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Choosing Reporter–Quencher Pairs for Efficient Quenching Through Formation of Intramolecular Dimers

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Summary

Fluorescent energy transfer within dual-labeled oligonucleotide probes is widely used in assays for genetic analysis. Nucleic acid detection/amplification methods, such as real-time polymerase chain reaction, use dual-labeled probes to measure the presence and copy number of specific genes or expressed messenger RNA. Fluorogenic probes are labeled with both a reporter and a quencher dye. Fluorescence from the reporter is only released when the two dyes are physically separated via hybridization or nuclease activity. Fluorescence resonance energy transfer (FRET) is the physical mechanism that is most often cited to describe how quenching occurs.

We have found that many dual-labeled probes have enhanced quenching through a nonFRET mechanism called static quenching. Static quenching, which is also referred to as contact quenching, can occur even in "linear" oligonucleotide probes that have no defined secondary structure to bring the reporter and quencher pair into proximity. When static quenching accompanies FRET quenching, the background fluorescence of probes is suppressed. This chapter describes how to pair reporter and quencher dyes for dual-labeled probes to maximize both FRET and static quenching. Data comparing various reporter–quencher pairs is presented as well as protocols for evaluation and optimization of the probes.

Key Words: FRET; intramolecular dimer; stacking interactions; fluorescent probes; biosensors.

1. Introduction

Fluorescent energy transfer within dual-labeled oligonucleotide probes is widely used in assays for genetic analysis. Nucleic acid detection/amplification methods, such as real-time polymerase chain reaction (PCR), use dual-

labeled probes to measure the presence and copy number of specific genes or expressed messenger RNA. Fluorogenic probes such as TaqMan®, molecular beacons, Amplifluors, and Scorpions are labeled with both a reporter and a quencher dye. Fluorescence from the reporter is only released when the two dyes are physically separated via hybridization or nuclease activity. Fluorescence resonance energy transfer (FRET) is the physical mechanism that is most often cited to describe how quenching occurs *(1)*.

We have found that many dual-labeled probes have enhanced quenching through a non-FRET mechanism called static quenching. Static quenching, which is also referred to as contact quenching, operates through formation of a ground-state complex. The reporter and quencher dyes can bind together to form a reporter–quencher ground-state complex. The same forces that control dye aggregation presumably control this formation. When a ground-state complex forms the excited-state energy levels of the donor and quencher dyes couple giving the ground-state complex its own electronic properties, such as being nonfluorescent and having a unique absorption spectrum. A ground-state complex that is an intramolecular dimer can form even in "linear" oligonucleotide probes that have no defined secondary structure to bring the reporter and quencher pair into proximity. **Figure 1** compares the static and FRET quenching mechanisms in "linear" reporter–quencher dual-labeled oligonucleotides.

When static quenching occurs accompanies FRET quenching, the background fluorescence of probes is suppressed *(2,3)*. Dual-labeled oligonucleotide probes that only have quenching via the FRET–quencher pairs are selected such that they can form a ground state complex, the signal/background ratios are much higher, up to a 10- to 30-fold increase in fluorescence signal, because of more efficient quenching.

This chapter describes how to pair reporter and quencher dyes for dual-labeled probes to maximize both FRET and static quenching. Data comparing various reporter–quencher pairs is presented as well as protocols for evaluation and optimization of the probes. It is important to recognize that quenching efficiency can depend on many factors, including temperature and buffer composition. Therefore, quenching via intramolecular dimers is only one of many criteria that should be considered when designing dual-labeled probes.

1.1. Important Probe Design Considerations

1.1.1. Positioning of Dye Labels

Standard convention places the quencher on the 3' and the reporter on the 5'-end of the probe. This is primarily because in oligonucleotide synthesis, all failure sequence fragments contain only the 3' label. Therefore, if the quality of synthesis and/or purification is poor, there will be an excess of 3'-quencher-
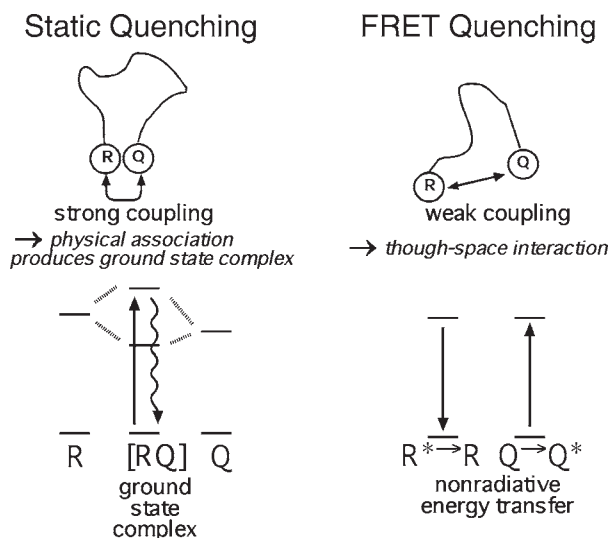
Static Quenching   FRET Quenching

Fig. 1. Static and Förster resonance energy transfer quenching mechanisms in "linear" reporter–quencher dual-labeled oligonucleotides.

labeled oligos. This is a better alternative to having failure oligos containing only a 3'-reporter, which will raise the background fluorescence of the probe *(4)*.

Dye labels may be available as a controlled pore glass (CPG), amidite, or active ester. The ease and yield of dye incorporation follows the order of CPG, amidite, ester, and this is generally reflected in probe price. A CPG dye is used to make 3' labels, whereas amidites and esters can be used for internal or 5' labels. Thus, to make a conventional 5'-reporter–3'-quencher probe, a reporter–amidite and a quencher–CPG are used. In **Table 1**, reporter dyes that are available as amidites are shown in bold. In the manufacturing of dye-labeled oligonucletides, it is more convenient and cost-effective to use dye amidites rather than succinimdyl esters. However, not all dye labels can be prepared as phosphoramidites that can withstandard oligo synthesis conditions.

1.1.2. Inherent Quenching by Bases

Another important consideration in the placement of the reporter label is that the oligo bases, especially guanine, quench many fluorophores. Therefore, reporter dyes should not be placed directly next to G residues *(5,6)*.

2. Materials

Table 1 is a listing of reporter dyes that are commonly used to label oligos. The dyes that are available as an amidite are shown in bold. Some of the dyes

Table 1
Common Reporter Dyes for Oligos

Dye name	Absorption max (nm)	Emission max (nm)	Dye type
BODIPY FL	502	510	O
FAM	**495**	**520**	**F**
Oregon Green 488	494	517	F
Rhodamine Green	503	528	R
Oregon Green 514	506	526	F
TET	**521**	**536**	**F**
Cal Gold	**522**	**544**	**F**
BODIPY R6G	528	547	O
Yakima Yellow	**526**	**548**	**F**
JOE	520	548	F
HEX	**535**	**556**	**F**
Cal Orange	**540**	**561**	**R**
BODIPY TMR-X	544	570	O
Quasar-570 /Cy3	**550**	**570**	**C**
TAMRA	**555**	**576**	**R**
Rhodamine Red-X	560	580	R
Redmond Red	**554**	**590**	**O**
BODIPY 581/591	581	591	O
Cy3.5	**581**	**596**	**C**
ROX	575	602	R
Cal Red/Texas Red	593	613	R
BODIPY TR-X	588	616	O
BODIPY 630/665-X	647	665	O
Pulsar-650	**460**	**650**	**O**
Quasar-670/Cy5	**649**	**670**	**C**
Cy5.5	**675**	**694**	**C**

Dyes in bold are available as amidites and/or CPGs. F, fluorescein; R, rhodamine; C, cyanine; O, other (*see* **Fig. 2**).

in **Table 1** are proprietary and others, such as FAM, are ubiquitous. There are many commercial sources for fluorescent dye labels and for oligonucleotide probes. The core structures of the fluorescein, rhodamine, and cyanane dyes are shown in **Fig. 2**.

Table 2 lists dark quenchers that are commonly used in fluorogenic oligo probes. The quenchers that are available as an amidite as well as a CPG are shown in bold; these quenchers can easily be incorporated as 5', 3', or internal modifications. Many of the quenchers listed in **Table 2** are proprietary, yet, they may be licensed and sold by several different companies. **Figure 2** shows the core structures of dabcyl and Black Hole Quencher™ (BHQ).
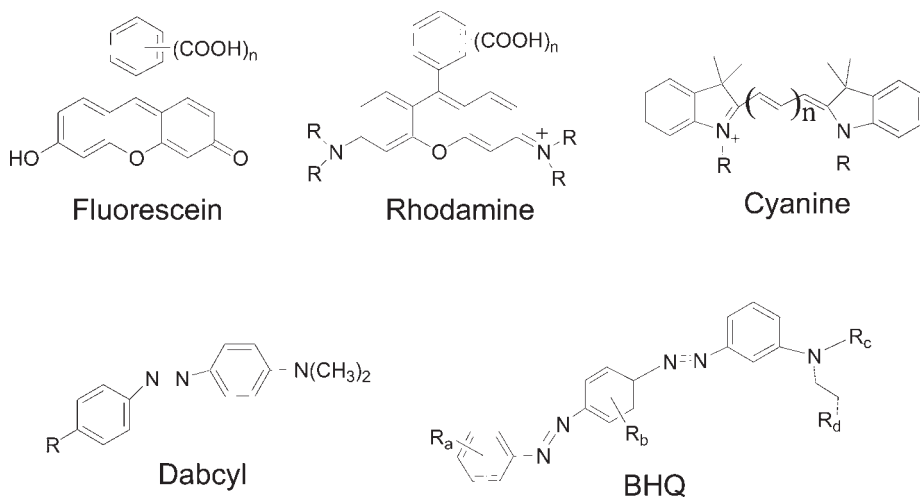
Fig. 2. Core structures for fluorescein, rhodamine and cyanine dyes, dabcyl, and Black Hole Quenchers™.

Table 2
Dark Quenchers
for Oligonucleotide Reporters

Dye name	Absorption max (nm)
Dabcyl	**453**
QSY 35	475
BHQ-0	**495**
Eclipse	**530**
BHQ-1	**534**
QSY 7	560
QSY 9	562
BHQ-2	**579**
ElleQuencher	630
Iowa Black	651
QSY 21	661
BHQ-3	**672**

Quenchers in bold are available in both CPG and amidite forms.

3. Methods

3.1. Choice of Reporter–Quencher Pairs

1. The first consideration in choosing a reporter dye is to check that the excitation and emission wavelengths are compatible with the instrumentation used to read

fluorescence. For example, FAM is generally used with 488 nm light sources. There are now many choices for reporter dyes across the visible spectrum, and new products regularly appear (**Table 1**) *(7)*. For multiplexing experiments, which use several reporter dyes to track different oligo sequences, the emission maxima of reporter dyes should be separated by at least 15 nm (*see* **Notes 1** and **2**).

2. There are a few criteria to keep in mind when choosing a quencher dye (**Table 2**). For FRET quenching, the absorption spectrum of the quencher must overlap with the fluorescence spectrum of the reporter. The first generation of dual-labeled oligo probes used the TAMRA dye (absorbance at 558 nm) as a quencher for FAM (emission at 517 nm). However, TAMRA has its own emission at 577 nm that can contribute to background fluorescence signal. Therefore, it is advantageous to use dark quenchers, such as the BHQs, which have no native fluorescence.

3. To pair reporters and quenchers for static quenching, one has to consider the structures of the dyes (**Fig. 2**). Static quenching involves formation of an intramolecular dimer; the dyes aggregate and stick together. Dye aggregation in aqueous solvents is controlled by electrostatic, steric, and hydrophobic forces *(2)*.

4. Cyanine (Cy3, Quasar 670, and others) and rhodamine (Cal Orange, Cal Red, ROX, and so on) dyes are quite planar, hydrophobic, and have delocalized charge because of quaternary nitrogens. It has been observed that these dye structures undergo more static quenching with the BHQ dyes than fluoresceins (e.g., FAM, JOE, TET, Cal Gold, HEX) (**Figs. 3–5**).

3.2. Measuring Quenching Efficiencies

See **Notes 3**–**5** and **Fig. 6** for examples of assays measuring quenching efficiencies for probe evaluation. Experimental conditions, such as temperature and buffer composition, can dramatically affect quenching efficiency and dye fluorescence (*see* **Notes 6** and **7**).

3.2.1. Sample Preparation and Analysis

1. Probe concentrations in samples for fluorescence measurements should be less than 0.5 μ*M*. This is because fluorescence measurements can become distorted owing to the re-absorption of emitted light *(8)*.

2. All fluorescence intensities should be corrected by subtracting the fluorescence intensity of a buffer blank.

3. Quenching data is often reported as a signal/background ratio where the background is from the intact (quenched) probe and the signal is recorded after hybridization or nuclease treatment (dequenched). Percentage quenching is calculated by dividing the signal of the dequenched probe (minus buffer blank) by the signal of the quenched probe (minus buffer blank), multiplying the result by 100, and then subtracting the result from 100.

3.2.2. Hybridization Assay

1. In a hybridization assay, it is important to use a buffer that contains Mg^{2+}, such as PCR buffer (10 m*M* trizma hydrochloride, 50 m*M* KCl, and 3.5 m*M* $MgCl_2$).
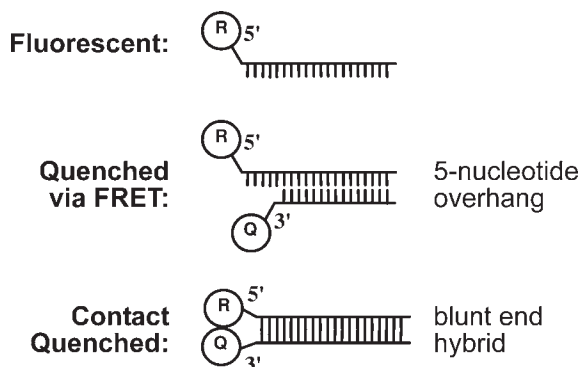
Fig. 3. Structures used by Marras et al. *(5)* to measure percentage quenching via Förster resonance energy transfer and contact quenching.

2. If a complementary sequence has extra bases on each end, the binding is stronger than if exact complement is used. (The complementary sequences used in **Fig. 5** have three extra T bases on each end.)
3. A probe should be used that has a melting temperature above room temperature (*see* **Note 8**).
4. After adding a fivefold molar excess of complement, the fluorescence intensity should be monitored until it reaches a final value. This usually takes between 5 and 15 min.

3.2.3. Nuclease Digestion Assay

1. There are several nucleases that can be used for digestion assays. Snake venom phosphodiesterase with bacterial alkaline phosphatase yield the nucleoside monomers through exonuclease digestion *(9)*. DNase I and Bal 31 are both endonucleases that degrade both single and double-stranded DNA (USB, cat. no. 14367 and 70011Y).
2. The probe should be dissolved in the buffer for enzyme digestion and split into two fractions, one of which is a control that does not receive the digestion enzyme.
3. The extent of oligo digestion can be monitored by anion-exchange high-performance liquid chromatography.
4. After incubation, both the control and the digested sample should be diluted with buffer (e.g., PCR buffer) and the fluorescence intensities can be measured.

3.2.4. Determination of Quenching Mechanism

In order to distinguish definitively between FRET and static quenching it is necessary to measure fluorescence lifetimes. The fluorescence lifetime is the average time before a dye emits a photon after the dye has absorbed a photon.

Dynamic quenching, which includes FRET, decreases both fluorescence intensity and fluorescence lifetime of the reporter dye by the same factor. In
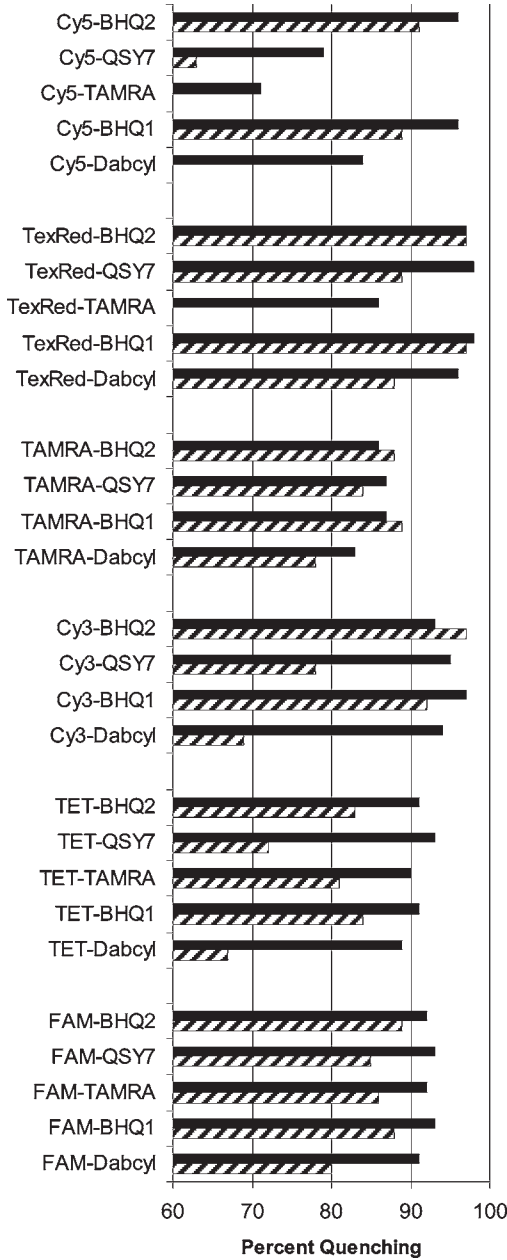
Fig. 4. Selected percentage quenching data from Marras et al. *(5)*. Dark lines show contact quenching and striped lines show Förster resonance energy transfer quenching.
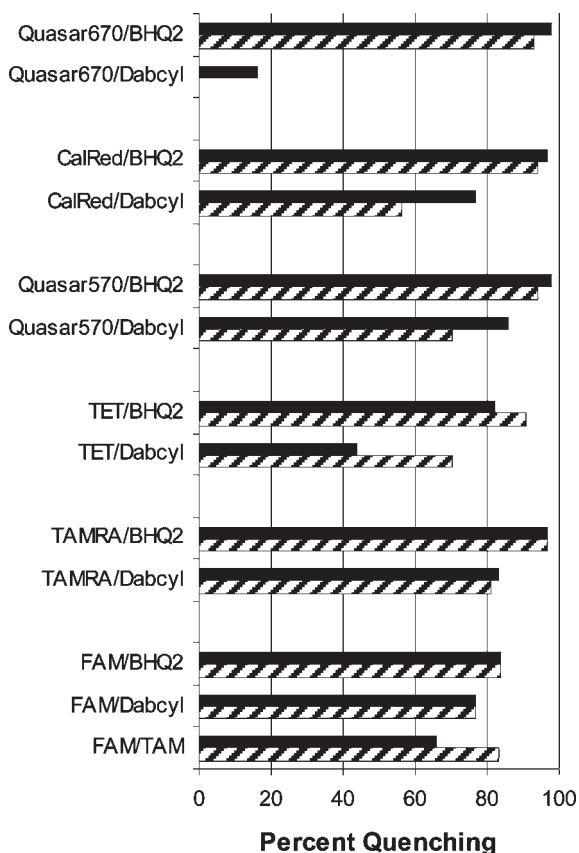
Fig. 5. Percentage quenching with dual-labeled β-actin probes. The β-actin sequence is 5'-d-ATG-CCC-TCC-CCC-ATG-CCA-TCC-TGC-G-3'. Dark lines show hybridization and striped lines nuclease digestion.

contrast, static quenching involves formation of a dye–quencher dimer. This effectively decreases the concentration of the fluorescent dye by creating a new, nonfluorescent reporter–quencher dimer. Because this dimer is formed before the dye absorbs a photon, static quenching does not change the reporter dye's fluorescence lifetime.

1. In a fluorogenic assay with a dual-labeled probe, if both the fluorescence intensity (I) and fluorescence lifetime (τ) change by the same amount going from the quenched to dequenched species, i.e., $I_{quenched}/I_{dequenched} = \tau_{quenched}/\tau_{dequenched}$, it can be concluded that dynamic quenching is the mechanism at work.

2. If $\tau_{quenched} = \tau_{dequenched}$ while $I_{quenched}/I_{dequenched}$ is less than one, static quenching is the mechanism at work. Another indication of static quenching is a change in the absorption spectrum of the probe in the quenched vs dequenched states (**Fig. 7**).
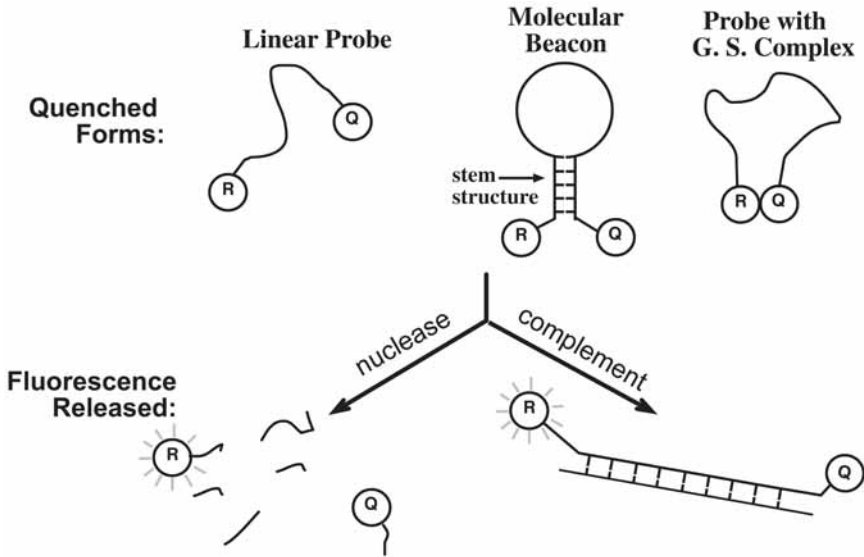
Fig. 6. Quenched probe structures and the release of fluorescence via digestion by nuclease and hybridization.

This is because the reporter–quencher dimer has its own unique absorption spectrum. FRET quenching does not effect the probe's absorption spectrum *(2)*.

4. Notes

1. There are several commercial sources for fluorogenic oligonucleotide probes and some companies offer proprietary dyes. The BHQs, Quasar, and Cal Dyes were developed at Biosearch Technologies.
2. Most real-time PCR instruments use filters to selectively monitor reporter fluorescence. The ABI PRISM® 7700 and 7900 perform spectral deconvolution. The instrument user manual may suggest combinations of reporters for multiplexing experiments.
3. There are several ways to screen combinations of reporter/quencher pairs. A series of quencher–reporter pairings were recently tested by Marras, Kramer, and Tyagi. They used complementary oligos with 5'-reporters and 3'-quenchers to bring the dyes directly together or at staggered distances to measure, respectively, contact-mediated and FRET quenching efficiencies *(5)* (**Fig. 3**). This method of bringing the dyes together in order to measure contact (static) quenching holds the reporter and quencher in a fairly constrained and fixed relative orientation. Furthermore, in this model, the reporter-quencher interaction may strongly depend on the length and rigidity of the oligo-dye linkers. **Figure 4** shows the Marras et al. *(5)* quenching efficiencies for a series of reporter/quencher pairs with emission maxima spanning from 441 to 702 nm.
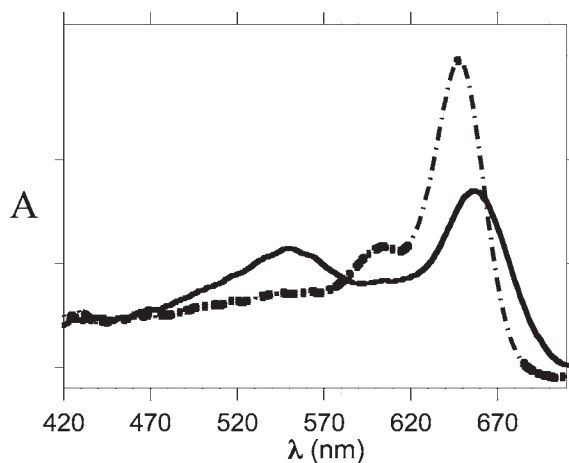
Fig. 7. Absorption spectrum of a Cy5/BHQ-1 probe alone (solid line) and hybridized to a complementary oligonucleotide (dashed line)

4. The data in **Fig. 5** show percentage quenching that has been measured in a series of "linear" 5'-reporter–3'-quencher probes. The efficiency of static (also known as contact-mediated) quenching depends on the affinity of the reporter and quencher for each other (i.e., association constant). In a linear probe, the strength of this affinity is more critical than in a probe in which the reporter and quencher are held together through hybridization *(10)*. Also, a linear probe can be thought of as a flexible linker that will allow the dyes to associate in a wider variety of conformations. Therefore, reporter–quencher pairs held in a fixed configuration, such as a molecular beacon or a hybrid, might have different quenching efficiencies than the same pair in a "linear" dual-labeled probe. These different structures are illustrated in **Fig. 6**. The quenching efficiency values measured by nuclease digestion differ from those measured via hybridization because the hybrid structure has an effect on the fluorescence intensities of many reporters *(11)*. The 25mer linear probes with BHQ-2 as the quencher and Quasar-670, Cal Red, Quasar-570, and TAMRA as reporters all have quenching efficiencies greater than 90%. Such high quenching efficiencies in "linear" probes are indicative of static quenching.

5. The BHQ are aromatic and quite hydrophobic (in fact, many dyes are only water-soluble after conjugation to oligos). One might expect that a hydrophobic reporter dye should be used to increase reporter–BHQ association. Some reporter dyes have phosphonate or sulfonates appended in order to increase water solubility. However, there is not very much data on intramolecular heterodimers with water-soluble dyes. Surprisingly, a sulfonated Cy 3 (Amersham Biosciences, cat. no. PA13101)/BHQ-2 β-actin probe has a quenching efficiency of 93% on both hybridization and nuclease digestion. Changes in the absorption spectra suggest formation of a reporter–quencher intramolecular dimer.

6. Quenching efficiency within dual labeled probes can be temperature dependent. Association between the reporter and quencher that controls formation of the intramolecular dimer decreases with increasing temperature. Thus, efficient static (or contact) quenching at room temperature may significantly decrease at higher temperatures.
7. The fluorescence quantum yields of some dyes, such as the cyanines, decrease significantly with increasing temperature. Fluorescence intensity can also depend on pH and the local environment of the dye *(12)*.
8. If a reporter–quencher pair that form a strong ground state complex are used in a molecular beacon (or other type of self-hybridizing probe), the additional stabilization owing to the reporter–quencher association can inhibit the molecular beacon from hybridizing to the complementary sequence at room temperature.

Acknowledgments

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