

The Mechanistic Side of Fluorescence Quenching: A Discussion of Specialized Quenching to Enable Multiplexing

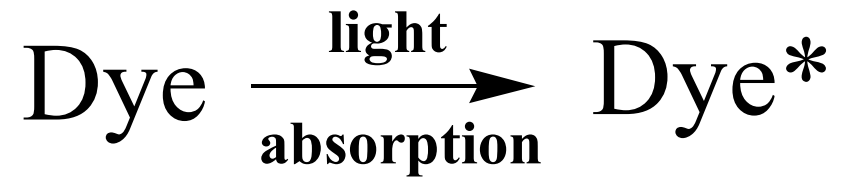
Cynthia Potter, Biosearch Technologies Inc., 81 Digital Dr., Novato CA, 94949-5750.

- ◆ Dynamic vs. Static Quenching (ground-state complex formation)
- ◆ Quenching & 5' nuclease assay
- ◆ TAMRA vs. dark quenchers
 - ◆ quenching ability and facilitation of multiplexing
 - ◆ instruments
 - ◆ probe designs



FRET Quenching

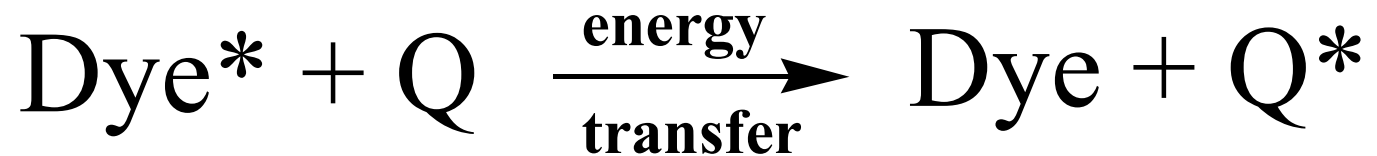
1st Step: dye molecule absorbs light & generates dye excited state.



2nd Step in absence of quenching:
Fluorescence!



2nd Step when FRET occurs: Dye excited state TRANSFERS ENERGY to Quencher ground state generating quencher excited state.



3rd Step: The Quencher then returns to the ground state.



FRET

r = distance between fluorophore and quencher

$J(\lambda)$ = overlap integral = spectral overlap of emission of fluorophore and absorption of quencher.

Q_D = Quantum yield of fluorophore (Donor).

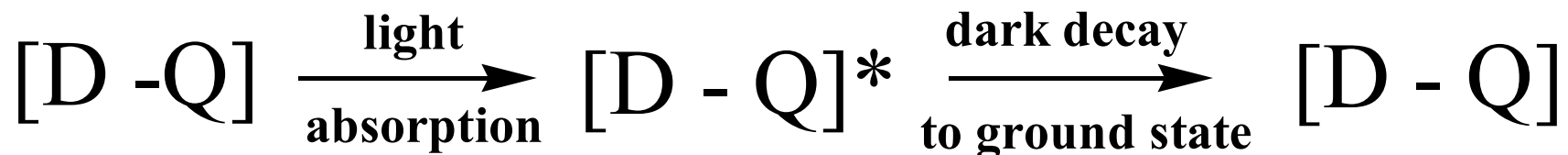
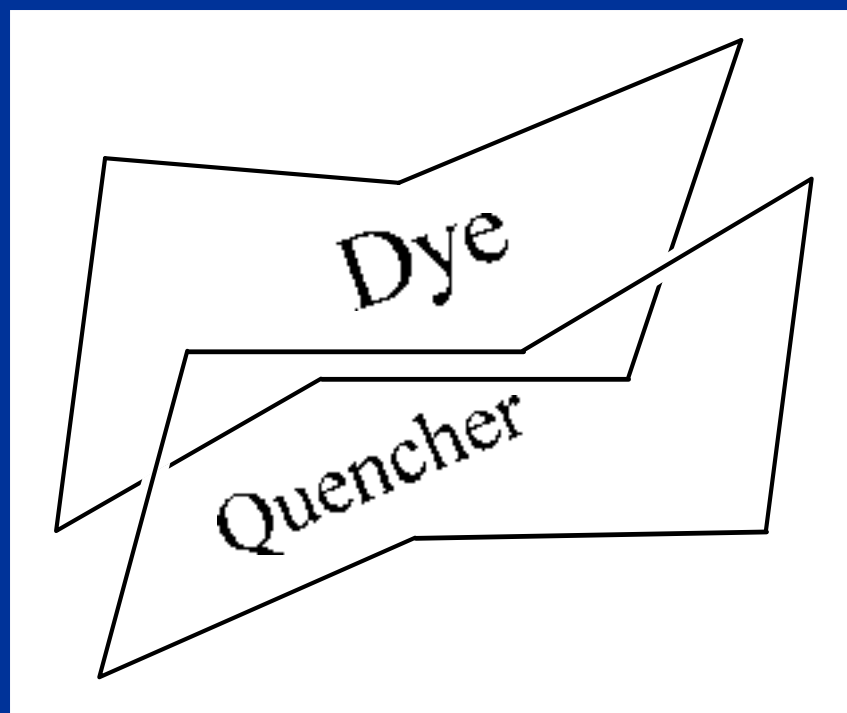
(relative orientation of transition dipoles)



Quenching by Ground State Complex Formation

1st Step: A nonfluorescent complex is formed between the dye and quencher.

- ◆ Hydrophobic & Electrostatic effects cause the dye and quencher molecules to stack together.
- ◆ The complex has its own unique properties. It has different absorption and fluorescence spectra than its component dye and quencher molecules.



Comparison of Quenching Mechanisms

FRET	vs.	G. S. Complex formation
Type of dynamic quenching		Static quenching
Förster/Coulomb mechanism		Dexter mechanism
Long distance 40-100 Å		Short distance < 20 Å
Depends on $1/R^6$		Depends on e^{-R}
Not so temperature dependent		very temperature dependent
Fluorophore absorption spectrum unchanged		Fluorophore absorption spectrum distorted

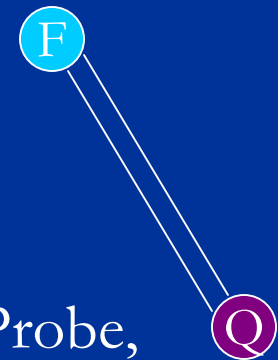
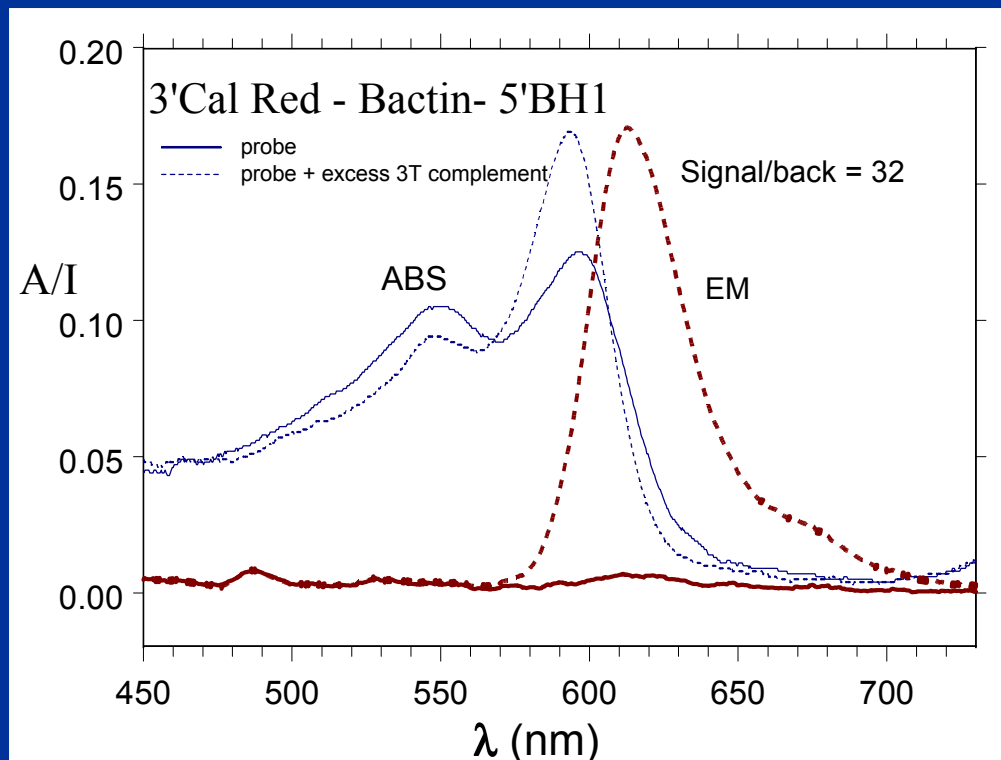


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Probe,
no
comp

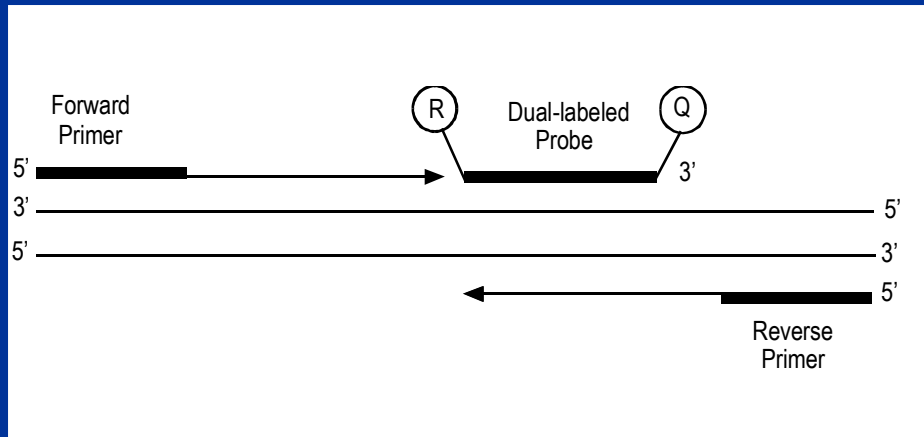


Probe,
with
comp

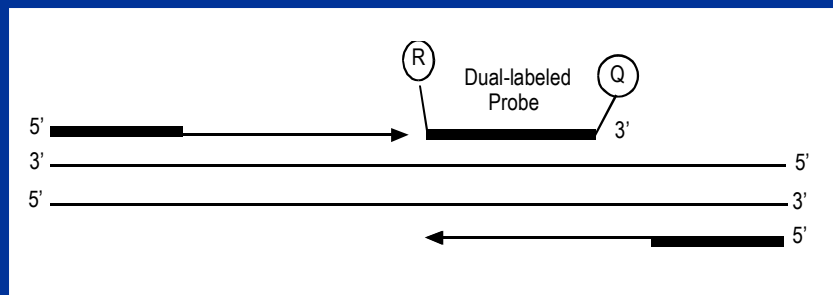


5' nuclease assay

(1) Reporter and the Quencher labeled probe is attached to the strand of DNA, along with the forward and reverse primers.

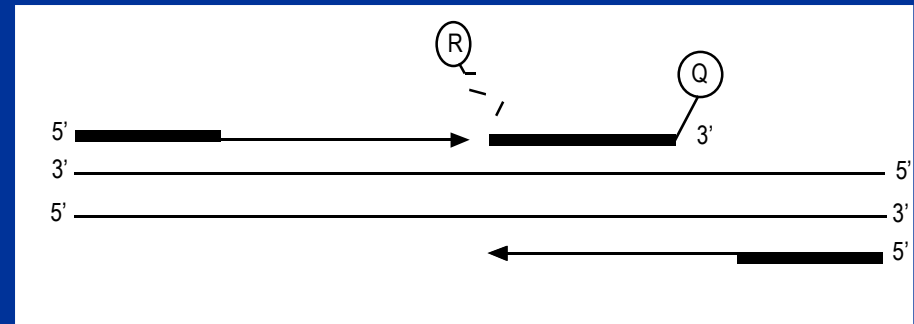


(2) Polymerase commences extension of the Primers.

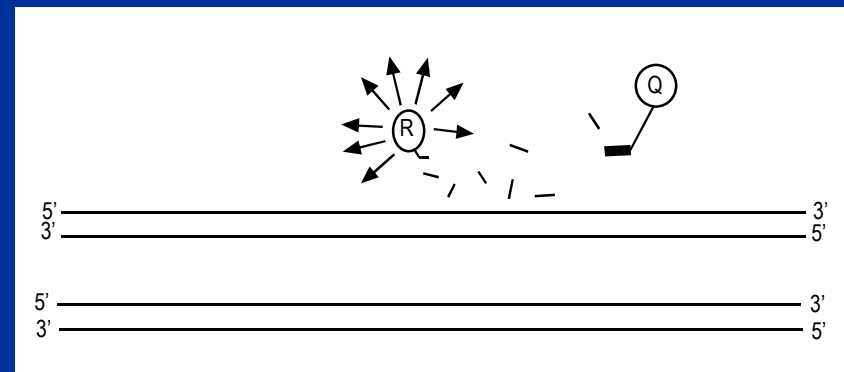


(3) dual-labeled probe is displaced by the extension of the primer.

reporter separated from quencher => increase in the reporter fluorescence intensity.



(4) The DNA Polymerase continues the extension to form a duplex for each original DNA strand.



TAMRA as a quencher

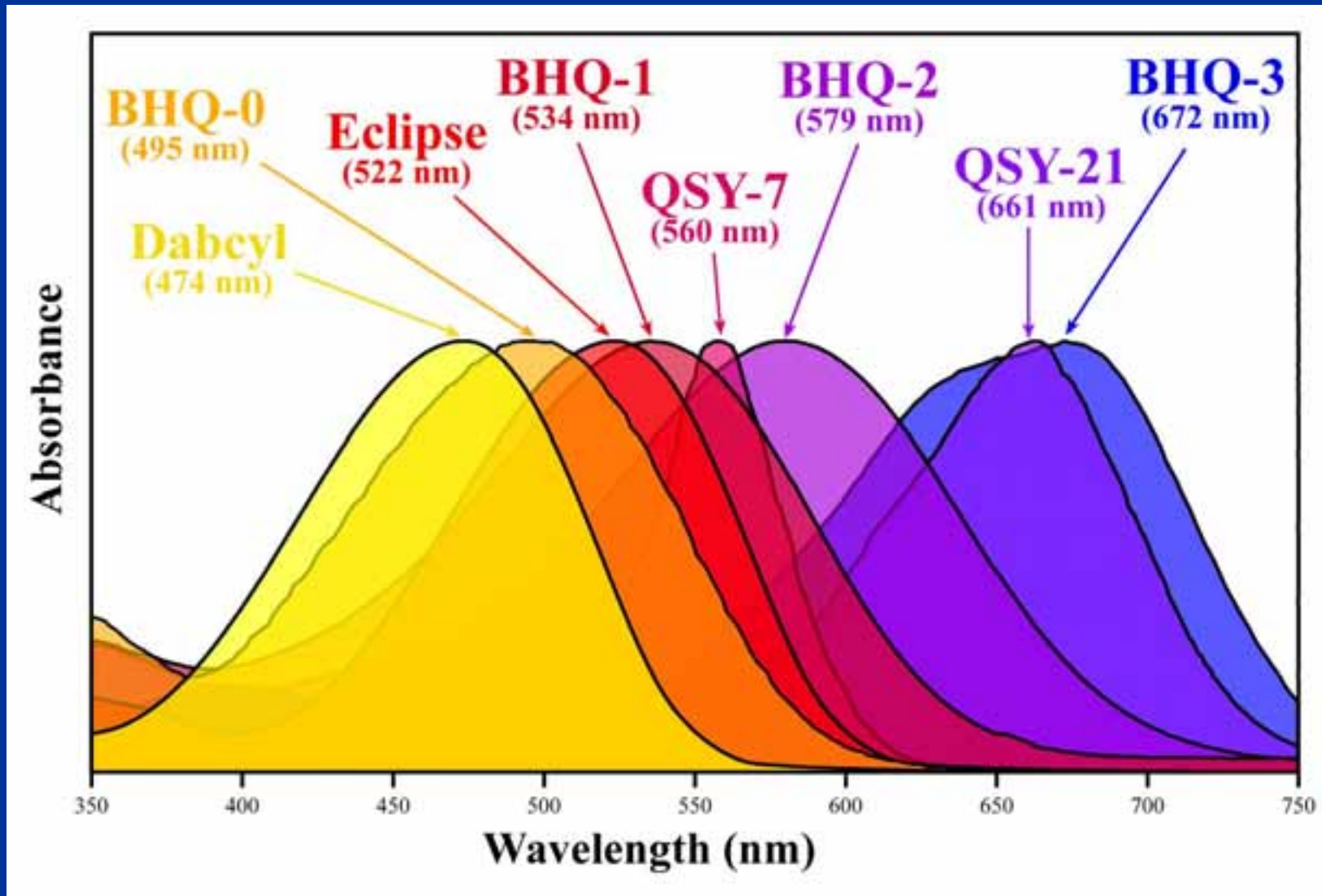
Why? TAMRA has spectral overlap with FAM, TET, HEX, JOE (VIC)

Problem: multiplex difficult because of broad emission spectra

Problem: TAMRA is fluorescent, so contributes to background



Dark Quenchers



Reporters & Dark Quenchers

Reporters					Dark Quencher				
Dye	Form available		Excitation	Emission	Absorption max	Dye	Form available		
	Amidite	Ester					Amidite	Ester	CPG
					474	DABCYL	X	X	X
					497	BHQ-0	X		X
FAM	X		495	520	522	Eclipse	X	X	X
TET	X		521	536	534	BHQ-1	X		X
JOE		X	520	548					
HEX	X		535	556	560	QSY7		X	
R6G		X	524	557					
Cy3	X		550	570					
TAMRA	X		555	576	579	BHQ-2	X		X
Cy3.5	X		581	596					
ROX		X	575	602					
Texas Red		X	583	603					
Cy5	X		649	670	661	QSY9		X	
Cy5.5	X		675	694	672	BHQ-3	X		X

All esters can be coupled to a free amine placed internally on a modified nucleotide.

Generally thymidine is used because it has exhibited the best behavior, i.e. it does not halt PCR

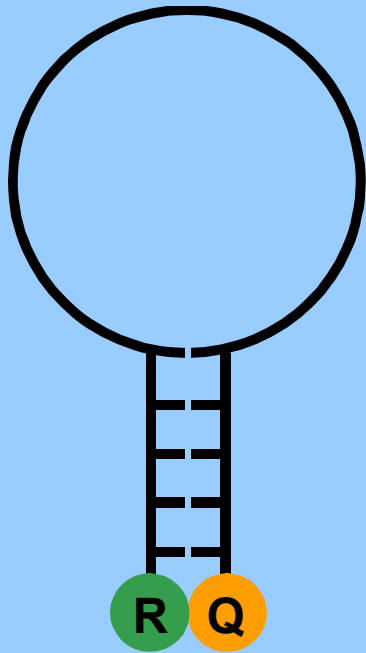
All CPGs allow easy 3' labeling that generally has larger yields and lower prices

Multiplexing Instrument Comparison

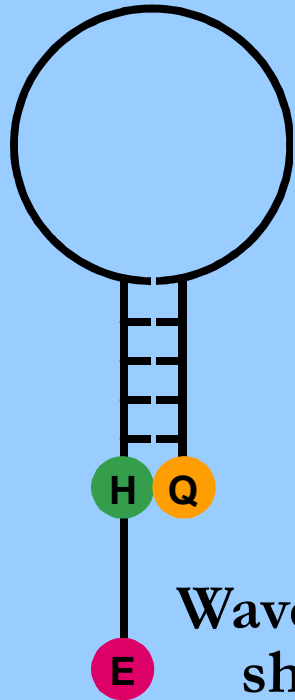
Filter systems (multiple excitation and emission filters)					
Stratagene MX4000	Dye dye available as:	FAM amidite	HEX/JOE amidite/ester	ROX/Tex Red ester/ester	Cy5 amidite
Corbett RotorGene	Dye dye available as:	FAM amidite	HEX/JOE amidite/ester	ROX/Tex Red ester/ester	Cy5 amidite
Bio-Rad iCycler	Dye dye available as:	FAM amidite	HEX/JOE amidite/ester	ROX/Tex Red ester/ester	Cy5 amidite
Cepheid SmartCycler	Dye dye available as:	FAM amidite	HEX/JOE amidite/ester	Alexa 546 ester	ROX/Tex Red ester/ester
ABI 7000	Dye dye available as:	FAM amidite	HEX/JOE amidite/ester	TAMRA amidite	ROX/Tex Red ester/ester
Algorithm systems (excitation source 488 nm)					
ABI 7700 & 7900	Dye dye available as:	FAM/TET/HEX/JOE amidite/amidite/amidite/ester		limited excitation	TAMRA/Cy3 amidite/amidite



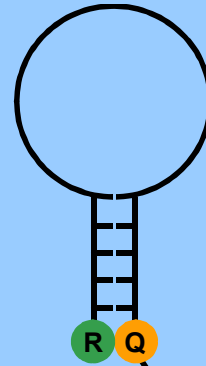
Probe Designs



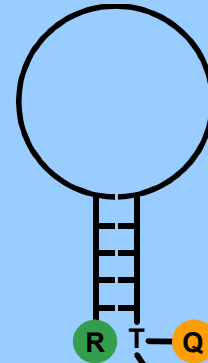
Molecular Beacon



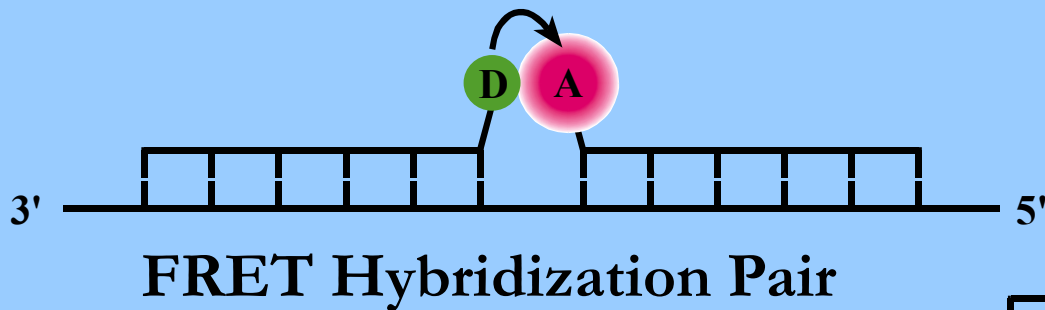
Wavelength-shifting Molecular Beacon



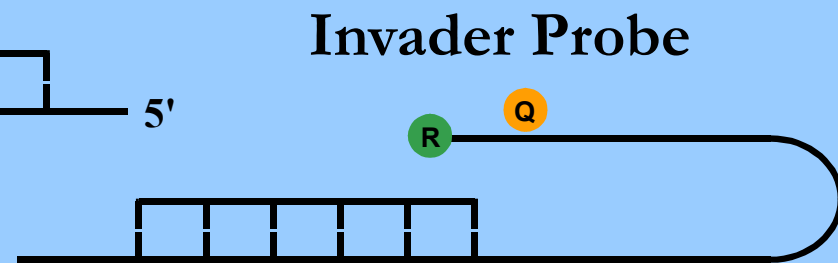
Scorpion Primer



Amplifluor



FRET Hybridization Pair



Invader Probe



References & Acknowledgements

Principles of Fluorescence Spectroscopy, 2nd edition, Lakowicz, J. R., 1999, Kluwer Academic/ Plenum Publishers, New York.

Modern Molecular Photochemistry, Turro, N. J., 1991, University Science Books, Sausalito, California.

Intramolecular Dimers: A New Strategy to Fluorescence Quenching in Dual-labeled Oligonucleotide Probes, Johansson, M. K.; Fidler, H.; Dick, D.; Cook, R. M. (submitted manuscript).

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Excellent online forum:

[http://groups.yahoo.com/group/
qpcrlistserver](http://groups.yahoo.com/group/qpcrlistserver)



Energy Transfer Efficiency

The energy transfer efficiency (E) is defined by:

$$E = \frac{R_o^6}{R_o^6 + r^6}$$





3.4 Å helix rise per base pair,
 so $25 \times 3.4 = 85 \text{ Å}$
 plus linkers assume 100 Å

For most R-Q pairs, $R_0 = 10\text{-}60 \text{ Å}$

The distance between R and Q in a single-stranded linear probe is sequence dependent.



R-Q distance = r
 $r = 30 \text{ Å}$

If $R_0 = 60 \text{ Å}$, then using the energy efficiency equation, you can calculate the signal to noise...



$$E = \frac{R_o^6}{R_o^6 + r^6}$$

The r value can be expressed in terms of a fraction of R_o . Since R_o is 60 Å, for the unbound linear probe where $r = 30$ Å, replace the r value with $((30/60) R_o)$. For the bound linear probe, where $r = 100$ Å replace the r value with $((100/60) R_o)$. The R_o value is divided out in both cases, leaving the energy transfer efficiency:

$$E = \frac{R_o^6}{R_o^6 + ((30/60)R_o)^6} = \frac{1}{1+0.015} = 98\%$$

the % fluorescence that can be measured is 2%

$$E = \frac{R_o^6}{R_o^6 + ((100/60)R_o)^6} = \frac{1}{1+21} = 4.4\%$$

the % fluorescence that can be measured is 95.6%

So, the Signal/ Background = 95.6% / 2% = 47.8



Energy Transfer Efficiency & S/N

$$E = \frac{R_o^6}{R_o^6 + r^6}$$

r	E	%Fluorescence
30Å	98	2
60Å	50	50
100Å	4.4	95.6

So, the Signal/ Background = 95.6% / 2% = 47.8

