic Jurkat cells. Caspase-3 activity was reproducibly detected. The relationship between fluorescence intensity and cell number was linear over a wide range of cell numbers, and the small standard deviations in the data indicated that the assay was very reproducible. The limit of detection for the assay was ~1500 camtothecin-treated cells, or ~3000 anisomycin-treated cells. This cell-based IRDye assay is sensitive, reproducible, and quantitative, and may be useful in drug screening or apoptosis research.

The quenched IRDye caspase substrate has also been used to screen Xenopus oocytes undergoing apoptosis. The substrate was microinjected into oocytes and imaged with the Odyssey® system at high resolution to identify apoptotic events (Kornbluth *et. al*, personal communication). Individual oocytes undergoing apoptosis were easily identified.

CONCLUSION

IRDye infrared dyes are excellent fluorescent labels for protein and cellular assays, biochemical assays, microscopy, and *in vivo* molecular imaging. These dyes are bright, have excellent water solubility, and exhibit low non-specific binding. Background fluorescence from membranes, plastics, tissues, biological samples, and chemical compounds is substantially reduced at NIR wavelengths, enabling a variety of fluorescent applications that were previously impractical. In addition, the ability of NIR light to penetrate deep into animal tissue opens new windows of opportunity for *in vivo* imaging of small animals (24).

The addition of a quencher dye, IRDye QC-1, to this dye family brings quenched FRET applications into the near-infrared. IRDye QC-1 is an excellent quencher with essentially no inherent fluorescence and good water solubility, for use in many biological applications. This quencher dye also has the widest available quenching range, with the ability to quench both visible and near infrared fluorophores (~500 nm to ~900 nm). IRDye QC-1 promises to bring the advantages of the near-infrared to a host of applications.

For more information:
http://www.licor.com/bio/reagents/irdye.jsp

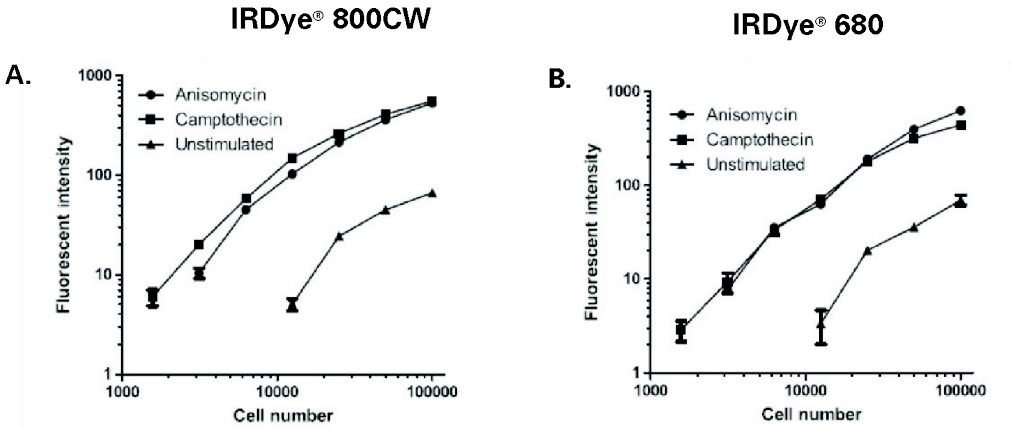

Figure 6. Detection of endogenous caspase-3 activity in Jurkat cells that were either untreated, or treated with anisomycin (1 µg/ml) or camptothecin (1 µg/ml) to induce apoptosis (16). A.) IRDye 800CW caspase-3 substrate quenched with IRDye QC-1. B.) IRDye 680 caspase-3 substrate quenched with IRDye QC-1. Error bars are ± standard deviations.

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